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Human and Animal Health Applications

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Professor Pandey is the recipient of many national and international awards and fellowships, which include Elected Member of the European Academy of Sciences and Arts, Germany; Fellow of the International Society for Energy, Environment and Sustainability; Fellow of the National Academy of Science (India); Fellow of the Biotech Research Society, India; Fellow of the International Organization of Biotechnology and Bioengineering; Fellow of the Association of Microbiologists of India; honorary doctorate degree from the Université Blaise Pascal, France; Thomson Scientific India Citation Laureate Award, United States; Lupin Visiting Fellowship; Visiting Professor at the Université Blaise Pascal, France, the Federal University of Parana, Brazil, and the École Polytechnique Fédérale de Lausanne, Switzerland; Best Scientific Work Achievement Award, Government of Cuba; UNESCO Professor; Raman Research Fellowship Award, CSIR; GBF, Germany, and CNRS, France fellowships; Young Scientist Award; and others. He was chairman of the International Society of Food, Agriculture and Environment, Finland (Food & Health) during 2003–04. He is the Founder President of the Biotech Research Society, India (www.brsi.in); International Coordinator of the International Forum on Industrial Bioprocesses, France (www.ifibiop.org); chairman of the International Society for Energy, Environment & Sustainability (www.isees.org); and vice president of the All India Biotech Association (www.aibaonline.com). Professor Pandey is editor-in-chief of *Bioresource Technology,* Honorary Executive Advisor of the *Journal of Water Sustainability* and *Journal of Energy and Environmental Sustainability,* subject editor of the *Proceedings of the National Academy of Sciences (India),* and editorial board member of several international and Indian journals, and also a member of several national and international committees.

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Preface

This book is a part of the comprehensive series, *Current Developments in Biotechnology and Bioengineering* (Editor-in-Chief: Ashok Pandey), composed of nine volumes. Within this series, the current volume is dedicated to human and animal health applications in biotechnology. In the 1970s, researchers developed DNA recombinant technology, which facilitated the production of recombinant proteins. One of the first molecules produced by this technology was somatostatin. Through genetic engineering techniques, the gene coding for somatostatin was inserted into *Escherichia coli* cells, which expressed a recombinant protein with activity similar to the human hormone. Over the ensuing years, other heterologous systems were introduced for producing recombinant human molecules, including yeast, insect, and mammalian cells. The new class of biologically derived therapeutic molecules was called *biopharmaceuticals*.

Actually, 60% of the new drugs approved by the Food and Drug Administration are biopharmaceuticals that were developed for the treatment of cancer and infectious, cardiovascular, and inflammatory diseases. Biopharmaceuticals now comprise a diverse number of molecules, such as coagulation factors, hormones, enzymes, monoclonal antibodies, and vaccines. Owing to their complexity in three-dimensional structure and size, each process applied to produce a molecule will result in a biopharmaceutical with specific physicochemical properties and biological activity. The biotechnological industrial sector has made outstanding contributions to medical progress resulting in improvements to life.

This book is composed of nine parts. In the first part, Chapters 1 and 2 present the global market and perspectives on biotechnology health, in which different classes of biopharmaceutical products are described and the manufacturing technologies used to obtain these products are discussed. Part 2 is devoted to research and development in immunotechnology, such as interferon production by the biotechnology industry for viral infections and cancer treatments, and monoclonal antibodies that have revolutionized the fields of research and medicine. Chapter 5 introduces and explores new perspectives in recombinant antibody production, a unique source of biomolecules that are used as pharmacologic tools and which also should contribute to a revival of immunodiagnostic and biomedical applications as yet unimagined.

In the third part, combinatorial libraries are described, which include the impacts of the aptamer technology on diagnostics, biotechnology, and therapy; combinatorial preparation; and high-throughput screening of arrays of compounds that are the basis of numerous platforms of pharmaceutical drug discovery processes. Finally the concept, innovations, applications, and future of phage display technology are presented.

The fourth part is on gene and cell therapy. Chapter 9 discusses the power of mathematical networks and modeling for the identification of key components related to rheumatoid arthritis and how to predict the response of different individuals to infections. Chapter 10 reviews the biogenesis pathways and general functions of microRNAs, PIWI RNAs, and long noncoding RNAs. It presents current knowledge concerning the use of noncoding RNAs in diagnosis, prognosis, and therapeutics and discusses their role in the development of

the immune system and the regulatory functions of H_2O_2 during the course of metazoan evolution.

Part 5 deals with tissue engineering. Chapter 11 presents biomaterials, which are derived from naturally occurring molecules and those that recapitulate key motifs of biomolecules within biologically active synthetic materials. The most significant biological features of the extracellular matrix are discussed and several engineering methods currently being implemented to design and tune synthetic scaffolds to mimic these features are presented. Chapter 12 presents protocols to isolate adipose-derived stem cells, strategies to characterize these cells, an example of stem cell differentiation into bone cells (generating material to perform bone repair in vivo) and some techniques that can be applied to verify the structure of the newly generated bone tissue. In Chapter 13, the current state of bioengineering and regenerative medicine is discussed with respect to each abdominal organ. Chapter 14 offers new approaches to reprogramming mesenchymal stem cells for tissue engineering on a biofunctionalized scaffold for tissue repair and for restoring its function.

The next part is about biofilms and biosurfactants in health, which describes the most promising technology using microbial surfactants, which have attracted attention as potential substitutes for, additions to, currently employed antimicrobial compounds, mainly for biofilm infection control. Chapter 15 discusses the potential use of these biomolecules in formulating drug delivery nanocarrier systems, including liposomes and polymer-based carriers. Chapter 16 highlights the use of bacteriocins as a new strategy for planktonic cells and biofilm control.

The field of vaccinology has yielded several effective vaccines that have significantly reduced the impact of a number of important diseases. Biotechnology has made the development of new vaccines safer and less costly. Chapter 17 reviews technologies that are currently available for the development of recombinant veterinary vaccines. Chapter 18 describes new technologies related to the production of acellular pertussis vaccine against whooping cough; Chapter 19 presents a case description about the biotechnological development of a diphtheria subunit vaccine using diphtheria toxin subunit B as the immunogenic protein. Chapter 20 discusses serum-free rabies vaccine production and Chapter 21 discusses the lyophilization process.

Biotechnological strategies needed to develop better pharmaceuticals against leishmaniasis are presented in Chapter 22. Finally, Chapter 23 focuses on maternal recognition of pregnancy and genes controlling maternal recognition of pregnancy events that facilitate the development of optimal reproductive management strategies and paradigms to augment embryo survival.

We hope that this book will be of special interest to academicians, researchers, graduate students, and industry scientists working in the field. We would like to thank the authors and reviewers of the chapters for their cooperation and for their preparedness in revising the articles on a scheduled timeline. We thank Dr. Kostas Marinakis, book acquisition editor, Ms. Anneka Hess, and the entire production team at Elsevier for their help and support in bringing out this volume. Without their commitment, efficiency, and dedicated work, this volume could not have been accomplished.

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1

Biopharmaceutical Products: An Introduction

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1.1 Introduction

The term "biopharmaceuticals" was coined in the 1980s specifically to refer to human health products generated or produced by means of modern molecular biological methods using engineered organisms, and to distinguish these products from traditional biological products directly extracted from natural biological sources such as proteins obtained from plasma or plants [1]. Techniques usually considered to be the landmarks in "modern Biotechnology", recombinant DNA technology and hybridoma technology, both developed in the 1970s, were important for the development of this class of products. However, besides "biopharmaceuticals," several other terms, such as biotherapies, immunotherapies, biologics, and biological products, have been used to refer to these products [2,3]. In 2002, in an attempt to avoid the problem that the various terms have different meanings for different people, Walsh [4] discussed the issue of nomenclature and proposed the use of the following definition:

> *A biopharmaceutical is a protein or nucleic acid-based pharmaceutical substance used for therapeutic or in vivo diagnostic purposes, which is produced by means other than direct extraction from a native (non-engineered) biological source.*

Currently, according to this definition by Walsh [4], "biopharmaceuticals" is the most widely adopted term, but it is often used interchangeably with "biologicals," "biologics," and "biological products" [3]. In terms of the biopharmaceutical market, recombinant therapeutic proteins largely dominate. Thus, it is also common to use the term "biopharmaceuticals" to refer to them specifically. However, if one refers to "biopharmaceutical products" or to the "biopharmaceutical sector" as an industrial segment, recombinant vaccines and nucleic acid–based therapies are usually included. Besides these, other biotechnological products for human health are cell therapies, in which the cells are the product used to regenerate or engineer tissues, and molecules used for in vitro or in vivo diagnostics.

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1.2 The Biopharmaceutical Sector: Market Aspects

The first biopharmaceutical product to be approved for human use in 1982 was human insulin produced in recombinant *Escherichia coli*. Four years later, there were other biopharmaceutical landmarks: the approvals of recombinant tissue plasminogen activator (t-PA) produced in animal cells (Chinese hamster ovary cells, CHO), of the monoclonal antibody OKT3 produced in hybridoma cells and of recombinant hepatitis B vaccine produced in *Saccharomyces cerevisiae* [5,6].

Since the commercialization of insulin almost 35 years ago as the first recombinant therapeutic protein, many other products followed, leading to a continuous increase in the sales of the biopharmaceutical sector. Until July 2014, 246 biopharmaceutical products had been licensed in the United States and in the European Union (EU), containing 166 different active ingredients. In the 20 years between 1995 and 2014, the number of new biopharmaceutical products approved in these regions was approximately constant, between 50 and 60 every 5 years. Among the 54 products licensed between 2010 and July 2014, about 40% were not truly new products (i.e., they were biosimilars, improvements of existing products, or products previously approved in other regions). Thus, only 32 of them, containing 30 different new active ingredients, can be considered genuinely new products [1].

Despite the decades that have passed since approval of recombinant insulin, the biopharmaceutical sector cannot yet be considered a mature industry, and many new developments regarding new products and new technologies are under way.

In the period 2010−14, there were several important landmarks:

- A first biosimilar monoclonal antibody (mAb) (i.e., a product comparable to the innovator biopharmaceutical product whose patent has expired) was approved in the EU: infliximab biosimilar products (Remsina and Inflectra) from two companies (Celltrion and Hospira) were licensed in 2013.
- In the so-called “highly regulated markets,” for the first time, there was approval of a gene therapy product: Glybera (alipogene tiparvovec), produced by the company UniQure, licensed in the EU in 2012.
- For the first time, a biopharmaceutical produced in plant cells was approved: Elelyso (glucocerebrosidase/taliglucerase alfa), expressed in recombinant carrot cells propagated in bioreactors and licensed by Protalix Biotherapeutics/Pfizer in 2012 in the United States.
- Ruconest, produced by the company Pharming using transgenic rabbits, was the second biopharmaceutical product obtained from the milk of transgenic animals to be licensed. It has been approved in 2010 in the EU and in 2014 in the United States. The first product had been Atryn, a recombinant human antithrombin III produced from the milk of transgenic goats, approved in 2009 in the United States.

The pharmaceutical sector as a whole, and more specifically the biopharmaceutical sector, has been steadily growing over past decades. This is related, among other factors, to the continuous increase in the average life expectancy of the world population. This is

particularly true in the case of biopharmaceuticals, because many of them are indicated for disorders that are more prevalent at advanced ages, such as cancer and inflammatory disorders. Between 1980 and 2012, for example, life expectancy grew from 75 to 82 years in Australia, 65 to 75 in China, 73 to 81 in Germany, 58 to 73 in Turkey, 67 to 77 in Mexico, and 61 to 68 years considering the worldwide average [7]. In 2013, for example, the pharmaceutical sector grew 3% in the United States (data by IMS Health, cited in EFPIA [8]). In emerging economies, such as Brazil, growth was even more pronounced (14% in Brazil in 2013), because in these countries economic and social factors such as a reduction in the inequality of income distribution, can have a significant impact on access of the population to drugs.

Around the world, according to a report by the McKinsey company [9], the annual growth rate of the pharmaceutical industry was 4%; it reached 8% in the case of the biopharmaceutical sector alone. Investments in R&D of biopharmaceuticals usually give a better return than in the case of synthetic drugs, because the success rate of biopharmaceuticals is usually two times higher than that of small molecules (13% of biopharmaceuticals that enter phase I of clinical trials reach the commercialization phase). Maybe motivated by this, the number of submitted biopharmaceutical patents has been growing 25% per year since 1995, ensuring continuous growth in the number of new biopharmaceuticals being developed [9].

This continuous growth of the biopharmaceutical pipeline will certainly further increase the importance of the biopharmaceutical sector, which in 2014 accounted for approximately 20% of all pharmaceutical sales [9]. This forecast is further corroborated by the fact that among the approximately 10,000 therapeutic products under development and about 41,000 clinical trials under way in 2012, about 40% referred to biopharmaceuticals [10]. However, to develop a new biological entity for human health, as of 2012 an investment of approximately USD 1.5 billion and a period of 12−13 years were needed. Thus, the biotech industry is known to be one of the most R&D-intensive sectors, spending approximately 14.4% of net sales in R&D [8]. The biopharmaceutical sales, which in 2014 reached USD 163 billion, exceed the reported gross domestic product (GDP) of 75% of the economies (156 out of 214 countries) included in the World Bank GDP ranking database [1,9]. A large part of these sales is due to 37 biopharmaceutical products that are considered blockbusters, i.e., that have sales higher than USD 1 billion per year.

Data displayed in Table 1.1 [11] show the important role of biopharmaceuticals in the global pharmaceutical industry. All 10 top-selling pharmaceutical products, which together accounted for over USD 75 billion sales in 2013, individually reached over USD 5 billion sales. Furthermore, it is noteworthy that:

- the top-selling pharmaceutical product was the mAb Humira (adalimumab), indicated for arthritis treatment
- among the five top-selling pharmaceuticals, four were biopharmaceuticals, all of which were mAb-based products
- among the 10 top-selling pharmaceuticals, seven were biopharmaceuticals, six of which were mAb-based products

Table 1.1 Ten Worldwide Top-Selling Pharmaceutical Products

Product	Main Indication	Company	Sales in 2012 (10^6 USD)	Sales in 2013 (10^6 USD)	Growth Rate 2012–13 (%)
Humira	*Arthritis*	*AbbVie*	*9265*	*10,659*	*15.0*
Remicade	*Arthritis*	*J&J/Merck & Co*	*8215*	*8944*	*8.9*
Rituxan	*Non-Hodgkin lymphoma*	*Biogen Idec/Roche*	*8266*	*8583*	*3.8*
Enbrel	*Arthritis*	*Pfizer/Amgen*	*7973*	*8325*	*4.4*
Seretide/Advair	Asthma/chronic obstructive pulmonary disease	GlaxoSmithKline	7887	8243	4.5
Lantus	*Diabetes*	*Sanofi*	*6586*	*7589*	*15.2*
Avastin	*Colorectal cancer*	*Roche*	*6217*	*6746*	*8.5*
Herceptin	*Breast cancer*	*Roche*	*6352*	*6557*	*3.2*
Crestor	Dyslipidemia	AstraZeneca	6253	5622	−10.1
Abilify	Schizophrenia	Otsuka/BMS	5308	5158	−2.8

Entries that are biopharmaceuticals are shown in italics.
Adapted from FirstWord Pharma. Pharma's 50 Biggest Selling Drugs: AbbVie's Humira Joins the $10 Billion Club, 2014 http://www.firstwordpharma.com/node/1194000#axzz3Y2ixQ0qJ (access in June 2015).

1.3 Types of Biopharmaceutical Products for Human Health

Among the different types of biopharmaceutical products, recombinant therapeutic proteins, vaccines, and products for in vitro and in vivo diagnostics are most mature, whereas nucleic acid–based therapies and cell therapies are less consolidated, with a still low number of approved products. In terms of clinical trials (phases I, II, and III), as of 2013, 245 were ongoing focusing on cell therapy, 99 focused on gene therapy and 127 focused on antisense therapies [12].

1.3.1 Recombinant Therapeutic Proteins

During the 20th century, advances in knowledge about the molecular basis of diseases revealed that many disorders were related to deficiencies in specific proteins. Because in healthy organisms the physiologic levels of such proteins are usually low, commercial manufacture of these proteins by isolating them from material obtained from healthy donors was shown in almost all cases to be unfeasible. In the 1970s, the development of recombinant DNA (rDNA) technology made it virtually possible to produce any desired human protein by means of cloning and expressing the respective human gene in a host cell line then to be cultivated on a large scale under controlled conditions.

Walsh [13] proposed the classification of recombinant therapeutic proteins as first- and second-generation biopharmaceuticals. The former are proteins that have an amino acid sequence identical to the native protein found in healthy organisms, intended for simple replacement or an increase in circulating levels in deficient patients. On the other

hand, second-generation biopharmaceuticals are engineered versions of human proteins which have been modified by protein engineering techniques to achieve improved therapeutic properties such as:

- generation of a product with an action that is either quicker or slower
- manipulation of the product half-life
- alteration of immunogenicity
- development of hybrid or fusion proteins

Protein engineering techniques used to develop second-generation biopharmaceuticals can involve different strategies:

- modification of the amino acid sequence, such as in commercial insulin and t-PA products
- deletion of domains, such as in commercial blood coagulation Factor VIII and also t-PA products
- generation of hybrid or fusion proteins, such as chimeric and humanized antibodies, bispecific antibodies, and proteins fused to immunoglobulin G (IgG) or albumin
- posttranslational engineering, such as the creation of additional glycosylation sites in erythropoietin or the pegylation of several proteins

In addition to the seven groups that have traditionally been used to classify therapeutic proteins [5], the approval of several fusion proteins in past years and the large number of such products in development have resulted in a further group. Thus, biopharmaceutical proteins can be divided into:

1. recombinant cytokines
2. recombinant hematopoietic growth factors
3. other recombinant growth factors
4. recombinant hormones
5. recombinant blood factors
6. recombinant enzymes
7. monoclonal antibodies and antibody-drug conjugates (ADCs)
8. fusion proteins

Among the wide range of products, some such as interleukins and erythropoietins seem to have attained saturation in terms of supply and demand, because no new product with such active ingredients was approved in 2010−14 [1]. On the other hand, therapeutic mAbs have gained increasing relevance in terms of new approvals, the total number of products under commercialization, production volume, and market value. Among the more recent products, almost all are humanized or fully human. Main therapeutic indications are different types of cancer and inflammatory/autoimmune diseases, such as rheumatoid arthritis and lupus. From 2010 until July 2014, mAbs represented 26.5% of the new biopharmaceutical products that received approval.

Sales of mAbs reached USD 63 billion in 2013, accounting for approximately 40% of biopharmaceutical sales. Because mAbs are usually administered in high doses to a relatively broad number of patients, their production volume is also significant compared with other products: It was estimated that in 2010 approximately 7 tons of purified mAb active ingredients were produced and the forecast for 2016 was 13.4 tons [1]. Among mAbs approved and under development, a small fraction consisted of ADCs [14]. The two most recent ADC products were Kadcyla (trastuzumab emtansine), sold by Roche and approved in the EU and the United States in 2013, and Adcetris (brentuximab vedotin), sold by the company Takeda and approved in the United States in 2011 and in the EU in 2012.

Fusion proteins are nonnative molecules obtained by the molecular combination of the gene sequences coding for two or more proteins, protein fragments, or peptides. The aim is to confer a longer half-life, manipulate cytotoxicity, or enable novel delivery and targeting routes [15]. Usually a linker (e.g., a peptide) is used in the construction of stable, bioactive fusion proteins [16,17]. The first fusion protein to be approved for human use, in 1998, was Enbrel (etanercept), which consists of two molecules of the 75-kDa fragment of tumor necrosis factor (TNF)-α receptor fused to the Fc portion of an IgG1, which is responsible for extending the half-life of the resulting fused product. Because etanercept binds reversibly to TNF-α, it is used to treat TNF-dependent inflammatory diseases such as arthritis. Right after its introduction to the market in 1998, Enbrel presented a great sales performance, which at first led to difficulties in production capacity to meet the unexpectedly high product demand. Enbrel continues to be the fourth most sold pharmaceutical product worldwide, with sales in 2013 at over USD 8.3 billion [11].

According to Belsey and Somers [16], of 43 projects involving fusion proteins, 19% were either already on the market or under analysis by regulatory agencies, 53% were in clinical phase I/II or II, and 28% were in clinical phase II/III or III. In terms of therapeutic indication, 63% of these projects focused on oncology or autoimmune diseases. Mellado and Castilho [5] compiled therapeutic recombinant proteins approved until that moment, indicating also information related to the production technology, such as the cell line/expression system and process details (bioreactor type, operation mode, etc.). A compilation up to July 2014 can be found in Walsh [1], but fewer process details have been included.

1.3.2 Nucleic Acid–Based Therapies

Nucleic acid–based therapies are composed of gene therapies and antisense therapies. In both cases, the main targets are different types of cancer and monogenic disorders. The aim of these therapies is to modulate expression of a given gene that is associated with the disease. By means of gene therapy, the genetic material of patient cells is modified. A gene that is inappropriately activated can be blocked, a gene that has a defect can be corrected or replaced, or a heterologous gene can be introduced to enable the cell to produce some useful protein [18].

Gene therapy can be carried out ex vivo or in vivo. In the former case, cells are collected from the patient and genetically manipulated in the laboratory, and then expanded in vitro and reintroduced to the same patient. In the case of in vivo therapy, the genetic material is introduced to the patient and gene delivery can be either systemic or site-directed. In this case, the main challenges are related to directing the genetic material adequately to the target cells or tissues, as well as to achieving an effective expression of the desired gene. Viral vectors are commonly used to carry the gene, aiming at efficient and stable expression.

The first gene therapy product to be approved for human use was the product Gendicine, licensed in China in 2003. The product is manufactured by the company Shenzhen Sibiono GeneTech and is based on an adenoviral vector containing the p53 tumor supressor gene. In up to 50−70% of human tumors, the p53 gene is mutated and the p53 pathway is inactivated [19]. The expression of heterologous p53 protein helps control the tumor. Gendicine is produced using HEK 293 cells in fixed-bed bioreactors.

Long after the approval of Gendicine, the first gene therapy product was licensed in the so-called highly regulated markets: Glybera was approved in 2012 in the EU. It is based on an adeno-associated viral vector (AAV1) containing a gene that codes for lipoprotein lipase. This is a key enzyme in the metabolism of triglyceride-rich lipoproteins, and its deficiency causes hypertriglyceridemia and pancreatitis, and can be lethal. Considering that since 1989 approximately 2000 gene therapy clinical trials have been approved (64% of which were in the United States and 26% of which were in the EU [1]), the approval of Glybera in the EU can be considered a landmark. However, the cost of EUR 53,000 per vial or EUR 1.1 million per treatment (21 vials per patient) creates doubts as to whether the product will be successful and whether it will open the path to an increase in the number of licensed gene therapy products [20].

On the other hand, antisense therapies are based on a different mechanism for modulating the expression of a gene related to a disorder: Nucleic acid molecules are used to block the target mRNA molecules. Single-strand oligonucleotides, small interfering RNAs, micro RNAs, ribozymes, and other antisense compounds can be used to block expression of a given gene: for example, an oncogene in the case of cancer therapies [21].

In 2013 the US Food and Drug Administration (FDA) approved an antisense therapy, the first to have a systemic delivery and to target a chronic disorder, and thus to be administered lifelong. The active ingredient is an antisense oligonucleotide and the therapeutic indication is the rare disorder “homozygous familial hypercholesterolemia,” which results in very high cholesterol levels and may cause heart attacks and even death in very young patients (e.g., at age 30 years). The product is named Kynamro and is commercialized by the companies Isis and Genzyme. It blocks the production of apolipoprotein B, an atherogenic protein that transports cholesterol in the bloodstream [22].

Although Kynamro is a single-strand oligonucleotide with just 20 nucleotides, and thus is produced by chemical synthesis and not rDNA, antisense therapies have historically been referred to as biopharmaceutical products [1]. Many other antisense

products are currently under clinical development and an increase in the number of approved products is expected to occur.

1.3.3 Cell Therapies

Cell therapies consist of a medical intervention aiming at reestablishing the structure and function of a tissue by means of using cells; i.e., in this case the cells are the product. Cell therapy is applied to treat dysfunctions caused by trauma or disease, as well as by degeneration processes which can be premature or result from aging. Some cell therapies have been used successfully for a long time, such as in cardiac diseases, eliminating the need for transplantation; in serious burns, decreasing hospitalization time and mortality; in cerebrovascular accidents (CVA), reducing cases, reducing hospitalization time and sequelae; and in autologous bone marrow transplant.

Many advances related to cell therapies are intrinsically related to advances in stem cell research. A landmark for therapies based on adult stem cells was the first transplantation of bone marrow cells in the 1950s [23]. However, the derivation of mouse embryonic stem cells in 1981 [24] and of human embryonic stem cells in 1998 [25] attracted unprecedented and widespread scientific and clinical interest in the potential use of these cells for regenerative medicine and tissue engineering, because the pluripotency of these cells allows them to be differentiated into any cell type in the organism.

A further landmark consisted of the development of cell reprogramming techniques by Takahashi and Yamanaka [26,27]. These authors showed that adult, fully differentiated cells can be reprogrammed to the pluripotent state by means of the heterologous expression of genes associated with pluripotency. These reprogrammed cells, known as induced pluripotent stem (iPS) cells, are a major focus of research for the development of cell therapies and are also used as tools to screen for new drugs and in basic studies of diseases. A further advance of great scientific and clinical interest is the direct generation of functional differentiated cells by reprogramming, e.g., fibroblasts. Vierbuchen et al. [28] showed that mouse fibroblasts can be directly converted to functional neuronal cells through expression of three transcription factors (Ascl1, Myt1l, and Brn2). These neurons were termed induced neuronal cells. Until 2015, no therapies based on cells derived from pluripotent stem cells had been approved by the FDA [29]. The only clinical trial using cells derived from pluripotent stem cells, sponsored by the company Geron, was approved in 2009 by the FDA, but it was canceled by the sponsor in 2011 [30].

1.3.4 Vaccines

In public health, there is no tool as effective (and also cost-effective) as vaccination. Not even antibiotics have had the effect of reducing population mortality as pronouncedly as have vaccines. Through vaccination, nine of the main human diseases have been controlled in several regions of the world: diphtheria, tetanus, yellow fever, pertussis, poliomyelitis, measles, mumps, rubeola, and smallpox. The latter was officially

eradicated worldwide in 1979 as a consequence of effective vaccination actions leaded by the World Health Organization. In addition, great progress has been achieved through vaccination against influenza, hepatitis B, pneumococcus, rotavirus, and *Haemophilus influenza* type B.

The origin of vaccine development dates to the end of the 18th century, when Edward Jenner observed that milkmaids who were in contact with the cowpox virus, which does not cause the disease in humans, were protected against infection with the human smallpox virus [31]. Traditional vaccines that were developed since then, and especially during the 20th century, can target bacterial or viral infections, and generally have as active ingredient the corresponding pathogen in either an attenuated or inactivated form, eliciting an immune response and protective antibody formation in vaccinated individuals, but without causing the disease.

In past decades, advances have been made in the development of recombinant vaccines based on the expression of genes coding proteins of the pathogen. The first recombinant vaccine to be approved was the one against hepatitis B in 1986. It contains the hepatitis B surface antigen, which forms multimers containing about 85−155 molecules of the antigen, forming tridimensional virus-like particles (VLPs) that mimic the virus and are immunogenic [6]. Two recombinant vaccines (Cervarix and Gardasil) against human papilloma virus, produced by GSK and Merck & Co., respectively, have been approved. These contain the recombinant form of the main protein of the virus capsid (the L1 protein). This protein forms in vitro tridimensional VLPs that consist of 72 pentamers of L1. These VLPs are immunogenic when administered to humans.

Recombinant vaccines currently account for approximately 10% of approved biopharmaceutical products. Besides an increase in approved VLP-based vaccines, new vaccines based on plasmid DNA or on mRNA are expected to be licensed in the next years. Between 2010 and July 2014, recombinant vaccines that were approved include [1]:

- Bexsero (Novartis), a multicomponent subunit vaccine against meningitis B produced in *E. coli* and approved in the EU in 2013
- Flublok (Protein Sciences), a vaccine based on VLPs of recombinant hemagglutinin from three different influenza virus strains, produced in insect cells and approved in the United States in 2013
- Provenge (sipuleucel-T, Dendreon), a vaccine approved in 2010 in the United States and in 2013 in the EU, consisting of autologous peripheral blood cells combined with prostate acid phosphatase and recombinant G-CSF produced in insect cells

1.3.5 In Vitro and In Vivo Diagnostic Products

Hybridoma technology, developed by Koehler and Milstein [32], made the generation of antibodies with predefined specificities possible. These antibodies were named monoclonal antibodies (mAbs). Besides their therapeutic use (previously discussed), these antibodies are widely employed for in vitro and in vivo diagnostics.

In the case of in vitro diagnotics, antibodies are mainly used in laboratory immunoassays, such as enzyme-linked immunosorbent assays (ELISA), blots and others. mAbs of murine origin are frequently used. Owing to the small quantities used in the assays, the production scale of these antibodies is significantly lower than that of therapeutic mAbs. Moreover, the purity requirements, although high, are considerably lower than for injectable therapeutic mAbs.

In the case of in vivo diagnostic mAbs, labeled antibodies (e.g., radiolabeled) are injected into patients, aiming at the in situ detection of tumors and other conditions. In this case, because the mAb is injected into the patient, quality and manufacturing requirements resemble those applicable to therapeutic mAbs [33]. Because there is usually no repeated administration of the product, murine mAbs are acceptable.

The number of approved in vivo diagnosis mAb-based products is not large [34]. One example is the product ProstaScint, approved for commercialization in 1996 in the United States, which is a murine mAb labeled with In^{111}, used as an in vivo diagnostic imaging agent in patients diagnosed with biopsy-proven prostate cancer who are at high risk for metastases. Another example is LeukoScan, approved in 1997 in the EU, which is a technetium-99m–labeled mAb fragment specific for NCA-90, a surface antigen found on activated granulocytes, and is indicated to localize infections and inflammations in bone in patients with suspected osteomyelitis.

1.4 Production Processes for Recombinant Therapeutic Proteins

Recombinant protein therapeutics represent the most mature and economically most important class of biopharmaceutical products. Therefore, the focus of this section is on the technologies used to manufacture them. However, many similarities exist among the production processes for the different classes of biopharmaceutical products, so much of the discussion here also applies to the production of gene therapy vectors, viral vaccines, diagnostic mAbs, and cell therapies.

1.4.1 Product Complexity Versus Expression System

Recombinant proteins used as therapeutic agents or as vaccines are frequently glycoproteins and are usually large and complex, with different posttranslational modifications such as glycosylation, γ-carboxylation, sulfation, phosphorylation, acetylation, methylation, and cleavage of peptides [35]. These posttranslational modifications are important because they affect the biological activity, stability, and immunogenicity of the recombinant products. Posttranslational modifications which either lack or result in structures that differ from corresponding human standards can result in recombinant proteins with low biological activity, decreased stability, and high immunogenicity, which make their commercial use unfeasible. Glycosylation can further affect protein folding, transport, target recognition, and binding. For these reasons, for the production

of a recombinant protein, it is crucial to know its structure beforehand and carefully select an adequate expression host.

Among different hosts, the structure of glycosylated structures attached to the amino acid chain can vary a lot, because the potential diversity of chemical structures is enormous owing to:

- the sequence of monossacharides
- the position of glycosidic bounds
- the configuration (alpha or beta) of the glycosidic bond
- the number of ramification points
- the position of ramifications

Human glycoproteins usually hold glycans of the so-called complex type, with several ramifications that yield a "multiantennary" structure. Different monosaccharides are added to an initial core rich in mannose, and the antennas terminate with N-acetyl neuraminic acid molecules, also known as sialic acid. When a human protein is produced in its recombinant form for replacement therapies, the aim is to produce a copy that is as similar as possible to the native protein found in healthy organisms. Thus, the glycosylation pattern of the host cell should be as similar as possible to the human glycosylation pattern.

The glycan structure depends on the availability of precursor sugars, culture conditions (e.g., medium composition, temperature, pH, metabolites), and the pool of glycosidases and glycosyltransferases present in the host cell. Differently from transcription and translation, in glycosylation there is no template for biosynthesis.

Because *E. coli*, the workhorse among bacterial expression systems, does not have the cellular machinery for glycosylation processing [36], it has been used as the host for the expression of nonglycosylated proteins such as insulin, or of glycosylated proteins whose activity is not strongly affected by the lack of glycans, such as occurs with granulocyte colony stimulating factor (G-CSF).

Mammalian cells have a glycosylation pattern similar to that of human cells, and therefore represent the most widely used expression system for the production of biopharmaceutical products. Yeast, insect, and plant cells typically produce high-mannose glycostructures which can be immunogenic to humans.

One example of highly glycosylated protein is erythropoietin (EPO), in which 40% of the molecular mass refers to the glycidic portion and 60% to the amino acid chain. Changes in the glycosylation pattern strongly affect the isoelectric point, biological activity, and stability of EPO molecules. Thus, international pharmacopoeias establish which glycoforms of EPO can be present in the final product, according to their isoelectric point. Undesirable glycoforms must be minimized during the cell culture process and those eventually present need to be removed during the purification process.

In specific cases in which the presence of exposed mannose residues is exceptionally required for protein action, such as in native glucocerebrosidase, it is interesting to produce the recombinant glycoprotein in an expression system that yields high-mannose glycostructures. In the case of glucocerebrosidase, the product

manufactured by Genzyme in CHO cells needs to have an additional cleavage step after purification, to expose mannose residues of the glycan core. Therefore, Protalix developed a recombinant glucocerebrosidase expressed in cultured carrot cells, so that the recombinant protein is directly synthesized bearing exposed mannose residues [37]. However, this is an unusual situation, because almost all human glycoproteins have a complex-type glycosylation pattern characterized by terminal sialic acid molecules.

The effects of glycosylation can also be explored to improve product properties: for example, using protein engineering techniques to generate hyperglycosylated molecules. Amgen developed a hyperglycosylated EPO which has two extra glycosylation sites in addition to the four sites present in native EPO. The resulting molecule has a threefold higher half-life, which decreases the frequency of required injections.

Other relevant aspects regarding the choice of the expression system are:

- if the host cell line is able to secrete the recombinant protein or if it accumulates the product intracellularly
- if the cell line has robust growth in large-scale stirred bioreactors
- if the cell line has characteristics that increase safety against contamination, e.g., of viral origin

Mammalian cells are the main expression system in the biopharmaceutical industry; as of 2014 they responded to the production of 52% of approved biopharmaceuticals. Bacteria represented 19%, yeast 16.5%, human cells 4%, and other hosts 8.5% [1]. Among the mammalian cells, the CHO cell line predominates, responding to 35.5% of approved biopharmaceuticals (or 68.2% of those produced in mammalian cells).

This is due to different factors in addition to the adequate glycosylation pattern conferred by CHO cells. These cells secrete recombinant proteins in the culture medium, which makes the purification processes easier and cheaper. Furthermore, they are robust, can be cultivated in suspension in large-scale bioreactors, and are a poor substrate for the replication of human viruses, which represents an additional safety factor in the event of viral contamination of the process.

1.4.2 Manufacturing Technology: Upstream Processing

Despite the more sophisticated bioprocess requirements presented by animal cells, including the need for complex, expensive culture media composed typically on the order of 50 components, they account for approximately 60–65% of all approved biopharmaceuticals, if mammalian, human, and insect cells are taken together [1,38]. Owing to the growing demand for biopharmaceuticals, there are continuously great efforts to improve mammalian cell-based technologies, with the aim of increasing productivity and reducing costs [38,39]. Mammalian cells have been cultured in vitro in the laboratory for over 100 years, but it was just in the past 60–70 years that research aiming at technological aspects and scale-up has been intensified, at first motivated by advances in the area of viral vaccines produced by mammalian cell culture [40,41].

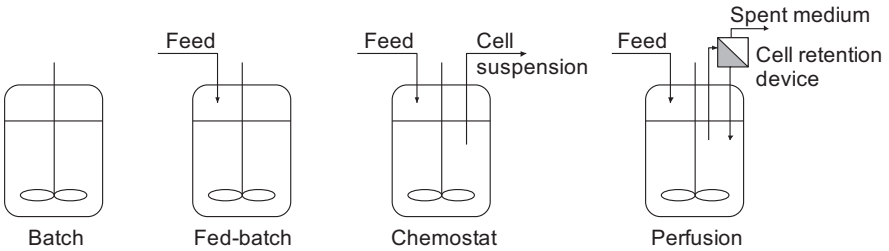

FIGURE 1.1 Overview of different bioreactor operation modes: batch, fed-batch, simple continuous mode (chemostat), and continuous mode with cell recycle (perfusion).

Different types of bioreactors can be used for animal cell culture, such as roller bottles and hollow-fiber bioreactors. However, most cell culture processes aiming at producing biopharmaceuticals both on a laboratory and industrial scale are based on the use of stirred-tank bioreactors for the cultivation of suspension-adapted cells [38,42].

Bioreactors can be run under different operation modes, which differ basically in the way in which medium is fed to them (Fig. 1.1). Most commercial products are still produced by batch and fed-batch processes, owing to their lower complexity. In fed-batch processes, the addition of a concentrated nutrient solution extends the culture time and allows higher titers to be obtained. The development, in the past 10–20 years, of fed-batch feed solutions with optimized composition, added at optimized feed rates to the bioreactor, enabled obtaining high cell concentrations (on the order of 30×10^6 cells/mL) and high product concentrations (on the order of 10 g/L).

Simple continuous cultures, on the other hand, have traditionally been used primarily for physiologic studies [43] and do not represent a good alternative to production processes. Because of the low specific growth rate of animal cells, the highest dilution rate that can be used with no cell washout is relatively low, and thus the maximum concentration of cells that can be achieved is not high.

However, if a cell retention device is coupled with a continuous bioreactor, resulting in a continuous process with cell recycle, also known as a perfusion process, continuous replacement of culture medium can be carried out at increasing flow rates, supplying a progressively increasing cell concentration with nutrients [44]. Cell retention in the bioreactor allows very high cell densities to be achieved, resulting in the continuous harvest of large product amounts at high dilution rates, thus giving volumetric productivities that are usually 10 times higher than in fed-batch processes. When a desired high cell concentration is achieved, a controlled cell bleed can be carried out, avoiding oxygen transfer limitations and allowing a steady state to be achieved under which the bioreactor can be operated for weeks or months [45,46]. The residence time of product in the bioreactor is short, which contributes to its quality and consistency. The high cell density in the bioreactor also allows the culture medium to contain fewer nutritional supplements, because the cells secrete to the medium growth factors and other autocrine factors [47]. However, maintenance of a continuously operating system attached to

a cell retention device (usually placed in an external recirculation loop) for a long period increases both the operational complexity and contamination risk [48].

In the past 2 decades, for stable proteins such as mAbs, fed-batch processes have become popular as a low-complexity process alternative that, when adequately developed, allows high concentrations of cells and product to be obtained. The use of perfusion processes on an industrial scale was mainly restricted to sensitive proteins such as enzymes and blood coagulation factors, which needed to be quickly removed from the bioreactor to maintain their functionality. However, a change in the technological paradigm has begun in the biopharmaceutical industry, and there is a growing trend to adopt continuous perfusion processes for both stable and unstable molecules, eventually integrated into continuous purification steps. This trend has arisen from the growing need for process intensification, cost reduction, and the development of more flexible plants.

1.4.3 Manufacturing Technology: Downstream Processing

Purity requirements for injectable products for human use are very high. Strict regulatory guidelines establish limits for critical contaminants, such as DNA and host cell proteins. For these reasons, purification processes in the biopharmaceutical industry consist of many sequential steps, including several sophisticated chromatography steps, to reach the degree of purity required for the product. The need for sequential downstream processing with many steps, exploiting differences in properties between the protein of interest and other biomolecules (such as the charge, size, surface hydrophobicity, and biospecific affinity), results in losses of product along the processing steps. Thus, as product purity becomes higher along processing, product yield and recovery decrease.

For biopharmaceutical products produced in bacteria, generally found as insoluble aggregates (inclusion bodies) in the cytoplasm, there are additional steps of cell lysis, protein renaturation, and separation of cell debris, as well as increased difficulty owing to the large amount of intracellular contaminants released upon cell lysis. For these reasons, cumulative product loss is significant, leading to decreased global yields that can be as low as 10%. Even when mammalian cells that usually secrete the recombinant protein to the extracellular medium are used, thus with no extra load of intracellular contaminants, global yield is not high, generally in the range of 30−70%. Global recoveries achieved in the downstream process have a direct impact, given a desired production capacity, on the bioreactor scale and on the sizing of separation equipment, thus affecting both capital and operational costs.

Thus, considering the manufacturing process as a whole, downstream processing steps can represent up to 90% of production costs of therapeutic proteins [49,50]. The fraction of manufacturing costs owing to product purification are strongly related to the number of steps required, because the processing time increases and the product yield decreases with an increase in the number of purification steps.

A way to reduce the number of downstream processing steps is to replace low selectivity techniques with more selective ones, such as affinity chromatography, thus achieving the same result with a lower number of steps. Affinity adsorption relies on the selectivity of reversible interactions between the protein of interest and a ligand, frequently resulting in very high purification factors at relatively high recoveries. However, affinity ligands are usually expensive, and the number of cycles that can be used is often limited because they are usually of biological origin and can be prone to degradation. On the other hand, ion exchange and hydrophobic interaction chromatography steps are carried out using adsorbents that are more stable and less expensive. Thus, the use of these techniques can contribute to decreasing downstream processing costs. Separation of molecules from a mixture in these cases is based on the differences of charge and surface hydrophobicity, respectively. Despite being less selective than affinity chromatography, they can result in efficient purification processes which are satisfactory in terms of both cost and performance.

1.5 Technological Challenges and Future Perspectives

Many changes are expected in the near future in the biopharmaceutical industry [9]. Global innovative companies will have to focus on product innovation to remain at the technological frontier, exploring new operational designs. Biosimilar companies will have to focus on costs, quality, and scale, because for them speed, innovation in manufacturing processes, and operational excellence will determine their survival. Contract manufacturing organizations (CMOs) will have to keep at the frontier of operational efficiency and innovation in manufacturing processes, warranting a high reputation in terms of services and performance.

Although the biopharmaceutical industry has traditionally been conservative and has been characterized mainly by incremental innovations [51], true evolution in the production technologies and operational capacity will be required in the future, because simple technological and operational gains will not be enough to increase productivity and quality and to reduce costs as much as needed. Companies will have to take different issues into consideration [9]:

- decrease costs related to the manufacturing process and to quality issues, improving technology from cell line development to downstream processing
- increase operational agility and equipment use at no expense of quality aspects, to eliminate bottlenecks, produce multiple products in fewer lines, and respond quickly to the demands of a volatile market
- increase production capacity and adopt new technologies enabling the design of flexible plants to suit specific product and market conditions, which can be based on stainless-steel equipment, disposable systems, or hybrid combinations of them
- be able to introduce new products and new technologies quickly to push a greater number of molecules toward commercial launches

- decide which activities should be carried out internally and which should be outsourced to CROs (contract research organizations) or to CMOs
- be able to keep talented human resources who should be able to handle these issues and future challenges that will emerge in this rapidly evolving sector

Indeed, a major technological shift currently seems to be under way [52]. According to Warikoo et al. [53], by the beginning of 2010 approximately 90% of the biopharmaceutical products were produced on an industrial scale through batch or fed-batch processes, but almost all innovative companies and suppliers were studying continuous bioprocessing [54]. The business context, with rising competition from off-patent biopharmaceuticals, broad pipelines of products, and rapidly evolving market demands, started challenging industry conservatism and pushing technological development toward continuous processes, which could provide higher productivity, improved product quality, lower costs, and enhanced product/scale flexibility [51,53].

Advances in disposable systems for upstream and downstream processing, as well as encouraging signs given by regulators, have further contributed to increasing industry interest in a new, continuous-flow production platform [52]. This new technology should be applicable to both mAbs and nonantibody proteins and should integrate upstream and downstream operations, allowing extra-low production cycles. A new paradigm toward integrated continuous biomanufacturing is currently being established in the biopharmaceutical industry, and the facilities of the future will be efficient, simple, compact, automated, mobile, and cost-effective, distributed around many countries and offering multiproduct and multiphase (clinical/commercial) manufacturing capability [53,55].

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2 Biopharmaceuticals and Biosimilars

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2.1 Introduction

Until the 19th century, pharmaceuticals provided to the population were based on plant-derived natural compounds and were applied in the form of infusions, teas, and creams. In 1885, Louis Pasteur attenuated the rabies virus in the laboratory and applied it to a boy who was bitten by a dog with rabies disease, thereby saving his life [1]. It was the first report of virus manipulation as medicine in the history of human health sciences.

Later, in 1928, Alexander Fleming developed the penicillin antibiotic. Immediately, pharmaceutical companies such as Pfizer, Squibb, and Merck tried to synthesize it for large-scale production without success. Hence, the North Agriculture Regional Search Laboratory in the United States developed a methodology to produce penicillin but did not have a specific application. The need for the large-scale production of penicillin occurred during the Second World War (1939–45), because the number of American soldiers dying from bacterial infections and gangrene, accounting for 12–15% of all men. The administration of penicillin to wounded soldiers reduced mortality to 3%, saving hundreds of lives [2,3].

The post–Second World War period was the beginning of the biopharmaceutical industry, when the production of large-scale antibiotics started, as did the development of novel drugs from animal sources [4]. During the 1970s, researchers developed DNA recombinant technology, which facilitated the production of recombinant proteins. One of the first molecules produced by this technology was somatostatin [5]. Through genetic engineering techniques, the gene coding for somatostatin was inserted into *Escherichia coli* cells, which expressed a recombinant protein with activity similar to the human hormone [5]. In 1982, the same procedure was applied for the production and commercialization of the first recombinant insulin [6].

Over the ensuing years, other heterologous systems were introduced for producing recombinant human molecules, including yeasts, insects, and mammalian cells. The new

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class of biologically derived therapeutic molecules was called biopharmaceuticals [7]. Hence, 60% of new drugs approved by the Food and Drug Administration (FDA) until 2013 were biopharmaceuticals that were developed for the treatment of cancer and infectious, cardiovascular, and inflammatory diseases [8]. Biopharmaceuticals have been on the market for the past 20 years and now comprise a diverse number of molecules, such as coagulation factors, hormones, enzymes, monoclonal antibodies (mAbs), and vaccines. Because of their complexity in three-dimensional structure and size, each process applied to produce a molecule will result in a biopharmaceutical with specific physicochemical properties and biological activity [9].

2.2 Biopharmaceuticals: Classes, Variations, and Applications

The composition of biopharmaceuticals can be peptides, proteins, or nucleic acids [10]. Cell-based systems can also be considered biopharmaceuticals for cell therapy applications [11]. Thus, we can group the broad nature of biopharmaceuticals into distinct classes (Figs. 2.1 and 2.2).

Among the protein-based biopharmaceuticals, mAbs comprise one of the most structurally and functionally complex classes. They are produced using in vitro display techniques [12] (i.e., phage, ribosome, bacterial, yeast, or mRNA display), hybridoma technology [13], or heterologous expression systems [14]. Most are used to prevent and treat cancer (e.g., Rituximab, Bevacizumab, Trastuzumab, Cetuximab) and autoimmune diseases (e.g., Adalimumab, Infliximab, Golimumab) [15,16].

Enzymes are another complex class and include molecules such as glucocerebrosidase, α-galactosidase, hyaluronidase, and asparaginase. In the case of hormones, the structural complexity is even wider and ranges from single-chain polypeptides and large heterodimeric proteins such as human growth hormone (somatotropin) and follicle-stimulating hormone (FSH), respectively, to simpler proteins and small peptides such as insulin and glucagon [15,17].

The blood factors group is composed of blood-clotting proteins (coagulation factors) such as factor VIII and factor IX, which are applied in the treatment of hemophilia A and B, respectively. Similarly, thrombolytic proteins (tissue plasminogen activator), anticoagulants (hirudin), hematopoietic growth factors (erythropoietin (EPO)), and granulocyte colony-stimulating factor (G-CSF) are biopharmaceutical classes used to treat cardiovascular diseases [15,17,18].

Furthermore, growth factors for non−blood cell types are used as therapeutic molecules. They include the vascular endothelial growth factor and somatomedins (insulin-like growth factors). Other molecules from this group include osteogenic proteins (bone morphogenetic protein) such as eptotermin-α for bone tissue regeneration at a clinical level. Finally, cytokines comprising cell signaling modulator molecules in immune responses are composed of two main subclasses: interferons (IFNs), such as IFN-alfa, IFN-beta, and IFN-gamma, which are used to treat hepatitis B and C, multiple

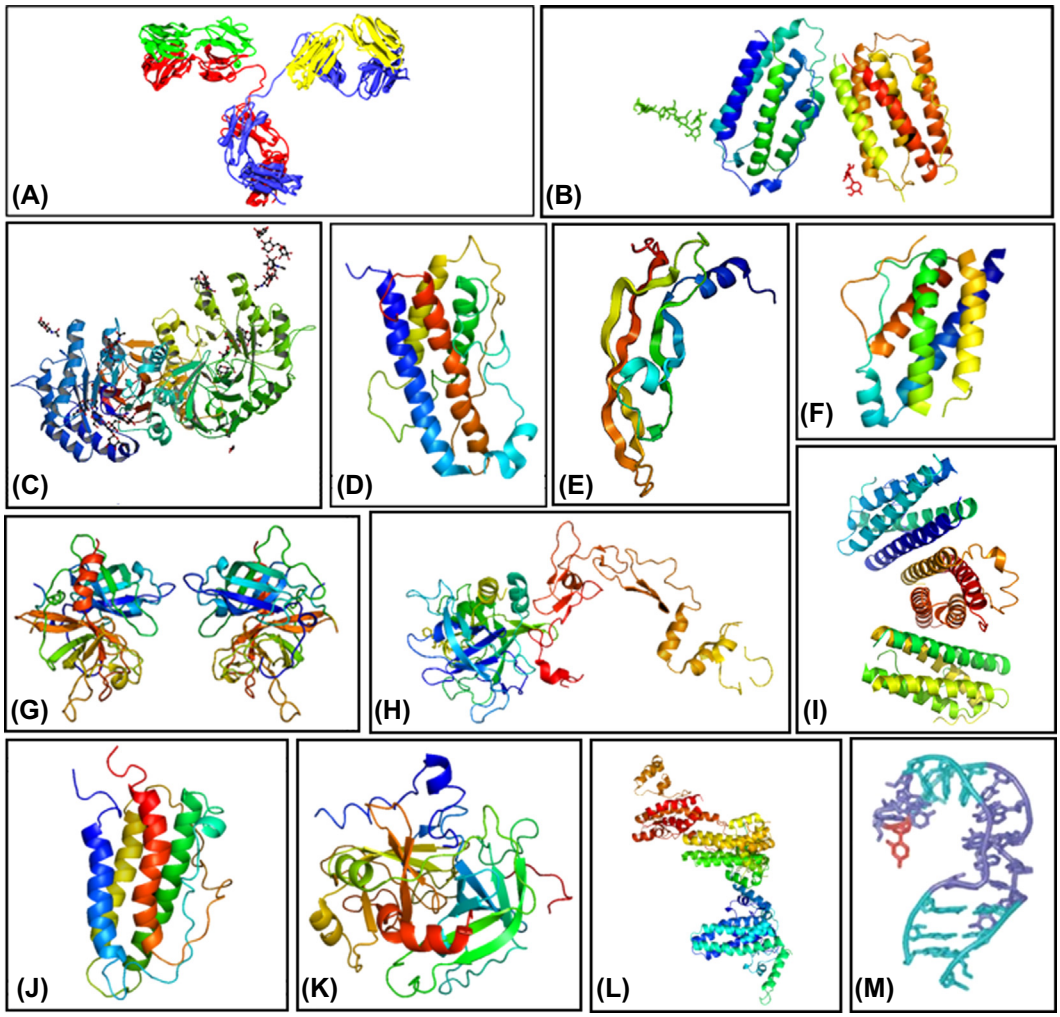

FIGURE 2.1 Groups/classes of biopharmaceuticals: some examples of each: (A) mAb: immunoglobulin G2a (PDB ID 1IGT); (B) IFNs: alfa, beta, and gamma (*left*), and three-dimensional structure of IFN-beta (*right*) (PDB ID 1AU1); (C) enzyme: α-galactosidase (PDB ID 1R46); (D) hormone: somatotropin (PDB ID 1HGU); (E) cell growth factor: vascular endothelial growth factor (PDB ID 1WQ8); (F) IL-2 (PDB ID 1M47); (G) thrombolytic: tissue plasminogen activator (PDB ID 1A5H); (H) blood factor: coagulation factor IX (PDB ID 1PFX); (I) colony-stimulating factor: G-CSF (PDB ID 1RHG); (J) hematopoietic growth factor: EPO (PDB ID 1BUY); (K) anticoagulant: hirudin (PDB ID 1DWC); (L) vaccine: hepatitis B surface antigen; (M) aptamer: pegaptanib. *PDB ID*, Identification code in *Protein Data Bank*. *PDB (http://www.rcsb.org/pdb/home/home.do); E. NG, D.T. Shima, P. Calias, E.T. Cunningham, D.R. Guyer, A.P. Adamis, Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nature Reviews Drug Discovery 5 (2006) 123–132; F.J. Van Hemert, H.L. Zaaijer, B. Berkhout, V.V. Lukashov, Occult hepatitis B infection: an evolutionary scenario. Virology Journal 5 (2008) 1–13.*

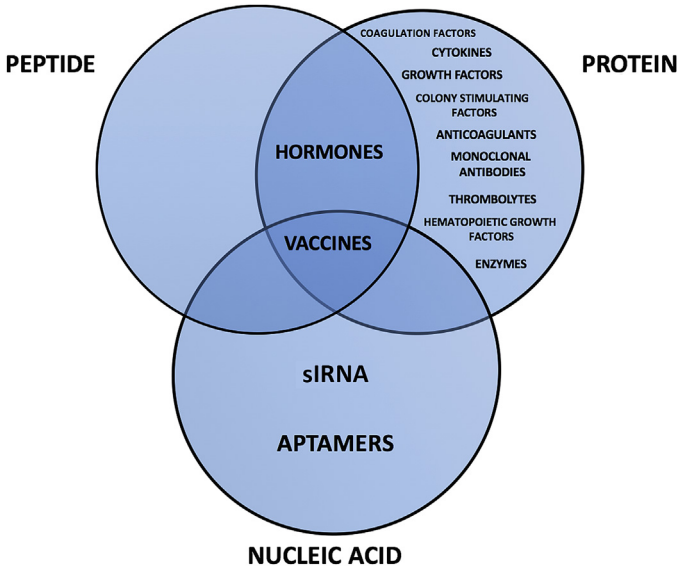

FIGURE 2.2 Venn diagram grouping biopharmaceutical classes according to their nature. Among the proteins, there are coagulation factors, cell growth factors, colony-stimulating factors, monoclonal antibodies, hematopoietic growth factors, anticoagulants, thrombolytics, and enzymes. Among the nucleic acids, there are siRNAs and aptamers. Hormones can be divided into peptides (e.g., glucagon) or proteins (e.g., insulin), and vaccines can be divided into all groups, although DNA and peptide-based vaccines have reached only the clinical phases to date and are not currently available on the market for use in humans. *A.M.M. Basso, P.B. Pelegrini, F. Mulinari, A.B. Viana, L.P. Silva, M.F. Grossi-de-Sa, Recombinant glucagon: a differential biological activity. AMB Express 5 (20) (2015) 1–9.*

sclerosis, and some types of cancer; and interleukins (ILs), such as IL-1, IL-2, and IL-12, which are used to activate immune cells, especially T-cells, and to treat autoimmune diseases [15,17,19].

Therefore, there is great diversity in protein therapeutic classes. However, many of the molecules used to prevent several diseases fit into the vaccines group. Prophylactic vaccines are made of peptides, DNA, or recombinant proteins, and some of these recombinant proteins have been produced and licensed for the market [20]. The hepatitis B surface antigen, for instance, is a well-known protein-based vaccine used against hepatitis B and has already been commercialized, but no peptide-based vaccine has reached the market yet [21].

Moreover, there are three types of nucleic acid biopharmaceuticals: DNA vaccines, small interfering RNA (siRNA)-based formulations, and oligonucleotide-based formulations (also called aptamers). These classes comprise fewer biopharmaceuticals, although their production is gradually and increasingly used to prevent and treat several human and animal diseases [10,22].

However, DNA vaccines generally have decreased immunogenicity compared with other vaccine types, and there are still some ethical concerns about their effects and

mechanism of action [23]. Nonetheless, aptamers are oligonucleotides composed of DNA or RNA and have binding affinity to proteins, which enables them to modulate protein activity through, e.g., pathogenic protein inhibition. This makes them attractive and flexible molecules for a wide range of therapeutic goals [24]. Pegaptanib sodium (Macugen) is a relatively recent RNA-based aptamer and the first licensed for clinical use in humans to treat neovascular age-related macular degeneration, with considerable and proven success [25].

Considering all of the biopharmaceutical classes and subclasses presented to date, they can be divided into numerous types according to their structure modifications. This increases their therapeutic applicability, coverage, safety, efficacy, and/or accuracy in mechanism of action. Hence, some incremental variations in the active site must be considered to produce higher-quality and advantageous pharmaceuticals, which are called second- and third-generation biopharmaceuticals [28].

In general, these variations result in immunogenicity reduction or elimination, pharmacokinetic profile changes, increases in half-life time, or even the generation of new proteins (hybrid proteins), such as fusion proteins, composed of different polypeptide chains fused to each other [28]. Etanercept is one example. It is a fusion protein composed of a cytokine receptor that contains an Fc antibody region fused to its C-terminus end [29].

In addition, many pharmaceutical proteins, such as mAbs, FSH, EPO, and G-CSF, are glycoproteins [30]. Their glycosylation pattern, that is, the set of sugar residues attached along the polypeptide chain at glycosylation sites, may positively or negatively influence the biological activity and immunogenicity, which also depend on the organism from which the molecule is obtained [31]. In most cases, variations concerning glycosylation or punctual amino acids lead to active ingredients that maintain the same function but exhibit a novel nomenclature and activity level. In this way, there are different forms of the glucocerebrosidase enzyme (i.e., imiglucerase, taliglucerase-α, and velaglucerase-α) owing to these differences in chains and sources [32].

New technologies, including PEGylation [33], and nanostructuration [34], however, have been used to increase the stability and consequently the half-life of medicines, or to create an address through nanostructured systems-based delivery, respectively. Another approach is to attach protein (mAb-like) scaffolds on pharmaceutical structures to increase the specificity and improve biological or physicochemical properties [35,36].

Therefore, the development of various biopharmaceuticals has also improved the safety of biotechnological products and increased their application in preventing, controlling, or curing several diseases such as cancer, rheumatoid arthritis, Alzheimer's disease, heart attacks, diabetes, dermatitis, Crohn's disease, multiple sclerosis, cystic fibrosis, hemophilia, hepatitis, heart failure, thrombosis, leprosy, and lupus. For multiple sclerosis, for example, recombinant β-interferon has improved the quality of life of many patients, reducing the number of relapses in 30% of individuals [37]. For rheumatoid arthritis, the new biotherapies reversed joint degenerations only 48 h after the beginning

of treatment [37]. Moreover, new biopharmaceuticals can selectively attack cancer cells and have shown less toxic effects than have chemotherapy procedures [37].

Biopharmaceuticals are only some of the pharmaceutical industry products. Chemical and natural medicines differ from biopharmaceuticals owing to their low molecular weight and structure because they are simple molecules with well-defined physicochemical properties that are administered orally and are quickly absorbed by the blood through capillaries. Unlike them, biopharmaceuticals have complex molecular structures and high molecular weight (200- to 1000-fold higher than traditional medicines). Because of their size and sensitivity, most biopharmaceuticals require a parenteral administration route and are primarily absorbed by the lymphatic system [38,39].

Originally, biopharmaceuticals were obtained by purifying molecule-containing tissue extracts and were named active components of a biological source [37,40]. After the advent of modern biotechnology, genetic engineering techniques allowed the production of biopharmaceuticals from heterologous expression of genes by using genetically modified organisms and microorganisms, and became active components of a biotechnological source [37,41].

2.3 Biosimilars

After 20 years, a generation of patents expired, which led to the production of a second generation of biopharmaceuticals, the so-called biosimilars [42]. Although the definition of biosimilars differs among countries, depending on the regulations applied by national agencies, in general terms, biosimilars correspond to drugs that are identical to the original biopharmaceutical products [43]. Biosimilars are developed using production processes which show differences in cell lines or production hosts regarding the reference biopharmaceutical process, to produce higher concentrations of the final product or a molecule with increased biological activity [44,45]. However, it is difficult to demonstrate that the biosimilar product is truly similar to the biopharmaceutical of reference (including structural differences and manufacturing variations). In this way, the current demand proposes a specific evaluation and approach to safety development for each biosimilar produced compared with the biopharmaceutical of reference [42].

In 2012, 26 biosimilars were in advanced production stages, following current guidelines (Table 2.1). Furthermore, 47 other biosimilars were in clinical study phases in 2015 (Table 2.2).

Over the past several years, pharmaceutical companies have demonstrated increased interest in commercializing biosimilar products [47]. Therefore, in the near future, it is expected that each developed biosimilar will be evaluated individually to better confirm its quality, safety, and efficacy compared with the biopharmaceutical of reference.

Table 2.1 Biosimilar Products and Stages of Development. Adapted from [45]

Molecule (Brand Name)	Developing Company	Current Status
G-CSF (Neutroval)	Teva Pharmaceuticals	BLA filed
G-CSF	Mochida/Fuji Pharma	BLA filed
Somatropin	JCR Pharmaceuticals	BLA filed
Trastuzumab	Celltrion	BLA filed
Infliximab	Celltrion	BLA filed
Follitropin-alfa (Gonadopin)	Dong-A Pharmaceuticals	Market[a]
Long-acting G-CSF (Neugranin)	Teva Pharmaceuticals	Phase III
Follitropin-alfa	Teva Pharmaceuticals	Phase III
Follitropin-alfa	Finox Biotech	Phase III
Etanercept	Celltrion	Phase III
Etanercept	LG Life Sciences	Phase III
Etanercept	Hanwha Chemical; Merck	Phase III
Darbepoietin-alfa (Avent)	Avestagen	Phase III
Erythropoietin	Hospira	Phase III
Interferon-beta	Reliance Life Sciences	Phase III
Interferon-alfa-2b	Amoytop Biotech	Phase III
G-CSF	Dong-A Pharmaceuticals	Phase II
Rituximab	Teva Pharmaceuticals	Phase II
Etanercept	Cephalon	Phase II
Etanercept (TuNex)	Mycenax	Phase II
Trastuzumab	GreenCross	Phase I
G-CSF	Hospira	Phase I
Pegylated G-CSF	Hospira	Phase I
RecombinanthCG	Dong-A Pharmaceuticals	Phase I
Interferon-beta	Dong-A Pharmaceuticals	Phase I (Brazil)
Folitropin-alfa	JCR Pharmaceuticals	Phase I

BLA, Biologic license application (biological products approved for marketing under the provisions of the Public Health Service (PHS) Act); *G-CSF*, granulocyte colony-stimulating factor.

[a]Phase III for ovulation induction.

S. Ariyanchira, Biosimilar market posts steady gains. BioMarket Trends 32 (12) (2012). Avaiable: http://www.genengnews.com/gen-articles/biosimilar-market-posts-steady-gains/4137/ (accessed 12.04.15).

2.4 Heterologous Systems for Protein Expression

To produce biopharmaceuticals and biosimilars via biotechnological routes, various heterologous systems can be used that are based on several microorganisms and macroorganisms, each of which has intrinsic traits in its protein expression and processing machinery (Table 2.3) [48–50]. Thus, selecting an organism as an expression system is a crucial and definitive step for the production and activity of recombinant biopharmaceuticals, once the system predicts the molecule's biological properties.

Hence, bacterial systems and mammalian cells are the most commonly used vehicles for expressing recombinant biopharmaceuticals. Bacteria are selected preferentially for simpler molecules, which do not require complex structure changes to keep their

Table 2.2 Biosimilars Approved for Commercialization in 2015. Adapted from [46]

Market	Number of Approved Biosimilars
Australia	8
Canada	3
Europe	20
India[a]	3
Japan	6
Latin America	4
South Korea	2
United States[b]	1

[a]Does not include biosimilar approved prior to implementation of regulatory guidelines.
[b]Omnitrope (somatropin/HGH) was launched by Sandoz in 2007 under special ruling.

Table 2.3 Comparison of Recombinant Biopharmaceuticals and Biosimilars Expression Systems, Based on Different Parameters [49–51]

System Parameters	Bacteria	Fungi	Mammalian Cells	Transgenic Animals	Transgenic Plants
Global cost	Low	Medium	High	High	Very low
Production time	Short	Medium	Long	Very long	Long
Scale-up capacity	High	High	Very low	Low	Very high
Product quality	Low	Medium	Very high	Very high	High
Glycosylation	Absent	Incorrect	Correct	Correct	Correct
Risk of product contamination	High (toxins)	Low	High (pathogens)	High (pathogens)	Low
Production scale	Limited	Limited	Limited	Limited	Unlimited
Production cost	Medium	Medium	High	High	Low
Storage cost	Medium	Medium	High	High	Low
Scale-up cost	High	High	High	High	Low
Insert size	—	—	Limited	Limited	Somewhat limited
Multimeric protein assembly	No	Partial	Yes	Yes	Yes
Spread	Easy	Easy	Difficult	Possible	Easy
Molecule homogeneity	Low	Medium	Medium	High	High
Protein folding	Low	Medium	High	High	High
Molecule yield	Medium	High	Medium/High	High	High
Safety	Low	Medium	Medium	High	High

J.K. Ma, P.N.W. Drake, P. Christou, The production of recombinant biopharmaceutical proteins in plants, Nature Reviews Genetics 4 (2003) 794–805; D.A. Goldstein, J.A. Thomas, Biopharmaceuticals derived from genetically modified plants, The Quarterly Journal of Medicine 97 (2004) 705–716; A.A.B. Viana, P.B. Pelegrini, M.F. Grossi-de-Sá, Plant biofarming: novel insights for peptide expression in heterologous systems, Biopolymers 98 (2012) 416–427.

biological activity. In other cases, mammalian cells are chosen generally for more complex molecules with longer polypeptide chains that require accurate post-translational modifications. Genes can be expressed in different biological systems; defining which system is advantageous for expressing the recombinant molecule form is a first step. In this respect, the most suitable expression system is the one that enables the production of a biologically active and safe therapeutic biopharmaceutical at the lowest cost [14].

Different microorganisms can be used to produce biopharmaceuticals at high expression levels. Among bacteria, *E. coli* is the most notorious and is used to produce a wide set of molecule types [51]. Filamentous fungi (e.g., *Aspergillus* spp., *Fusarium* spp., *Trichoderma* spp.) [52] and yeasts (e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*) [53] are also common choices of fungal systems for the expression of therapeutic proteins. In addition, protozoans such as *Leishmania tarentolae* [54] are becoming useful heterologous expression systems, and insect cells (*Spodoptera frugiperda* and *Trichoplusia ni*) [55] are another choice when performing baculovirus-mediated protein expression. Among mammalian cells, Chinese hamster ovary cells, human embryonic kidney, NS0, and SP2/0-Ag14 cell lines are the most commonly employed, especially for the expression of mAbs [56–58].

Furthermore, transgenic animals and plants are the most widely adapted expression systems for the large-scale production of recombinant molecules, once the available biomass quantity is sufficient to ensure high amounts of the molecule of interest [19]. In animals, many heterologous proteins are expressed and secreted into the fluids or organic systems of fishes, birds, rabbits, pigs, cows, goats, and sheep. Blood, milk, urine, semen, eggs, and embryos can be the final destination from which molecules are purified [59].

However, among all of the expression systems, transgenic plants seem to be advantageous in almost all parameters related to satisfactory yield and functionality [60,61]. Therefore, plants tend to become the most promising and economically feasible alternatives of biofactories in the large-scale production of biopharmaceuticals, considering their numerous advantages [62–64]. However, some systems, including algae (*Chlamydomonas reinhardtii*) [65,66] and mosses (*Physcomitrella patens*) [67,68], are new alternatives and have been used to express growth factors and mAbs.

2.5 Financial Market

During the 1970s, most pharmaceutical industries were located in the United States. However, lately, the participation of European and Asiatic pharmaceutical companies has increased in the market because multinationals have opened factories in different countries and because of increasing interest in patenting pharmaceutical processes and molecules in several places at once [69].

Currently, the cost required to produce pharmaceuticals is around US $1 billion, because it takes up to 10 years from the development of the process to produce a biomolecule until the release of the drug to the market [70].

Moreover, the current pharmaceutical global market is US $300 billion per year, and this amount might reach US $400 billion within the following 3 years. Ten major pharmaceutical companies control one-third of this market, with sales exceeding US $10 billion per year and with a 30% profit margin. Six of the 10 richest pharmaceutical industries in the world are located in the United States and four are in European countries. In this way, Japan and European and American countries will be responsible for 85% of the world pharmaceutical market in the 21st century [69].

In the pharmaceutical industry in Europe, the largest companies are located in Germany, France, Italy, Spain, and the United Kingdom. However, in 2012, the leading manufacturers were located in Switzerland, Germany, Italy, the United Kingdom, France, and Ireland; together, they had a value of €30 million, employing more than 700,000 people [71]. In 2014 European pharmaceutical companies invested €30,5 million in research and development, employing 707,000 people, which generates three to four times more employments indirectly, thus demonstrating the importance and interest in investments in the sector worldwide [72].

Considering other countries, the biopharmaceutical sectors in China, India, and Israel are increasing considerably, because investments in internal strategies for developing their own products are enhanced annually and they are promoting advances at a national level. Similarly, Brazil is developing its own strategies to overcome expenditures of biopharmaceuticals by the Ministry of Health, because the country still depends on imports of these products owing to the lack of national production [38].

In 2008, the 30 most commercialized biopharmaceuticals yielded US $42 billion to the companies, with the expectation of a 56.5% increase by 2014 [73]. mAbs occupy the first positions (39% of total sales) as biopharmaceuticals most in demand, followed by insulin and its analogues, EPO and IFNs, respectively [17,38]. Nevertheless, the high expenditures required for the development process to produce biopharmaceuticals and for preclinical and clinical testing are not the only reason why these drugs are commercialized at such an expensive value. Patenting biopharmaceuticals, which enables companies to have exclusivity and licensure for the commercialization of certain drugs, is a main cause for high prices [74]. In 2014, 81.4% of all patent applications deposited were from pharmaceutical companies, followed by universities (10.1%) and biotechnological industries (8.5%). Among the companies, Merck occupied the first position in patent submission, followed by Eli Lilly, Pfizer, Roche, and Upjohn [8] (Table 2.4).

Hence, the marketing of biopharmaceuticals was enhanced from only 10% of the total pharmaceutical market in 2002, increasing to 18% in 2010. For 2016, the share of biopharmaceuticals is expected to reach 21% of the total market [75]. Companies such as Amgen, Genentech, Genzyme, Johnson & Johnson, Novo Nordisk, Serono, Novartis, Sepracor, Chiron, Celgene, Bayer, Merck & Co., Abbott, Pfizer, Eli Lilly & Co., Roche, MedImmune, Gilead Sciences, Shire Pharmaceuticals, Cephalon, and Biogen have become established as the main biopharmaceutical companies worldwide and are the

Table 2.4 Biopharmaceutical Companies and Number of Patents Deposited. Adapted from [76]

Company	Patents
Merck	65 (4.7%)
Eli Lilly	41 (3.3%)
Pfizer	39 (2.8%)
Roche	38 (2.8%)
Upjohn	38 (2.8%)

Five companies with major patents in 1374 examined (M.S. Kinch, J. Raffo, Economic Research Working Paper N°.24. Economics and Statistics Series, WIPO (Word Intelectual Property Organization), 2015 pp. 1—14.

reason for the exponential growth of biopharmaceuticals in the market (Table 2.5) [39,77]. In Brazil, Oswaldo Cruz Foundation (Fiocruz) set up a laboratory to produce biopharmaceuticals as a strategy to overcome the expenditures incurred by importing biological medicines.

Drugs whose global revenues exceed billions of dollars per year are denominated as blockbusters, such as Remicade (US $7 billion), Avastin, Enbrel, and Humira (US $6 billion), which were developed during the 1990s [78]. Production of these biopharmaceuticals started in the 1990s. Thus, the patents for some began to expire in 2011, and others will expire by 2015 once the validation for each patent is reached, that is 20 years [79]. In 2011, biological products yielded US $142 billion, which is equivalent to 19% of the total biopharmaceutical market [80] and is expected to reach 20% by 2016 [81].

Because several biopharmaceuticals were initially produced in the 1990s, many patents are now expiring [79,82]. This situation opens opportunities for the development of new processes for the production of biosimilars. Compared with the drug of reference, biosimilars have similar quality, safety, and efficacy [74].

Hence, over the past several years, both biopharmaceutical companies and chemical pharmaceutical industries have become interested in producing biosimilars. Whereas biopharmaceutical companies are investing in biosimilars to diversify their portfolio and create new revenue streams [83], chemical pharmaceutical industries aim for growth in manufacturing synthetic drugs at the lowest possible cost through economies of scale owing to patent litigation skills [84]. Thus, the biosimilars market offers chemical and pharmaceutical industries an opportunity to increase their manufacturing support value and to diversify their portfolios.

The first biosimilar that was developed and licensed for therapeutic use (Omnitrope), a human growth hormone medicine produced by Sandoz, reached the market in 2006 and was approved for commercialization in the European Union (EU) to treat growing disorders such as Turner's syndrome [85,86]. Since then, various biosimilars have been developed and licensed and were expected to represent approximately 2% of total biological medicine sales by the end of 2015 [38]. During this year, there were already 246 biosimilars in the EU and the United States alone [86].

Table 2.5 Top 25 Biotechnology Medicines Based on Reported Worldwide Sales. Adapted from [87]

Product	Active Ingredient	Company	Therapeutic Indication	Annual Sales ($US Millions)		
				2008	2009	2010
Remicade	Infliximab	Merck & Co.	Autoimmune diseases	5856	6631	7324
Enbrel	Etanercept	Pfizer	Autoimmune diseases	6191	5916	6808
Humira	Adalimumab	Abbott	Autoimmune diseases	4500	5500	6500
Avastin	Bevacizumab	Roche	Cancer (several types)	2908	5837	6061
Rituxan	Rituximab	Roche	Non-Hodgkin's lymphoma, leukemia, rheumatoid arthritis	2852	5710	5962
Herceptin	Trastuzumab	Roche	Breast cancer; gastric cancer	1819	4940	5093
Lantus	Insulin glargine	Sanofi-Aventis	Diabetes	3259	4096	4668
Epogen/Eprex	Epoetin-α	Amgen/J & J	Anemia	4916	4814	4458
Novolog	Insulin aspart	Novo Nordisk	Diabetes	2503	3020	3666
Neulasta	Pegfilgrastim	Amgen	Febrile neutropenia	3318	3355	3558
Aranesp	Darbepoetin-α	Amgen	Anemia	3137	2652	2486
Novolin	Insulin	Novo Nordisk	Diabetes	2194	2103	2198
Humalog	Insulin Lispro	Eli Lilly & Co.	Diabetes	1736	1959	2054
Pegasys	Peginterferon-alfa-2a	Roche	Hepatitis C	1534	1553	1543
Novo Seven	Factor VIIa	Novo Nordisk	Hemophilia A and B	1189	1314	1493
Lucentis	Ranibizumab	Roche	Macular edema and degeneration	887	1124	1368
Sandostatin	Octreotide acetate	Novartis	Several tumors	1100	1155	1291
Neupogen	Filgrastim	Amgen	Febrile neutropenia	1341	1288	1286
Neo Recormon	Epoetin-α	Roche	Anemia	1664	1463	1205
Humulin	Insulin	Eli Lilly & Co.	Diabetes	1063	1022	1089
Synagis	Palivizumab	Astra Zeneca	Prevention against RSV	1230	1082	1038
Gardasil	Quadrivalent HPV vaccine	Merck & Co.	Prevention against HPV	1403	1118	988
Norditropin	Somatotropin	Novo Nordisk	Growth failure	718	818	893
Genotropin	Somatotropin	Pfizer	Growth failure	989	887	885
Forteo	Teriparatide	Eli Lilly & Co.	Osteoporosis	779	816	830
			Total annual sales	59,086	70,174	74,746

HPV, human papillomavirus; *J & J*, Johnson & Johnson; *RSV*, respiratory syncytial virus.

Adapted from R.J.Y. Ho, Biotechnology and Biopharmaceuticals: Transforming Proteins and Genes into Drugs, second ed., Wiley Blackwell, New Jersey, 2013.

Biosimilars are a promising market for investment [88] because their final cost and prices on the market can be 50–80% less than those of the original biopharmaceuticals [89,90]. Thus, more than 250 companies are currently involved in the development and/or commercialization of biopharmaceuticals and biosimilars worldwide [86,91]. Estimates from the market indicate that biosimilar inclusion will become 4% of total biopharmaceutical sales over the next decade, amounting to a savings of US $44.2 billion. This global market is estimated to reach US $2 billion in 2018 and to have

a growth rate of more than 20% between 2013 and 2018. However, this market depends on decisions from the FDA [43,90].

In emerging countries such as Brazil, Russia, India, China, and South Korea, biopharmaceutical companies receive incentives and have advantages in producing biosimilar products, including a large internal demand for biopharmaceuticals, lower labor costs, and government support [91]. With the increase in small local biopharmaceutical companies, big pharma industries are facing a challenge to continue selling their products at higher prices while searching for and developing new products.

In fact, big pharma will confront the challenge of losing market exclusiveness once some companies develop biosimilar products from other original industries while searching for new biopharmaceuticals in their development pipeline [43].

However, there are commercial disputes among pharmaceutical companies that may cause an increase in the final price. Currently, the main concern in the US market is whether companies producing biosimilars conflict with any previously granted patents that belong to biopharmaceutical-producing companies whose main patents have already expired [92].

Hence, to control this process, the Innovation Act and Price Competition of Biosimilars (BPCIA) was created in 2010 (part of the Affordable Care Act and the Patient Protection). BPCIA's purposes are to help companies reach a consensus concerning patent disputes and to share important information. According to the BPCIA essential standards, biosimilar producers must deliver, within 20 days, documents to the FDA and to the company that developed the reference drug such that the sale of the new drug containing the biosimilar molecule can be legalized. Nevertheless, biosimilar producers usually deliver the documents merely to the FDA, claiming that sharing information with other companies will occur only under certain conditions, which is often not accepted by the company that developed the reference drug [92]. Consequently, the judicial processes from those disputes may return more expensive drugs to the market and retard patent submissions [92].

2.6 Legislation History and World Regulations

2.6.1 History

The first legislations and regulations regarding the pharmaceutical industry were released in the United States at the end of the 19th century. During the Mexican–American war (1846–48), the US government issued the first law related to pharmaceuticals, called the "Import Drugs Act of 1848," to control the quality and purity of imported drugs used to treat several infectious diseases. Fifty years later, the first law regarding biological products was created. The Biologics Control Act of 1902 demanded previous licensing for the production and distribution of any biological product (vaccine, sera, toxins, and viruses) and established rules about product labeling, license number, and expiration dates [93].

The Food and Drugs Act of 1902 was a landmark in the creation of the current FDA, which occurred later in 1906. The Act stipulated the mandatory labeling of ingredients to all drugs and food that was commercialized in the United States. The law also controlled and prohibited the transportation and adulteration of food and drugs in the country and, for the first time, had an impact on the overall national system of drug and food safety [93].

As the pharmaceutical industry of the United States continued to grow, new rules were established. Hence in 1938, after the discovery of the deadly effects of sulfanilamide in several patients, the government released the Food, Drug, and Cosmetic Act, in which every drug had to be proven safe before being authorized for commercialization in the country. In 1962, even after being approved as safe by the FDA, thalidomide caused deformities in babies born to mothers who consumed the drug during pregnancy. Therefore, the Kefauver–Harris Amendments were released, in which both the safety and efficacy of a drug had to be confirmed before marketing [93].

Currently, the FDA is the main agency worldwide that is responsible for drug quality control. Along with the Current Good Manufacturing Practice (CGMP) regulations, agencies in the United States guarantee safety in the manufacturing, processing, packaging, and use of every new drug released to the market. Moreover, the federal american agency determines whether the pharmaceutical industry contains the necessary facilities, equipment, and technical team to produce the new drug that is in process for approval [93].

To standardize all of the steps to produce and commercialize new drugs, the FDA follows four federal regulations (Federal Food, Drug, and Cosmetic Act; 21 Code of Federal Regulations Part 201 and Part 211; Federal Register Notices for Proposed Changes; and Final Changes to CGMP), as well as Guidance, Manuals, and Compliances [94]. The Guidance and Guidelines are instructions for industries on how to process, manufacture, produce, test, and evaluate the drug that has been approved or is in the approval phase [94]. Nevertheless, industries should follow the Guideline on the Preparation of Investigational New Drug Products (issued November 1992, posted March 2, 1998), the Guidance for Industry: Investigating Out-of-Specification Test Results for Pharmaceutical Production (October 2006), and the Standing, Operating Procedures for New Drug Application/Abbreviated New Drug Application Field Alert Reports (issued October 30, 1998; posted November 2, 1998).

In this way, the Hatch–Waxman Act regularizes rules for the chemical pharmaceutical market (add in Section 505j of the Federal Food, Drug and Cosmetics Act). The Act stimulates independent pharmaceutical companies to create novel synthetic drugs at affordable and accessible prices for the population. Thus the Hatch–Waxman Act was a success because it maintained the incentive for drug discovery and development. However, this act does not apply to biomolecules [94].

Therefore, other regulations, such as the Life-Saving Medicine Act, were released to determine whether the infrastructure of a pharmaceutical company is appropriate for the production of biosimilar drugs [94,95]. Hence, the effort resulted in the creation

of the Biologics Price Competition and Innovation Act, which allowed the FDA to authorize the review and approval of biosimilars to the market by evaluating biosimilarity or interchangeability with the reference product in terms of animal studies and clinical trials in addition to an analytical evaluation [41].

Furthermore, the FDA uses the Biological License Application to evaluate and approve new biological products. In this process, industries must submit a report demonstrating all research and results developed during preclinical and clinical studies, biological activity evaluation of the drug, and physicochemical and manufacturing information [96]. The purpose is to prove that the product is "safe, pure, and potent." After approval, the pharmaceutical industry must follow CGMP regulations to standardize the facilities, production process, packaging, quality control procedures, stability and activity tests, expiration date analyses, and production records, among others (CFR Parts 210, 211, and 600) [97].

2.6.2 World Regulations

Each country's administration department that handles these manners regulates all changes in importing and exporting biopharmaceuticals. These organizations and administrations are important political players in the pharmaceutical industry. Politics has a role in regulating the manufacture, distribution, and sale of pharmaceuticals for the safety of the consumer. Governments want to ensure that the medicines are safe and effective for all users. Each nation has its own system for ensuring the safety and distribution of drugs.

Hence, over the past decade, biopharmaceuticals have been responsible for an expansive increment in the biotechnology and pharmaceutical industry. Despite the high costs involved in research and in biopharmaceutical development and production, regulations were created to balance intellectual property and companies' investments to patients who depend on free access to medical drugs [94].

In this way, the European Medicines Agency (EMA) has established and documented a robust legal regulatory guideline for the approval of biosimilars. Since the legalization of the first guideline in 2005, 14 biosimilars have been approved for commercialization in European countries (Table 2.6). Nevertheless, not all biosimilars reach the market even after a long period of investment and research. For example, the drug Alpheon (BioPartners GmbH), which consists of the biosimilar interferon-alfa-2a, had purity incompatibilities and unexpected side effects compared with the drug of reference, and therefore was rejected by the EMA for release to the market [43,94].

The technological changes that occurred as a result of troublesome experiences with previous cases led to the release in 2013 of the Committee of Medical Products for Human Use. The EMA has a robust regulatory process and guidelines are frequently revised as a result of experiences with the approval of biosimilars over time [43,98].

Table 2.6 Approved Biosimilars in Europe up to 2010. Adapted from [94]

Product	Common Name (International Nonproprietary Name)	Company	Reference Product	Year of Approval
Omnitrope	Somatropin	Sandoz International Limited	Genotropin	2006
Valtropin	Somatropin	Biopartners GmbH	Humatrope	2006
Binocrit	Epoetin-alfa	Sandoz International Limited	Eprex	2007
Epoetin Alfa Hexal	Epoetin-alfa	HEXAL AG	Eprex	2007
Abseamed	Epoetin-alfa	Medicine Arzneimittel Putter GmbH Co. KG	Eprex	2007
Retacrit	Epoetin-zeta	Hospira, Inc.	Eprex	2007
Silapo	Epoetin-zeta	SAASA Arneimittel AG	Eprex	2007
Biograstim	Filgrastim	CT Arzneimittel GmbH	Neupogen	2008
Filgrastim Ratiopham[a]	Filgrastim	Ratiopham GmbH	Neupogen	2008
Ratiograstim	Filgrastim	Ratiopham GmbH	Neupogen	2008
Tevagrastim	Filgrastim	Ratiopham GmbH	Neupogen	2008
Zarzio	Filgrastim	Sandoz International Limited	Neupogen	2009
FilgrastimHexal	Filgrastim	HEXAL AG	Neupogen	2009
Nivestim	Filgrastim	Hospira, Inc.	Neupogen	2010

[a]Note that Filgrastim Ratiopharm was withdrawn on April 20, 2011, at the request of the sponsor. Up to December 2010.

In 2014, in the United States, the FDA drafted Guidance to support biopharmaceuticals and biosimilar industries and created a list of biologicals called the "Purple Book." Two biosimilar applications were filed in this book during 2014: filgrastim and infliximab [43].

Other countries such as Japan, Switzerland, Canada, and Australia, as well as the World Health Organization (WHO) adopted the same scientific principles from the EU for biosimilar approval. Although some countries are based on the same regulation guides, some items are distinct (Table 2.7). In addition, in 2010 the workshop entitled "WHO Guidelines on Evaluating Similar Biotherapeutic Products" took place to facilitate the "global harmonization" of biosimilar evaluation [94]. In this event, the discrepancies in regulations between the WHO and those of countries such as Canada, Korea, Japan, and the EU were highlighted and discussed.

Because regulations and guidelines for biosimilars are still under optimization, several scientific factors remain unresolved: (1) how similar molecules are considered a biosimilar; (2) the degree of similarity (individual or collective) between molecules; (3) statistical models showing total biosimilarity between molecules; (4) the criteria used to assess biosimilarity (average against variability; one criterion or flexible criteria); (5) interchangeability; and (6) standardization of the design of studies (potential uses for adaptive design in biosimilar studies). Therefore, with some factors unresolved, the development of new regulations and guidelines to "global harmonization" is necessary [94].

Table 2.7 Comparison of Biopharmaceutical Regulations Among Different Countries. Adapted from [94]

	World Health Organization	Canada	Korea	European Union	Japan
Term	Similar Biotherapeutic Product SBPs	Subsequent Entry Biologics SEBs	Biosimilars	Biosimilars	Follow-on biologics
Scope	Recombinant protein drugs			Mainly recombinant protein drugs	Recombinant proteins, drugs
Efficacy	Double-blind or observed-blind; equivalence or noninferiority design		Equivalence design	Comparability margins should be prespecific and justified	
Reference product	Authorized in jurisdiction with well-established regulatory framework			Authorized in European Union	Authorized in Japan
Stability	Accelerated degradation studies Studies under various stress conditions				Not necessary
Purity of manufacture	Process- and product-related impurities				
Physicochemical	Primary and higher-order structure Posttranslational modifications				
Biological activity	Qualitative measure of function Quantitative measure (e.g., enzyme assays or binding assays)				
Nonclinical studies	In vitro (e.g., receptor-binding, cell-based assays) In vitro (pharmacodynamic PD activity, at least one repeat dose toxicity study, antibody measurements, local tolerance)				
PK study design and criteria	Single dose, steady-state studies, or repeated determinations of pharmacokinetics PK Crossover or parallel Include absorption and elimination characteristics Traditional 80—125% equivalence range is used				
PD	PD markers should be selected and comparative PK/PD studies may be appropriate				
Safety principles	Prelicensing safety data and risk management plan Generic approach is not appropriate for Follow-on Biologic FOB Follow-on biologics should be similar to the reference in terms of quality, safety, and efficacy Stepwise comparability approach: Similarity of the SBP to RBP Reference biotherapeutic product in terms of quality is prerequisite for reduction of nonclinical and clinical data required for approval Case-by-case approach for different classes of products Pharmaco-vigilance is stressed				

SBP or SBPs - similar biotherapeutic products. SEB - subsequent-entered biologics. PD - pharmacodynamics tests. PK - pharmacokinetics tests. FOB - follow-on biologics. RBP - Reference biotherapeutic product. S.C. Chow, Biosimilars: Design and Analysis Follow-on Biologics, first ed., CRC Press, Florida, 2014.

Currently, the guidelines of regulatory agencies from the EU, United States, and Japan, among others, regulate general principles of biosimilars, including fabrication and preclinical and clinical tests. Moreover, comparability tests to reference products in all development phases are made to ensure the quality, efficacy, and safety of biosimilars [44,49]. Furthermore, to avoid serious adverse effects, pharmaco-vigilance and immunogenicity tests are required [44,49].

2.6.3 Regulations in Brazil

The biosimilar market is growing in emerging countries; in Brazil, the costs of importing biopharmaceuticals as estimated by the Ministry of Health represent 50% of all annual health care expenditures. Considering that the Brazilian government is responsible for the health care of 205 million people, the amount spent on biopharmaceuticals reaches billions of dollars every year [43,91].

The pharmaceutical industry in Brazil has been developed with the goal of the national independence of drug production, but local research and development have had less than 10 years of application. Nevertheless, the first policies created in the country date to 1973, when Law 5991 initiated a new step in drug safety regulation. Three years later, Law 6360 demanded new procedures for the registration of any pharmaceutical product commercialized in Brazil. At that time, there was nothing in place concerning the bioequivalence or interchange of drugs [99,100].

The new Constitution of Brazil, established in 1998, included Laws 8080/90 and 8142/90, which created the Unique Health System (Sistema Único de Saúde - SUS), whose purpose was to "provide health to all." With these laws, access to health treatments and several medicines could be obtained free or for a charge by the population. However, the pharmaceutical industry still depended on international companies and the import of medicines, and thus Ministry of Health expenses increasing yearly [101,102].

In 1993, the Brazilian government set up the Program for Specialized Medicines. In this context, drugs commercialized in the country were categorized into three groups based on their price and application. Moreover, it was possible to estimate the amount of a specific drug needed for Brazilian patients and to predict the value to be spent with pharmaceutical products by the government. Five years later, the National Drug Policy showed that more than 50% of the population actually had access to only 20% of all commercially available drugs [103].

Therefore, to decrease the high government expenditures with the acquisition of pharmaceutical products, enhance the access of drugs to the population, and increase the local production of medicines, the Law of Generics was released in 1999. With this law, the prices of drugs decreased, a wider variety of medicines went on the national market, and rules on interchangeability with new medicines and bioequivalence started to be part of Brazil's health priority [104].

Hence, the need for novel resolutions and laws as a result of the fast development of the pharmaceutical sector in Brazil led to the release of Collegiate Director Resolution (RDCs) 133/134 and 16/17 by the National Agency for Sanitary Vigilance (ANVISA), during 2003 and 2007, respectively. These resolutions included the requirements for pharmaceutical equivalence and pharmacokinetic studies, as well as for the registration of generic drugs. However, although the registration of generic products has increased approximately 3600% since then, the registration of biopharmaceuticals has increased four times less. Furthermore, the dependence of Brazil on importing specialized

medicines and biological products reached almost US $3 billion dollars in 2012 alone, corresponding to 30% of all expenditures by the Ministry of Health. Although biological products corresponded to less than 10% of all drugs commercialized in the country, they represented 50% of the cost of all medicines in Brazil [41,105–108].

Thus, in 2004, the government introduced the Law for Technological Innovation (Law 10,973) and in 2007, the Politics for National Biotechnology. The purposes were to incentivize the development of innovative products and processes and stimulate the improvement of Brazilian pharmaceutical companies. By 2005, the ProPharma Programs, with the financial support of BNDES (National Development Bank), invested 44% of the amount into the increase of national companies, 40% into production, and 16% into research and development [41,109].

Therefore, considering that the commercial balance showed a deficit of more than 9 billion dollars on the national production, export, and import of biopharmaceuticals in Brazil, public institutions started looking for partnerships with international private companies to speed up the release of national biopharmaceutical products into the market. Until 2011, 64 partnerships were settled among 15 national laboratories and 40 private companies, working on producing drugs. In 2014, there were 14 new pharmaceutical companies in partnership with six Brazilian public laboratories investing in the production of 14 different biosimilar molecules [110].

In Brazil, first-generation biotechnological products (e.g., insulin, growth hormones) were under constant development and registration by national companies, especially Bio-Manguinhos. However, second-generation products are the new future for pharmaceutical industries worldwide. The first patents are expiring and others will expire in approximately 2020. This reflects a global race for the development of novel technologies to produce biosimilars at the same time that specific regulations are being released for these products, including in Brazil [111]. Moreover, Brazilian private companies such as the FarmaBrasil Group are investing in technology and training and prioritizing the development of biosimilar products, although the focus on producing new molecules has not been discarded [111].

Along with the politics involved in the improvement of the biopharmaceutical industry and following the demand for updated regulations, ANVISA released new RDCs (49, 50, 55) and Ordinances (506/2012 and 837/2012) to establish registration and postregistration requirements for biological products. It also requires stability studies for the registration and postregistration of biological products. Guidelines and criteria for development of a productive development partnerships and the construction of a Health Industrial Complex and its Managing Committee were included in the new ordinances [112–116].

The legal basis for the production and registration of biotechnological products in Brazil has been under consolidation since 2010, and its rules are in accordance with international standards. These regulations include guidelines from EMA and FDA, with modifications that fit the Brazilian market. Those guidelines are constantly being revised

according to the technical complexity, economic and financial importance the regulations demmand for the registration and maintenance of biotechnological products into the market [117].

The Ministry of Health invested $17 billion in the development of 100% national drugs [119]. There are already two biosimilar molecules on the market in Brazil because of the National Politics for Biotechnology Development: botulinum toxin and the vaccine H1N1, which benefit more than one million patients in the country. Last year, the biopharmaceutical Etanercept (Enbrel), which is used against rheumatoid arthritis and was developed by partnerships between Brazilian and international companies, obtained registration for commercialization by ANVISA. Moreover, at least nine biosimilar molecules and five vaccines produced in Brazil will be released into the market by 2017 [118].

In addition to boosting the production and development of public laboratories and national private companies, the partnerships have provided savings for the Ministry of Health of $2 billion. Furthermore, they reduced Brazilian Social Policy problems and strengthened the country's Industrial Complex and Health Innovation, thus allowing SUS to transform its productive structure and permitting the Health System of Brazil to be compatible with new technological world standards [111]. The expectation for 2015 was that the new drugs would provide savings for the Ministry of Health of more than $500 million, which could be spent in other areas of the country's health system.

2.7 Perspectives: Pharmacogenomics and Next-Generation Biopharmaceuticals

With advances in modern biotechnology, the possibility of developing biomarkers for the diagnosis and treatment of several diseases has become a reality. Moreover, improvement in technologies has allowed the use of pharmacogenomics to indicate personal treatment for a certain disease [119].

In parallel with the development and expansion of novel automated techniques, obtaining the personal genetic profile of a large number of individuals and correlating this profile with their pharmacodynamics profile has also become a reality. The results will lead to the ability to determine exactly which biopharmaceutical doses and formulations are more suitable for administration to each patient [37,120].

Along with pharmacogenomics studies, the development of next-generation biopharmaceuticals together with meaningful contributions of nanotechnology are even more promising because they will provide safer and more effective medicines against many diseases. As a result, biologics may soon become even more effective, which will also allow for the reduction of side effects, especially for serious diseases such as cancer, and will significantly increase quality and expectancy life. [34,37].

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3 Interferons

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3.1 Interferons: Biological Functions and Clinical Uses

Although the phenomenon of interference in the process of viral infection caused by a previous (viral) infection had been known for several decades, in 1957, Isaacs and Lindenmann [1] decisively characterized the responsible factor that was released by chick chorioallantoic membrane after exposure to heat-inactivated influenza virus. That factor interfered with the growth of live virus in fresh pieces of membrane and was called "interferon" (IFN). Intensive research showed that, apparently, all viruses can induce IFN in most types of vertebrate animal cells and, in principle, IFN can inhibit all types of viruses. IFNs were shown to possess potent antiviral and antiproliferative activities, and also immune and inflammatory modulation roles in the human body through cell—cell interactions [2]. Such activities inspired high hopes for the development of medical applications, and the genes for human IFN-alfa, -beta, and -gamma were cloned and characterized in the early 1980s [3—10].

Their expression [11—17] and clinical investigation contributed to the development of the biotechnology industry with Genentech, Biogen, Hoffmann-La Roche, and others. Indeed, the expression, purification, and clinical trials set high hopes that human IFN would be a magic bullet against viral infections, and especially against cancer.

After the IFN-alfa, beta, and gamma encoding genes, many other interleukins (ILs) were cloned and characterized, starting with IL-2 [18], and initiated important biotechnological and biopharmaceutical portfolios. Many were also cloned from other vertebrate organisms; later, genome projects and bioinformatic analyses amplified the scientific knowledge and cytokine product portfolio.

IFN-alfa proved to be one of the most important biopharmaceutical products [19], especially because of its application in the treatment of viral hepatitis [20] and also because of its oncologic indications, with a market value of about US $1 billion per year, whereas IFN-beta has progressed toward more widespread use for the treatment of multiple sclerosis. IFN-gamma, also called "immune IFN," was expected to be the most prominent of the IFNs, but has not found much clinical use thus far. It is applied in

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chronic granulomatosis and rheumatic diseases. A lot of research was invested in the discovery and further characterization of additional molecules with IFN-like activities, including the bioinformatic analyses of genomic sequences; a "consensus interferon" was marketed, and synthetic modified variants of IFN with better stability, systemic half-life, and modified activities are being engineered.

To date, three main types of IFN have been described: type I IFNs, composed of IFN-alfa, -beta, -epsilon, -kappa, -tau, -omega, -zeta (the latter is also called limitin [21]), and -delta (only in mice); type II IFNs, with IFN-gamma as its sole member; and type III IFN-lambda (also called IL-28 and -29). Their respective biological activities, physiologic roles in the host defense system, mechanism of action, and gene/protein structures have been studied in greater detail, and are briefly described subsequently.

IFNs can be described as a group of cell−cell signaling proteins that are produced and released by host cells in the presence of a variety of pathogens ranging from viruses to bacterial and fungal products, parasites, and tumor cells [22,23], besides natural and synthetic nucleic acids, several polymers, and mitogens. They are secreted and trigger the deployment of protective measures in neighboring cells. As such, IFNs can be considered cytokines [24] that activate innate and acquired immune responses such as natural killer cells and macrophage activities and increased expression of the major histocompatibility complex, which promotes antigen presentation and mediates cellular immunity. IFN proteins range from 19 to 45 kDa, with 143−172 amino acids. All IFNs are monomers, except for IFN-gamma, which is a homodimer. They are frequently modified by glycosylation, but these posttranslational modifications are often not critical for their biological function.

Human IFNs are classified according to the receptors to which they bind, provoking a signaling cascade in the target cell [24].

Type I IFNs [25] are found in all mammals, and orthologs have been found in birds, reptiles, amphibians, and fish species [26]. Type I IFNs are defined according to their binding to a specific multichain receptor complex located on the cell surface, and known as IFNAR (for IFN-a/b receptor), consisting of at least two different subunits, IFNAR1 and IFNAR2 [27]. They are encoded by at least 23 IFN genes with a length of 1−2 kb, which are clustered on the short arm of human chromosome 9, at band p21 [28,29], resulting in 13 subgroups: IFN-a1, -a2, -a4, -a5, -a6, -a7, - a8, -a10, -a13, -a14, -a16, -a17, and -a21. It is not clear whether all of these genes are expressed after cell stimulation, and the expression varies in different cell types [30]. Only two species of human alfa-IFNs are glycosylated: IFN-a2b is *O*-glycosylated at the threonine residue in position 106 and IFN-a14c is *N*-glycosylated at the asparagine residue at position 72 [31]. IFN-alfa is a major cytokine produced by fibroblasts and monocytes in response to a viral infection [32]. Furthermore, type I IFNs produced by malignant cells or tumor-infiltrating dendritic cells seem to have a role in anticancer immunity by controlling the autocrine or paracrine circuits that underlie cancer immune surveillance [33]. The only type II IFN in humans is IFN-gamma, which is secreted by type I T helper (Th) cells (Th1 response) and has a pivotal role in the immune system because it is activated by IL-12 and suppresses type II Th cells (Th2). IFN-gamma binds to the oligomeric IFN-gamma receptor (IFNGR) consisting of IFNGR1 and IFNGR2 chains [34].

Type III IFNs bind to a receptor complex composed of IL10R2 (or CRF2-4) and IFNLR1 (CRF2-12) chains and is important in some types of virus infections [35].

Binding of secreted IFNs to their specific cell surface receptors on neighboring cells leads to the activation of signal transducer and activator of transcription complexes (STATs), which constitute a family of specific transcription factors responsible for the regulation of specific genes related to the immune response [34]. In the case of the IFNs, STATs are activated via phosphorylation by receptor-associated Janus-activated kinases and associate with other factors to move to the nucleus of the cell, where the complex binds to specific nucleotide sequences called IFN-stimulated response elements in the promoter regions of IFN-stimulated genes, inducing their transcription and subsequent expression. Several additional signaling complexes can bind to IFN-activated site elements in gene promoters, some specific for type I or II IFNs [34], and the expression of a wide variety of genes is thus influenced [36,37].

Subsequent antiviral activity of the type I IFN response is mediated by several defense systems in the cell, such as protein kinase RNA-activated and 2′,5′-oligoadenylate synthetase/RNase L system, the adenosine deaminase ADAR1, the Mx GTPases, and cellular microRNAs [38]. In infected cells, expression of IFN itself is activated by both viral RNA and DNA through sensing of double-stranded RNA by membrane-bound Toll-like receptor, triggering activation of transcription factors such as IRF3 and nuclear factor-κB, and retinoic acid-inducible gene 1, melanoma differentiation-associated protein 5, and DAI cytosolic viral nucleic acid sensors [38,39].

In addition, mitogens and several other cytokines can induce IFN production. In response to IFN, cells slow down protein synthesis, degrade mRNA (both viral and host), and end up promoting apoptosis of the infected cells [40–42].

3.1.1 Interferon Protein Structures

Human IFN-alfas are synthesized as a 188- to 189-residue-long precursor protein. The first 23 residues correspond to a signal peptide, yielding a protein with a calculated molecular mass of 18.5 kDa. The experimentally determined molecular masses range from 19 to 26 kDa, owing to the presence of posttranslational modifications [23]. The gene sequences encoding the IFN-alfa subfamily show a high level of overall sequence similarity and the corresponding protein sequences share between 70% and 80% homology and about 35% identity with human IFN-beta [43]. Several highly conserved residues in IFN-alfa proteins were shown to be crucial for their function (lysine residues close to the N-terminus and a tyrosine residue near the C-terminus) [44]. Conformational stability is enhanced through four cysteine residues at positions 1, 29, 99, and 139 that are involved in the formation of two disulfide bonds between the Cys29 and Cys139 residues that are necessary for biological activity, and between Cys1 and Cys99, which is functionally nonessential [45,46].

After crystallization of IFN-alfa, its three-dimensional structure could be elucidated [47,48]. IFNs-alfa and -beta are structured in a single domain, which is mostly alpha-helical.

The globular structure is composed of five major alpha-helices connected by a long loop and three shorter loops. The amino acids essential for the structuring of the protein, as well as for interaction with the IFN receptor, are known [49,50]. Several intrinsically disordered regions seem to have a functional role in the protein [51].

IFN-gamma shows little homology with type I IFN protein sequences and is encoded on chromosome 12, map 12q24.1. Unlike the other IFNs [4,7], its gene has three intervening sequences [10], the first of which contains a repetitive element. The monomeric molecular weight (MW) of the protein is around 35 kDa. The precursor protein is 166 amino acids, of which 24 N-terminal residues form a signal peptide. It is homodimeric in solution, with an MW of 70 kDa. The IFN-gamma protein has a structure similar to the type I IFNs, because it is constituted primarily by alpha-helical structures, with six helices in each subunit, without beta sheets. The dimeric structure is stabilized by the intertwining of helices with multiple intersubunit interactions [52].

3.1.2 Clinical Applications

Although several cases of viral infection resistance to IFN are known [53,54], as also demonstrated in the case of HIV [55] and in several cancers [56], many preparations of IFNs have been tested in clinical trials.

IFNs are potent biopharmaceuticals but they pose many hurdles for clinical applications. Because they are synthesized in the body in low quantities and show variable levels of species specificity, only human recombinant IFN can be used for medicinal applications. Moreover, it was soon evident that because IFNs act mainly on the level of cell−cell interactions, their systemic application, mostly by intramuscular or sometimes subcutaneous injection, is not straightforward, although fairly well tolerated.

IFN treatment has frequently been shown to be on the limit of the compromise between therapeutic effect and toxicity. Often, flulike symptoms are experienced within hours of IFN-alfa administration, which can be alleviated by paracetamol administration and tolerance later on. However, in a significant percentage of patients, treatment has to be interrupted owing to cardiovascular, central nervous system, or autoimmune reactions.

3.1.3 Interferon-Alfa in Antiviral Treatment

The most successful and widespread clinical application of (alfa)-IFN has been in the treatment of chronic hepatitis infections, especially hepatitis C (HCV) (formerly non-A/non-B). HCV infection is a global threat; an estimated 130−170 million people are chronically infected worldwide [57] and over 350−1000 deaths occur each year as a result of detrimental HCV-related liver diseases such as cirrhosis and cancer [58]. Given either as monotherapy or combined with ribavirin, IFN-alfa has been the treatment of choice for patients with chronic HCV infection [59]. New chemotherapeutic tools have become available and the use of IFN may be quickly coming to an end [60].

The IFN-alfa2 subtype has been the only IFN in use for the treatment of chronic hepatitis B and C, and leukemia [19,61]. Natural recombinant alfa-IFNs approved by the Food and Drug Administration (FDA) as therapeutics are mainly IFN-alfa2a (Roferon A1) and IFN-alfa2b (Intron A1) subtypes, and their PEGylated forms (Pegasys1 and PEG-Intron1). Introduction of the PEGylated IFN in 2001 led to the improvement of its antiviral effectiveness, mostly owing to the longer half-life in the bloodstream, which required only one injection per week instead of the previous two to three and combination with ribavirin therapy [62]. However, variable success rates have been observed in different countries, especially with different HCV subtypes [63]. Thus, there has been an intensive search for new therapies. Often, additional antiviral drugs such as protease inhibitors Telaprevir or Boceprevir, or the nucleotide analog Sofosbuvir are given in combination treatments. Interestingly, human genetic polymorphism near the gene IL28B that encodes IFN lambda3 was shown to influence patients' response to treatment and also natural clearance of the HCV genotype 1 virus [64,65]. The high cost and frequency of relapse led to a renewed effort for the development of alternative drugs, which have since reached the market [20,66].

IFN-alfa was also shown to be successful in the treatment of several papilloma viral infections such as genital warts and laryngeal papillomatosis [67].

3.1.4 Antitumor Treatment

The antiproliferative activities of crude preparations of IFN-alfa reported in the late 1970s and early 1980s elicited high hopes for anticancer treatments [68]. Encouraging results were obtained for breast cancer, lymphomas, and some myelomas and sarcomas. Clinical studies with purified recombinant IFN-alfa in the 1980s were less successful mostly owing to collateral effects, and the clinical use of IFN-alfa became more restricted. Schering Plough's Intron A (IFN-alfa2b produced in *Escherichia coli*) has been on the market since 1986. IFN-alfa2a and 2b remained successful in the treatment of hairy cell leukemia (a B-lymphocyte neoplasm) and also malignant melanoma, condylomata acuminata (genital warts), ovarian carcinoma, follicular lymphoma, metastatic renal-cell carcinoma, and AIDS-related Kaposi sarcoma [69]. IFN-alfa is also used as an adjuvant with other therapeutics to treat patients with bladder and renal cancers, and melanoma [70].

3.1.5 Interferon-Beta

IFN-beta, which was first cloned, sequenced, and expressed in 1982 [6,7], did not immediately find a satisfactory medical application, but was later used to treat relapsing-remitting multiple sclerosis, and slowed disease progression [71,72]. IFN-beta is thought to inhibit IFN-gamma and TNF-alpha production, thus down-regulating the proinflammatory response. The mechanism of action of IFN-beta is complex, involving effects at multiple levels of cellular function. IFN-beta appears to increase expression and concentration of antiinflammatory agents directly while down-regulating the expression

of proinflammatory cytokines. IFN-beta treatment may reduce the trafficking of inflammatory cells across the blood–brain barrier and increase nerve growth factor production, leading to a potential increase in neuronal survival and repair. Overall, therapy with IFN-beta leads to a reduction of neuron inflammation [73].

Recombinant IFN-beta can be expressed and purified with relative ease in *E. coli* or in Chinese hamster ovary (CHO) cells (Avonex and Rebif). Production in *E. coli*, such as Betaferon/Betaseron, yields a nonglycosylated product, which has not affected its biological activities negatively [74]. In Betaferon, the cysteine 17 was substituted by a serine residue.

Type I IFNs are also promising for their use as vaccine adjuvants, but the subtype of IFN for this purpose could have a significant influence on the adaptive immunity generated at specific immune compartments. For instance, IFN-beta could be used to enhance systemic T cell immune responses, whereas IFN-epsilon could be more promising as an adjuvant to enhance mucosal T cell immunity in the lung and the gut mucosae [75].

3.1.6 Interferon-Gamma

IFN-gamma, a type II IFN, formerly called immune IFN, is a critical cytokine in innate and adaptive immunity against infection by a variety of viral pathogens, and some bacterial and protozoal infections. It activates other immune cells, such as macrophages and natural killer cells, and has an essential immune-stimulatory and immune-modulatory effect. However, it is also involved in several autoimmune diseases [76]. Because of its complex and pivotal role in the immune system, clinical applications have not been straightforward, and IFN-gamma is used on few occasions, such as for chronic granulomatous disease. It is normally produced in *E. coli* and is purified with standard chromatography techniques.

3.1.7 Other Interferons

IFN-epsilon is a type I IFN that was identified more recently. It is highly and constitutively expressed in the brain, but its biochemical and biological characteristics are not fully understood. It has less antiviral, cytotoxic, and antiproliferative activity, but the differential regulation of genes related to the central nervous system by recombinant human IFN-epsilon has been reported, which suggests a role for this IFN in the structure and operation of the brain [77]. However, one of its main functions seems to be in the protection of the female reproductive tract against viral and bacterial infection [78]. Contrary to other type I IFNs, IFN-epsilon is not induced by known pattern recognition receptors that signal to induce effector cytokines in response to pathogens that normally trigger the innate immune system. However it was shown to be constitutively expressed by epithelial cells of the female reproductive tract and also in the male reproductive organs, and to be hormonally regulated, possibly with a crucial role in the defense against sexually transmitted pathogens [79].

IFN-kappa is also a member of the type I IFN family and is expressed in keratinocytes. The gene is found on chromosome 9, adjacent to the type I IFN cluster [80,81].

IFN-omega has only one functional form described to date (*IFNW1*), but has several pseudogenes: *IFNWP2*, *IFNWP4*, *IFNWP5*, *IFNWP9*, *IFNWP15*, *IFNWP18*, and *IFNWP19* in humans. Many nonprimate placental mammals express multiple IFN-omega subtypes [82].

IFN-zeta, also called limitin, displays antiviral, immunomodulatory, and antitumor effects, but it has much less lympho-myelosuppressive activities than does IFN-alfa, and a narrow range of biological activities [83].

Additional type I IFNs, such as alfa-omega, nu, and IFN-delta, are found in several mammal species [84], as well as IFN-tau [85], which is a multifunctional type I IFN secreted by trophectoderm, and acts as the pregnancy recognition signal in ruminants. It also displays antiviral, antiproliferative, and immunomodulatory bioactivities [86].

The most recently discovered type III IFN group consists of four IFN-lambda proteins called IFN-lambda 1, 2, 3, and 4, (also called IL-29, IL-28A, and IL-28B) [87,88], but their biological activity has been less well characterized.

3.2 Interferon Production and Formulation

Initial IFN production systems were established using mammalian cell lines in culture flasks; soon after, immortalized cancer cell lines were discovered to secrete larger amounts of IFN-alfa. For example, a human lymphoblastoid cell line called Namalwa, stimulated with Sendai virus, was used as a large-scale production system, yielding a mixture of at least eight different IFN-alfa subtypes [89]. This mixture was then purified from the culture supernatant using standard process chromatography systems. Much improvement was also made with the use of monoclonal antibodies for the sensitive detection of IFN in immunoassays. Wellferon was one of the first products produced in this way. It contained a mixture of nine IFN-alfa subtypes. Such “natural” IFN preparations were later shown to elicit less relapsed or refractory clinical responses, at least partly owing to the much lower frequency of the production of IFN-neutralizing antibodies [90]. However the search for purified single-IFN species, large-scale and cheaper production systems, and the newly developed recombinant DNA technologies that became available in the early 1980s quickly led to the cloning and sequencing of IFN-alfa, -beta, and -gamma complementary DNAs (cDNAs) from humans, mice, and other mammals, also followed by the characterization of their genomic gene sequences. Many cDNAs were expressed in a variety of heterologous systems such as *E. coli*, yeast, fungi, and mammalian cell lines (CHO and monkey kidney cell lines). However *E. coli* remained the system of choice because most human IFN-alfa and -beta is not glycosylated, and although IFN-gamma is glycosylated, the form expressed in *E. coli* shows similar biological activity. Cloning gene sequences and expressing them in high volumes

and cell densities with relatively cheap culture media is easy, fast, and accessible, but has as a major drawback extensive and costly purification steps that are needed to remove unwanted contaminants from the product, and it is often difficult to achieve full biologically active protein conformation. *Escherichia coli* does not normally secrete heterologous proteins into the culture medium, which poses the challenge of removing all unwanted nucleic acids, contaminating proteins and pyrogenic activities from the protein solution to reach more than 99% purity. Also, many heterologous proteins are trapped in inclusion bodies in *E. coli* expression systems, and much work has been invested to avoid or minimize this phenomenon. In some cases in which biologically active conformations are not needed, inclusion bodies can be an advantage, providing a partial purification step, for example in the production of antigens with non-conformational epitopes. In other cases, however, different promoter systems were developed to minimize inclusion body formation through better transcriptional control, slower expression, or induction and expression at lower temperatures, for example at 25°C or 20°C. Synthetic gene construction and codon use adaptation of the heterologous sequences to the *E. coli* codon preference has been added to the arsenal of tools. However, careful research into optimization of downstream processing technologies is needed, such as inclusion body cleanup and subsequent solubilization with specialized buffers, followed by renaturation of the recombinant protein. The optimization of renaturation protocols must be achieved case by case, because many factors have a role in this process, most of them poorly understood, and variable degrees of loss of the active protein must be accounted for [91–93]. Purification protocols must be optimized, and usually include precipitation steps, and chromatographic techniques ranging from gel filtration and ion exchange, metal affinity, hydrophobic interaction, and also affinity chromatography using immobilized anti-IFN monoclonal antibodies can be employed, besides ultrafiltration, crystallization, and more specific techniques. Large-scale chromatographic systems are expensive; they use process scale columns, with medium pressure and often a final high-pressure high-performance liquid chromatography (HPLC) step. The purified protein can be crystallized before final formulation, which adds to purity and stability, and micro- and nanocrystals are increasingly used as part of the formulation technology (Fig. 3.1).

Many different approaches have been used to overexpress human IFN-alfa in *E. coli* using a variety of promoter systems and even more purification schemes [94–96]. New tools for optimization of codon use have been employed, with promising success [97,98], although many factors influencing protein translation and folding, including codon bias, are still only partially understood. Alternatively, other expression systems, such as *Bacillus subtilis* [99,100], *Pichia pastoris* [101,102], mammalian cells such as CHO [103] or Human Embryonic Kidney cells (HEK 293) [104], insect cells using baculoviral vectors [105], and cell-free protein synthesis systems [106], were employed and evaluated for the production of recombinant human IFNs.

Several IFN-alfa 2a formulations have been marketed, and even more for IFN-alfa 2b such as Intron A from Schering Plough, and many new formulations mostly from new or

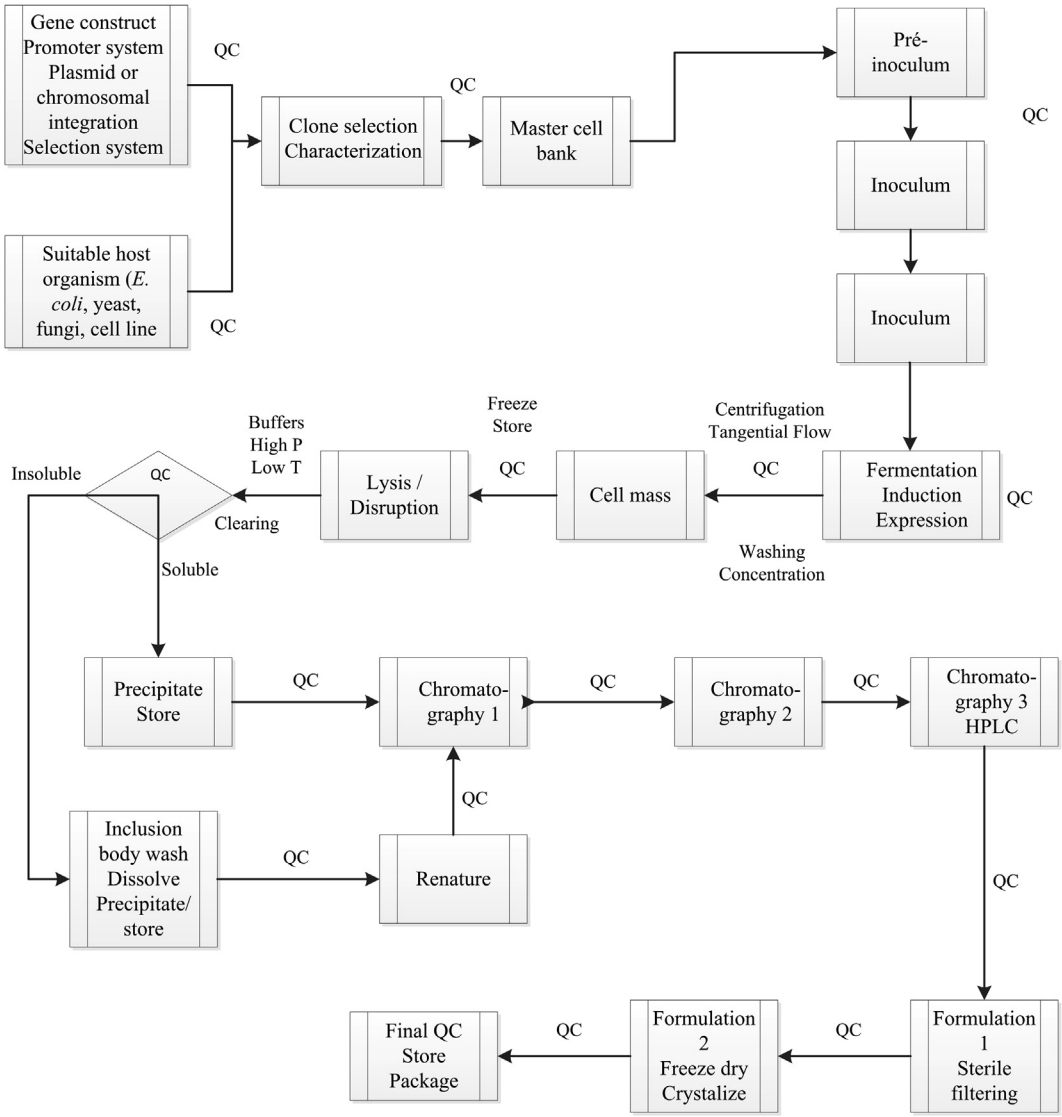

FIGURE 3.1 Overview of recombinant protein expression and purification scheme suitable for production of IFN in a bacterial, yeast, or cell system.

regional companies, such as Realderon from Teva-Israel, and many formulations: Viferon, Alpharona, Reaferon, Infagel, Recolin, Altevir, Kipferon, Giaferon, Genferon, Grippferon, Opthalamoferon, and Gerpferon, from several Russian companies [107]. To increase serum half-life, high–molecular weight groups such as polyethylene glycol (PEGylation) can be added through reaction of free amino groups of the IFN protein with activated PEG such as methoxy-polyethylene glycol or other variants, resulting in more

effective drugs, which contain, in addition to mono-PEGylated molecules, some free and di-PEGylated forms [108,109]. Typically, some 40 kDa of linear or branched PEG chains are added. They are thought to provide a slower release into the bloodstream, but also a slower clearance resulting, on average, in a threefold longer half-life. Examples are Pegasys, Reiferon Retard, Peg-Intron, Pegetron, and ViraferonPeg, among others (Table 3.1).

Table 3.1 Types of Interferon and Commercial Pharmaceutical Forms

Type of Interferon	Trade Name	Company	Observations
IFN-alpha-n1	Wellferon	GSK	A "natural" mixture of >20 IFN-alpha, purified from a Sendai-virus induced lymphoblastoid cell line, for treatment of hairy cell leukemia, chronic hepatitis B and genital warts.
IFN-alpha-n3	Alferon-N	Interferon Sciences Hemispherx Biopharma	A "natural" mixture of up to 14 IFN-alpha, purified from Sendai-induced pooled human leukocytes.
IFN-alpha-Le	Multiferon	Swedish Orphan Biovitrum	A mixture of 6 IFN-alpha
IFN-alpha 2a	Roferon A	Roche	Neoplasms of the lymphatic and hematopoietic system; chronic hepatitis B and C. Produced in *E. coli*
Interferon-alpha 2b	Intron A Viraferon Reliferon Uniferon	Schering/Merck Shering Plow Reliance life Sciences (India) Getz life Sciences (Pakistan)	Hairy cell leukemia, melanoma, follicular lymphoma, chronic hepatitis B and C, genital warts. Produced in *E. coli*
INF-alpha 2c	Berofor	Boehringer Ingelheim	Hairy-cell leukemia, viral eye infections
PEGylated IFN-alpha 2a	Pegasys Reiferon- Retard	Hoffmann-La Roche Egypt	Chronic hepatitis B and C
PEGylated IFN-alpha 2b	Peg-Intron Viraferon-Peg Pegetron	Schering Plow Shering Plow Canada	Chronic hepatitis B and C Combination with ribavirin
IFN-beta 1a	Rebif Avonex	Merck Serono Biogen	Relapsing forms of multiple sclerosis. Produced in CHO cells, glycosylated
IFN-beta 1b	Betaseron Betaferon	Chiron Shering	
Interferon-gamma 1b	Actimmune	Horizon Pharma	Chronic granulomatous disease, Severe malignant osteopetrosis
	Imukin	Boehringer Ingelheim	
Consensus IFN-alpha	Infergen (IFN-alfacon-1, CIFN or IFN-Con1)	Amgen/Intermune	

3.3 Modified or New (Synthetic) Interferons and Formulation

IFNs have many drawbacks as therapeutic molecules, as mentioned previously. Improving activity, eliminating toxicity and unwanted side effects, increasing serum half-life, stability, and shelf life are among the desired modifications, besides optimization of heterologous expression through codon use modifications. Indeed, growing tools for gene synthesis and high content screening make the design and selection of optimal variants accessible, but they are limited by the high costs of clinical trials. Synthetic variants of IFN-alfa are commercially available, and several other modified IFNs are under development. The first such IFN that reached the market will be discussed briefly as an illustration of the overall development and production process for such molecules.

The best known synthetic IFN-alfa, called consensus IFN-a (IFN alfacon-1; Infergen), was launched by Amgen in 1997 [110,111]; it claimed to improve the antiviral activity of IFN-alfa [112,113]. Consensus IFN is thus an artificial recombinant second-generation type I IFN. Its gene sequence was derived after a multiple alignment analysis of 14 human IFN-alfa subtype protein sequences that assigned essentially the most frequently occurring amino acid in each position. The resulting sequence differs from that of IFN-a2 at 20 of 166 amino acids (approximately 88% homology), and has about 34% homology with human IFN-beta. The resulting protein sequence was then back-translated using codon optimization into a DNA sequence, cloned in suitable expression plasmids, and introduced in *E. coli*. Induction through the use of adequate promoter systems by either the lambda P_L system or a T7 polymerase system and isopropyl β-D-1-thiogalactopyranoside induction resulted in mostly inclusion bodies, with variable yields. These are then recovered through centrifugation and washing steps, solubilized, refolded, and IFN purified through chromatography. Evaluation of safety, biochemical, and pharmacologic activity was done in rodent and primate models, as well as in vitro on a number of human and animal cell lines. Preclinical tests included pharmacokinetic studies of Infergen in hamsters and rhesus monkeys, demonstrating similar absorption and elimination profiles using several administration routes (intramuscular, subcutaneous, or intravenous). Peak serum concentrations in hamsters and monkeys were measured at several time points. Systemic exposure was evaluated, as well as bioavailability and clearance. Pharmacologic and toxicologic profiles were compared with those of other IFN-alfas. Adverse effects at different doses were also monitored, as well as reproductive toxicology. Initial clinical trials were encouraging, and the consensus IFN was claimed to display higher biological activity (antiviral, antiproliferative, and cytokine-inducing activity) than the native type I IFNs, also in patients [114]. Infergen was approved by the FDA for use in treating chronic HCV infection in 1997, with Amgen as the producer, who passed the rights to InterMune in 2001.

The product is supplied in a sterile liquid dosage form as single-use vials containing 9 μg IFN alfacon-1 in 0.3 mL 100 mM NaCl and 25 mM $NaPO_4$. The production process

[115] follows a typical general design for recombinant protein production (Fig. 3.1). A Master Cell Bank of the plasmid-transformed recombinant *E. coli* strain is constructed and verified for contamination using microbial tests for contaminant strains of bacteria and phages. The presence of bacteriophages would invalidate the cell bank for several reasons.

Fermentation of *E. coli* is monitored for pH, temperature, dissolved oxygen, cell density, and eventually nutrient monitoring. After cell growth to the desired density, induction and expression take place for the optimum time. Cells are then collected, usually through (continuous) centrifugation or tangential flow filtration and the obtained cell paste is stored for quality assurance testing. This normally includes bioburden and product yield evaluation and testing for contamination, viability, protein concentration, genotype verification, plasmid retention, and paste yield. At this point, validation of plasmid stability has been confirmed by continuous monitoring of peptide mapping and N-terminal sequence of end-of-production protein, and complete DNA sequencing of the target gene at the end of fermentation. This ensures that the strain, plasmid, IFN coding gene, and resulting protein continue as expected. IFN purification starts with cell lysis (usually through a large-scale pressure homogenizer), centrifugation (of inclusion bodies) or clearing steps, contaminant removal through inclusion body washing, solubilization, oxidation and refolding, precipitation, and clarification, followed by chromatography steps, formulation into final buffer and eventual addition of preservatives, and sterile filtration of purified bulk before filling. The purification process is controlled by monitoring homogenizer pressure, time and temperature limits, bioburden, chromatogram records, and process yield.

The process thus developed must be validated through production of several purified bulk lots derived from at least three cell paste lots, and verification of the absence of host cell and product contamination, purity [HPLC, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and peptide mapping], identity, and specification of the product, including full biological activity of the IFN, and consistent process yields. Biological activity is measured in validated assays with reference material (natural or reference IFN). Full biochemical and biophysical characterization of the recombinant IFN must, of course, be performed, including complete amino acid sequence determination by sequencing of peptides and mass spectrometry; peptide mapping; amino-terminal isoform analysis by isoelectric focusing; anion exchange and reverse-phase chromatography (HPLC); size exclusion chromatography and laser light scattering; SDS-PAGE electrophoresis; circular dichroism; fluorescence spectroscopy; acid sensitivity; and UV absorption curves (calculation of extinction coefficient). Product stability must be demonstrated, both on bulk and final product, the latter for a shelf life of 24 months at 2–8°C. Therefore, stability assays must be performed for several biological and biochemical parameters in a comprehensive stability program at −70°C, −20°C, 4°C, and 37°C. Experimental stability tests must also simulate shipping conditions and freeze-thaw and temperature cycling.

Manufacturing facilities must fully comply and be certified for Good Manufacturing Practices, including environmental impact evaluation and control for the production facility and processes.

Several other modified IFNs are under development, such as a second-generation IFN-gamma with high activity, through shortening of the C-terminus of the protein, and a third generation with higher stability through introduction of a disulfide bridge in the molecule, leading to a tighter packed protein [116]. Also, several groups have invested continued efforts in the optimization of IFN expression, especially in *E. coli*, through the use of new promoter or induction systems, and even through expression in cell-free systems, although this is currently not a viable large-scale production alternative. However, cell-free systems can be used to obtain sufficient biological material for functional assays in a fast approach, for example, for screening purposes.

3.3.1 Future Perspectives

The main application of IFN-alfa, as an antiviral agent in the treatment of chronic HCV, seems to have come to an end with newer, more effective drugs reaching the market that have fewer side effects. IFN-beta is still promising in the slowing down of the progression of multiple sclerosis. IFNs and IFN-gamma are also used for clinical treatment of some specific tumors. Will this lead to a full winding down of IFN as a drug? Because of its intrinsic cell—cell interaction profile and inherent toxicity and often only partial success when used on a systemic basis, this would not be surprising. However, a number of questions remain unanswered related to the clinical use of IFN. Several options are still available for further development, including a more detailed characterization of the functional role and applications of less known IFNs, such as type III IFN-lambdas and type I IFN-epsilon, -omega, -kappa, and -tau. Indeed, it is possible that specific niche treatments can be discovered for these cytokines.

Equally important could be the construction of a number of variant IFNs based on (parts of) natural sequences, but combining different structural and functional elements into the molecules, and systematic screening for desired characteristics in adequate functional assays. Tools now available in synthetic gene construction and the availability of high-content screening systems should facilitate such an endeavor.

However, in our view, the most promising applications of IFNs have yet to be developed as elements in the construction of targeted cell therapy. One can envisage that many future medicines will target specific cell types: for example, immune cells, cancer cells, and neurons. More and more tools are being developed to target such cells physically using drug or effector conjugated antibodies. These can be single-chain antibodies, classic monovalent antibody fragments (Fab and scFv), and engineered variants such as diabodies, triabodies, minibodies, and single-domain antibodies, or aptamers. Coupling IFN molecules, engineered or not, to such constructs could be a strategy to obtain the unique benefits of these classes of cell signaling proteins, unharnessing their full strength in the complex pathways of immune and host defense systems.

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4 Monoclonal Antibodies

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4.1 Introduction to the Immune System

4.1.1 Origins of the Immune System

The evolution of multicellularity in organisms can explain the origin and function of the immune system. The interaction of different organisms or the cohesion of cells of unicellular beings resulted in multicellular organisms. The evolutionary process of cell recognition of the same origin and interdependence with others from a different origin has been happening since the first microorganisms got together to form the first multinuclear organism. In addition to larger size, multicellularity increased biological complexity through the formation of new biological structures [1].

It may seem as if the immune system is active only in a disease state. In reality, it is always active [2]. Therefore, the absence of disease does not mean a lack of interaction with microorganisms. In a homeostatic condition, the immune system constantly interacts with other cells such as resident microbiological flora and organisms from the external world.

Studies have revealed the unexpected importance of host and microorganism interactions for evolution. Resident microbes in the human body, which form the microbiota, can be viewed as an integrated system [3]. Resident microbes are continuously interacting with the immune system, and depending on their location, their compositional diversity, or the lack of it, they can affect the health state of the organism. This interaction between a host and microbes has prompted the possibility of considering the microbiome as another organ [4]. The discovery of the importance of the microbiome for human health was a crucial advancement in evolutionary biology in the first decade of the 21st century [5]. Advances in genomic mapping allowed insight into microbiome–host interactions, information that was previously unavailable to immunologists. With this new information, current understanding of the role of microbes is changing; they are not assumed not be pathogens or symbionts, but cohabitants of the same genetic milieu of the host. Each organism is the result of a combination of germs and host DNA integrated into a complex system [6]. During evolutionary history,

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microbes and their hosts have cohabited, with natural selection acting upon the entire superorganism to drive evolution [7].

The current understanding of host–microbe interactions engenders the possibility that infection and immunity are an important factor in organismal evolution. This hypothesis favors the paradigm that there is a need to preserve the coexistence of organisms in the same biological structure for lateral gene exchange among microbes. Thus, infection is necessary for both microbe and host evolution. The immune system can thus be considered the metagenomic immune system (MIS).

The idea of the MIS is a new interpretation of immunity. It is a different perception of the basics of the immune system as part of a new evolutionary theory. The evolution of biological systems by integrating genetic material is an emergent theory that takes into account recent scientific facts. The data show that life results by assimilating one genomic system into other genomic arrangements. Therefore, genetic exchange among organisms is a priority instead of a competitive process. This is a satisfactory interpretation for understanding biological diversity by evolution and the immune system origin. Therefore, the main driving force of immune system development is the process of interaction among biological beings. This new paradigm includes the interpretations that the immune response of one organism does not react, but rather facilitates interrelation with other organisms.

4.1.2 Innate and Adaptive Immunity

Evolutionary processes have led to the generation of two types of immune processes: innate and adaptive [8,9]. The difference between the innate and adaptive immunity is not because the cells have a different role in each system, but rather how they recognize and interact with microorganisms and other cells. Whereas the adaptive immunity recognizes an antigen by its specific epitope, the innate immunity relies on evolutionarily conserved targets such as the pathogen-associated molecular pattern (PAMP). They are recognized by the pattern recognition receptors. PAMPs are found in both pathogenic and nonpathogenic microorganisms. Therefore, the term “PAMPs” is instead more aptly characterized as microbe-associated molecular patterns. The innate immune system serves functions beyond recognizing and clearing pathogens. The innate immune system also serves as a clearing mechanism for damaged cells that are no longer viable, as well as transformed cells that do not operate within their normal parameters. Unique molecules displayed on injured or transformed cells, referred to as danger-associated molecular patterns, can be recognized by the innate immune system. The innate immune system can recognize approximately 10^3 molecular patterns.

Although the development of the adaptive immune system has led to an increase in genomic complexity of vertebrates, this complexity appears to be irrelevant in terms of the species survival. Invertebrates did not develop adaptive immunity and are also able to survive the pathogen interactions successfully with only the innate immune system [10].

Whereas the targets that the innate immune system recognizes are common in any microorganism structure; such as double-stranded DNA, the adaptive immune system

"learns" from exposure to microorganisms and can form "memory" to mount a more adept immune response with subsequent exposures. The main cells in the adaptive immune system are the T-cells and B-cells. Both T-cell and B-cell receptors have the ability to recognize a particular target on a microorganism, typically a segment of an extracellular protein. The segment that is recognized by B-cells and T-cells is known as an epitope.

The primary activity of B-cells is the ability to secrete their receptor in a soluble form, producing antibodies that are specific to epitopes on antigens. Once tagged, antibodies are able to recruit other immune cells to carry out a subsequent function. Antibodies act as molecular tags, directing other immune cells to destroy the tagged substance. Antibodies are Y-shaped proteins with a variable and a constant region (Fig. 4.1). The variable region, as the name suggests, varies between antibodies, and it is where antibody–antigen interactions occur. In the variable region, the antibody specificity is determined.

Antibodies can be divided into five classes: immunoglobulin (Ig)M, IgD, IgG, IgA, and IgE, which are determined by their constant region. The IgG class of Igs contains four subtypes, named IgG1–4 [11]. The constant regions interact with fragment crystallizable region receptors (FcR), which have a high affinity for each particular class. FcRs are named in relation to the class of antibodies for which they have the highest affinity: FcαR, FcγR, FcδR, and FcRμR, respectively. Different categories of Igs are thought to have various functions. IgM and IgG3 have a strong affinity for complement fixation. IgG1 can cross the placental barrier and has mild complement fixation ability. IgE demonstrates high affinity to mast cell and basophils [12]. The specificity of an antibody in tagging proteins has made it an invaluable tool in the field of biotechnology.

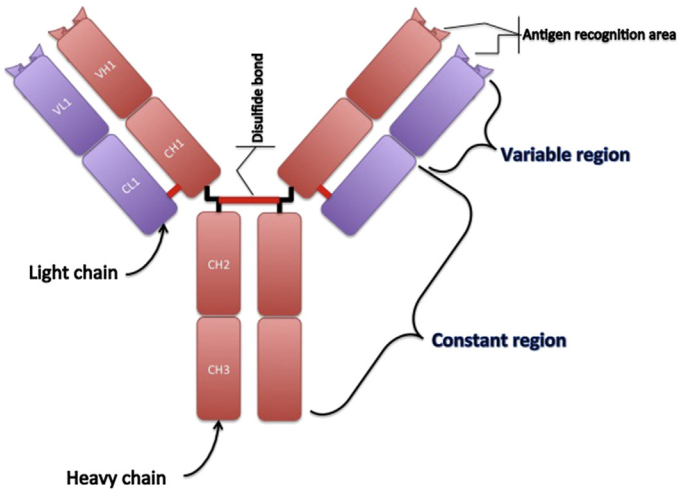

FIGURE 4.1 An immunoglobulin protein is composed of two proteins called the light and heavy chains, which are joined by disulfide bonds. Each heavy and light chain has a constant and a variable region. The antigen recognition area is found in the variable region.

4.2 Monoclonal Antibody Development

The modern era of immunology began in 1890 with the discovery of antibodies as key components of protective immunity. However, it was not until the early 20th century when the use of antibodies was brought to fruition by Paul Ehrlich, one of the fathers of immunology, in his "magic bullet" theory. Ehrlich rationalized that because the interactions of antigen and antibody were already known, it stood to reason that a drug could be bound to a distinct carrier exhibiting pharmacologic activity only in the target tissue. Thus the undesirable effects of its action in other tissues would be greatly reduced. As an added benefit, the efficacy of the drugs could be increased, which would decrease the necessary dose to achieve therapeutic effects.

Although antibodies seem like an ideal candidate, the immune response to any antigen is polyclonal. The polyclonal nature of the immune response makes it difficult to purify antibodies with a desired specificity in mass quantities. It was not until 1975, with the discovery of cell hybridization techniques by J. F. Georges Kohler and Cesar Milstein, who created hybridomas, that the use of mAbs could be mass produced. The creation of hybridomas involves extracting splenocytes of mice immunized with a desired antigens and fusing them with myeloma cells. This technique makes use of an agent, such as polyethylene glycol, which facilitates the fusion of the adjacent plasma membranes. Myeloma cells are screened for two key features: They do not produce their own antibody and lack the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene. HGPRT is used in nucleotide synthesis and is essential to cell division. The HPGRT gene has a redundancy because there is a de novo synthesis route through dihydrofolate reductase (DHFR). By using the molecule aminopterin, which inhibits DHFR, the cell is unable to divide without supplementation.

In the creation of hybridomas, three cell populations are present: myeloma cells, hybridomas, and splenocytes. When these cell cultures are grown in hypoxanthine, aminopterin, and thymidine (HAT) culture medium, the myeloma cells are unable to divide because there is no HPGRT, and they die. Splenocytes are unable to grow indefinitely by the average finite life, and eventually die off. This results in only hybridomas remaining. Hybrids can grow indefinitely and begin to multiply, with rapid formation of colonies (Fig. 4.2). The hybridoma cells are cloned and supernatants are tested for antibody production. Extensive tests are conducted to ensure the specificity of the antibodies and are seeded from a single cell colony so that cells in one medium are from one clone and thus produce only one type of antibody. They can be produced large amounts of antibody in vitro (in culture medium) or in vivo (in ascites in mice). This discovery earned Kohler and Milstein the Nobel prize for Medicine and Physiology in 1984.

With the invention of hybridomas, it became possible to mass produce large amounts of mAbs for clinical use. One could produce mAbs specific to a microorganism or tumor specific antigen, to interfere with immune system capacity. However, because these antibodies originated from a mouse source, the human immune system recognized

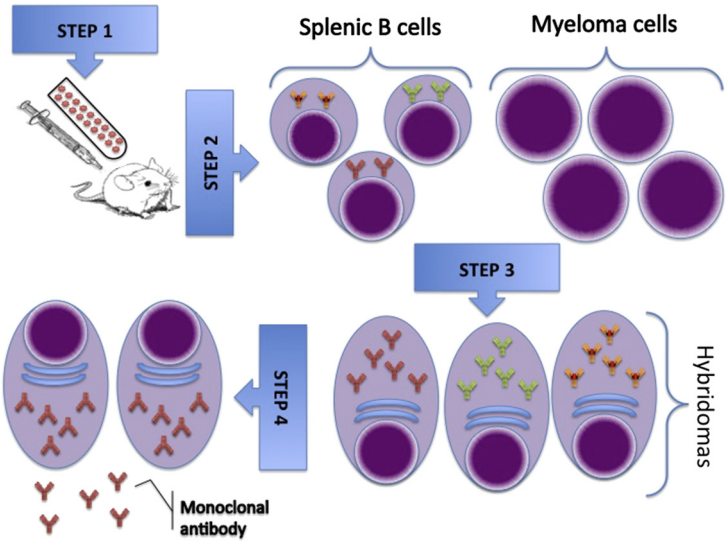

FIGURE 4.2 Breakdown of myeloma creation. Step 1: a murine carrier is immunized and splenocytes are extracted. Step 2: The murine splenocytes are fused with the splenocytes. Step 3: The hybridoma cells are cultured in HAT medium and the hybridoma producing the desired antibody is isolated. Step 4: The isolated hybridoma cell colony is expanded.

these antibodies as foreign and produced human anti-mouse antibodies that neutralized the efficacy of mAbs. With the advent of genetic engineering, it became possible to replace the murine antibody constant region gene for the same human genomic sequence. These "chimeric" antibodies are more tolerable to the human immune system while providing the same specificity [13]. However, to minimize immune activation, the less murine the antibody has, the more efficacious it is. Further technologies have been developed to create mAbs that are more human, in which part of the variable region is human; that is, they are humanized, or the entire antibody is human: it is a fully human antibody [14] (Fig. 4.3). The ability to mass produce mAbs with a desired specificity has revolutionized both the clinical and research fields.

4.3 Monoclonal Antibody Nomenclature

The seemingly infinite possible different targets, different classes, and different sources of antibodies require a naming convention for mAbs. Conventions for naming antibodies and antibody fragments have been established in the United States. Four or more syllables in each name reveal much information about each product. mAb nomenclature includes the suffix "mab", for monoclonal antibody. One or two letters preceding the "mab" suffix provides much information about the source of the antibody: "u" for human source, "o" for mouse, "a" for rat, "i" for primate, "xi" for chimeric, and "zu" for humanized.

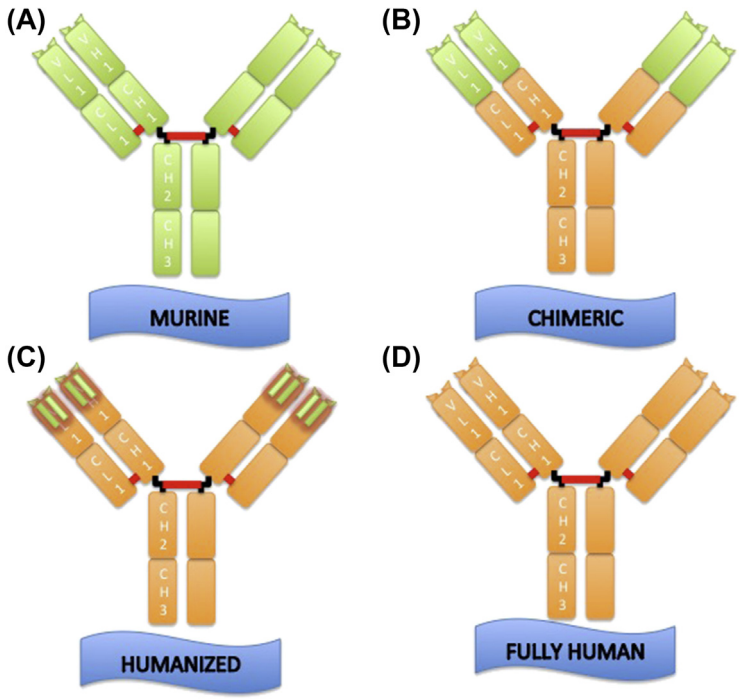

FIGURE 4.3 Murine antibodies are completely foreign and unmodified (A). In a chimeric antibody, the constant region is modified to be human (B). Humanized antibodies have part of the variable region edited to be of human origin (C). A fully human antibody contains constant and variable regions of human origin (D).

The target of the antibody or the indication of disease is designated by an additional internal syllable in the jargon of the governing body of nomenclature, called "infix," which is the prefix. These include syllables: "ba" for bacterial, "li" for the immunomodulator, "mel" for melanoma, "pr (a)" for the prostate tumor, "gov" for the tumor gonad (ovary), "ci (r)" for cardiovascular, "vi" for viral, "col" for cervix tumor, "Sea" for breast tumor, and "got" for gonad tumors (testicle); however, all of these have been combined under the infix "tu" for tumor. The prefix is preceded by a compatible syllable that designates the end of the product name. With the nomenclature convention, words such as biciromab, sevirumab, edobacumab, and nebacumab start to make sense.

If another molecule is added to the antibody, a separate word is added to the name. If a toxin is added, the "tox" should be part of the second word. For example, "aritox" refers to the toxin ricin, and "sudotox," the exotoxin of *Pseudomonas aeruginosa*. Pendetide or pentetate words are also used for holding all linker antibodies to a toxin or isotope.

A summary of the nomenclature would be thus: Endings with "mab" are mAbs; endings in "ximab" are monoclonal chimeric and can form neutralizing antibodies; final "zumab" is humanized mAbs and is less able to generate neutralizing antibodies; final "cept" involves fusion with the Fc portion of human IgG1 (Table 4.1).

Table 4.1 Monoclonal Antibody Nomenclature

Prefix	Target infix		Source infix		Suffix
Variable	*- ba -*	Bacteria	*- a -*	Rat	*- mab*
	- ci -	Cardiovascular	*- e -*	Hamster	
	- fu -	Fungal	*- i -*	Primate	
	- li -	Immunomodulation	*- o -*	Mouse	
	- os -	Bone	*- u -*	Human	
	- vi -	Viral	*- xi -*	Chimeric	
	- tu -	Tumor	*- zu -*	Humanized	

4.4 Monoclonal Antibodies in Research

With their ability to target a desired protein specifically, mAbs have revolutionized the research field by allowing the quantification of protein assays. Through the use of molecular biology techniques, it is possible to conjugate the constant region of antibodies with another product. Fluorescent and colorimetric labels are commonly tagged onto antibodies. Conjugated antibodies, coupled with spectrometers, allow researchers to quantify or confirm the presence of a protein target in a sample. Using mAbs, researchers have developed many techniques.

4.4.1 Enzyme-Linked Immunosorbent Assay

One commonly used technique in both in the laboratory and clinic, is the enzyme-linked immunosorbent assay (ELISA). As the name suggests, an enzyme, commonly horseradish peroxidase (HRP), is linked to a specific antibody [15]. The benefit of conjugating HRP is that it can undergo a chemical reaction with substrates such as 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) and 3,3′,5,5′-tetramethylbenzidine to induce a color change. The change in color can be used visually to confirm the presence of a protein or, by using a reference assay and spectrophotometers, quantitatively to measure the concentration of a given protein. Typically the conjugated antibody is a secondary antibody specific to a class of antibodies. This allows for the use of the conjugated antibody in different tests.

A common form of ELISA is the sandwich ELISA. In this model, antibodies unique to the desired antigen, the substrate, are coated on a plate. The substrate is added to the plate and enough time is allotted for it to bind to the antibody. The sample is then washed to remove any unbound substances. A detection antibody is then added, which then binds to the substrate that is bound to antibodies that were coated on the plate (Fig. 4.4). After an allotted time, the plate is washed to remove any unbound antibodies. A secondary antibody specific to the constant region of the detection antibody is then added. The secondary antibody is conjugated with HRP. After any unbound secondary

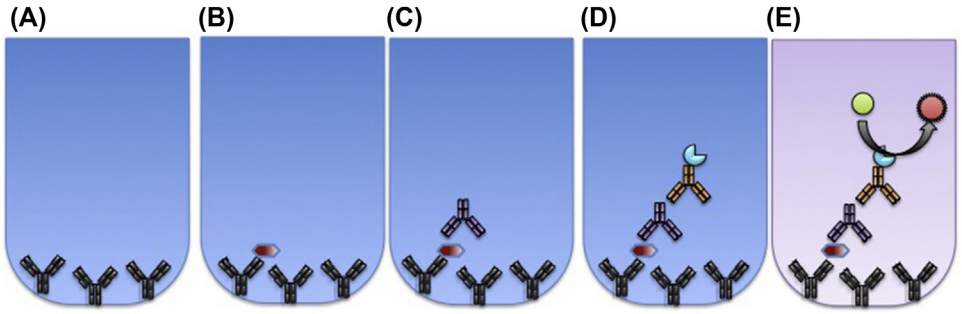

FIGURE 4.4 A plate is first coated with the capture antibody (A). The substrate to be detected is added to the plate (B). The detection antibody is added to form a sandwich (C). The conjugated secondary antibody is added (D). Substrate is added to induce a color change (E).

antibodies are washed off, a substrate that induces a color change in HRP is added and the color change is observed.

4.4.2 Western Blot

Similar to ELISAs, western blot (WB) can be used to detect the presence of a protein through the use of antibodies [16]. However, WB protocol can also give insight into the molecular weight of a protein as well as the relative concentration. Cell or tissues are lysed to gain access to cellular proteins. The lysates are then separated by gel electrophoresis according to their molecular weight. After separation the proteins are transferred to a membrane where antibodies are applied to probe the protein of interest.

4.4.3 Flow Cytometry

Antibodies tagged with a fluorescent molecule have uses beyond ELISA. The use of fluorescent-conjugated antibodies is a powerful tool in flow cytometry. Flow cytometry relies on the principle of fluorescence resonance energy transfer (FRET). FRET relies on two closely positioned fluorescent molecules. In this configuration, the proximity of the

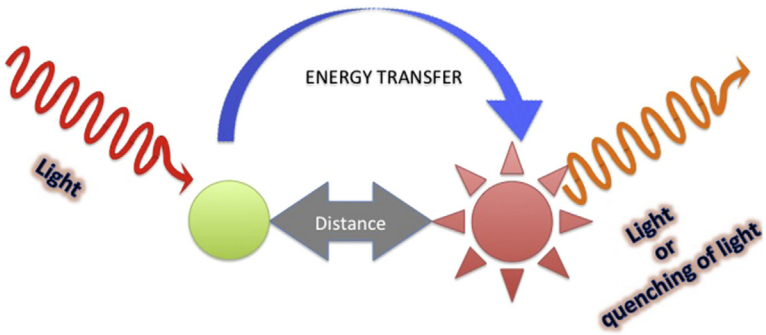

FIGURE 4.5 FRET allows for energy transfer of the emission spectrum of the first molecule (green (gray in print versions)) to be absorbed by the second molecule (red (dark gray in print versions)).

two molecules allows the emission spectrum of the first molecule to be the absorption spectrum of the second molecule (Fig. 4.5). As energy is lost between absorption and emission, this results in the emanation gamut of the whole system to be different from the absorption molecule, allowing for easy differentiation between the two spectra.

In flow cytometry, antibodies are conjugated to a fluorophore, which takes advantage of the FRET paradigm. Cell samples are stained for the desired molecular markers and suspended in a medium, which prevents cell clumping. More than one molecular marker can be detected at the same time. However, a limitation exists in the differentiation of spectra owing to spectral overlap. The emission spectrum of the fluorophores has a range, and these spectra can overlap each other, making it difficult to determine which fluorophore is being activated. Once stained, the samples are run through the machine, which uses hydrodynamic focusing to move the cells in a single line (Fig. 4.6). A laser is then used to activate the appropriate fluorophores and a detector measures the emission spectra.

Once cells are in a single line, a laser is used to excite the fluorophores conjugated to the antibodies. The emitted light is then recorded for each cell, and by using multiparametric analysis, a detailed understanding of the cellular composition of a sample can be inferred. Beyond measuring the presence of an antigen, flow cytometry can be used to purify samples. By preparing a fluorescence-conjugated mAb tag to identify the desired cell population, a desired population of cells could be isolated from a heterogeneous sample. In this paradigm, the solution in which the cell samples are suspended is run through the detector. Immediately after the sample is measured it is split into droplets that can contain one more many cells, depending on the size. The droplets are given a charge, and using electrostatic deflection, the sample can be isolated into a desired tube.

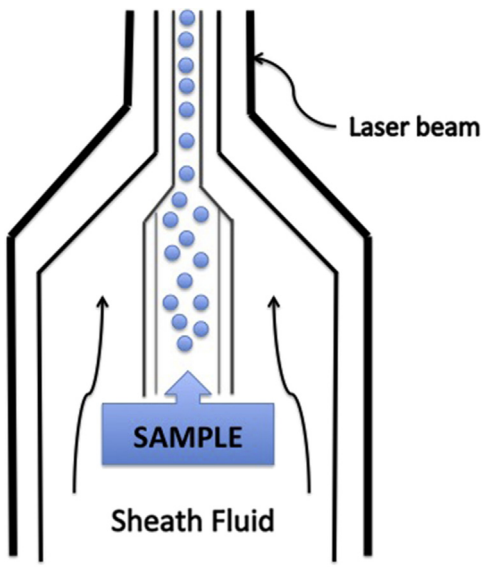

FIGURE 4.6 Hydrodynamic focusing allows a sample of cells to be run in a single line for analysis.

4.4.4 Mass Cytometry

Similarly to flow cytometry, mass cytometry allows insight into cell or tissue composition. The main difference between mass cytometry and flow cytometry is that in mass cytometry antibodies are conjugated to heavy metal ions. One advantage of using heavy metal ions is that the output peaks are more discreet and therefore less prone to spectral overlap. This allows more sensitivity in the measurements and allows for a greater number of simultaneous probes.

4.4.5 Immunohistochemistry

Immunohistochemistry uses antibodies to visualize targets on a tissue level [17]. There are various techniques for staining tissues, but the principle is that antibodies specific for an extracellular protein or protein complex are used on a tissue sample. The antibodies contain either radiative or fluorescent conjugates. The stained tissue is then visualized to give a visual representation of the target in the tissue.

4.5 Clinical Uses of Monoclonal Antibodies

mAbs can be applied in various areas of medicine. mAbs are capable of recognizing specific molecules present on the cell surface or in body fluids, affecting their function without affecting the remaining cells of the organism. Therefore they can mark or destroy tumor cells, inactivate enzymes, stimulate or turn off receptors, activate and reduce physiologic functions, and stop pathologic processes. mAbs have significant application in oncology, the treatment of autoimmune diseases, and transplant rejection. They can act as markers for diagnostic tests and as scientific research tools in medicine. The following section is devoted to providing an overview of some Food and Drug Administration (FDA)-approved mAbs used to treat diseases in the fields of clinical immunology, dermatology, hematology, and rheumatology (Table 4.2).

4.5.1 Alemtuzumab

Alemtuzumab is a humanized mAb specific for CD52. The CD52 glycoprotein is expressed on the surface of B- and T-lymphocytes and is not present on progenitors, monocytes, and granulocytes [18]. Two forms of Alemtuzumab are approved: murine and humanized. In vitro studies suggest that alemtuzumab mechanisms of action are mediated through antibody-dependent cell-mediated cytotoxicity, the complement pathway, as well as direct induction of apoptosis [19,20]. Whereas in vitro studies have suggested possible mechanisms, in vivo mechanisms for therapeutic effects remain to be elucidated. The specificity of CD52 to B-cells and T-cells as well as the commonality between the two allow CD52 to be an ideal target for decreasing B-cell and T-cell counts when they are too high, such as in malignancies. Alemtuzumab is currently used to treat hematologic malignancies, such as chronic lymphocytic leukemia, cutaneous T-cell

Table 4.2 Food and Drug Administration–Approved Monoclonal Antibodies

Nonproprietary Name	Trade Name	Target	Format	Indication First Approved or Reviewed	EU year	US year
Adalimumab	Humira	TNF	Human IgGl	Rheumatoid arthritis	2003	2002
Ado-trastuzumabemtansine	Kadcyla	HER2	Humanized IgGl £	Breast cancer	2013	2013
Alemtuzumab	Lemtrada	CD52	Humanized IgGl	Multiple sclerosis	2013	2014
Alirocumab	Praluent	PCSK9	Human IgGl	High cholesterol	In review	In review
Basiliximab	Simulect	IL-2R	Chimeric IgGl	Prevention of kidney transplant rejection	1998	1998
Belimumab	Benlysta	BLyS	Human IgGl	Systemic lupus erythematosus	2011	2011
Bevacizumab	Avastin	VEGF	Humanized IgGl	Colorectal cancer	2005	2004
Blinatumomab	Blincyto	CD 19, CD3	Murine β	Acute lymphoblastic leukemia	In review	2014
Brentuximab vedotin	Adcetris	CD30	Chimeric IgGl £	Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2012	2011
Canakinumab	Ilaris	IL-1β	Human IgGl	Muckle–Wells syndrome	2009	2009
Catumaxomab	Removab	EPCAM/CD3	Rat/mouse bs mAb	Malignant ascites	2009	NA
Certolizumab pegol	Cimzia	TNF	Humanized Fab PEG	Crohn disease	2009	2008
Cetuximab	Erbitux	EGFR	Chimeric IgGl	Colorectal cancer	2004	2004
Denosumab	Prolia	RANK-L	Human IgG2	Bone loss	2010	2010
Dinutuximab	Unituxin	GD2	Chimeric IgGl	Neuroblastoma	In review	2015
Eculizumab	Soliris	C5	Humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2007	2007
Evolocumab	Repatha	PCSK9	Human IgG2	High cholesterol	In review	In review
Golimumab	Simponi	TNF	Human IgGl	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009	2009
Ibritumomab tiuxetan	Zevalin	CD20	Murine IgGl	Non-Hodgkin lymphoma	2004	2002
Idarucizumab	(Pending)	Dabigatran	Humanized Fab	Reversal of dabigatran-induced anticoagulation	In review	In review
Infliximab	Remicade	TNF	Chimeric IgGl	Crohn disease	1999	1998
Ipilimumab	Yervoy	CTLA-4	Human IgGl	Metastatic melanoma	2011	2011
Mepolizumab	(Pending)	IL-5	Humanized IgGl	Severe eosinophilic asthma	In review	In review
Natalizumab	Tysabri	a4 integrin	Humanized IgG4	Multiple sclerosis	2006	2004
Necitumumab	(Pending)	EGFR	Human IgGl	Non-small cell lung cancer	In review	In review
Nivolumab	Opdivo	PD1	Human IgG4	Melanoma	In review	2014
Obinutuzumab	Gazyva	CD20	Humanized IgGl§	Chronic lymphocytic leukemia	2014	2013

Continued

Table 4.2 Food and Drug Administration—Approved Monoclonal Antibodies—cont'd

Nonproprietary Name	Trade Name	Target	Format	Indication First Approved or Reviewed	EU year	US year
Ofatumumab	Arzerra	CD20	Human IgGl	Chronic lymphocytic leukemia	2010	2009
Omalizumab	Xolair	IsE	Humanized IgGl	Asthma	2005	2003
Palivizumab	Synagis	RSV	Humanized IgGl	Prevention of respiratory syncytial virus infection	1999	1998
Panitumumab	Vectibix	EGFR	Human IgG2	Colorectal cancer	2007	2006
Pembrolizumab	Keytruda	PD1	Humanized IgG4	Melanoma	In review	2014
Pertuzumab	Peijeta	HER2	Humanized IgGl	Breast cancer	2013	2012
Ramucirumab	Cyramza	VEGFR2	Human IgGl	Gastric cancer	2014	2014
Ranibizumab	Lucent is	VEGF	Humanized IgGl	Fab macular degeneration	2007	2006
Raxibacumab	(Pending)	B. anthrasis PA	Human IgGl	Anthrax infection	NA	2012
Rituximab	MabThera, Rituxan	CD20	Chimeric IgGl	Non-Hodgkin lymphoma	1998	1997
Secukinumab	Cosentyx	IL-17a	Human IgGl	Psoriasis	2015	2015
Siltuximab	Sylvant	IL-6	Chimeric IgGl	Castleman disease	2014	2014
Tocilizumab	RoActemra, Actemra	IL-6R	Humanized IgGl	Rheumatoid arthritis	2009	2010
Trastuzumab	Herceptin	HER2	Humanized IgGl	Breast cancer	2000	1998
Ustekinumab	Stelara	IL-12/23	Human IgGl	Psoriasis	2009	2009
Vedolizumab	Entyvio	$\alpha 4\beta 7$ integrin	Humanized IgGl	Ulcerative colitis, Crohn disease	2014	2014
Alemtuzumab	Campath-1H	CD52	Humanized IgGl	Chronic myeloid leukemia	2001	2001
Daclizumab	Zenapax	IL-2R	Humanized IgGl	Prevention of kidney transplant rejection	1999	1997
Efalizumab	Raptiva	CD11a	Humanized IgGl	Psoriasis	2004	2003
Gemtuzumab ozogamicin	Mylotarg	CD33	Humanized IgG4	Acute myeloid leukemia	NA	2000
Murom on ab-CD 3	Orthoclone Okt3	CD3	Murine IgG2a	Reversal of kidney transplant rejection	1986	1986
Tositumomab-1131	Bexxar	CD20	Murine IgG2a	Non-Hodgkin lymphoma	NA	2003

β, bispecific tandem scFv; *bs*, bispecific; *EGFR*, epidermal growth factor receptor; *EU year*, first European Union approval year; *Fab*, fragment antigen binding; *Ig*, immunoglobulin; *IL*, interleukin; *NA*, not approved; £, immunoconjugate; *PEG*, pegylated; *TNF*, tumor necrosis factor; *US year*, first US approval year; *VEGF*, vascular endothelial growth factor; *WD*, withdrawn from market; §, glycoengineered.

Modified from: J.M. Reichert, Ph.D., Reichert Biotechnology Consulting LLC; table updated March 19, 2015.

lymphoma (CTL), and T-cell lymphomas. The murine (rat) form of Alemtuzumab, marketed as Campath 1G, has been used extensively in allogeneic transplant bone marrow in minimizing rejection.

The main side effects are fever, hypotension, tremors, skin rash, and dyspnea, most common in the first weeks of drug infusion. Significant hematologic toxicity and immunosuppressive clinical features are observed. Alemtuzumab induces profound lymphopenia, with wide range of infections opportunistic observed in patients who are using this drug. There are reports of severe and prolonged myelosuppression and, in some studies, aplasia and hypoplasia bone marrow, even in the recommended dose. The incidence of these complications is greater with increasing doses. Serious autoimmune anemia and thrombocytopenia have also been reported.

4.5.2 Anti-CD20: Rituximab and Ibritumomab Tiuxetan

Rituximab is a chimeric monoclonal IgG1 antibody with specificity for CD20. The variable region of a murine antibody specific to CD20 is fused with a constant human κ light chain and heavy chain. The antibody heavy chain has been selected to induce antibody-dependent cytotoxicity. CD20 is a transmembrane protein located on pre–B-lymphocytes and mature B-lymphocytes, but not on stem cells, pro-B cells, normal plasma cells, or other normal tissues. The CD20 antigen is present in more than 95% B-cell non-Hodgkin lymphomas (NHLs), which makes it an ideal target for identifying cancerous cells. The mechanisms of rituximab in fighting NHL are thought to be through direct signaling, cytotoxicity complement-dependent cellular cytotoxicity (CDCC), and antibody-dependent cytotoxicity (ADCC) [21,22].

Significant indirect results suggested that rituximab has direct immune system–independent cytotoxic effects. Rituximab binding has been theorized to induce apoptotic cellular signals through mechanisms such as lipid rafts, Src-family kinases, and caspases [23–25]. Rituximab immune-mediated cytotoxic effects are carried out through its Fc region. Two main effects are CDCC and ADCC. After antibodies have tagged an antigen, through their Fc region, they induce complement fixation that activates the complement pathway [26]. ADCC uses secondary immune cells to eliminate targets tabbed by antibodies [27]. Cells such as macrophages, natural killer (NK) cells, and eosinophils have Fc receptors that can recognize a tagged cell. Once recognized, they perform downstream functions that destroy the tagged cell.

Rituximab was the first unconjugated antibody approved by FDA in 1997 for the treatment of relapsed lymphomas. In 2002, it became the main anticancer drug in the world. It is indicated for cases of lymphoma and non-Hodgkin low-grade and follicular CD20. It is effective when combined with chemotherapy and has demonstrated promise in long-term response in treating aggressive lymphoma. The combination with cyclophosphamide–hydroxydaunorubicin–oncovin–prednisone (CHOP) therapy increased response rates and survival. Toxic events observed with the combination are not greater or more toxic than those present in therapy isolated with CHOP.

The use of rituximab against autoimmune diseases such as rheumatoid arthritis (RA), immune thrombocytopenic purpura, autoimmune hemolytic anemia, systemic lupus erythematosus (SLE), pemphigus vulgaris, and multiple sclerosis (MS) is under investigation. The success of this mAb has prompted the development of conjugated antibodies. Ibritumomab tiuxetan (Zevalin) is a combination of ibritumomab, a CD20-specific mAb, and tiuxetan, a chelator with a radioisotope. The benefit of ibritumomab tiuxetan is that, in addition to direct signaling, CDCC, and ADCC, it is able to kill cells using the radioactive isotope, which improves its ability to eliminate target cells [28,29].

4.5.3 Anti—Tumor Necrosis Factor: Infliximab, Adalimumab, Certolizumab Pegol (Cimzia), and Golimumab

Tumor necrosis factor (TNF)α is a molecule part of the TNF family. As the name suggests, this class of molecule has the ability to induce cellular apoptosis. TNFα, the canonical TNF molecule, is highly active in host defense [30]. When TNF function is impaired, the host's ability to fight off intracellular bacteria is greatly impaired. TNFα is a potent proinflammatory cytokine with the capacity to recruit immune cells to the site of infection. For circulating immune cells to reach the site of infection, they must cross through the endothelial walls of the circulatory system, a process known as extravasation. TNFα has been shown to inhibit Fas ligand expression, an important molecule in the inhibition of leukocyte extravasation [31]. The overproduction of TNFα present in autoinflammatory diseases has been long observed [32] to be specifically associated with T-cell—mediated tissue damage [33]. Blocking TNFα biological activity carries out therapeutic effects of anti-TNFα mAbs. Two anti-TNF mAbs, infliximab and adalimumab, are currently in the market. Infliximab is a chimeric mAb (IgG_1) with specificity to TNFα. The FDA approved it in 1998 for use in patients with Crohn disease with moderate to severe activity that responded inadequately to conventional therapy. In 1999, its approval was widened for use in the treatment of RA in combination with methotrexate therapy. In 2000, its use was expanded for other inflammatory diseases. Indicated for adults with moderate to severe active RA, its use was approved for reducing signs and symptoms of the disease and inhibition of structural progression damage of joints for patients who have not obtained effective response to disease-modifying antirheumatic drugs. After its use in patients with RA, there is a rapid fall in serum acute phase inflammatory markers: C-reactive protein, erythrocyte sedimentation rate, and cytokines [interleukin (IL)-6]. The success of infliximab has prompted the creation of other anti-TNFα mAbs.

Adalimumab is a fully human mAb IgG1 that specifically binds to TNFα and neutralizes the biological function of blocking TNF. Its interaction with TNF receptors on the p55 and p75 cell surface also modulates the biological responses induced or regulated by TNF, including changes in the levels of adhesion molecules responsible for leukocyte migration (ELAM-1, VCAM-1, and ICAM-1). An advantage of adalimumab is that its fully human nature makes it less immunogenic, unlike infliximab, which is a chimeric mAb [34].

Another fully human anti-TNFα mAb is golimumab (Simponi). Because it is an entirely human mAb, it shares similar characteristics with adalimumab. In vitro studies suggest that golimumab possess a higher affinity to soluble TNFα than does adalimumab; however, this has not been tested in vivo [35]. Studies suggest that adalimumab and golimumab possess similar efficacy after indication. However, golimumab demonstrated superior outcomes for sustained outcomes [36].

Certolizumab pegol (CZP) (Cimzia) is a pegylated mAb. Unlike a classical mAbs with a variable and constant region, CZP is composed of a variable region fragment known as fragment antigen binding, with polyethylene glycol conjugated to it. The addition of polyethylene glycol increases the half-life of the antibody fragment comparable to that of unmodified mAbs [37]. CZP lacks a constant region and is therefore unable to perform ADCC or complement binding. The similar therapeutic effects of CZP compared with infliximab, adalimumab, and golimumab suggest that Fc, mediated, and complement fixation are not large contributors to the therapeutic effects of mAb therapy [38] (Fig. 4.7).

4.5.4 Belimumab

Belimumab is a human IgG1 mAb specific for the B-lymphocyte stimulator (BLyS), sometimes known as B-cell activating factor. It has been approved for use in SLE. SLE is a heterogeneous autoimmune disease with a wide array of clinical symptoms ranging from

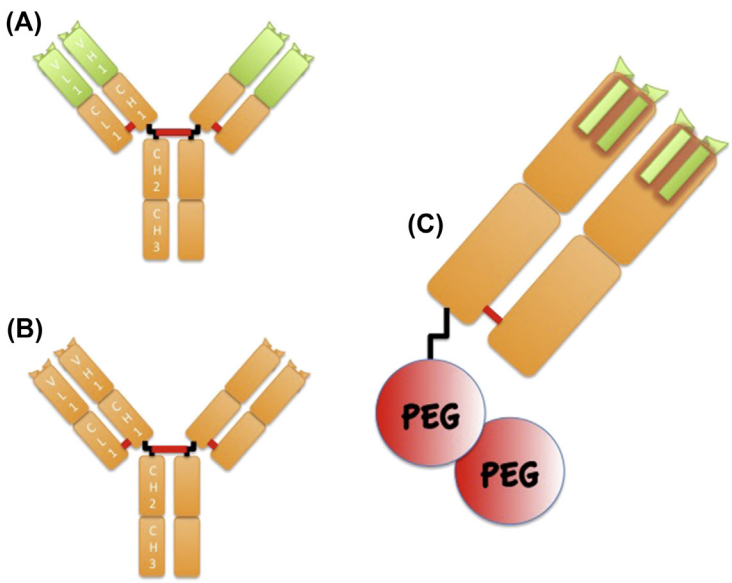

FIGURE 4.7 Three anti-TNFα biologics. Infliximab is a chimeric mAb with a murine variable region and a human constant region (A). Adalimumab is a fully human mAb (B). Certolizumab pegol is a humanized mAb fragment with two polyethylene glycol molecules attached to it (C).

fever to fatigue rash. Over time, SLE can affect major organs, resulting in renal, cardiac, pulmonary, and central nervous system toxicities. Although the pathogenesis of SLE is not well understood, there are indications that B-cell malfunction is involved. Autoantibodies, particularity anti−double-stranded DNA antibodies, are present in 70% of patients. Immune complexes are thought to contribute to the clinical manifestations seen in SLE, and higher BLyS levels are correlated with a worsening of clinical symptoms [39]. BLyS has been shown to inhibit B-cell apoptosis and promotes differentiation of B-cells into antibody-producing plasma cells. Moreover, patients with SLE have elevated BLyS levels, which makes it an ideal target when designing mAbs for patients with SLE [39,40]. By binding to the soluble form of BLyS, Belimumab prevents BLyS from binding to its receptors and inhibits its downstream functions.

4.5.5 Ipilimumab

Ipilimumab is a human IgG1 mAb specific for cytotoxic T-lymphocyte antigen 4 (CTLA 4). CTLA 4, like CD 28, binds to the CD 80:86 (B7.1:B7.2) ligands [41]. However, unlike CD28, which sends a stimulatory signal, CTLA 4 acts as an immune regulator and inhibits T-cell activation. One of the defining characteristics of cancer is its ability to hide from the immune system [42]. Normally, when cells are transformed into cancerous cells, the immune system can eliminate them early on [43]. However, the ability of cancerous cells to not induce immune activation when they proliferate uncontrollably makes it difficult for the immune system to destroy abnormal cells. By blocking CTL 4, an immune suppressant, it is thought to make the immune system more sensitive and able to find and eradicate cancerous cells. Currently, Ipilimumab is accepted for treatment of metastatic melanoma [44].

4.5.6 Mepolizumab

Mepolizumab is a humanized IgG1 mAb specific for IL5. It is approved for management of severe eosinophilic asthma. Severe eosinophilic asthma differs from day-to-day asthma symptoms in that it does not respond to typical inhalants [45]. Asthma exacerbations are associated with airway inflammation and morbidity. Mepolizumab, an IL5 mAb, had shown disappointing results in asthma treatment in three clinical trials [46−48]. However, interest emerged in the use of mepolizumab in a subset of patients with asthma who are unresponsive to prednisone treatments. These patients demonstrate a high concentration of eosinophils in their sputum [49]. Eosinophils have been thought to be implicated in severe asthma owing to their presence in postmortem examinations of those who died from an acute severe asthma attack [50].

4.5.7 Natalizumab

Natalizumab is a humanized IgG4 mAb specific for the α4 integrin molecule. It is used in the treatment of MS. MS is a demyelination disease of the central nervous system. Axonal

loss and plaques in the central nervous system characterize MS. Lymphocyte passage across the blood–brain barrier is thought to be an important factor in disease etiology [51,52]. α4β1 is an integrin glycoprotein expressed on leukocytes that interact with vascular-cell adhesion molecule 1 (VCAM-1). α4β1–VCAM-1 interactions are essential to diapedesis. Natalizumab is a humanized mAb specific for the α4 subunit of the integrin proteins (both α4β1 and α4β7). Natalizumab blocks the interaction of the integrin and the endothelial receptors, inhibiting diapedesis and in turn inflammation [53]. Randomized trials of natalizumab have demonstrated promise as a treatment for MS [54,55].

4.5.8 Omalizumab

Omalizumab is a humanized monoclonal anti-IgE antibody with a high degree of human characteristics greater than 95% (IgG1-k). It recognizes the constant region of IgE antibodies, Fcε, and prevents it from binding to the high-affinity FcεR receptors. FcR interactions can elicit various downstream effects. When these effects go awry, pathologies can occur. The ε region of IgE antibodies has been strongly correlated in asthma and allergic rhinitis. These two diseases are thought to have effects mediated through the interaction of IgE antibodies and their corresponding Fc receptors: in the case of IgE, the Fc receptor is Fcε. When IgE constant region is recognized by the high -affinity Fcε receptor, on cells such as mast cells, and neutrophils, there is a surge of activity that is implicated in this pathogenesis. The importance of IgE antibodies in disease pathology has prompted the creation of anti-IgE mAbs. By preventing IgE antibodies from being recognized by Fcε receptors, omalizumab prevents the release of inflammatory mediators from mast cells and basophils such as histamine, leukotrienes, and cytokines. Omalizumab prevents asthma attacks after provocation test by inhalation allergens in both immediate and delayed response in patients with the mild allergic disease. Other applications have been suggested. Effective use in atopic dermatitis has been reported.

Omalizumab can reduce asthma exacerbations in patients with moderate and severe stages of the disease. It can reduce symptoms and improve parameters of pulmonary function, as well as reduce necessary doses of corticosteroids and beta-agonists, resulting in improvement in the quality of life for patients. In patients with eosinophilia, omalizumab has been shown to improve numbers to a clinical level. In patients with seasonal allergic rhinitis, omalizumab has been shown to decrease nasal and ocular symptoms as well as reduce the need for drugs such as antihistamines.

Administration of omalizumab can be done intravenously or subcutaneously. After administration, there is a rapid dose-dependent drop in serum free IgE (>99%). The blockage of IgE–FcεR interaction results in a down-regulation of high-affinity FCεR1 expression and subsequent release of histamine receptor by antigen-stimulated basophils. The dosage of anti-IgE needed must be individualized according to the total IgE levels and the weight of each patient. The formation anti-IgE–IgE complexes results in the disappearance of circulating free IgE. Because these complexes cannot bind the IgE

receptors, they are devoid of IgE-specific biological activity. Owing to antibody humanization and the small=size anti-IgE−IgE complex, omalizumab does not trigger autoimmune diseases because these complexes are unable to activate the complement system.

4.5.9 Programmed Death 1 Blockers: Nivolumab and Pembrolizumab

Like CTLA 4, programmed death 1 (PD1) inhibits immune activation. Its ligand, PD-L1, is a B7 family ligand. However, unlike B7, it cannot interact with CD28 and CTLA4 [56]. PD-L1 has been shown to be up-regulated in various cancer tissues [57], one system where the PD-L1 blockade has shown promise is in melanoma [58,59]. Nivolumab is a human IgG4 mAb specific for PD1 [60]. It is approved for management of melanoma. By blocking PD1, nivolumab blocks the PD-L1−PD1 interaction, and dampens immune inhibition by PD-L1 on melanoma cells. It is hypothesized that blocking PD1 melanoma cells are no longer able to avoid immune surveillance. Therefore, the immune system can destroy the malignant cells. Although monotherapy with nivolumab has shown promise, combination therapy of nivolumab with ipilimumab shows strong results displaying rapid tumor progression [61]. Pembrolizumab is a humanized IgG4 mAb with specificity for PD1 [62]. Pembrolizumab is an approved mAb under the FDA's fast-track program. It showed promise in patients for whom nivolumab therapy had failed.

4.5.10 Secukinumab

Secukinumab is a human IgG1 mAb specific for IL17A. Psoriasis etiology is poorly understood. Beyond IL12 and IL23, IL17 has been implicated in psoriasis [63,64]. IL17A is a proinflammatory cytokine essential for microbial pathogen defense. It is released primarily by a subset of CD4 cells known as Th17 cells [65,66]. IL17 has been shown to interact directly with keratinocytes to stimulate the release of molecules that are elevated in psoriasis [67]. Secukinumab, an mAb specific for IL17A, is an approved mAb drug that has shown promise in the treatment of moderate to severe psoriasis [68−70]. By binding to IL17A, secukinumab can inhibit the interaction of IL17A with its receptors and reduce its immunologic functions.

4.5.11 Tocilizumab

Tocilizumab is a humanized mAb specific for the IL6 receptor. It is indicated for treatment of RA. IL6 is a potent proinflammatory cytokine affecting leukocytes, neutrophils, and osteoclasts [71]. IL6-activated cells are found in high numbers in RA. Beyond affecting immune cells, IL6 is a main player in activating the acute phase reaction, a feature correlated with disease activity in RA [72]. The presence of IL6 effects in RA has made blocking the IL6 receptor an attractive candidate in the treatment of RA. Tocilizumab has shown promise as an effective treatment of RA in randomized control trials [73], and has even shown to be more efficient as a monotherapy compared with the

standard methotrexate regiment [74]. A systematic review of the literature has shown support for the efficacy of tocilizumab as a therapeutic agent for RA [75].

4.5.12 Ustekinumab

Ustekinumab is a human IgG1 mAb specific for the p40 subunit common to IL12 and IL23. It is used to treat psoriasis. Psoriasis is a common chronic skin autoimmune disease. It is characterized by a thickening of the epidermal layer caused by the proliferation of keratinocytes [76]. Skin plaques, a common manifestation, can be found in the elbows, knees, umbilical, and cosmetically sensitive areas such as the face, feet, hands, scalp, and genitalia [77]. Beyond the dermatologic manifestations, psoriasis is associated with comorbidities such as depression, cardiovascular disease, Crohn disease, obesity, and psoriatic arthritis [78]. Although the etiology of psoriasis is not understood, elevated cytokines levels have been highly correlated with disease severity [64]. Beyond cytokine levels, the T-helper subtype 1 (Th1) is commonly seen in psoriatic lesions [79,80]. One of the main cytokines driving a Th1 phenotype is IL12 [81]. IL12 is a heterodimer protein composed of IL12A and IL12B, released by antigen-presenting cells in response to antigen presentation [82]. IL12 is joined by two disulfide bonds that are glycosylated, termed p35 and p40. IL12 activates NK cells and has been shown to polarize CD4 response to a Th1 response. IL23 is another heterodimer protein that shares the same p40. IL23 has demonstrated activity on NK cells as well as T-cells [83]. Ustekinumab has shown significant promise in a double-blind randomized controlled trial at reducing signs and symptoms of psoriasis [84]. A long-term follow-up of 1500 patients treated for over 4 years demonstrated safety and tolerability [85].

4.5.13 Vedolizumab

Vedolizumab is a humanized IgG1 mAb specific to $\alpha 4\beta 7$ integrin protein. It is used in the treatment of ulcerative colitis (UC), and Crohn disease. $\alpha 4\beta 7$ is an integrin glycol protein expressed on T- and B-cells [86] that binds to mucosal addressin-cell adhesion molecule-1 (MAdCAM-1) [87], which is found on intestinal vasculature [88] and is highly expressed in an inflamed gut tissue. The interaction of integrin and MAdCAM-1 is essential to leukocyte migration into the gut [89]. UC is a chronic form of an inflammatory bowel disease characterized by abdominal cramps and bloody diarrhea [90]. One proposed theory for the pathogenesis of UC is thought to be an overactive T-cell population [91]. In theory, by blocking T-cell diapedesis into the gut, UC symptoms should subside, which makes MAdCAM-1—integrin interactions an attractive candidate. Targeted therapy for $\alpha 4\beta 7$ has been shown to be effective as an acute treatment for UC [92] as well as for long-term maintenance [93]. Crohn disease is another form of an inflammatory disease of the intestine. It is characterized by thickening of the colon wall, with inflammation localized to patches anywhere in the digestive track, unlike UC, in which inflammation is largely continuous in the large intestine [94]. An overactive T-cell population is thought to underlie the etiology of both Crohn disease and UC [95].

Vedolizumab has shown efficacy in the induction and maintenance therapy for Crohn disease in randomized control trials [96].

4.6 Conclusion and Perspectives

The development of mAbs by genetic engineering has provided new treatment to numerous diseases. The ability of mAbs to bind to a predetermined epitope stimulated the development of these new drugs. The complexity of the molecule made of polypeptide chains with large−molecular weight domains associated with multifunctionality was the initial hurdle to their production and use. The mouse origin and the difficulty in selecting the appropriate target structures on the cell surface can be added to the list of initial difficulties in the development of mAbs. The replacement of most murine sequences with equivalent human sequences, a concomitant decrease in immunogenicity, and identifying cell surface components of mAbs have become valuable resources in the treatment of infectious, inflammatory, autoimmune, and neoplastic diseases. In addition, mAbs have significant application as markers for diagnostic tests and scientific research tools in medicine.

Continued success in the development of therapy-based antibodies will require extensive clinical research detailing how to use these compounds properly, which patients will benefit, and long-term side effects. Immunologic research also benefits because it will reveal important aspects of the mechanisms' basic action of antibodies and the fundamental immunologic mechanisms involved.

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5

Recombinant Antibodies: Trends for Standardized Immunological Probes and Drugs

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5.1 Introduction

In 1975, G. Kohler and C. Milstein (Nobel Prize in Medicine, 1984) described hybridoma technology, which consists of immortalizing antibody-secreting cells [1]. Fairly simple to achieve with mouse or rat plasma cells, this technology has led to the selection of a multitude of monoclonal antibodies directed against various antigens. Soon after that, a wide range of applications have emerged. Every month and every week has seen new monoclonal antibodies appearing with well-established properties including high antigen-specificity and that can be produced continuously in a stable form [2]. These antibodies have led to the identification of cell markers (CD). They also have contributed to the understanding of the structure–function relationship of complex proteins and to elucidating the mechanisms of interaction between biomolecules. The immunoassay sector has greatly taken great advantage of this discovery by developing robust immuno reagents (Fig. 5.1). On July 8, 2015, a simple search for the term "immunoassay" in the PubMed database revealed more than 4.5×10^5 original articles. The market of "immunodiagnostic" represents today $1.6 billion with over two million antibodies sold for research by more than 300 companies [3].

However, the use of these monoclonal antibodies of animal origin has been a dramatic failure for therapeutic applications in humans [4,5]. Only three antibodies have been approved by the Food and Drug Administration (FDA): an anti-CD3 IgG (Orthoclone OKT3; Muromomab), which is used in the prevention of acute allograft rejection, and also two radiolabeled anti-CD20 antibodies (Bexxar, ^{131}I-Tositumomab; and Zevalin, ^{111}In and ^{90}Y Ibritumomab) prescribed as second-line in the treatment of particular lymphomas. This setback has several origins [6]. Indeed, serious reactions including fatal allergic ones

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1960: First radioimmunoassay (RIA) of circulating hormones. The principle comprises precipitation of plasmatic hormone, using polyclonal antibodies in competition with a low concentration of radiolabeled hormone. These tests are sensitive but long (24 hours).

1971: First sandwich immunoassays in which the target antigen is captured by an antibody immobilised on a plastic surface. The immune complexes formed are detected by a secondary antibody chemically coupled to an enzyme (peroxidase, phosphatase alkaline β-galactosidase).

1975: The hybridoma technology will revolutionise the immunoassay sector. It becomes possible to immortalise rodent cells able to secrete a monoclonal antibody unique. Thousands of murine monoclonal antibodies are thus produced against antigens. Simultaneously, new materials are developed to immobilise antibodies (microtiter plates, beads). Assays are simplified, become faster and allow the simultaneous analysis of several biological samples.

1980-1990: The methods consisting in coupling antibodies to non-isotopic tracers generalise and become more effective. New luminescent or fluorescent tracers amplify signals and contribute to increase the sensitivity of the assays. They offer many advantages over isotopes. In the 1990s, these immunoassay techniques are automated and quickly spread

Early 2000: New formats mono- or multivalent antibodies, mono- or bispecific are created by molecular engineering. Directed evolution methods modulate the specificity of these antibodies and adapt to highly specific immunoassays. The technology of phage antibody enables the selection of specific antibodies *In vitro* against any antigen without recourse to prior immunisation of animals.

2015: More than 30 recombinant antibodies have been approved for pharmacological treatments. The rapid growth in demand for therapeutic antibody production has been well accommodated by the industrialization of antibody manufacturing. However, the areas of immunoassay and diagnostic have not yet experienced the same growth.

FIGURE 5.1 Antibody and immunoassay over time.

have occurred during or after administration of these molecules owing to their xenogenic nature. In addition, it is now well established that the effector function of the mouse antibody Fc domain is not optimal in humans. Finally, the mouse antibody half-life in human plasma may be too short for pharmacologic effects.

In this context, the past 30 years have seen continuous and progressive innovations in the design of new antibody formats and in vitro selection and maturation of high-affinity antibody fragments specific for any sort of antigens. Complementary DNA (cDNA) cloning and protein expression in recombinant host have enabled us to create new formats of antigen-binding molecules including single-chain antibody fragment (scFv), which are simply made of the light chain variable domain fused via a short peptide linker to the heavy chain variable domain of an antibody. scFvs can be used to design more sophisticated antibody formats often better adapted to specific uses than conventional antibodies [7,8]. In particular, they are templing tools for antibody drug designers because their small size enables the generation of fusion proteins without limiting tissue penetration and with pharmacologic properties optimized for specific applications. Owing to their small size, they have higher tissue permeability, do not elicit Fc-associated cytotoxicity, and still exhibit antigen-binding properties. In addition, combinatorial scFv-phage libraries have been created [9]. They allow in vitro selection of antibody fragments against any antigen. Finally, mice have been genetically engineered to produce fully human antibodies [10]. These transgenic mice are now used as platforms for the discovery of high-affinity antibodies with inherent qualities for successful drug development [11] (Fig. 5.2).

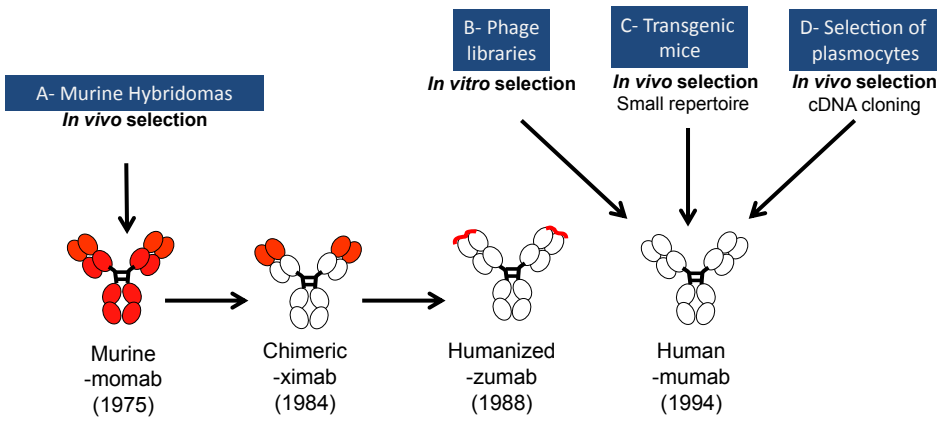

FIGURE 5.2 Design of therapeutic antibodies: a long quest for more humanity. Therapeutic antibodies can be produced after reengineering murine monoclonal antibodies, whose domains are represented in red (dark grey in print versions) (A), leading to chimeric or humanized antibodies that are less immunogenic, while preserving antigen-binding properties (antibody domains from human origin are represented in white). Alternatively, in vitro panning of phage libraries displaying antibody fragments followed by fusion with human antibody constant domains allows in vitro selection of fully human antibodies (B). In vivo selection of fully human antibodies can also be performed by using transgenic mice (C) or single peripheral blood B-cells and cDNA cloning (D). The suffix used in the international nonproprietary name (INN) for murine, chimeric, humanized, and fully human antibodies are indicated as well as the year of first approval.

Thirty-six antibodies have been marketed in the United States and Europe for therapeutic applications (January 2015). The FDA approved seven in the past year and no slowdown is expected. In 2014, global sales of all approved therapeutic antibodies were US $78 billion. Sixteen antibodies achieved blockbuster status, each generating a net annual turnover higher than US $1 billion. In addition, five launches nearly reached sales of US $1 billion in 2015 year [12].

In the immunodiagnostic sector, inputs of these recombinant technologies are still modest, probably because conventional poly- or monoclonal antibodies all meet current needs. Their immunogenicity is not a drawback for in vitro use and their production is cheap. However, recombinant DNA technologies may offer new resources and suggest the design of innovative reagents with even unsuspected scope.

5.2 Natural Antibodies: Structure and Applications

In response to the injection of a foreign antigen, vertebrates produce antibodies, which are proteins secreted by plasma cells, and that serve as effectors of humoral immunity. These antibodies are usually heterogeneous, with each B-cell clone producing a monoclonal antibody that recognizes a particular structural motif (epitope). All of these monoclonal antibodies are part of the polyclonal antibody serum.

The structure of an antibody such as immunoglobulin G (IgG) is well understood from early studies carried out by R. Porter and G. Edelman (Nobel Prize in Medicine, 1972) between 1950 and 1960 [13]. IgG is an Ig monomer consisting of four polypeptide chains: two identical polypeptide chains called heavy (H) of approximately 50 kDa, and two identical light chains (L) (25 kDa) (Fig. 5.3). Disulfide bonds, in a "Y" configuration, join the four chains in which the light chains bracket the heavy chains, starting at the mouth of the "Y" and continuing through the variable region. The nature of the heavy chain (γ, μ, α, δ, and ε) determines the "class" of the antibody as IgG, IgM, IgA IgD, or IgE, respectively. Differences in structures and functions in the constant domains of the heavy chains distinguish some subclasses among them (e.g., $\gamma 1-4$ for human IgGs). The light chains consist of two types (kappa or lambda).

Within an IgG, each heavy polypeptide chain (H) or light (L) is organized into domains: a variable domain of about 110 residues at the N-terminus of the light (VL) and heavy (VH) chains, combined with a constant domain (CL) for the light chain or three constant domains for the heavy chain (CH1, CH2, and CH3). In each heavy chain, peptides join the CH1 and CH2 domains. Disulfide bonds between the two peptides create a structure called the hinge region, which gives the molecule great flexibility and the ability to link: for example, two epitopes that are in opposite directions relative to the other [14].

The association of the variable domains VH and VL forms the binding site of an antibody to its target antigen. The sequence variability of the V domains is not equally distributed. There are four regions called framework regions or frameworks (FRs) in which well-conserved residues determine the domain structure (Fig. 5.3). Between the

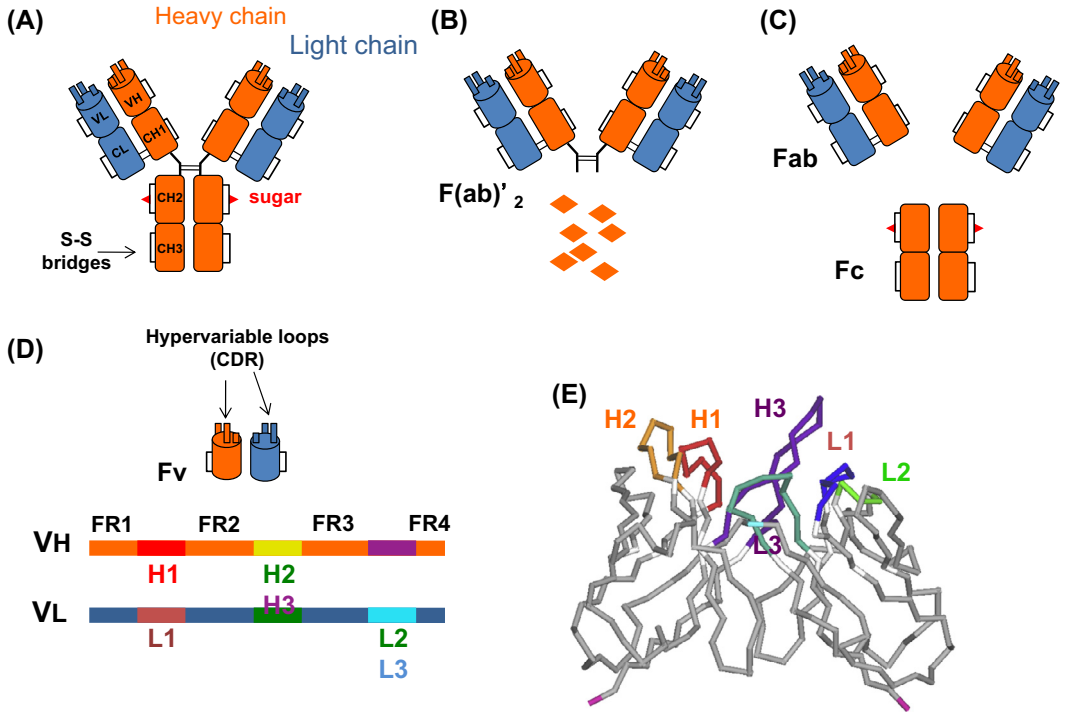

FIGURE 5.3 Immunoglobulin G and functional fragments. (A) Monomeric antibody (IgG) made of two light chains and two heavy chains each organized in domains. (B) Bivalent fragment $F(ab')_2$ obtained after limited enzymatic digestion with pepsin. (C) Monovalent Fabs and Fc fragments obtained after limited enzymatic digestion with papain. (D) Fv variable fragment consisting of the VH and VL domain. Each variable domain is organized in three FRs and three hypervariable loops or CDRs. (E) Three-dimensional structure of the Fv. Hypervariable loops L1, L2, and L3, and H1, H2, and H3 constitute a pocket (paratope) that interacts with the target antigen.

FRs of the same area, there are three "loops" held together in close proximity, also called hypervariable loops or "CDRs," owing to the variability of their particular sequence. The spatial approximation of the loops of both V-domains (six loops) is the paratope, which interacts with the epitope of the target antigen. Despite their high sequence variability, five of the six loops adopt just a small repertoire of main-chain conformations, called "canonical structures" [15]. These conformations are first determined by the length of the loops and second by the presence of key residues at certain positions in the loops and in the FRs that determine the conformation through their packing, hydrogen bonding, or the ability to assume unusual main-chain conformations.

The idea of using antibodies outside the natural humoral immune response was born even before their formal identification. First, von Behring, the first Nobel Prize in Medicine (1901), and Kitasato discovered the concept of serum therapy: that is to say, the treatment of diseases by the injection of blood serum from immune animals [16]. The first therapeutic serum was marketed in the early 1900s for the treatment of infectious diseases (diphtheria and tetanus), and soon after that for the specific treatment

of envenomings. Expansion of antibodies as immunoassay tools or diagnostic in clinical biology began in the 1960s with the polyclonal antibodies obtained by immunization animals (rabbits, goats, and sheep) against antigens of human origin. Even if successful, the batch-to-batch reproducibility of polyclonal antibody preparation is a major drawback to their use.

The possibility of producing an infinite homogeneous population of antibodies (monoclonal antibodies) by hybridoma technology was then used in the 1980s to generate an original murine antibody with constant and well-defined specificity and affinity [1].

Today, hybridomas and monoclonal antibodies have become an industrial product, and new concepts and technologies have emerged in biological sciences, in both research and in vitro diagnostics. Thus, immunocytometry has become a key diagnostic tool for blood cell differential analysis combining morphologic characteristics (size and relative granularity) with the presence of immunophenotypic markers. The same antibodies are also exploited in immunohistochemistry to label a particular antigen in tissue. The enzyme immunoassays that use antibodies coupled to a colorimetric enzyme also experienced an extraordinary expansion that is the result of their high sensitivity, the need for simple apparatus, and the possibility of being robotic.

The scope of monoclonal antibodies is wide [17]. However, there are situations in which they are not suited. In the field of in vivo diagnosis, as in therapy, murine monoclonal antibodies will be quickly recognized as a foreign substance by the patient's immune system. The presence of an Fc fragment, which has no role in antigen recognition, and effector functions, which are not effective in humans, is a major drawback. The fragments derived from the proteolysis of antibodies [Fab and $F(ab')_2$] as well as IgG (150 kDa) always include constant domains unnecessary in the recognition of the antigen. Their molecular weight remains high, limiting their ability to diffuse into tissues. Finally, the functional qualities of these antibodies are limited by the immune response of the animal that produced them and cannot be changed, altered, or enhanced [18].

5.3 Recombinant Antibodies: Wide Range of Alternative Antibody Fragments

Since the early 1990s, the development of molecular cloning techniques and the discovery of polymerase chain reaction have given new insights that led to the identification of antibody genes and their expression by genetically modified organisms. These technologies rapidly led to a wide variety of pieces that can be adapted to new applications (Fig. 5.4) [19].

5.3.1 Simple Monovalent Antibody Fragments

The variable fragment (Fv) of an antibody simply consists of the V-domains of light and heavy chains. Fvs are the minimal antigen-binding structure derived from antibodies.

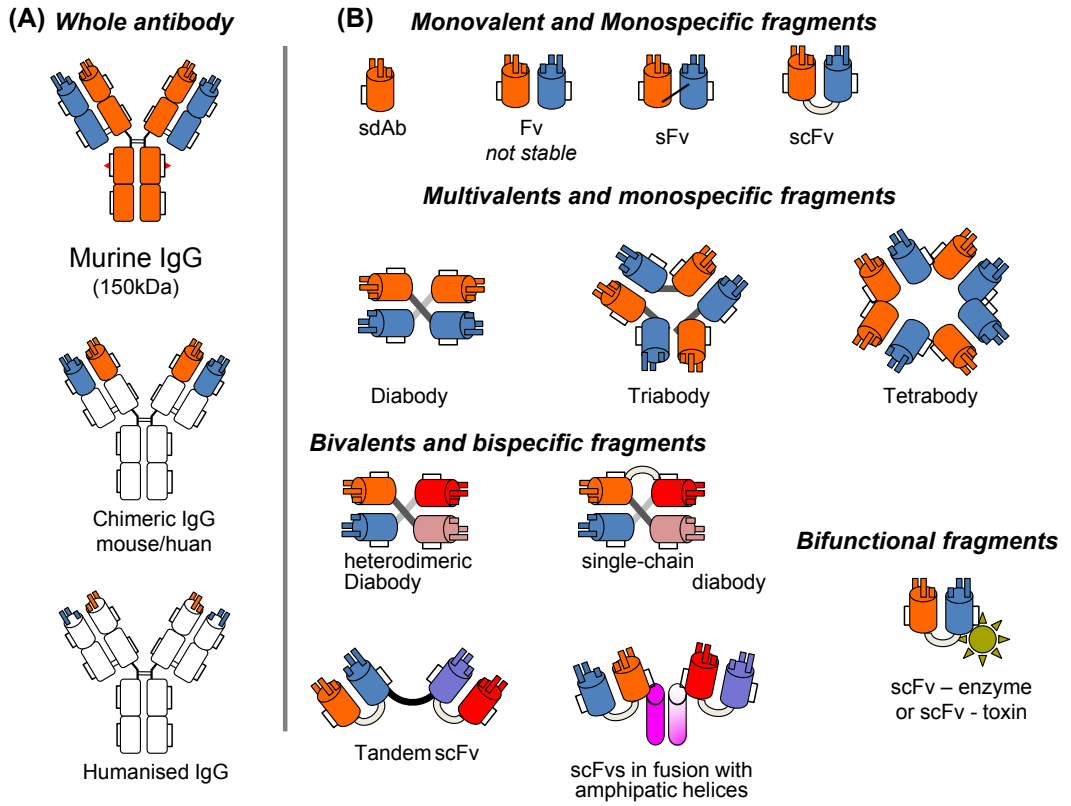

FIGURE 5.4 Recombinant antibodies: unedited structures for new applications. (A) Molecular engineering allows designing chimeric antibodies in which domains of human origins substitute for murine constant domains. In humanized antibodies, only the hypervariable loops are of murine origin. (B) Various formats consisting in antibody variable domains can also be created. These include monovalent and monospecific fragments, multivalent and monospecific fragments, bivalent, and bispecific fragments, and finally, bifunctional fragments.

However, these Fvs have rarely been used. They are not obtainable after limited proteolysis of whole antibodies. In addition, they are not stable owing to the lack of a covalent bond between the two antibody domains. Therefore, new monovalent antibody structures were invented. The first single-chain Fv was produced with Cys residues introduced by site-directed mutagenesis into each of the variable domains to induce disulfide cross-domain without altering the specificity of antigen recognition. Today, far more common are scFvs in which the variable domains of an antibody are produced by genetically modified organisms in the form of a fusion protein in which they are joined together by a peptide bond [20]. These molecules were created to facilitate phage display, where it is highly convenient to express the antigen-binding domain as a single peptide. As an alternative, scFv can be created directly from subcloned heavy and light chains derived from a hybridoma. The sequence and length of the peptide linker have to be adapted to balance the stability and functionality of the structure. The linker is usually

rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. Thus, the scFv represents the basic module from which it is possible to create monovalent or original multivalent antibody formats. It can be produced free or as a fusion protein suitable for a wide range of therapeutic or diagnostic applications.

Some single monomeric variable domains (VL or VH), also called single-domain antibodies (sdAb), have been reported with specific ability to bind to antigen [21]. In addition, new antibody classes consisting of heavy-chain homodimers with no associated light chains have been discovered in camelids and cartilaginous fishes. In both cases, the antigen binding is encapsulated within a single immunoglobulin domain 13−14 kDa in size, also called V_HH (in camelids) or VNAR (in sharks) containing three or two hypervariable loops, respectively [22,23]. These molecules have been shown to be just as specific as a regular antibody, and in some cases they are more robust. They show great structural similarity to the human VH and are easy to transform into bacterial cells for bulk production, which makes them ideal for research purposes.

5.3.2 Fragments of Monospecific Multivalent Antibody

In the scFv molecule, the length and flexibility of the peptide bridging both V-domains are important from a structural and functional point of view. Thus, substituting the pentadecapeptide $(Gly_4Ser)_3$ for a pentapeptide (Gly_4Ser) promotes the formation of dimers in which the VH domain of each subunit joins the VL domain of the other, thus leading to structures called "diabody," monospecific but bivalent, and whose size is equivalent to that of a Fab (50 kDa) [24]. Diabodies have been shown to have dissociation constants up to 40-fold lower than corresponding scFvs, meaning that they have a much higher apparent affinity to their target.

Reducing the linker to a single residue or eliminating it leads to trimeric, trivalent ("triabody") or tetramers, tetravalent ("tetrabody"), with the main consequence being an even higher avidity for the target antigen. Functional analysis in preclinical studies also showed a better retention in target tissues, and lower systemic clearance.

5.3.3 Bispecific Antibody Fragments

The idea to develop bispecific antibodies capable of binding different cells or molecules with potential applications, particularly in the field of cancer immunotherapy, dates back to 1980. The methods described at the time consisted mainly of developing hybrids hybridoma or chemically coupling two Fab derived from the proteolysis of different monoclonal antibodies, but they all experienced failure. However, molecular engineering has allowed the main difficulties to be bypassed [25]. It gives the ability to create bispecific antibody fragments consisting only of the variable domains of two different antibodies. Several arrangements are possible: heterodimeric diabody in which one of the subunits is composed of VH of a first antibody associated with the VL of the second antibody with a different antigen-binding specificity. The second subunit consists of the

VH of the second antibody associated with the VL of the first antibody. Such a structure is compact and rigid. Much more flexible are tandem-scFvs consisting in two scFv associated together with a short peptide bond. Other techniques involve molecular domains that have a propensity to self-assemble. Thus scFv can be produced by fusion with amphipathic α helices that are coiled together like the strand of a rope or constant domains of antibodies that, by associating with their partner, will lead to bivalent molecules.

5.3.4 Bifunctional Antibody Fragments or Fusion Proteins

Most immunoassays performed make use of antibody molecules previously chemically conjugated to a tracer (a protein or an enzyme). The chemical reactions employed involve nonspecific cross-linking agents, which lead to immunoconjugates whose structure is heterogeneous and tends to aggregate, leading to altered functional properties of one or both partners. For instance, luciferase has never been efficiently coupled to an antibody although its luminescence properties are commonly used in reporter gene techniques.

It is also a reason for the failure of immunotoxins for therapeutic purposes. Today, genetic fusion of an scFv to a protein tracer is possible and offers many advantages: the coupling is complete, equimolar, and homogeneous [26,27]. This homogeneous structure results in quick and simple purification processes. The technology is reproducible and the functional properties of the two entities may be subsequently improved by mutagenesis.

5.3.5 Alternative Nonantibody Scaffolds for Molecular Recognition

Even if antibodies and their recombinant fragments are considered the paradigm for binding proteins, miniproteins are currently developed as alternatives for the generation of novel binding motifs. Usually, these molecules consist of rigid scaffolds that are stabilized by alpha-helices, beta-sheets, or disulfide-constrained secondary structural elements. They are tolerant to multiple substitutions or to the incorporation of a customized affinity domain. Phage display techniques allow the expression of libraries and the selection of specific binders [28,29].

These antibody mimetics belong to various scaffolds also designated as affibodies, DARPins, anticalins, avimers, versabodies, and duocalin.

5.4 Main Sources of Recombinant Antibodies

Beside polyclonal and monoclonal antibodies, there are several strategies to obtain recombinant antibodies, two of which are widely used (Fig. 5.5). The first consists of starting from a monoclonal antibody–secreting hybridoma as a source of V-domains. The cDNA is cloned and sequenced. Then, a gene encoding an antibody fragment (e.g., scFv) is synthesized and incorporated into an expression vector, which will be used to

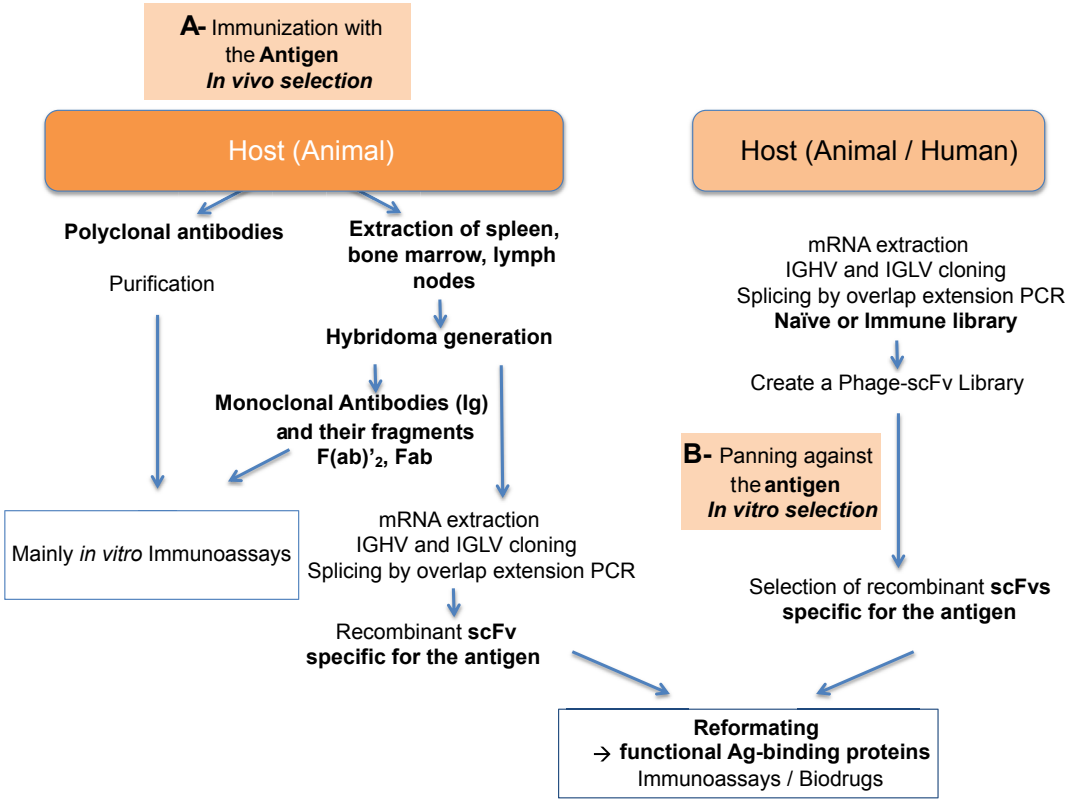

FIGURE 5.5 Sources of antibodies and applications, antibodies specific for an antigen can be prepared after in vivo selection (conventional immunization) (A) or after in vitro selection (biopanning of immune or naïve libraries) (B). Polyclonal antibodies are purified from the serum of immunized animals. Monoclonal antibodies are secreted from immortalized B-cells (hybridoma). Both are widely used for in vitro immunoassays. Recombinant antibodies are usually engineered after reformatting recombinant scFvs. They are mainly two sources of scFvs. First, they derive from well-characterized hybridomas. Alternatively, they are selected after in vitro panning of phage-scFv libraries. In both cases, the strategy can lead to antibodies with therapeutic potential (reformatted chimeric, humanized, or fully human antibodies).

transform host cells before being expressed as a soluble recombinant protein. Although this approach is successful, it remains limited to the original murine antibody for which the secreting hybridoma has been isolated previously [30]. We published a detailed step-by-step protocol allowing cloning and expression of recombinant antigen-binding molecules from hybridoma within 50 working days [31]. Coupled with directed evolution technologies, this strategy permits modulating affinity and antigenic specificity and also "humanizes" the molecule for in vivo applications [32]. In addition, these antibody fragments can easily be reformatted into whole immunoglobulin backbones in a high-throughput independent manner. Most of antibodies approved for therapeutic purposes have been designed according to this strategy.

Another technology consists of creating in vitro display methods such as a phage, yeast, and ribosome display [33,34]. The most widely spread technology consists of using combinatorial libraries of antibody fragments exposed on the phage surface and selecting phages expressing the antibody fragment specific to an antigen of interest (Fig. 5.6). This technique, originally developed for the presentation and selection of peptides, has been adapted to recombinant antibody fragments (scFv, Fab, and sdAb) [35]. It consists first of creating a library of antibody fragments, usually scFv, in which the variable domains are associated in random combinations. A VH can be married to any VL, and vice versa. It recreates the diversity of the natural repertoire.

Besides the fact that these libraries can consist of antibody fragments of both human and animal origin, there is interesting in offering highly complex libraries capable of reaching 10^{11} combinations. The principle bottlenecks are the transfection efficiency of the host and loss of antibody functionality owing to frameshift mutations [36].

Depending on the origin of the variable domains that will constitute the antibody fragments, there are three types of libraries. "naive" libraries are constructed from cDNA, encoding the antibody variable domains of nonimmunized donors. Antigen-biased libraries are constructed using cDNA from donors previously immunized so that

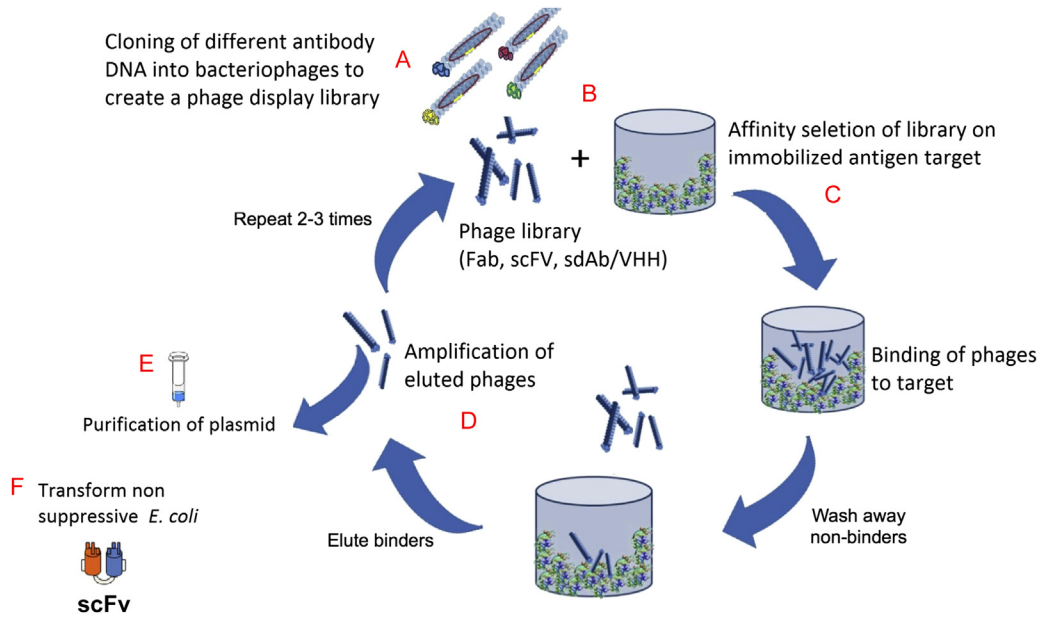

FIGURE 5.6 Affinity selection of antigen-specific scFvs from a phage display library. (A) DNA encoding millions of different scFv fragments is cloned into the genome of filamentous bacteriophage linked to one of the phage coat protein genes. (B) Each DNA variant is packed into a separate phage particle, and the antibody fragment displayed on the phage coat protein. (C) Phage displaying scFv that bind to the target antigen are selected using biopanning cycles of binding, washing, and elution. (D) Eluted phages are reinfected into suppressive *E. coli* cells and amplified for further rounds of affinity selection. (E) Clones from the enriched library are characterized for binding properties using appropriate technique. (F) Free scFvs are then produced after transformation of suppressive *E. coli* strain.

antigen-specific immunoglobulin genes are overrepresented. These libraries are mostly of animal origin; they may also come from individuals recovering from infectious diseases or producing autoantibodies. Finally, synthetic or semisynthetic libraries are constructed from synthetic genes in which the diversity of the framework regions is significantly reduced.

A naive repertoire avoids animal immunization and can produce recombinant antibodies in a shorter period than immune or synthetic approaches. However, affinities from these libraries are usually lower. In addition, naive libraries are often subjected to licensing fees with restricted access to protect intellectual property rights. Consequently, most of the time, recombinant antibodies with superior functional properties are produced from immunized animals.

The scFv repertoire subsequently created is cloned into a phage or a phagemid and the library is propagated in suppressive bacteria (Sup E and Sup F). The phage produced by bacteria contains the phagemid that encodes the antibody fragment to be exposed at the surface of a coat protein. Phages exposing at their surface the antibody fragments are then screened against the antigen of interest usually immobilized on a microtitration plate. Bacteriophages presenting an antibody fragment capable of interacting with the antigen on their surface are captured. They are eluted after the plate is washed and the clones selected are recovered by infecting new suppressive bacteria. This step, also known as "biopanning," is repeated four to five times and is used to enrich the population in bacteriophages carrying antibody fragments with affinity for the target antigen; it mimics the natural immune response. Once screening is completed, phagemids contained in the selected bacteriophages are cloned into nonsuppressive bacteria to generate the antibody fragment of interest as a soluble free molecule. It is the presence of an "Amber" codon at the end of the gene encoding the antibody fragment that allows this action.

Advantages of the phage-antibody technology are numerous. First, they offer faster production pipelines relative to conventional monoclonal antibodies. Their production avoids the need for animals, in accordance with legislation on animal protection. This strategy provides greater opportunity for postproduction improvements and alteration by in vitro genetic manipulation. Recombinant antibodies can be generated against extremely potent toxins that would not be possible in vivo [37,38]. Although phage display technology was established more than 20 years ago, and despite widespread use in early-stage antibody discovery, to date only a limited number of approved antibody products have been created via phage display.

5.5 Recombinant Antibodies as Emerging Molecules for Pharmacologic Treatments

Hybridoma technology gave rise to the hope for the rapid development of therapeutic applications. Indeed, it offers the opportunity to keep an unlimited amount of murine

antibody with unique specificity and reproducible affinity. Thus the first hybridoma-derived murine monoclonal anti-CD3 was introduced into clinical practice in the mid-1980s to prevent organ rejection. However, this strategy faced early failure owing to side effects, mainly immunologic [39]. Thus, modulation of the immune response has been studied by two approaches to reduce the human anti-mouse antibody (HAMA) responses. The first one consists of producing chimeric antibodies that are less immunogenic, in which constant domains of a murine monoclonal antibody are replaced by constant domains of human origin. The second approach is to produce humanized or entire human antibody fragments even less immunogenic. It took a decade for the first chimeric monoclonal antibody, abciximab for hemostasis, to be approved by the FDA in 1994 [40]. The first humanized monoclonal antibody; daclizumab (Zenapax) for kidney transplant rejection, was approved for clinical use by the FDA in 1997 [41]. Humanization alleviated the HAMA response to various degrees, but many other drawbacks became evident. For example, the humanization process is demanding of technology and the process may result in reduced antigen-binding affinity and decreased efficacy. Two major approaches were developed to avoid the human immune response to murine-derived monoclonal antibodies and to overcome the technical challenges associated with humanizing murine monoclonal antibodies. The first is to express human antibody fragments on bacteriophage surfaces [42]. Adalimumab (Humira), the first fully human monoclonal antibody derived from a bacteriophage display antibody library, was approved by the FDA in 2002 for the treatment of rheumatoid arthritis [43]. The second approach was the use of transgenic mice to produce fully human antibodies [44]. Panitimumab (Vectibix), an anti-epidermal growth factor receptor antibody approved for colorectal cancer therapy in 2006, was the first fully human therapeutic antibody derived from a transgenic mouse system [45].

Thus, to date, recombinant antibodies have attracted a great deal of attention by the pharmaceutical industry. Antibody therapeutics have reached higher approval success rates and similar development phase lengths compared with those of small-molecule drugs. They have become the centerpiece in the design of targeted therapeutic strategies and constitute a well-accepted class of therapeutics that now covers the fields of cancer, infectious diseases, transplantation, allergy, asthma, and some autoimmune diseases (Fig. 5.7) [46,47].

5.6 Recombinant Antibodies: New Tools for Research and Clinical Analysis

The areas of immunoassay and diagnostic use of recombinant antibodies have not experienced the same growth as the pharmaceutical market. The heterologous original murine monoclonal antibody is not a problem for in vitro applications and conventional antibodies currently meet expressed needs.

FIGURE 5.7 Timeline of antibodies and immunoadhesins approved for therapeutic application or imagery in humans, Approval limited to the United States (*), China (★), Europe (#), or withdrawn (⃠). Therapeutic class: oncohematology in red, immunology and inflammation in blue, cardiology in brown, ophthalmology in orange, infectiology in green, orphan diseases in pink, transplantation in black, imagery in light blue. Murine (-momab), chimeric (-ximab), humanized (-zumab), fully human (-mumab).

The unique specificity of monoclonal antibodies, the possibility to obtain unlimited amounts of murine antibody with unique specificity and reproducible affinity, to attach on solid supports, or to conjugate with radiolabeled tracers, fluorescent, or enzymatic tracers, led to a wide range of tests that allow in vitro titration of a wide variety of substances.

These tests are rapid and mostly sensitive and can be performed in a reproducible manner. They are used routinely in clinical biology for the titration of hormones, the search for tumor markers, infectious agents, and toxic substances. They are also used for veterinary diagnosis, environmental quality control in the food industry, and many other sectors.

However, there is room for improvement. Phage-antibody technology makes it possible to select scFvs or Fabs specific for nearly any antigen without depending on the immune response of animals, which allows the fields of their applications to be expanded. Difficulties experienced when coupling antibodies to protein tracers by

conventional chemical methods could be avoided by adopting gene fusion technology [48]. The gene of a functional antibody fragment can be fused in a reading frame with that of an enzyme, cloned into an expression vector, and introduced into a host cell that will produce the fusion protein. In this case, various host cells have been tested with success, including bacteria, yeast, insect, or mammalian cells.

The overexpression of the fusion protein in bacteria is fast, and certainly, from a practical and economic point of view, the most interesting. Secretion into the bacterial periplasm leads to soluble fusion protein, properly folded and bifunctional with a specific antigen recognition site and an active colorimetric enzyme. Several examples of antibody fragments, scFvs, or Fabs expressed in fusion with enzymes (alkaline phosphatase or acetylcholinesterase), peptides, streptavidin, or fluorescent protein have been designed for the detection and titration of clinically relevant antigens (toxins or hormones) [49]. Internal tags can even be designed [50]. The sensitivity of the immunoassays performed with these fusion proteins readily achieved a few picomoles. This sensitivity can certainly be increased further and reach that of radioimmunoassay. This may require improving the specificity and affinity of the antibody, and also the physical and catalytic properties of enzymes. By combining in vitro selection steps (directed or random mutagenesis) or in vivo, using bacterial strains causing mutations, the affinity of some antibodies has been increased by a factor of 1000. A high-affinity monoclonal antibody for progesterone, which also cross-reacts with steroid structural analogs, has been reformatted by these techniques. This led to an scFv strictly specific for progesterone without loss of affinity, giving it a strong potential to develop a rapid, sensitive, and specific titration kit [51].

Antibody engineering also offers the prospect of developing specific biosensors capable of recognizing an antigen in a heterogeneous medium such as biological fluids, and instantly transduces the event directly into a measurable signal. Thus, Bedouelle et al. established a method of processing recombinant antibody fragments in fluorescent biosensors [52]. They first identified residues of an scFv in the vicinity of the antigen-binding site that may be mutated to cysteine without affecting the antigen recognition function. Cysteine introduced into the mutants with these criteria was then coupled to a fluorophore and the mutants for which the antigen-binding causes a change in the structural environment of the fluorophore were selected. This structural change immediately induces an increase in fluorescence intensity proportional to the antigen concentration. The process has been patented and could be applied to the detection, dosing, and monitoring of many antigens, hormones, markers, or infectious agents in biological fluids using the biosensor solution free or immobilized on a protein array.

The antibodies used in in vivo diagnostics sector remain murine antibodies, even if they are few. Originally, it is their high specificity associated with the possibility of combining them with gamma emitters that stimulated their in vivo use in medical imaging to target specific antigens associated with tumors. A gamma camera is used to detect sites, which concentrate the radiolabeled probes. A multitude of antibodies have been developed for immunoscintigraphy but fewer than a dozen experienced

commercial development with limited application in monitoring metastatic colorectal, ovarian, or prostate cancer, and evaluation of myocardiac necrosis consecutive to infarction. Here, the limiting factor is not the potential immunogenicity of these molecules but rather the low contrast generally observed between healthy and pathologic tissues. The size of the probes (whole IgG or their Fab) limits the diffusion and their ability to reach the target antigen embedded in a tissue or a tumor. Their plasma half-life is relatively high even for Fabs that can be cleared by the kidney, so the specific signal−background ratio is low [53].

Studies in animal models have shown the potential benefits of recombinant antibody fragments much more diverse in size and valences. Thus, bivalent diabodies are rapidly eliminated from the plasma compartment and disseminate better to target tissues. The signal−noise ratio is thereby increased and more contrasted images are observed. The sensitivity could be improved by using positron emission tomography or antibodies coupled to fluorescent reagents [54]. However, no molecule has yet been placed on the market, perhaps because of the costs of development and the habits that make immunoscintigraphy unsuitable to be used as the first-line approach, but only in the case of relapses and when results of conventional examinations (scanner or ultrasound) are doubtful.

Another area in which the recombinant antibodies nourish high hopes is that of the proteomic study of biological samples. These hopes are fed in part by the enormous diversity of antibodies that carry combinatorial libraries and the possibilities offered by "microarray" technologies that consist of attaching proteins or antibodies to silicone glass nanosurfaces and developing rapid sensitive and multiplex analysis [55]. Random screening of nonimmune libraries has already demonstrated the ability to clone antibody fragments directed against conserved epitopes or nonimmunogenic antigens that had never been achieved by conventional hybridoma technology. Antibodies directed against the protein of the endoplasmic reticulum B-cells or antibodies capable of distinguishing the posttranslationally activated forms of intracellular proteins as Rab6 or guanosine-5′-triphosphate tubulin are interesting examples [56]. The screening of antibody libraries against proteins extracted from a healthy or diseased tissue by two-dimensional electrophoresis and characterized by mass spectrometry could lead to the selection of an antibody of interest (Fig. 5.8). These antibodies could be immobilized at specific positions on nanosurfaces capturing molecules of interest contained in a biological sample, and after washing, immune complexes of various disclosure systems could be implemented with the position of the signal on the chip and its intensity indicating the result for each of the parameters analyzed [57]. Several detection systems are being evaluated, including nanoelectrodes, sandwich reactions involving a second fluorescent antibody, surface plasmon resonance, or matrix-assisted laser desorption/ionization mass spectrometry. Simultaneous analysis of the healthy and pathologic samples (differential analysis) would provide added value in early diagnosis, prognosis, or therapeutic monitoring of complex pathologies. These objectives could be achieved by using technological alternatives: for example, microfluidic approaches in which the

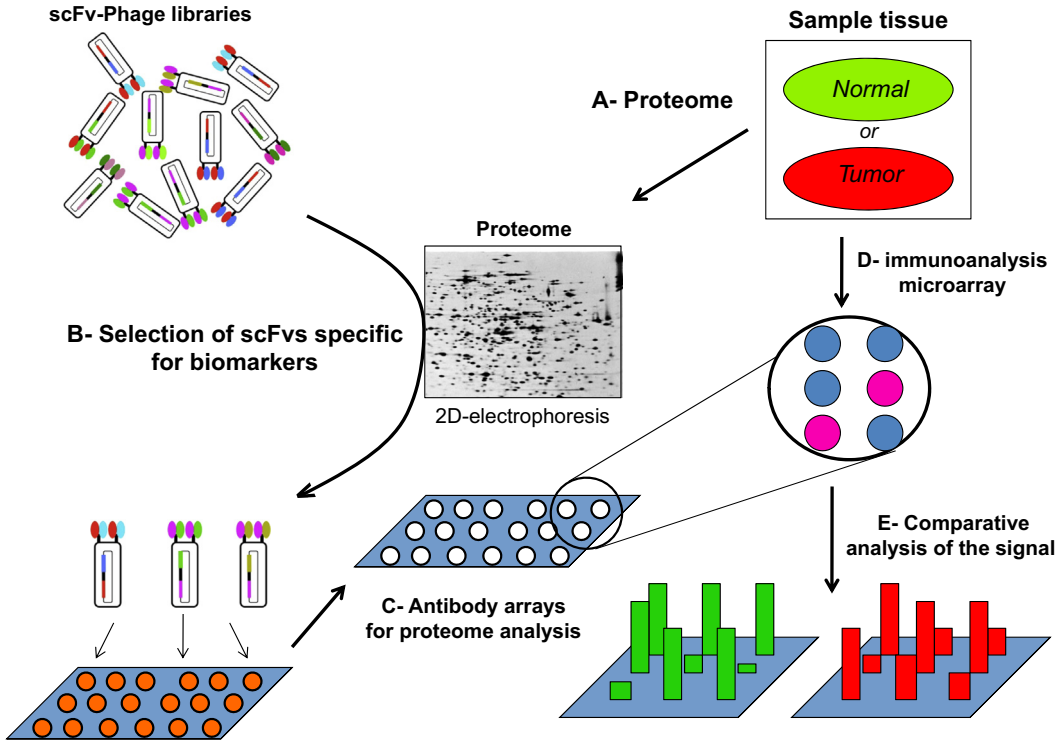

FIGURE 5.8 Potential application of recombinant antibodies for global analysis of tissue proteome. (A) The proteomes derived from normal and malignant tissue are isolated. (B) scFv-phage libraries are panned against these proteomes. Highly specific scFv-phages are selected and free scFvs are produced in nonsuppressive bacteria and purified from periplasmic extracts. (C) The antigen-specific scFvs are spotted on the chips. (D) The samples to be analyzed are then allowed to react with the functionalized chip. (E) The readout is shown as a three-dimensional histogram.

natural molecules in solution are captured by antibodies coupled to colored beads and then run in microcapillaries where other specific antibodies have been immobilized. These immune complexes retain color to a preset position, thereby allowing their detection.

5.7 Producing Recombinant Antibodies

As consequence of growing interest in recombinant antibodies, many different production systems have been evaluated for their expression, ranging from bacterial hosts to transgenic animals. The choice of the most suitable expression system depends on several factors. The first is the antibody format (whole antibody or nonglycosylated fragment). Other factors that will influence this choice are the scale and total annual production, the application (therapeutic, diagnostic, and experimental tools), and finally the downstream process, whose cost may have a dramatic impact on the product.

5.7.1 Prokaryotic Systems

Microbial expression systems are attractive for the expression of antibody fragments that do not require posttranslational modifications such as phosphorylation and glycosylation. The preferred host is *Escherichia coli* K12-derived strains, even if several gram-positive bacteria have been used for expression such as *Bacillus subtilis*, *Streptomyces lividans*, and *Staphylococcus carnosus* [58,59]. The expression is typically achieved by fusion to N-terminal signal peptides that address the protein to the periplasmic space of *E. coli*, where chaperones assist the correct folding and disulfide bridges formation that stabilize the structure (Fig. 5.9). This expression system is attractive. Large biomass density and quick growth can be reached using unexpansive serum-free media. However, recovery of the properly folded and functional antibody fragment strongly depends on the amino acid sequence. Most of the time, large amounts of antibody fragments remain sequestered in the cytoplasm as inclusion bodies. When exported to the periplasm the protein can undergo undesired degradation by periplasmic proteases. In an academic research environment, the yield is rarely higher than a few milligrams of functional antibody fragment per liter of shaken flask cultures, but it is sufficient to allow purification and characterization of early-stage material.

Few therapeutic antibody fragments have been marketed and no more than 50 antibody fragments are currently developed and tested in clinical studies [60,61].

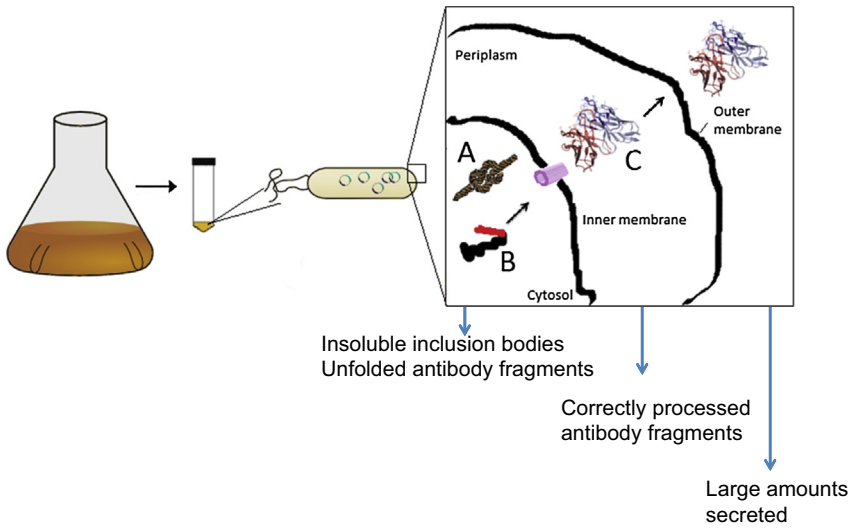

FIGURE 5.9 Expression of recombinant antibody fragment in *E. coli*. (A) When produced in the cytoplasm, antibody fragments form inclusion bodies and refolding is a tedious step difficult to standardize. (B) Under the control of a signal sequence the unfolded premature antibody fragment is transported across the inner membrane into the periplasm. The protein folds and forms disulfide bridges in the oxidative environment of the periplasm. (C) Using engineered strain, it becomes possible to translocate the correctly folded native protein into the extracellular medium. This strategy greatly simplifies the downstream process.

However, these studies have contributed to develop new *E. coli* expression technologies that allow the secretion of recombinant proteins in their native form directly into the culture medium. The expression yield can reach a couple of grams per liter, which is achieved without cell disruption. These features contribute to simplify the downstream process and reduce the amount of endotoxins that are considered to be the biggest drawback compared with nonbacterial expression technologies [62].

5.7.2 Yeast Systems

Yeast expression systems have been used for therapeutic protein expression since the early 1980s. They grow quickly in well-defined medium in the absence of animal-derived growth factors and can secrete large amounts of recombinant protein. In this way, they constitute a valuable alternative when antibody fragments are difficult to express in bacteria. Until 2009, several biopharmaceutical proteins were produced in *Saccharomyces cerevisiae*, but other expression systems were also successfully developed independently using the methylotrophic yeast *Pichia pastoris* [63,64]. Yeast are even more robust than *E. coli* in cell culture and can grow to high cell densities, which increases the yield per a given volume of cell suspension. Thus, protein production tends to be faster and cheaper than in mammalian systems. Typical expression levels after 120 h fermentation processes can reach 2−5 g/L for a wide range of proteins including Fab fragments. The main advantages of yeasts over bacterial expression systems are the absence of endotoxins and their ability to secrete recombinant proteins into the culture medium using an eukaryotic protein-synthesis pathway. In addition, protein folding and posttranslational modifications can occur in *Pichia* so that it could be used to express glycoproteins. However N-linked glycosylation patterns differ significantly from that of humans, which is a major drawback of yeasts compared with mammalian cells.

5.7.3 Mammalian Cell Systems

Mammalian cell expression systems have emerged as the method of choice for the production of whole monoclonal antibodies because they support expression of larger proteins than can bacteria (Fig. 5.10). They allow assembling multidomain proteins and the addition of sugar motifs that profoundly affect the function of the Fc fragment. Patterns of glycosylation are highly species-specific and mammalian cells have glycosylation repertoires most similar to humans.

Human embryo kidney (HEK-293) has long been considered to be the host of choice for transient expression of recombinant proteins. Improvements in media composition and host cell engineering have led to generate higher-yielding transient transfection systems based on suspension-adapted Chinese hamster ovary (CHO) K1 cells [65]. These systems allow the production of up to 100 mg/L of antibody within 10 days after transient transfection. However, protein expression remains an empirical process and results are never guaranteed even with the most seemingly appropriate expression system.

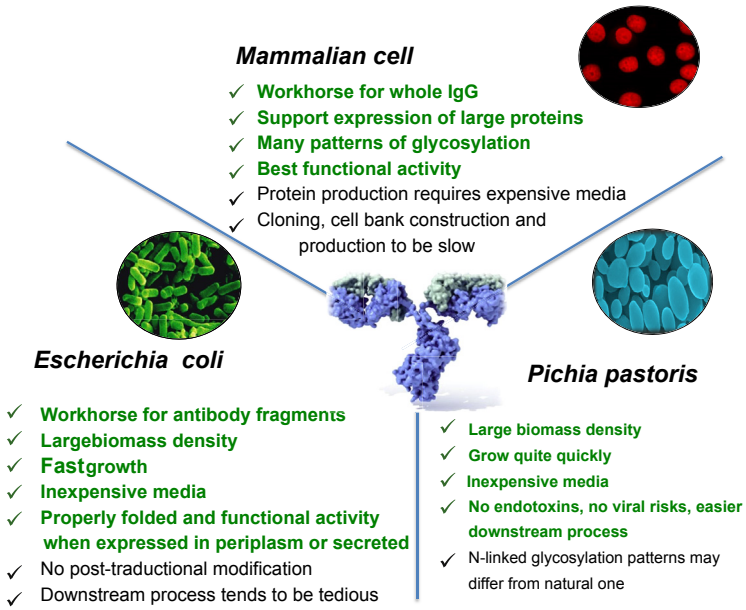

FIGURE 5.10 Overview of the most popular systems for the production of recombinant antibodies and antibody fragments.

In the biopharmaceutical industry, CHO has become the workhorse for the production of whole IgGs in front of other cell lines including mouse myeloma (NSO), HEK-293, and human embryonic retinoblast (PER.C6) [66,67]. Companies have mainly focused on CHO and have been able to optimize industrial upstream processes to reach typical titers ranging from 1 to 5 g/L after several days. Concerning expression of antibody fragments (Fab and scFv), interest in mammalian cells remains questionable. Indeed, they are more difficult to culture than are microbial cells and protein production tends to be slower and more expensive in mammalians versus microbial systems. In addition to the complexity of the protein folding, other points have to be considered, including the downstream process, whose cost may have a dramatic impact on the product. In this way, the relative purity of the protein secreted in CHO cell culture medium may be advantageous compared with *E. coli* periplasmic extracts but viral clearance is absolutely required and cost-effective.

5.7.4 Alternative Expression Systems

To date, approved biopharmaceuticals are produced in a limited number of expression systems, especially in *E. coli*, *P. pastoris*, and CHO cells. However, unconventional systems are catching up. An interesting one is the use of transgenic animals as bioreactors. They are capable of posttranslational modifications that closely match those of human proteins and offer most economical efficient technological models of production.

Recombinant antibodies were produced in transgenic animals for the first time at the end of the 1990s and many companies are actively working on developing this technology [68]. So far, antibodies have been produced mainly in transgenic goats at a relatively high concentration (0.5−10 mg/mL) without affecting the health and lactation of animals. These antibodies are stable and highly efficient, and one can estimate that herds of several dozen of transgenic goats whose milk contains a specific monoclonal antibody could fully meet the world demand for the treatment of most pathologies [69]. One of the main challenges in this strategy will be to develop an economically viable downstream process, because milk is a complex mixture that can still be infected with pathogenic agents.

5.8 Conclusions and Perspectives

The possibilities offered by molecular engineering to adapt the format and valence o recombinant antibodies to specific applications, increase their affinity, and modulate their specificity will greatly expand the range of their applications. First, as a therapeutic agent: nearly 40 molecules received marketing authorization in areas as diverse as infectious diseases, oncology, hematology, and treatment of cardiovascular, inflammatory, or autoimmune diseases. Nearly 500 are currently in clinical trials. The number of antibody fragments remains much lower. Only three Fabs have yet been marketed: Abciximab (Reopro) for thrombosis; Ranibizumab (Lucentis) for wet age-related macular degeneration; and Certolizumab pegol (Cimzia) for Crohn disease.

In the immunoassay sector, although conventional monoclonal antibodies remain essential tools, the panel of their targets is limited to a few thousand proteins. This sector should quickly take advantage of technological progress and see if its scope can be broadened.

Altering the intrinsic properties of an antibody (size, affinity, and specificity) is possible even if the methods used are still difficult to generalize. The preparation of recombinant bifunctional immunoconjugates that are homogeneous in structure and perfectly adapted to the requirements of the immunoassay is a reality. Intensive research is carried out in this area and recombinant immunoenzymatic conjugates will soon appear on the diagnostic market. Through in vitro selection technologies from combinatorial libraries, probes specific to various antigens may be isolated without immunizing animals. Thus the repertoire of available antibodies increases significantly regardless of the target proteins, including poorly immunogenic proteins. The microfluidics and the ability to create recombinant antibody arrays pave the way to the development of rapid assays with simultaneous detection of several predetermined antigens in biological samples. Chips could then be made to determine the protein expression profiles of healthy and cancerous cells, discover new biomarkers and the molecular mechanisms of diseases, or identify new therapeutic targets.

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6

Impacts of Aptamer Technology on Diagnostics, Biotechnology, and Therapy

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6.1 Systematic Evolution of Ligands by Exponential Enrichment Technique

The systematic evolution of ligands by exponential enrichment (SELEX) technique describes the reiterative screening of a combinatorial RNA or single-stranded (ss) DNA library for binding affinity and specificity against desired target molecules, followed by reverse transcription–polymerase chain reaction (RT-PCR) or PCR amplification of eluted target binders, renewing an already selected library for the next cycle. The SELEX process mimics natural selection, in which best-fitting species are amplified for the next generation. RNA or DNA molecules with high target-binding affinity and specificity obtained after completion of various *in vitro* selection cycles are denominated aptamers (from Latin *aptus*, which means "to fit").

The basic design of the initial library is similar to both RNA and DNA selection processes using a DNA template composed of a random inner region (20–70 random nucleotides) flanked by constant sequences containing the primer annealing sequences as well as RNA polymerase initiation sites (in case of RNA aptamer selection). For DNA aptamer selection, the double-stranded (ds) pool is denatured into its single strands, whereas for RNA aptamer selection, the dsDNA pool needs first to be *in vitro* transcribed into RNA.

For the formation of secondary and tertiary structures of ssDNA or RNA, pools are denatured and then presented to their targets, to which they bind in a sequence- and structure-specific manner.

Following the regular flow of the selection cycles and removal of unbound DNA or RNA molecules, target binders are eluted and amplified by RT-PCR or PCR procedures to restore the DNA library for the next cycle of *in vitro* selection. After consecutive SELEX

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cycles, the heterogeneous random library containing up to 10^{15} different sequences is purified to a homogeneous population of aptamers binding with high affinity and specificity to their selection target. Individual aptamers are identified from this final pool by DNA sequencing, and the obtained aptamers are characterized for binding affinity and biological activity (Fig. 6.1).

Resistance against nuclease activity can be achieved during the SELEX process by enzymatic incorporation of modified nucleotides using PCR or in vitro transcription reaction [1,2]. Stability and specificity of already identified aptamers can be further optimized by post-SELEX modifications, a particular feature of the SELEX process that is feasible to introduce modifications after selection. These modifications include truncation of the sequences, incorporation of modified bases by chemical synthesis, and attachment of biotin moieties and fluorescence reporters.

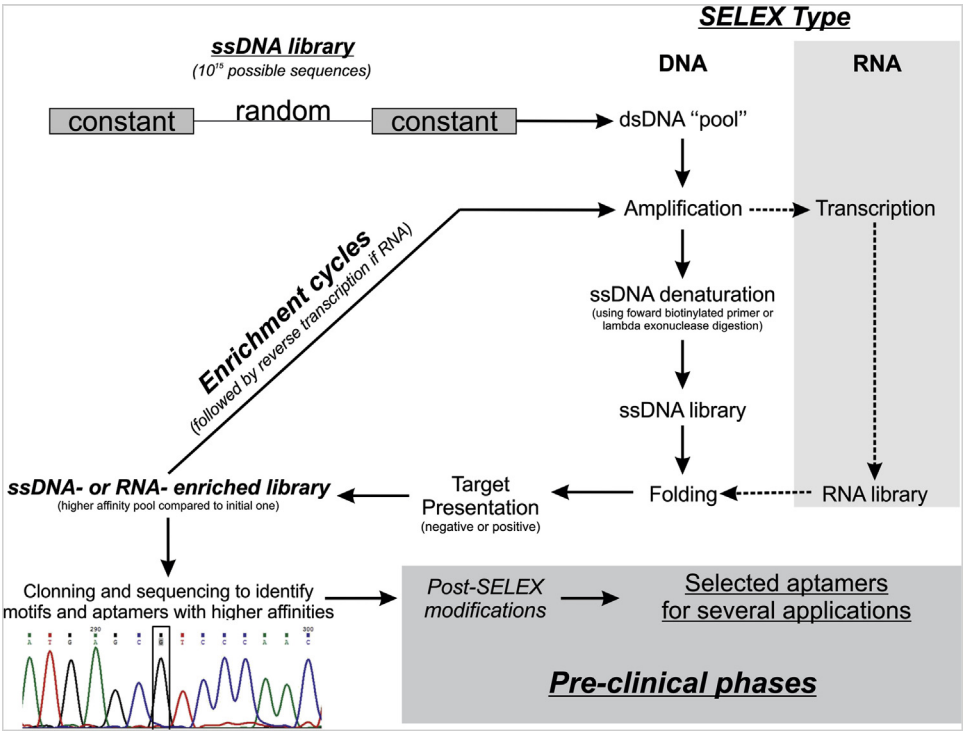

FIGURE 6.1 In vitro selection of high-affinity aptamers for *in vivo* applications. DNA and RNA aptamers are selected from a randomized oligonucleotide library by reiterative SELEX rounds. In case of the DNA aptamer, the double-stranded DNA pool needs to be denatured for purification of the single-stranded sense strand, which is then presented to its selection target. After removal of unbound and low-affinity bound DNA molecules, target-bound DNA molecules are eluted and amplified by PCR in the presence of a biotinylated primer. After denaturation, the unbiotinylated sense strand can be purified by polyacrylamide gel electrophoresis and used for the next SELEX cycle. In the case of development of an RNA aptamer, the double-stranded DNA pool is *in vitro* transcribed to the RNA pool, which is then used for SELEX. Eluted target binders are reversed-transcribed to cDNA, amplified by PCR, and again *in vitro* transcribed to give the RNA pool used in the next SELEX cycle. RNA and DNA molecules can be protected against nuclease attacks during the selection process by incorporation of modified nucleotides using enzymatic reactions, or they can be chemically modified after the SELEX process. *HPLC*, high-performance liquid chromatography; ss, single-stranded; ds, double-stranded.

The origin of SELEX studies goes back to the work of the research groups of Larry Gold and Jack Szostak [3,4], who first identified RNA molecules specifically interacting with T4 bacteriophage DNA polymerase or small molecules such as adenosine triphosphate (ATP). In fact, Ellington and Szostak showed that the selected RNA aptamers bound to ATP but not to related nucleotides such as deoxyadenosine triphosphate, indicating that this technology would generate more specific ligands than those present in nature.

These original works suggested that high-affinity RNA ligands could be developed for any target molecule. In the following years, subsequent studies revealed that aptamers possess target recognition features, which are similar to those of antibodies [5]. Because they are highly specific molecules, aptamers are able to distinguish between isoforms of the same protein, i.e., protein kinase C [6]. Moreover, they are able to distinguish by the matter of 10,000-fold affinity between theophylline and caffeine; the only difference between the two molecules is a methyl group at the nitrogen atom N-7 [7]. Aptamers are even able to distinguish different conformational forms of the same protein [8–10]. These capabilities position oligonucleotide molecules as potential candidates for replacing the antibody technology in several applications.

Aptamers are being developed into novel tools, such as therapeutics and/or diagnostic agents against a broad spectrum of bioactive peptides and growth factors, with tremendous impact on therapeutic and diagnostic approaches. As a result of these developments, starting in the early 1990s, the most prominent molecule was a therapeutic aptamer targeting the isoform 165 of vascular endothelial growth factor (VEGF), which was approved for the treatment of disease-related neovascularization in macular eye disease, commercially named MACUGEN [11,12]. A new aptamer aimed at ophthalmologic applications has been developed targeting platelet-derived growth factor (PDGF), commercially named FOVISTA [13].

SELEX applications were extended from targeting soluble proteins to complex targets, which are comprehensive structures such as transmembrane proteins needing the membrane environment to maintain activity, or entire membranes or even whole cells [14–18]. Remarkably, the use of complex targets for aptamer selection is strategically advantageous over targeting purified proteins, because there is no concern regarding conformational changes, which may occur in purified or recombinant-expressed cell surface proteins [19]. Cell SELEX, an improvement of the original technique, exploits differences in surface molecule expression between any two types of cells. Consequently, panels of aptamers can be obtained, which specifically recognize the molecular signature of the target cell type. A specific probe for a distinct cell type can be obtained with no knowledge of molecular differences between, for example, cancer cells or parasite-infected cells and normal cells.

For these applications, the combinatorial RNA or ssDNA library is incubated with target cells. Unbound oligonucleotides are removed by washing steps, and bound sequences are eluted from the cell surfaces and amplified by RT-PCR or PCR for subsequent SELEX cycles. To overcome limitations in aptamer selection owing to unspecific binding of RNA or DNA pools to sites on cell surfaces other than the desired target site,

subtractive SELEX cycles have been established. The preselected RNA or DNA pool is exposed to control cells and only the unbound RNA or DNA fractions are used for the next SELEX cycle.

Therefore, all sequences bound to cell surface epitopes commonly expressed by both cell types have been discarded. After various cycles of target selection and subtraction rounds, the final SELEX pool consists of a homogeneous population of aptamers exclusively binding to cells used as targets for *in vitro* selection [19]. Identified aptamers can then be employed as capture agents for cell separation and identification of their target proteins on the cell surface [20].

The basis of aptamer-target recognition relies on the fact that SELEX carried out with RNA or DNA combinatorial libraries consists of up to 10^{15} different sequences. As result, such molecules can fold into a vast number of different complex structures, with a high probability that from this library of possible structures an RNA molecule with an appropriate secondary structure can be identified for any given target protein.

Aptamers can also be developed from ssDNA libraries, although secondary structures formed by ssDNA are less stable than those of RNA molecules [21]. DNA aptamers have some advantages over RNA aptamers, because there is less need for chemical modifications to grant nuclease resistance. Furthermore, although they are synthesized by the PCR reaction, modifications can easily be introduced using modified primers.

Aptamers are known to interact with their selection targets with micro- to femtomolar binding affinities and identify the sequence and conformation of protein surface features (epitopes) such as antibodies do. Interactions between aptamers and a peptide target are disrupted by sequence substitutions [5].

Sequence-specific recognition result from the establishment of hydrogen (H-) bonds between bases of ribonucleotides or deoxyribonucleotides and amino acid side chains of the target peptide or protein. Structure-specific recognition results from the formation of H-bonds with oligonucleotide backbone sugars or phosphates and the protein chain. Furthermore aptamers are capable to bind their targets with different solution conformations [5], ensuring optimal molecular shape complementarities [22].

This structural plasticity enables small target molecules to be incorporated into the aptamer structure, whereas in the case of binding to larger proteins, aptamers integrate into binding pocket of the protein. This mechanism of induced fit also provides a mechanism for the observation that hidden target sites, which cannot be accessed by antibodies, are accessible for aptamers.

6.2 Therapeutics Features of Aptamers

Over the past three decades, aptamers have been generated against hundreds of molecular targets. DNA and RNA aptamers have been selected against proteins of therapeutic interest, such as cytokines, proteases, kinases, immunoglobulins, gene regulatory factors, cell-surface receptors, cell-adhesion molecules, and growth factors (Table 6.1). Moreover, aptamers were also developed against pathogenic bacteria [23],

Table 6.1 Dissociation Constants (K_d) of Aptamers Binding to Targets of Therapeutic Interest

Target	Dissociation Constant (K_d) (nM)	Possible Therapeutic Application	References
Growth Factor, Hormones			
Fibroblast growth factor 2, basic	0.35	Prevents angiogenesis	[31]
Substance P	40	Prevents tumor development	[32]
Epidermal growth factor receptor	2.4	Prevents tumor development	[33]
Gonadotropin-releasing membrane hormone 1	50	Prevents tumor development	[34]
Receptors			
Cocaine binding site of the nicotinic acetylcholine receptor	2 and 12	Prevents toxic effects of cocaine	[14]
Receptor tyrosine kinase RETC634Y	35	Prevents tumor development	[35]
Neurotensin receptor 1	1.5	Prevents tumor development/ manages pain	[36]
Nociceptin	110	Manages pain	[37]
Viral Proteins			
HIV-1 reverse transcriptase	1	Inhibits viral replication	[38]
HIV-1 rev	<1	Inhibits viral replication	[39]
HIV-1 integrase	10	Inhibits viral replication	[40]
HIV gp120	5	Inhibits viral infectivity	[41]
NS3 protease	10	For treatment of hepatitis C viral infection	[42]
Coagulation Regulatory Proteins			
α-thrombin	25	Prevents thrombosis	[43]
Factor IXa	0.65	Prevents thrombosis	[44]
Cell Adhesion Molecules			
L-selectin	3	Modulates inflammation	[45]
Epithelial cell-adhesion molecule	38	Prevents tumor development	[46]
Pathogenic Proteins			
Acetylcholine-specific autoantibodies	60	For treatment of myasthenia gravis	[47]
Bovine prion protein	6.8	For treatment of prion and Alzheimer disease	[48]
Whole Organism			
Trypanosoma cruzi	40–400	Diagnostics and treatment of Chagas disease	[49]
Trypanosoma brucei	60	Diagnostic and treatment of "sleeping sickness"	[50]

viral particles [24] and cancer cells [25], and are able to distinguish tumor tissue such as microvessels of rat glioblastoma from normal tissue [26]. The use of aptamers instead of antibodies is arising in almost every application, for which they are suitable, because they have many advantages over antibodies in engineering and medicine applications:

1. *Production and quality control:* The *in vitro* method for nucleic acid aptamer selection is simple compared with the complex *in vivo* antibody production process [27]. No organisms are required for aptamer production, resulting in no batch-to-batch variation among aptamers for a specific target. In addition, aptamers are chemically produced in a readily scalable process, which renders products, which are not susceptible to viral or bacterial contamination [28].
2. *Ligand specificity and affinity:* It has been shown that aptamers often exhibit high affinities toward the selected target compared with antibodies and often yield dissociation constants (K_d) of ligand−target complexes in the nano- or sub-nanomolar range (Table 6.1).
3. *Immunogenicity and toxicity:* Aptamers are reported to be low-immunogenic and low-toxic molecules. Whereas the efficacy of many monoclonal antibodies often depends on the antigen's ability to trigger an immune response, nucleic acid fragments are not typically recognized by the human immune system as nonself entities [29].
4. *Variety of targets:* Molecules that do not elicit strong immune response, such as toxins, ions, or small molecules, are not eligible for antibody production. However, aptamers can be generated with high affinity and specificity against targets that are not recognized by antibodies [30].
5. *Stability:* Oligonucleotides are more thermally stable and maintain their structures over repeated cycles of denaturation/renaturation whereas antibodies are easily denatured and lose their tertiary structure at high temperatures. Aptamers recover their native conformation and can bind to targets after reannealing, whereas antibodies easily undergo irreversible denaturation [27]. In addition, aptamers can be stored for extended periods (more than 1 year) as lyophilized powders [30]. These biochemical features of oligonucleotide aptamers allow for their use in a wide range of assay conditions.

Under biological conditions, unmodified RNA and DNA molecules are prone to nuclease-mediated degradation. Because oligoribonucleotides are particularly susceptible to the attack of nucleases present both, in the serum and inside cells, the development of chemical modifications to improve aptamer stability has been crucial for biological applications. Accordingly, modified nucleotides can be introduced into libraries before the initiation of SELEX by polymerases that accept modified nucleotide triphosphates as substrates. This modification step yields aptamers, whose nucleotide sequences are partially or completely substituted with one or more modifications. Among the most commonly used modifications are 2′-amino pyrimidines [51,52], 2′-fluoropyrimidines [53,44], and 2′-*O*-methyl ribose pyrimidines and purines [54,55].

Another modification is composed by internucleotide linkages often inserted at the 5′-ends of aptamers, such as high-molecular mass polyethylene glycol conjugation [56,57]. Such modifications result in an enhanced resistance to nuclease attacks, improving aptamers' half-lifes in biofluids from minutes to several hours [30]. In rat plasma, the half-life of the thrombin aptamer was extended from 24 min to 6 h with no effect on target-binding affinity [58].

To enhance stability, the sugar backbone of an oligonucleotide can also be substituted by enantiomers, built from nonnatural L-nucleotides, generating aptamers also known as Spiegelmers (from the German word for mirror, *spiegel*) [59–61]. Because SELEX cannot be directly performed with L-ribonucleotides, because wild-type RNA polymerases do not accept L-nucleotide triphosphate as substrates for PCR amplification and transcription reactions, the first step in the production of Spiegelmers is to synthesize an enantiomer of the target. After performing SELEX using oligonucleotides composed of natural D-nucleotides, once the aptamer is selected, the sequence is used to chemically synthesize the enantiomers of the nucleotide sequences with L-nucleotides. Because of this change in chirality of the nucleotides, Spiegelmers are more nuclease-resistant than D-nucleotide sequences. The synthetic production of the enantiomeric target is a limitation of this approach, because only relatively small proteins or peptides are eligible for Spiegelmer methodology [62].

6.3 From Basic Research to Therapeutics: Application of Aptamers

The feasibility of aptamer development for almost every type of molecular target makes this class of high-affinity class important not only for basic research but also for several therapeutic approaches, such as *in vivo* imaging diagnosis and therapy. Basic steps for aptamer development and possible applications in biotechnology and therapy are shown in Fig. 6.2.

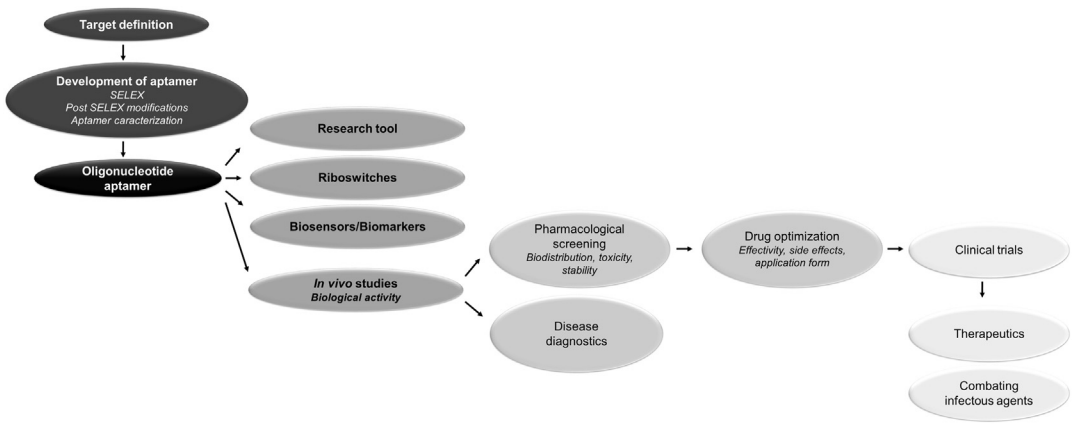

FIGURE 6.2 Flowchart for the development of aptamers and their potential applications.

Some aptamers are found in nature as ligand-binding sites of special RNA structures called riboswitches. Commonly found in bacteria, riboswitches play roles in modulation of many fundamental biochemical pathways by sensing metabolites, such as guanine, adenine, flavin mononucleotide, lysine and glycine [63]. The typical riboswitch contains two distinct functional domains. An aptamer domain binds the effector molecule, and the second domain, the expression platform, is responsible for the interface between the binding event and the change in gene expression [64]. Riboswitches are commonly found in the 5′-untranslated region (UTR) of mRNAs, exerting their regulatory control over the transcript in a *cis*-fashion upon binding of small molecule ligands [64,65]. Because no protein is required for binding of metabolites to the aptamer domain of riboswitches, this regulation offers an immediate feedback response. Insertion of aptamers into the 5′-UTR of mRNAs provides a specific handle for controlling gene expression [66,67]. The binding of the ligand promotes a reversible conformational change in the aptamer domain that regulates the translation of the mRNA [66]. Another promising approach is the reengineering of natural riboswitches, aiming at conditional control of gene expression and cellular processes [68–71]. A vaccination strategy against Venezuelan equine encephalitis virus using this method was successful in controlling the immune response, RNA replication expression, and viral replication [71].

Owing to their ability of recognizing a large variety of targets, aptamers can also be used as biosensors in biosecurity applications. Ions, such as lead (Pb^{2+}), potassium (K^{+}), and mercury (Hg^{2+}), can be detected by aptamers [72,73]. The detection of K^{+} ions can be used as an indicator of several kidney diseases. Chang and colleagues detected this ion in urine by the formation of a G-guadruplex, a secondary RNA structure, in the ATP-binding aptamer, because the folding of this aptamer depends on the concentration of K^{+} ions [72]. The same approach of ion-dependent aptamer folding was employed to detect both, Pb^{2+} and Hg^{2+}, in samples of ponds and soil [73]. Besides monitoring small molecules and drugs in biological samples, aptamer-based biosensors can be used to measure environmental contamination. For example, trinitrotoluene concentrations can be readily detected (within minutes) in water and soil samples [74]. The same aptamer technique is used for the screening of milk samples for the antibiotic neomycin [75].

Besides measurement and detection of specific targets, aptamers can be useful for imaging of biomolecules. Numerous analytical techniques, such as electrochemical, colorimetric, optical and mass-sensitive methods, can be employed to detect targets [76]. Aptamers specific for p68 in liver tumors and for small-cell lung cancer cells demonstrated potential as bioimaging probes [77–79].

Prostate-specific membrane antigen (PSMA), an important prostate cancer marker, is expressed in the vasculature of many solid tumors, especially in prostate. A dual-aptamer probe consisting of an A10 aptamer for PSMA(+) cells and a DUP-1 aptamer for PSMA(−), binds to prostate cancer cells (LNCaP and PC3), but not to normal (PNT2) or other cancer cells [80]. In addition, a drug-delivering system using the same A10 aptamer effectively introduced doxorubicin, an anticancer drug, into prostate cancer cells [81].

6.4 Aptamers in Clinical Trials

The search for the term "aptamer" in public clinical trials database http://clinicaltrials.gov lists 26 clinical trials as of August 2015, summarized in Table 6.2. These clinical trials demonstrate the clinical relevance of a range of aptamers and their targets in a variety of clinical conditions.

Table 6.2 List of Aptamers in Clinical Trial According to clinicaltrial.gov database

Trial Number	Aptamer	Target	Disease/condition	Phase
NCT00950638	ARC1905	Complement component 5 (C5)	Age-related macular degeneration	Phase I
NCT01089517	E10030	PDGF	Neovascular age-related macular degeneration	Phase II
NCT02397954	Zimura	Complement component 5 (C5)	Idiopathic polypoidal choroidal vasculopathy	Phase II
NCT00709527	ARC1905	Complement component 5 (C5)	Neovascular age-related macular degeneration	Phase I
NCT00569140	E10030	PDGF	Neovascular age-related macular degeneration	Phase I
NCT00021736	EYE001	VEGF	Neovascular age-related macular degeneration	Phase II/III
NCT00113997	REG1	Coagulation factor IXa	Healthy	Phase I
NCT02387957	Fovista	PDGF	Neovascular age-related macular degeneration	Phase II
NCT00056199	EYE001	VEGF	Von Hippel—Lindau disease	Phase I
NCT00215670	Pegaptanib sodium (Macugen)	VEGF	Neovascular age-related macular degeneration	Phase II/III
NCT01944839	Fovista	PDGF	Neovascular age-related macular degeneration	Phase III
NCT01940900	Fovista	PDGF	Neovascular age-related macular degeneration	Phase III
NCT00321997	EYE001	VEGF	Neovascular age-related macular degeneration	Phase II/III
NCT00312351	Pegaptanib sodium	VEGF	Neovascular age-related macular degeneration	Phase IV
NCT01940887	Fovista (E10030)	PDGF	Neovascular age-related macular degeneration	Phase III
NCT00040313	Pegaptanib sodium (Macugen)	VEGF	Diabetic macular edema	Phase II
NCT01034410	AS1411	Nucleolin	Acute myeloid leukemia	Phase II
NCT01194934	NOX-A12	CXCL12	Hematopoietic stem cell transplantation	Phase I
NCT00976729	NOX-E36	CCL2	Chronic inflammatory diseases/type 2 diabetes Mellitus/systemic lupus erythematosus	Phase I
NCT00976378	NOX-A12	CXCL12	Autologous stem cell transplantation	Phase I
NCT00694785	ARC1779	Von Willebrand factor	Von Willebrand disease	Phase II
NCT00632242	ARC1779	Von Willebrand factor	Von Willebrand factor—related platelet function disorders	Phase II
NCT02079896	Lexaptepid Pegol (NOX-H94)	Hepcidin	Erythropoiesis-stimulating agent—hyporesponsive anemia	Phase I/II
NCT01191372	ARC19499	TFPI	Hemophilia	Phase I
NCT01487044	Pegaptanib sodium (Macugen)	VEGF	Diabetic macular edema	
NCT01487070	Pegaptanib sodium (Macugen)	VEGF	Proliferative diabetic retinopathy	Phase I

PDGF, platelet-derived growth factor; *TFPI*, tissue factor pathway inhibitor; *VEGF*, vascular endothelial growth factor.

ARC1905 (Zimura): The target of this therapeutic aptamer is complement component, an important mediator of inflammation processes. A phase 1 study was completed with the objective of evaluating safety and tolerability in subjects with dry age-related macular degeneration (AMD) in both eyes. In other completed phase I study it was tested in combination with Lucentis, a monoclonal antibody fragment that binds and inactivates the VEGF. VEGF is a protein involved in angiogenesis. With the same strategy, a phase II study currently recruiting participants is testing the anti-C5 aptamer combined with anti-VEGF therapy in subjects with idiopathic polypoidal choroidal vasculopathy.

E10030 (Fovista): An anti-PDGF aptamer was tested in a completed phase I study in subjects with neovascular AMD to evaluate its safety, tolerability, and pharmacokinetics. In another completed phase II study the aptamer was tested alone or combined with Lucentis in subjects with subfoveal choroidal neovascularization resulting from AMD. In a phase II study that is recruiting participants, the anti-PDGF aptamer is being tested in association with anti-VEGF therapy (Avastin, Lucentis, or Eylea) in subjects with neovascular AMD. Similarly, in two phase III studies, one recruiting and other active but not recruiting, patients with neovascular AMD are being evaluated for the safety and efficacy of combined Fovista and Lucentis therapy compared with Lucentis monotherapy. Another phase III study is recruiting subjects with the same disease to test therapy with Fovista associated with Avatin or Eylea compared with Avatin or Eylea monotherapy. Preliminary results of a phase 1 study that had tested the combination of E10030 and ranibizumab showed partial regression of choroidal neovascularization [82].

Pegaptanib sodium (EYE001, Macugen): This anti-VEGF aptamer was coupled to a 40-kD polyethylene glycol to increase *in vivo* half-life. Pegaptanib sodium was tested in a completed phase II/III study with the objective of assessing the effectiveness of stabilizing and/or improving vision in patients with exudative AMD. Two other studies, one a completed phase II/III study and the other a terminated phase IV study, aimed to compare the safety and efficacy of three different doses of pegaptanib sodium in patients with wet AMD. The safety, tolerability, and pharmacokinetics of intravitreous injections of 1 or 3 mg/eye pegaptanib were also tested in subjects with exudative AMD in a completed phase II/III study. In other two studies pegaptanib was tested in patients with diabetic retinal edema. In the first, a completed phase II study, the purpose was to test pegaptanib's safety and effectiveness in stabilization or improved vision compared with sham injection. In the second study, whose status was identified as unknown and no phase specified, the goal was establish the efficacy of pegaptanib in restoring VEGF levels. The safety of multiple injections of pegaptanib was shown in a phase II study [83]. The results of another phase II clinical trial revealed improvements in visual acuity in subjects treated with pegaptanib [84]. González and collaborators showed that intravitreal pegaptanib injection leads to regression of diabetic retinal neovascularization [85]. The safety and efficacy of intravitreal injections of Macugen in subjects with proliferative diabetic retinopathy were the aim of a completed phase I study. The ability of EYE001 to reduce retinal thickening and improve vision in patients

with Von Hippel–Lindau syndrome, who usually have angiomas in the retina and optic nerve, was tested in a phase I study.

REG1: REG1 is an aptamer-based anticoagulation system composed of two components. The first is an aptamer that binds and inhibits factor IXa (pegnivacogin), and the second is a complementary sequence that inactivates the anticoagulant effect of the first component (anivamersen). In a phase I completed study, the system was tested in healthy volunteers to check the safety and dosing of REG1. In phase I studies it was shown in healthy volunteers that pegnivacogin inhibits factor IX activity in a dose-dependent way, quickly increases clotting, and activates partial thromboplastin time [86]. Subsequently it was shown that in patients with stable coronary artery disease, anivamersen quickly reversed the action of pegnivacogin [87]. Then in healthy subjects who had demonstrated previous results of safety and reproducibility, the researchers tested the use of multiple doses of the REG1 system [88]. Phase II studies evaluated the applicability of the REG1 system in elective percutaneous coronary interventions [89]. The REG1 system was able to suppress ischemic events and thrombotic complications [90].

AS1411: This aptamer targets nucleolin, one of the most abundant proteins. This protein is overexpressed in tumor cells and its presence in the cell surface is related to the growth and metabolic activity of cells. The AS1411 aptamer has been tested in combination with cytarabine in patients with primary refractory or relapsed acute myeloid leukemia in a phase II study.

NOX-A12: This aptamer binds and neutralizes CXCL12, a chemokine involved in metastasis, neovascularization, cell homing, and tissue regeneration. It was tested in two phase I studies in healthy volunteers to assess safety, tolerability, pharmacokinetics, and pharmacodynamics of an intravenous dose of NOX-A12 and to evaluate the effect on mobilization of hematopoietic stem cells. The intention is to use this aptamer in patients with lymphoma undergoing autologous stem cell transplantation.

NOX-E36: This aptamer targets the proinflammatory chemokine CCL2, which is involved in the recruitment of monocytes and T cells to sites of injury and inflammation. In a completed phase I study, safety and tolerability were shown in healthy subjects. Together with pharmacokinetic and pharmacodynamic data, this clinical trial aimed to establish the dose and route of administration of NOX-E36 in patients with chronic inflammatory diseases, type 2 diabetes mellitus, and systemic lupus erythematosus.

ARC1779: This aptamer targets the A1 domain of von Willebrand factor (vWF), inhibiting their binding to platelet glycoprotein Ib. The safety and tolerability of ARC1779 was tested in patients with vWF-related platelet disorders in a completed phase II study. Another phase II clinical trial using ARC1779 in patients with vWF-related platelet disorder was withdrawn before enrollment. Results of the phase I study showed safety and tolerability of ARC1779 in healthy volunteers. Moreover, the inhibition of vWF activity and platelet function occurred dose-dependently [91]. The results of a phase II study provided the proof of concept that ARC1779 inhibits vWF and prevents a

desmopressin-induced platelet drop in patients with type 2b vWF disease [92]. The aptamer was tested in patients with thrombotic thrombocytopenic purpura (TTP) for the safety, pharmacokinetics, and pharmacodynamics of ARC1779. Results showed that ARC1779 aptamer is well tolerated and inhibits vWF, leading to increased platelet count, which suggests that treatment with ARC1779 may be beneficial in patients with TTP [93,94].

NOX-H94: This aptamer binds hepcidin, a hormone produced in the liver that is involved in the control of plasma levels of iron. In addition, hepcidin has been identified as a possible mediator of anemia of chronic diseases. NOX-H94 is currently being tested in a clinical trial phase I/II, aiming to assess the safety and efficacy in increasing hemoglobin concentration in patients receiving dialysis who have functional iron deficiency and erythropoiesis-stimulating agent hyporesponsiveness.

ARC19499: This aptamer is an antitissue factor pathway inhibitor. It was used in a terminated study whose purpose was to test the safety and tolerability of ARC19499 in patients with hemophilia.

6.5 Conclusions

Aptamers have turned into potent tools in research with promising applications in diagnostics and therapy. These high-affinity binders developed by *in vitro* selection and chemical synthesis can be developed for almost every target molecule. Improved selection protocols have made possible the development of aptamers that recognize complex targets on cell surfaces or even entire cells or organisms. Aptamers are ideal for therapeutic and diagnostic applications in medicine and biotechnology, because they can easily be modified to enhance stability and nuclease resistance. The large number of aptamers in clinical trials suggests that they will soon compete with antibodies for therapeutic applications.

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7

Synthetic Peptide Libraries: Chemical Diversity to Reach Lead Compounds

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7.1 Introduction

A combinatorial peptide library is a method in which a vast number of various peptides are synthesized; its use was first reported by Geysen et al. in 1984 using the pin method. Since then, many papers describing different methods of synthesizing and screening peptide libraries have been published [1]. In this approach, a huge number of diverse peptides are produced and successively screened in:

1. drug discovery and development,
2. epitope mapping,
3. target validation, and
4. structure-activity studies.

This method revealed the ability to identify biologically active peptides successfully, including antibacterial peptides, ligands for cell surface receptors, opioid receptor antagonists, protein kinase inhibitors, and substrates. This combinatorial approach is mainly based on three methods by which peptides are selected in an unlabeled and soluble form or still linked to their support, either synthetic or biological, or to a labeling molecule.

These methods can be described as follows:

1. Peptide libraries are synthesized and cleaved from the solid support to be screened as free compounds.
2. Synthetic combinatorial libraries of peptides are assayed on their solid support.
3. A phage display is used, which enables the selection of clones of interest rather than screening, because large phage libraries can be panned against a target molecule by standard protocols.

In this chapter the first two synthetic methods will be examined.

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7.2 Preparation of Synthetic Peptide Libraries

Synthetic combinatorial libraries, especially peptide libraries, are generally prepared by two different methods. The first is the "divide, couple, recombine" method, also referred to as "pool/split, portion/mix, and **mix-and-split**." It was first conceived of by Furka in 1991 [2] and was further developed by the groups of Hruby and Geysen [3] as a robust methodology to create large arrays of peptides to be used for the selection of target ligands. Resin beads are used as microreactors onto which steps of amino acid coupling and redistribution are carried out to increase the number of new sequences exponentially at each step. It consists of three basic steps: splitting, coupling, and mixing. First, resin beads are split into a number of aliquots that equals the number of building blocks to be used in the synthesis (splitting). To each resin aliquot, one building block is coupled and the reactions are driven to completion (coupling). Successively beads are randomly and thoroughly mixed (mixing) and then resplit into the same number of aliquots, achieving a set of homogeneous and equimolar collections of compounds. Repetitive execution of these basic steps for "n" times will produce a rapid increase in the newly generated molecules whereas the bead number remains constant. Because the resin beads encounter one reactant at a time, no more than one compound per bead can be generated [one bead one compound (OBOC)]; therefore the total number of final compounds is limited by the number of initial beads. Furthermore, as reactions are driven to completion, all peptides are generated in equal ratios in the mixtures. The basic principles of the mix-and-split method are outlined in Fig. 7.1A in a simplified version, in which three different amino acids are used as building blocks (A−B−C). The same steps can be performed regardless of the number or types of monomer units, which enormously increases the number of new sequences and their chemical diversity. With this procedure, couplings are carried out on spatially separated samples, adding single amino acids to each separate resin aliquot. As a result, both the number of peptides and their equimolar ratio are preserved in each aliquot. By choosing suitable sets of acid-resistant linkers and side chain protections, the compounds can be left on the beads but freed of the protecting groups. In this way, peptides can be tested still attached to the beads, exploiting the high local concentration of compounds on their surface. However, the use of suitable linkers allows the partial release of molecules, also permitting testing in solution. A major drawback of OBOC libraries is the limited set of peptides. Thus, the library's complexity is dictated by the initial number of beads.

The second method is the "amino acid mixture" method, **PRE-MIX**, in which the amino acids chosen for the library's construction as building blocks are premixed and then coupled to a single resin batch. This method is faster than mix-and-split and allows the preparation of large libraries, thus producing wide chemical diversity. Conversely, the distribution of any single peptide within the final mixtures depends on the relative kinetic rates of individual protected amino acids. To overcome the kinetic differences, the use of "smart" mixtures prepared in molar ratios adjusted according to different coupling rates of preactivated amino acids was proposed [4]. However, alternative

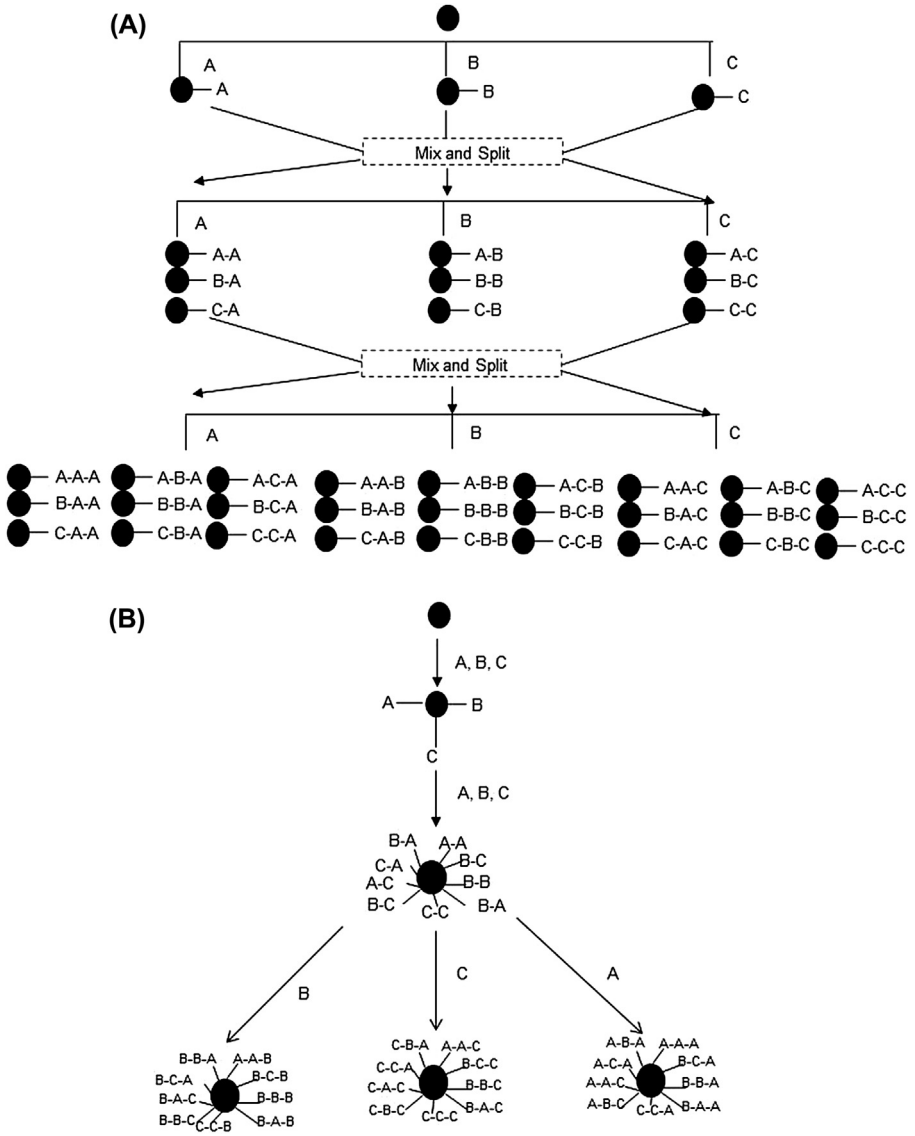

FIGURE 7.1 Scheme of basic principles of the synthesis of peptide library built with three randomized positions and three different amino acids as building blocks (for a matter of simplicity). Three amino acids, after three randomization steps, generate 27 different sequences. A, Mix and split method, repetitive cycles of resin mixing, and repooling are performed until the requested length and complexity are reached. By repeating this procedure n times, a complexity of 3^n is achieved. B, Premix method: the first $n - 1$ randomization steps are performed on a single resin batch by acylating with the premixed amino acids. Before the nth coupling, the resin is subdivided in three equal aliquots and isolated residues are coupled to each different aliquot.

methods can be applied, such as the use of large excesses of mixtures (50- to 100-fold) to compensate for the different reactivities. In Fig. 7.1B the basic principles of this approach are described in a simplified version in an iterative screening format.

Nevertheless, independently of the synthetic method, how are mixtures screened to arrive at one active compound? Various deconvolution methods have been employed, most commonly:

1. **Iterative deconvolution**, which consists of an iterative process of screening and resynthesis of smaller sublibraries in the attempt to fractionate a mixture into its most active constituent(s), as described in Fig. 7.2A [3]; and
2. **Positional scanning**, in which the mixtures are presynthesized in a number of sublibraries for each variable position. Each compound present in a given mixture has a common individual building block at a given position, whereas the remaining positions are composed of mixtures of all of the building blocks used to prepare the library [5]. Positional scanning synthetic peptide libraries (PS-SPCLs) are generated by making individual libraries with one amino acid held constant while the remaining amino acids are varied. The peptide sequence is scanned by creating additional unique combinatorial libraries, each holding a different amino acid constant. A tripeptide positional scanning library provides three distinct library subsets such that each holds one amino acid position constant, as described in Fig. 7.2B [6].

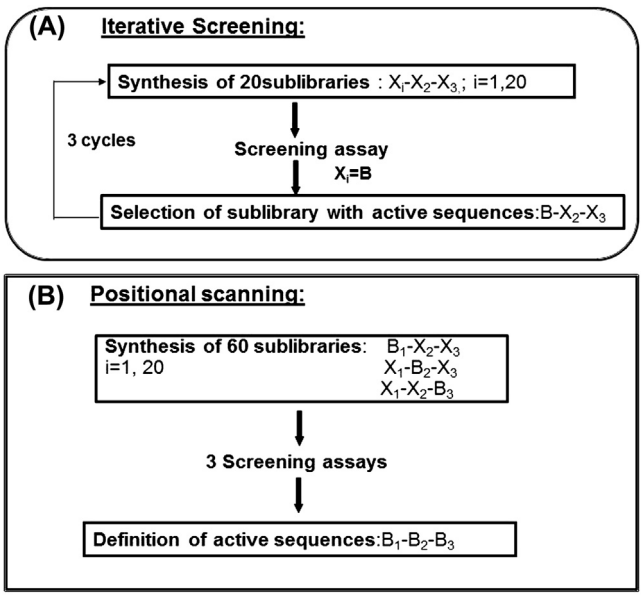

FIGURE 7.2 Scheme of the most common deconvolution methods of a library with three randomized positions and 20 building blocks. A, Iterative screening: Iterative process of screening and resynthesis of smaller sublibraries in the attempt to fractionate a mixture into its most active constituent(s). B, Positional scanning synthetic peptide combinatorial libraries: mixtures are presynthesized in a number of sublibraries for each variable position. Each compound present in a given mixture has a common individual building block at a given position, whereas the remaining positions are composed of mixtures of all of the building blocks used to prepare the library.

Besides deconvolution methods, libraries containing α-amino acids are easy to sequence by Edman degradation using automated protein sequencing. However, Edman degradation requires a free N-terminus, so libraries with more complex peptide structures, such as cyclic or branched peptides or peptides containing β- or γ-amino acids, necessitate inclusion of a chemical tag in the bead structure. These chemical tags can be incorporated into the interior of the bead so as to not interfere with binding of the library peptides to targets of interest, and then subsequently sequenced using either Edman microsequencing or mass spectrometry [7].

A novel method for generating beads with interior tags, termed bilayer beads, was proposed, in which the library compound resides on the outer layer of the bead and the coding tag remains in the bead interior [8]. Such topologically segregated bifunctional beads can easily be prepared with a biphasic method. In this method, TentaGel beads are first thoroughly swollen in water. After excess water is drained, a limiting amount of amino-protecting reagent (e.g., 0.25 Eq.), such as *N*-(9-fluorenylmethyloxycarbonyloxy)-succinimide (Fmoc-OSu) dissolved in an organic solvent mixture of dichloromethane–ether (55:45, v/v), is added to the water-swollen beads. Under these conditions, only the outer layer of the TentaGel bead is derivatized. These bifunctional beads can be applied to OBOC peptide libraries with structures that cannot be sequenced with conventional methods [9]. In such an approach the surface density of the bead is crucial. As an example, it was applied to select multimeric ligands in which the surface density of the displayed peptide resulted in a high local concentration of the ligand (about 100 mM on a TentaGel bead), which can lead to the selection of low-affinity peptides. To avoid this problem, the selection can be performed in the presence of compounds or the peptide density can be reduced through a bilayer bead encoding approach. Alternatively, the beads can be spatially separated [10].

Similar to a phage display, synthetic libraries are relatively inexpensive and easy to generate; however, they are not constrained to natural amino acids such as those generated by phage technique, and can include both unnatural and D-amino acids, in addition to secondary structures constraints not tolerated by the phage. Indeed, completely unnatural peptoid libraries have been used to select cell-binding ligands [11]. Because unnatural and D-amino acids are less susceptible to proteases and peptidases than are natural L-amino acids, synthetic libraries have the potential to identify stable peptide sequences rapidly, and posttranslational modifications such as glycosylation and phosphorylation can be incorporated into the library design as well. The design of synthetic libraries also makes them ideal for use in optimization of known ligands. Peptides previously isolated by phage display or structure-based methods can be used as lead compounds for synthetic peptide library construction, allowing for rapid generation of optimized peptides with higher affinity or specificity.

Synthetic libraries in soluble format offer a series of advantages such as the higher specificity of the interaction of bioactive selected peptides that can be synthesized as single library and aliquoted for use in multiple assays with multiple targets and lead sequences can be rapidly optimized. Furthermore, they are adaptable to almost any

selection technique. They can be incubated with cells or receptors, typically in a high-throughput fashion such as in a 96-well plate or a microarray. For example, screens can be made for binding versus competitor fluorescently tagged natural ligands, looking for loss of fluorescence [12] or using biotinylated peptide and streptavidin–horseradish peroxidase as a detection reagent [13]. However, they are not commercially available and usually require additional synthesis for retesting. Because this approach depends on the idea that each amino acid of single lead sequence contributes individually to binding to the target of interest, this may make it difficult to determine ideal peptide sequences when multiple peptide motifs exist for the given target [14]. Thus after the ideal amino acids at each peptide position are determined, all possible combinations of peptides using these ideal amino acids should be generated and further tested for binding. Although PS-SPCLs are not used as frequently for the initial isolation of cell-targeting peptides, they are an excellent way to optimize lead peptides isolated from a phage displayed or bacterial peptide library [15,16].

On the other hand, OBOC libraries have unique features such as the selection of peptides on whole cells in vitro or ex vivo assays, but the presence of the linker provides potential steric hindrance between the peptide sequence and cellular receptor and is not suitable for in vivo screening in animals.

Although synthetic procedures generate millions of peptides, they are partially limited by fact that the sequence of the peptide on a particular bead must be determined by either sequencing or using additional encoding procedures as described in deconvolution methods (Fig. 7.2).

In an alternative method, reaction reagents and amino acid monomers can be spotted (SPOT technique) onto a support surface such as a cellulose membrane, creating spatially addressable peptide arrays containing about 100 sequences/cm^2 [17].

However, often the most common cellulose membranes used for SPOT are acid sensitive and lack mechanical stability in the synthesis process [18,19].

Further miniaturization of peptide arrays relies on the parallel synthesis of individually addressable peptide microchips that appeared efficient and versatile. This approach is based on conventional peptide chemistry with in-solution removal of acid-labile protecting groups using photogenerated reagents and digital photolithography [20,21]. These peptide microchips resulted in assays suitable for epitope binding to screen quickly and systematically for sequences specific to the human p53 antibody Pab240 [22,23].

Cell-binding ligands that can deliver biologically active cargo to a specific cell type or a diseased cell are highly sought [24,25]. Soluble target proteins can be screened for binding to OBOC libraries using several different approaches, all of which rely on the ability to distinguish protein-bound beads selectively. The protein of interest can be labeled with a tag (i.e., fluorescent or colorimetric dye, biotin, enzyme, radionuclide, epitope tag) that allows the protein-bound beads to be detected. Most commonly, an enzyme-linked colorimetric assay is employed [7]. The protein target can be directly labeled with alkaline phosphatase, or if a primary antibody against the protein is

available, an alkaline phosphatase secondary antibody can be employed. The peptide content of isolated positive beads is then determined using Edman sequencing or mass spectrometry. The success of the screen depends on the stringency of the selection conditions and effective negative screens.

The greatest potential of combinatorial chemistry is represented by the number and variety of screenable compounds. Major efforts have focused on the development of methodologies to further increase molecular diversity: "dynamic mixtures" are based generally on reversible reactions in these; reactants and products are present in thermodynamic equilibrium. The most potent binding compound is selected among different combinations of mixture components, producing a shift of the equilibrium by subtracting products. However, although innovative and promising, these libraries have been limited so far to a small number of reversible reactions and libraries of moderate size [26,27]. Similarly, "libraries from libraries" represent an innovation respect to the traditional combinatorial chemistry. By this approach, combinatorial libraries of peptides once built on a solid phase are subsequently modified to maximize chemical diversity. Oxidations, reductions, alkylations, and acylations are performed, exponentially increasing the number of new compounds [28].

7.3 Peptides as Drugs

Peptide-based drug discovery processes have raised renewed interest because of the substantial failure, in most cases, of the screening of small molecules in discovering antagonists of protein−protein interactions (PPI) at the molecular basis of several human diseases [29,30].

Peptides are highly specific and show minimized risks of systemic toxicity, because their degradation products are amino acids and have a short half-life. The advantages of peptide-based therapeutics derive from unique chemicophysical properties including the modularity of amino acids and amide bonds; high target affinity, specificity, and potency; relatively low cost; ease of synthesis and storage; and reduced antigenicity [31]. Despite these favorable properties, they often have poor bioavailability and low metabolic stability. Thus major challenges for the development of peptide drugs appear to be optimization of their pharmacokinetics and setup of successful delivery strategies.

The most commonly used strategies to transform peptides into small peptidomimetics are outlined in Fig. 7.3, but process results are often difficult and intricate [32]. Once the primary structure of the biologically active peptide has been determined, the first step is to identify the minimum active sequence (MAS) required for the activity. This step involves testing truncated peptides from the C- and N-termini alternatively. Subsequently, the influence of individual amino acid on the biological activity is determined by systematically replacing each residue in the peptide with specific amino acids, typically alanine or D-amino acids.

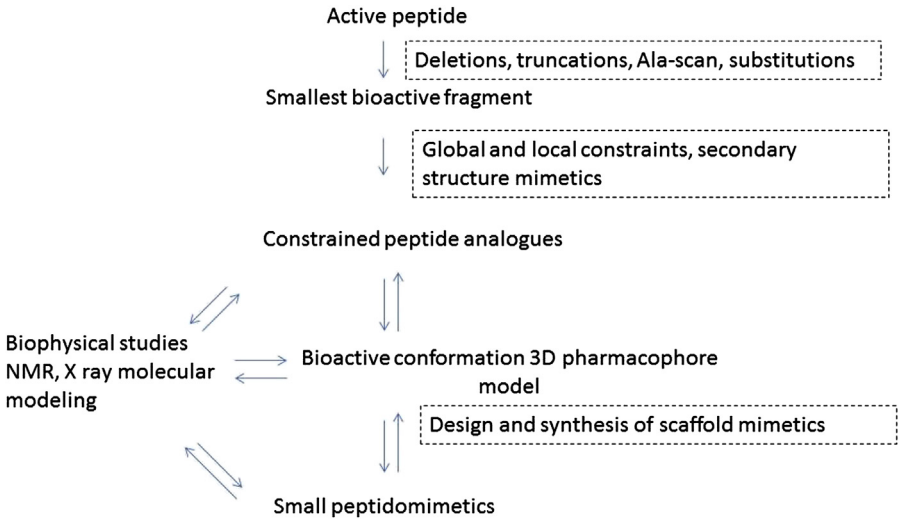

FIGURE 7.3 Flowchart of strategies for the conversion of peptides into small peptidomimetics.

Synthetic peptide libraries of single compounds are often employed in this context: Ala-, Pro-, and D-Aaa scanning truncated and overlapping peptidomimetic libraries are often synthesized and screened to identify MAS [15].

After the structure–activity relationship (SAR) of each amino acid in the sequence has been assessed, the bioactive conformation is investigated by introducing constraints at various positions to reduce the conformational flexibility of the peptide; indeed, to fix crucial features for biological activity, peptides should be as small and rigid as possible. On the length, generally peptides with fewer than six residues are good starting points [33], whereas for peptides between 6 and 15 residues are challenging even if they often contain a well-defined key core and can include other dispensable positions. Conformational flexibility can be reduced by introducing local or global constraints at various positions in the peptide. These constraints can be achieved by incorporating modified amino acids, chemical modifications of the N-, the C-termini, and of the backbone, as well as by side chain-to-side chain cyclization including the formation of disulfide bridges. Many cyclic peptides, pseudopeptides, and peptidomimetics preserving their biological properties have been further modified to increase their resistance to degradation and elimination, bioavailability, and selectivity to become good drug candidates [34,35]. Usually the exact binding conformation of both peptide and protein in a complex is difficult to determine by experimental structural techniques (X-ray crystallography or nuclear magnetic resonance), but also the docking of peptides to binding partners in silico is one of the most complex modeling problems owing to the intrinsic flexibility of the ligand [36]. SAR studies of the constrained analogs can be used in an iterative process to provide information about the receptor-bound and biologically active conformation.

7.4 Recent Applications

Synthetic peptidomimetic libraries have found many applications in select bioactive compounds in many fields, such as antigens, inhibitors and activators of PPIs, kinases, proteases and tyrosinases, and ligands of receptors and catalysts [37].

Here interesting applications will be described.

To evaluate the protease activity of several enzymes, often synthetic peptide libraries have been employed. A study was focused on high-temperature requirement A2 (HtrA2). This protein belongs to the HtrA family of adenosine triphosphate–independent serine proteases. The primary function of HtrA2 includes maintaining the mitochondria homeostasis, cell death (by apoptosis, necrosis, or anoikis) and contribution to the cell signaling. HtrA2 protease substrate specificity was delineated via a combinatorial chemistry approach that led to the selection of novel intramolecularly quenched substrates. A combinatorial chemistry approach and the mix-and-split method with iterative scanning performed in solution were employed. Two separate substrate libraries to characterize the nonprime and prime binding pockets of HtrA2 were synthesized. The general formula of the synthesized peptide libraries were:

1. the nonprime library, ABZ-X_4-X_3-X_2-X_1-ANB-NH_2, in which in position $X_4 = X_3 = X_2$ the set of proteinogenic amino acid residues, excluding Cys, was present, whereas Ala, Abu, Val, Nva, Ile, Leu, Nle, Ser, or Thr was introduced in position X_1; ABZ-2-aminobenzoic acid (fluorescence donor); ANB-NH2-amide of 5-amino-2-nitrobenzoic acid (quencher of fluorescence); and
2. the prime library, ABZ-peptide-X_{10}-X_{20}-X_{30}-Tyr(3-NO_2)-NH_2, in which in position $X_{10} = X_{20} = X_{30}$ the set of proteinogenic amino acid residues, excluding Cys, was introduced.

For all synthesized compounds, the highest HtrA2-mediated hydrolysis efficiency and selectivity among tested HtrA family members was observed for ABZ-Ile-Met-Thr-Abu-Tyr-Met-Phe-Tyr(3-NO_2)-NH_2, which displayed a specificity constant kcat/KM value of 14,535 M^{-1} s^{-1} [38].

In past decades peptides were considered a class of anticancer agents that could specifically target cancer cells with lower toxicity to normal tissues, offering new opportunities for cancer prevention and treatment [39]. Indeed considerable progress has been made to identify peptides that specifically bind to tumor cells and tumor vasculature, defined as tumor-homing peptides [40]. The principle behind the peptide homing strategy is that they should recognize only molecules that are up-regulated in tumors, and therefore would not recognize normal cells from the corresponding organ [41]. Numerous peptide ligands have been isolated against various types of receptors or cells, such as RGD-containing peptides against integrin receptors in angiogenic tumor vasculature [42], or specific for platelet-derived growth factor receptor-β receptor in pericytes and endothelial cells [43], KRK-containing peptides directed to angiogenic blood vessels and tumor cells [44], and a peptide recognizing thrombin receptor [45].

Tissue-specific homing peptides have also been reported for pancreatic β-cells, as well as specific peptides for tumor cells, especially lung tumor. There is also a peptide named GE11, which specifically recognizes the epidermal growth factor receptor [46]. The most widely used peptides in the targeted-delivery applications are integrin-targeting RGD-peptides, the first tumor-targeting peptides discovered. Integrins αvβ3 and αvβ5 are overexpressed in tumor endothelium and some epithelial cells during tumor growth, angiogenesis, invasion, and metastasis. Therefore, they represent an interesting molecular target for a tumor-homing approach [47]. RGD is a cell-adhesion motif present in many proteins of the extracellular matrix. This motif is recognized by αvβ3 and αvβ5 integrin receptors. The binding affinities of some of the RGD-containing derivatives for αvβ3 range from 3.2 to 100 nM. The addition of specific amino acid residues to peptide sequence motifs, such as RGD, induces binding to cell-attachment proteins and strongly enhances the binding affinity of the peptide. The injection of the modified peptides induced antigen-specific serum antibodies [48].

In the employment of synthetic peptide libraries, few peptides have been selected against purified cell surface cancer biomarkers using OBOC libraries [49]. However, these synthetic libraries have been more widely exploited for selections on whole cells such as breast cancer MDA-MB 231 toward receptor α3β1 [50], leukemia Jukart cells toward α4β1 [51], ovarian cancer cells such as CAOV-3, SKOV-3, ES-2 [7,52], lung cancer cells such as A-549, H-1650 [53,54], and prostate cells such as DU 145 [55,56]. This may be partly because of the ease with which positive beads can be isolated by visual inspection. Biased selections can be performed in which the target receptor is overexpressed and the selection occurs within the context of whole cells. For example, the cyclic peptide cGRGDdvc was isolated from OBOC screening using αvβ3 integrin-transfected K562 leukemia cells [57]. The parental K562 cell line was used for negative selections to remove ligands that bound to other receptors with respect to αvβ3. However, most OBOC screens have been performed on cell lines without overexpression of a specific receptor. Nonetheless, all of the peptides isolated from OBOC libraries for which receptors have been identified bind integrin receptors. Much of this is likely due to library synthesis, because many of the libraries are designed with a bias toward sequences that are known to bind integrins. As such, it cannot be ruled out that the peptides bind other cellular targets as well. Unlike peptides isolated from phage-displayed libraries, the ligands identified from OBOC libraries frequently bind multiple cancer cell lines. This is not surprising considering that integrins are often up-regulated in many cancers [58].

On the other hand, the PS-SPCLs are ideal for screenings for cellular effects induced by peptides. Kang and co-workers used integrin microarrays to select peptides that inhibit angiogenesis. From this screening a novel peptide ligand containing a PDZ-binding motif (Ser-Asp-Val), named P11, was selected. During the screening fluorescently labeled fibronectin was mixed with the peptide library before addition to the microarray. Library peptides that inhibited the receptor−fibronectin interaction were thus visible by a reduced fluorescent signal [59].

In a similar fashion, an α5β1 microarray and a PS-SPCL were used to screen for peptides that inhibit α5β1–fibronectin interactions [60,61]. RGDs have been found by panning on purified integrins, on whole cells, and in vivo [62]. The specificity of the peptide for a particular integrin varies depending on the flanking sequence around the RGD [63,64]. High-affinity binding to integrins is achieved with only three amino acids; any library with a diversity of 8.0×10^3 is statistically likely to contain the RGD binding motif. For this reason and the fact that integrins are often overexpressed in cancer cells and tumor vasculature, it is not surprising that RGD sequences are often isolated.

Similarly, numerous NGR-containing peptides have been found over the years [65]. This peptide motif binds to aminopeptidase N (CD13) but can also undergo a spontaneous deamination to form isoDGR, a ligand for αvβ3 and α5β1 integrins [66].

In an applicative perspective, the possibility of incorporating unnatural amino acids in synthetic peptide libraries makes them useful for organo-catalysis. Metallopeptide catalysts and artificial metalloenzymes built from peptide scaffolds and catalytically active metal centers possess a number of exciting properties that could be exploited for selective catalysis [67]. Control over metal catalyst secondary coordination spheres, compatibility with library based methods for optimization and evolution, and biocompatibility stand out in this regard. A wide range of unnatural amino acids (UAAs) have been incorporated into peptide and protein scaffolds using several distinct methods, and the resulting UAA-containing scaffolds can be used to create novel hybrid metal–peptide catalysts. Promising levels of selectivity have been demonstrated for several hybrid catalysts, and these provide a strong impetus and important lessons for the design of and optimization of hybrid catalysts [68].

New strategies involving computer-customized combinatorial libraries offer enormous potential for the design of more “focused” and “smart” chemical libraries with maximal diversity. Libraries can be seen as a source of bioactive molecules that are selected on the basis of the biochemical properties predefined by appropriate assay settings. These settings represent the fishing-out method. The more diverse the library will be, the higher will be the probability of selecting good “hits.” The concept of “diversity” is therefore of utmost importance in choosing a library for a particular screening, and good libraries must first fulfill the requirement of “highest diversity” instead of the “highest complexity.” Molecules with overlapping structures will not or only poorly contribute to the overall probability to find a positive hit. The diversity of a library is generally associated with its complexity (the number of different components). However, this is not always true, and low-complexity libraries can display a higher diversity than can libraries with a huge number of components. In fact, the synthesis of random combinatorial libraries of peptides generates a large number of “quasiduplicates” deriving from the strong similarity between several side chains.

Using common amino acids, in L- or D-configuration, sequences where Glu is replaced by Asp, Leu by Ile or Val, Gln by Asn, and so on, can display similar properties. Such residues, although different in their propensity to adopt secondary structures, can be considered almost equivalent in terms of intrinsic physicochemical properties, as for

example the capacity to establish external interactions or to fit in a crucial recognition site. On the contrary, in large libraries, the need to manage large arrays of tubes and codes can puzzle the way to identifying lead compounds and slow down the synthetic and deconvolution steps. In the perspective of simplifying the synthesis and deconvolution procedures without affecting the probability of finding active peptides, our approach focused on the general properties of L- and D-amino acids, reaching a compromise between the need to maintain the highest possible diversity and reducing the number of building blocks. We called these libraries "simplified libraries," intending with this any new ensemble of possible sequences achievable with a reduced and nonredundant set of amino acids. The distribution of residue molecular weights is also an important parameter in cases of deconvolution of the library by mass spectrometry approaches [39].

We have further evolved this approach by applying "focused simplified libraries" in which, through an Ala-scan approach, we defined hot spots of a natural peptide, the KIR domain of SOCS1, in the interaction with JAK2 and randomized few positions within it, to find a more active peptidomimetic named PS5 [15,16]. Indeed the chances of finding a hit are much higher in a focused combinatorial library than in a completely random library because the structural diversity is already positively biased for a given problem, e.g., binding to a specific target protein. Thus to identify new ligands it is often sufficient to use much smaller libraries with only a couple of hundred different members. The use of small but focused libraries, rather than completely random mixtures, has already been successfully applied in pharmaceutical and medicinal chemistry [33,69].

7.5 Conclusions and Perspectives

Major advantages of peptides rely on their typical high affinity and selectivity, their high potency, minimized drug–drug interactions, and low accumulation capacity and toxicity.

Peptide libraries can have a pivotal role in the rapid identification of high- or medium-affinity target ligands that can be subsequently optimized in terms of potency, selectivity, and stability. Indeed the construction of peptide libraries is commonly used to identify and characterize ligand receptor-specific interactions and search for novel ligands for protein purification. The easiness of preparation, characterization, and robustness of the available chemistry have pushed tens of laboratories around the world to use synthetic peptide libraries as a source of chemical diversity and a versatile way to select new active molecules.

Further improvement in chemical and biological resistance of affinity ligands encouraged the "intelligent" design and synthesis of chemical libraries of low–molecular weight bio-inspired mimic compounds. For instance, high-throughput synthesis and screening of compound collections through phenotypic or biochemical assays often yields useful compounds discovered, relative to the high cost in time and resources expended [70].

The large complexity achievable by using nonnatural amino acids, by introducing secondary structure diversity elements or changing the basic scaffolds to enhance molecular surfaces and target avidity, represents an indisputable advantage with respect to small-molecule libraries. These major points, coupled with the ease of preparation, characterization, and handling, will increasingly set forth synthetic peptide libraries to the attention of the drug discovery chemists community, making them an invaluable tool for drug discovery.

The use of novel automated synthesis and screening strategies, such as simplified focused peptide libraries, can increase the process throughput and speed the discovery procedure, making the discovery of new active peptides a fast and efficient process.

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8

Biotechnological Role of Phage-Displayed Peptides for the Diagnosis of Neglected Tropical Diseases

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8.1 Introduction

Thirty years ago at the University of Missouri, George P. Smith [1] reported, for the first time, an in vitro strategy for identifying peptide ligands for antibodies. Based on the specificity and affinity between molecules, filamentous phages displaying peptides (phage display) on the surface were selected from a combinatorial library of random peptides.

Although the phage display technology has evolved in the context of protein bioengineering, the potential of this new approach was noted years later, taking into consideration the increase in articles related to the technique.

Over the years, owing to its simplicity, cost effectiveness, reproducibility, and versatility for generating a wide range of biological ligands to almost any target, phage display has become a well-established platform for a variety of applications including screening of antibody repertoires [2], selection of binders in vivo [3], discovery of new therapies [4], inhibitors of pathologic antibodies [5], design of vaccines [6,7], studies about receptor–ligand interactions [8], epitope mapping [9], and identification of immunodominant epitopes for serologic diagnosis including neglected diseases [10–12].

This practicality in identifying binders with desired properties from any number of possibilities is feasible because this technology is based on linkage between the surface-displayed peptide or protein and the genetic material that encodes it, i.e., the physical association between the phage phenotype and its genotype is guaranteed. In fact, as an interconnection between molecular biology and bioengineering, the gene encoding the displayed molecule is fused to that of a phage coat protein. This strategy provides an efficient exposure of the candidate ligands to the target molecules, ensures the selective

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isolation of identical bacteriophages from a single host cell clone, and allows the primary structure of the phage display–derived binder to be easily determined by nucleotide sequencing.

Considering the efficiency of phage display as a selection method, regardless of research purpose, layout and strategy of screening, or which library is used (peptide, antibody, or protein), the selection of ligands requires some rounds of panning. This selective process is composed of an incubation that allows interaction with the target and posterior removal of nonspecific phages. The recovery of particles with potential affinity occurs after an efficient elution process and sequential enrichment of specific binding phages through the infection of host cells, usually *Escherichia coli*. Successful screening of phage-display libraries after each panning can be evaluated by titering the infective phages before each amplification [13]. In this phase, each infected host cell is individualized as a clone that can be recovered and stored. Thus, unamplified phages can be assayed individually just after each panning to assess their reactivity or affinity with the target.

Undoubtedly, the library quality in terms of complexity, diversity, and stability has a direct influence on efficient screening. Nevertheless, other technical elements, such as the use of high-quality affinity reagents and the process of elution, have to be considered before starting the selection of phage-display random peptide libraries.

The epitope mapping of polyclonal antibodies from patients is an alternative to obtain antigens and/or immunogens that are able to be used as binders in immunodiagnosis or to elicit specific and efficient immunologic response against an etiologic agent, especially if this agent is the cause of a neglected disease. Taking into account that usually these patients have concomitant diseases, the antibodies, as good targets, should be as specific as possible, i.e., should be able to be recovered after binding to the original antigen with high affinity.

In this same context, another important element is the elution of the bound phage. Typically, when the ligand is not known or there is insufficient quantity of antibody binders to be used in a specific competitive elution, phages are eluted with severe pH alteration, i.e., in a nonspecific manner. Employing two different models: a *bacillus* (*Mycobacterium leprae*) [11,12] and a metazoan parasite of tissues (*Taenia saginata*) [14], in this chapter, an overview about some important aspects relating to each of these procedures will be discussed.

8.2 Hansen Disease: Example of Rarity of Antigens and Patients' Antibodies

"I have examined a great number of them—small staff-like bodies, much resembling bacteria, lying within the cells." One hundred forty years ago, these were the words used by Gerhard Henrik Armauer Hansen (1841–1912), a Norwegian physician, to describe for the first time the *M. leprae*, the causative agent of leprosy [15]. As a tribute to him, this

chronic infectious illness is also known as Hansen disease and affects mainly the skin, mucosa, and peripheral nerves.

Although the skin and neurologic examination is fundamental, the diagnosis is confirmed by the presence of acid-fast bacilli in skin smears or biopsies. Noninvasive procedures are not available. For this reason serologic assays using the major antigenic glycolipid in the *bacillus*, phenolic glycolipid 1 (PGL-1) have been developed. Although PGL-1 is considered a good antigen [16], its application on a large scale is unfeasible because *M. leprae* is an uncultivated bacteria. Considering the epidemic character of Hansen disease in developing countries, more than ever the development of synthetic antigens [11,12,17] is desirable.

A possible alternative to the use of a native antigen in diagnosis would be the identification of peptide mimotopes (or mimic epitopes) from a phage-displayed peptide library [18] using patients' antibodies as the target. However, the successful screening of a phage-display library depends on the specificity and affinity between the ligand and its target. Thus obtaining specific antibodies after binding to the antigen is necessary.

Particularly in relation to *M. leprae*, two problems are evident:

1. the difficult of obtaining the crude antigen, because it is a noncultivable bacteria cultured only on the footpad of nine-banded armadillos [19], and
2. the low level of antibodies found in patients with type 1 T helper—dominant immune response against the bacilli [20].

The affinity purification of patients' antibodies using *M. leprae* antigens bound to polyvinylidene difluoride (PVDF) [21] membrane was the alternative to using minimal amounts of bacilli to obtain specific and rare antibodies from patients' sera. The next section discusses the most important steps of the protocol used by Alban and colleagues [11,12] to provide anti—*M. leprae* antibodies used as targets for the phage-display peptide library.

8.3 Antibody Purification Based on Affinity to Membrane-Blotted Antigens

8.3.1 Obtaining Crude Human Immunoglobulin by Ammonium Sulfate Precipitation

Precipitating human immunoglobulins by using ammonium sulfate [22] reduces contaminants and concentrates antibodies from a large starting volume of serum. Besides being easy to perform, it is a rapid and inexpensive method.

Protocol 1.

1. The ammonium sulfate saturated solution (4.3 M) should be prepared at least 24 h before use.
2. Centrifuge serum for 30 min at 10,000 × *g* at 4°C. Remove any remaining lipids that would compromise the salt precipitation.

3. Determine the total sample volume and transfer it to a beaker containing a stir bar. Place the beaker into an ice bath and on a magnetic stirrer.
4. Stir gently and add the ammonium sulfate–saturated solution drop by drop slowly and continuously to produce a 50% final saturation, i.e., add the saturated solution to the sample in the proportion of 1:2.

   Adding the saturated solution very slowly guarantees the homogeneity of the salt concentration at the dripping site and consequently prevents the precipitation of contaminants.

5. After dripping the entire volume of ammonium sulfate, keep the beaker in the stirring plate at 4°C overnight to ensure complete precipitation.
6. Transfer the milky solution to conical tubes and centrifuge at 10,000 × *g* for 15 min at 4°C.
7. Remove the supernatant and carefully invert the tube on an absorbent paper to drain the residual liquid.
8. Initially, dissolve the pellet in 20% of the original volume in phosphate-buffered saline (PBS) (0.05 M, pH 7.4) by drawing the solution into and out of the pipette carefully.
9. After total dissolution, complete the volume by adding PBS up to 40% of the original volume and dialyze it against PBS (100 vol) at 4°C for 3 h. New buffer changes should be done after 6 and 12 h of dialyzing (overnight) to remove any residual ammonium sulfate from the antibody solution.

   In the last dialysis, to prevent bacterial contamination, it is recommended to add 0.03% thimerosal.

10. Centrifuge to remove aggregates, verify the concentration, make aliquots to prevent repeated freeze–thaw cycles, and keep it at −70°C or make working aliquots and keep them at 4°C for a few months.

8.3.2 Affinity Purification of Human Antibodies Using Antigens Immobilized on Blot Membranes

An efficient method of antibody purification results in the selective enrichment of specific molecules based on their functionality. This process depends on the stability of the antigen and of the preservation of its antigenicity when immobilized on a matrix.

The purification of human antibodies from PVDF [21] or nitrocellulose [10] membranes, as illustrated in Fig. 8.1, containing immobilized antigens has been shown to be a useful and convenient method when both antibodies as antigens are scarce. Especially in relation to ethical aspects, sometimes the serum volume collected from patients is limited and it is necessary to work with minute quantities.

Although some renaturation of proteins occurs during the process [23], antibodies whose recognition depends on the antigen conformation might not be purified using this methodology.

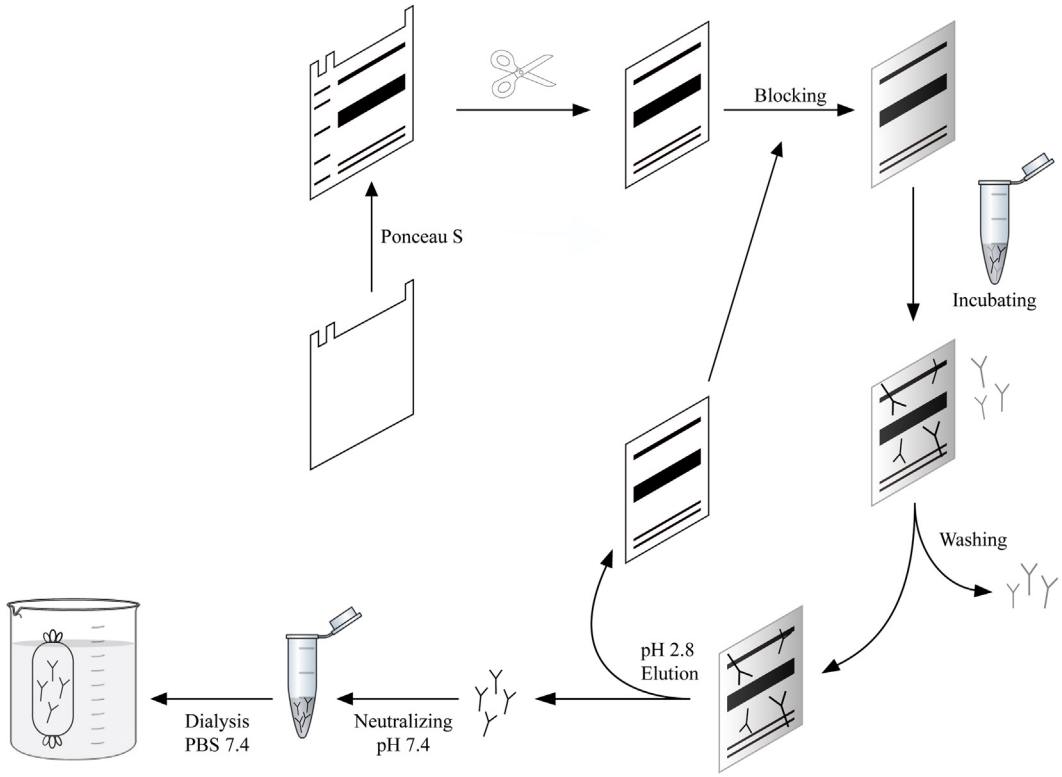

FIGURE 8.1 Overview of some steps to purify immunoglobulins from human serum based on the affinity to membrane-blotted antigens.

Protocol 2.

1. First, prepare a polyacrylamide gel (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) in terms of acrylamide concentration, according to the specifics and characteristics of the sample.
2. If the sample consists of crude antigen, run 1–10 mg of protein on one large lane in a 1.5-mm tick, 10-cm-wide gel size. On the other hand, if the antigen is a purified protein, 20–100 μg may be used.
3. Once the protein(s) has been resolved, transfer them to a PVDF membrane prewetted with methanol or ethanol according to the manufacturer's instructions.

   Protein transfer to a membrane support is done to make the proteins accessible to ligands such as antibodies. Therefore this support could also be a nitrocellulose membrane. Although nitrocellulose is cheaper than the PVDF membrane, the latter is recommended for the affinity purification of antibodies because of its higher protein binding capacity (180 μg/cm^2 against 100 μg/cm^2) and because of its higher resistance to mechanical damage and to a wide operating range of pH, which makes it resilient to the elution and reprobing process.

4. Visualize the proteins by staining the PVDF membrane within freshly prepared Ponceau solution [0.5% (w/v) in 1% (v/v) acetic acid] and rinse it with 50% methanol to destain.

   Nitrocellulose membrane must be rinsed with distilled water until the bands become clearly visible.

5. Trim away any excess membrane and block it with 0.3% (v/v) Tween-20 in PBS [24] for 1 h at room temperature.

   In the case of using purified proteins, cut out the band of interest after staining and destaining and transfer the small membrane pieces to a 1.5-mL microtube.

6. Wash the membrane with PBS containing 0.05% (v/v) Tween 20 (PBS-T) three times for 5 min.
7. Place the membrane into a appropriately sized plastic bag containing 1 mg/mL crude immunoglobulin fractions in PBS-T and close it using a plastic bag sealer. Incubate at 4°C overnight with gentle agitation.

   Using this method of antibody purification, it is reasonable to assume that small amounts of antibody will be recovered, considering that the serum concentration of an antibody against an immunodominant antigen is generally between 50 and 200 μg/mL [25] and that recovery of a specific antibody is between 25% and 33% [21].

8. Remove the membrane from the plastic bag and wash it with PBS-T six times for 5 min.

   More stringent washes by using PBS and 0.1% Tween 20 can be used to reduce nonspecific binding and to select high-affinity antibodies.

9. Elute the antibodies by incubating the membrane with 2 mL of 0.1 M glycine, 0.15 M NaCl, pH 2.8, at room temperature for 30 min, and agitation.

   If the elution is done from small membrane pieces, add around 200 μL of 0.1 M glycine, 0.15 M NaCl, pH 2.8, to the microtube or in enough of a volume to maintain them completely submerged in solution.

10. Transfer the glycine solution to a tube containing a volume of 1 M Tris−HCl, pH 9.0, to neutralize the pH (7.0−7.5).
11. Repeat the purification process; the membrane can be reprobed several times.
12. Pool eluates, dialyze it against PBS, and verify the antibody concentration spectrophotometrically by absorbance at 280 nm.

    This dosage method is interesting because the sample is recoverable. At 280 nm, human antibody solution at concentration of 1 mg/mL gives an absorbance of 1.38.

13. Add bovine serum albumin (BSA) to 1 mg/mL to reduce loss from adsorption to tubes and to stabilize molecules. Make aliquots to prevent repeated freeze−thaw

cycles and keep at −70°C for long-term storage or working aliquots and keep at 4°C for a few months.

8.4 Parasitic Infections: Elution Strategy of Phage-Displayed Peptides When Antigens Are Available

The World Health Organization estimates that more than a quarter of the total population in the world, approximately 1.4 billion people, are affected by helminth infections, especially in the developing countries [26]. These data, which are alarming in themselves, do not include protozoan infections. It is not rare that besides been caused by more than one parasitic worm, helminth infections coexist with other etiologic agents such as *Plasmodium* spp. [27], a phenomenon that increasingly requires the use of specific antigens for differential immunodiagnosis.

Considering the number of patients, the helminth dimension, and the efforts for developing techniques for in vitro cultivation of these parasites [28], limitations in obtaining a crude antigen would not be expected in some fundamental steps of phage-display library selection, as occurs with *M. leprae bacillus*. However, one of the most relevant problems relating to search of immunodominant proteins from parasitic helminths lies in the fact that most of them have complex cycles and different evolutionary stages.

The taeniosis/cysticercosis complex is one example. *T. saginata*, a tapeworm species classified within the Platyhelminthes phylum, is a parasite in the small intestine of humans (taeniosis), the only definitive host. The ingestion of *T. saginata* eggs scattered in pastures or water by cattle, the intermediate host, culminates with the development of the infective larval stage in the musculature (cysticercosis). The life cycle is perpetuated when a human eats raw or improperly cooked bovine meat containing a viable larval stage [29].

Habits of consuming undercooked steaks facilitates the continuity of the taeniosis/cysticercosis complex and makes taeniosis an important social and public health problem in developing countries. Some efforts must be done to break the parasite cycle, such as to establish wider health education regarding the prevention of parasitosis is and high standards of veterinary control over slaughter practices, and to adopt modern and more sensitive diagnostic tools [30].

To propose an alternative to the current method of inspection by visual detection, the phage-display technique was employed as a biotechnological approach able to screen epitopes recognized by antibodies from bovines infected with *T. saginata* larvae to develop a new generation of antigens. To this end, specific antibodies purified against larvae antigens were used for immunopanning phage-displayed libraries [14]. The libraries used in this work were described by Bonnycastle and colleagues [18] and obtained from J. Scott (Simon Fraser University, Burnaby, BC, Canada).

In this topic, by using the bovine cysticercosis as model, the reactivity of ligands after two different elution protocols (acid and competitive) was verified. The results obtained

with both strategies were compared after probing the original antibodies against synthetic sequences corresponding to each peptide phage using the SPOT method [31,32].

8.4.1 Screening Phage Displayed Peptide Library: Acid × Competitive Elution

Generally, considering that bacteriophages are able to tolerate extreme conditions and remain infective, the elution of high-affinity binders from phage libraries can be done by changing drastically the pH, i.e., by using 0.1 M glycine–HCl, pH 2.2 [9,10] or 0.1 M triethanolamine, pH 11 [33], solutions.

Although the acid–basic elution can result in the selection of binders to denatured antibodies or targets [34], this nonspecific elution is convenient if the original antigen or ligand of the antibody or target is not known, is not available, or is obtainable in low amounts, conditions that restrict the elution based on the binding characteristics of the target.

To demonstrate the utility and differences of both of these elution strategies, the following provides a step-by-step guide of how to perform the selection procedure for screening phage-displayed peptide libraries.

8.4.2 Panning Phage Display Library

Protocol 3.

1. For the first panning round, coat an immunotube with 5 μg/mL bovine immunoglobulin purified against *T. saginata* crude antigen diluted in 1.5 mL 0.1 M carbonate buffer, pH 8.6. Seal the tube with parafilm and incubate overnight at 4°C.

   To select binders with high affinity and specificity to the antibodies, in the subsequent pannings it is recommended that the target concentration be gradually decreased. Thus in the following selections, the immunotube must be coated with 1.0 and 0.5 μg/mL of antibodies, respectively.

2. Using a tube rotator, wash the immunotube five times for 5 min with TBS/0.05% Tween 20, pH 7.5 (TBS-T) and fill the tube completely with TBS-T containing 3% BSA previously filtered on a 0.22-μm-pore-size membrane filter. Seal the tube and incubate for 2 h at 37°C to block nonspecific binding sites.
3. After washing, add an appropriate titer, generally 100-fold the library complexity [18] of each library in a final volume of 1.5 mL TBS-T containing BSA 1 mg/mL. Seal the tube and incubate overnight at 4°C by using a tube rotator.

   Because BSA is used as a blocking agent, it is recommended to use the protein in the binding buffer to avoid the recovery of BSA-specific binders.

4. Remove unbound and nonspecific phages by washing the immunotube 10 times with TBS-T 0.5%, five times for 5 min each with TBS-T 0.05%, and finally twice with TBS.

If a nonspecific elution, such as drastic changes in pH, is chosen as a strategy, an appropriate washing stringency must be done to result in a successful affinity selection. Practically, the washing conditions must eliminate target-unrelated binders so that the selecting process is not compromised.

5. Specific and nonspecific elution

5.1 For nonspecific elution, elute the bound phage from the antibody by adding 1.5 mL of 0.1 M glycine−HCl, pH 2.2, containing 1.0 mg/mL BSA for 30 min at room temperature. Transfer the eluted phages to a fresh 2-mL microtube and neutralize the pH immediately by adding 70 μL 2 M Tris−HCl, pH 9.0. Check the pH by using test strips.

The introduction of a sonication process has shown good results [35]. The adapted protocol consists of removing nonspecific phages by washing the immunotube with TBS-T as described and rinsing it four times with glycine−HCl (0.1 M, pH 2.2). Another 1.5 mL of glycine−HCl solution is added to the tube. After sealing, the tube is inserted into a sonicator water bath (50 kHz) for 10 min and the eluate is neutralized as described previously.

5.2 For a specific elution, elute bound phage-displayed peptides by adding a concentration of antigen 100-fold higher than that of immunoglobulins immobilized in the immunotube. After incubation for 2 h at room temperature using a tube rotator, the eluted phages can be cloned by infecting host cells.

To remove lower-affinity binders, a previous elution can be done with the competitive antigen for 60 min at room temperature. After removing the eluate, a second elution is carried out under the same conditions for 24 h [36].

Typically, when the competitor antigen is a known protein, a recombinant protein, for example, specific elution is performed in the presence of 10- to 100-fold excess molar protein. In the competitive elution just described, the competitor agent was not known; thus the crude parasite antigen concentration used was 100-fold (w/w) higher than that of purified immunoglobulins.

6. Before amplification, save an aliquot of the eluted phages for titering (around 50 μL) to evaluate the output input ratio of phages after each round of selection.

8.4.3 Amplification

After each round of panning, the amplification procedure is necessary to enrich bacteriophages carrying peptides with high affinity and specificity to the target. However, some promising clones may have difficulty infecting host cells; consequently, they may not be efficiently amplified.

On the other hand, some peptides have characteristics that do not interfere with the replication process. Thus some resultant peptide sequences selected with more frequency are not necessarily those with higher affinity to the ligand, but those whose infection process may be easier if compared with others.

Protocol 4.

1. On the day before phage elution, pick a single colony of host cell from a freshly Luria Bertani (LB) agar plate and inoculate it into 10 mL LB medium without antibiotics in a 50-mL aerated tube. Grow the preculture overnight, shaking at 225 rpm at 37°C.

   Each day a new culture of host cells is necessary, prepare a preculture from a newly prepared plate.

   It is possible to prepare a preculture from a thawed glycerol stock of bacteria stored at −80°C by using 100-fold dilution into autoclaved LB medium. Avoid rethawed glycerol stocks; this can reduce the bacterial viability.

2. During the elution process, place 5 mL from the preculture in a 500-mL Erlenmeyer flask containing prewarmed 100 mL LB to prepare an exponentially growing culture of host cells: in this case, *Escherichia coli* K91 cells. Incubate cells at 37°C, shaking at 225 rpm until the culture reaches 1.8 at 600 nm (approximately 3−4 h). After that, incubate the culture for 30 min, shaking at 50 rpm at 37°C to pili formation.

   After approximately 2 h 30 min, start reading the bacterial suspension diluted one-half in LB medium. When the A_{600} of the dilution reaches approximately 1.6, incubate the culture slow the rotation (50 rpm) for 30 min to pili formation

3. Incubate eluted phages with 5 mL of pilated bacteria for 10 min at 37°C without agitation, and add to prewarmed 95 mL LB medium containing a low concentration of the appropriate selective antibiotic: in this case, 0.2 μg/mL tetracycline. After new 30-min incubation at 37°C at 225 rpm, add tetracycline to yield a final concentration of 20 μg/mL and grow the culture overnight.

   Tetracycline hydrochloride is not soluble in water. For a stock solution of 20 mg/mL, add 200 mg powder in a final volume of 10 mL 60% ethyl alcohol. Vortex until completely dissolved, filter, and stock in the dark at −20°C. Wrap tetracycline containing plates in aluminum foil to prevent light exposure.

4. Centrifuge cell culture suspension (3000 × *g*, 10 min) and transfer the supernatant to a 250-mL centrifuge flask and add 15% (v/v) of autoclaved polyethylene glycol (PEG) 8000−NaCl solution (20% PEG 8000, 2.5 M NaCl). Homogenize by shaking and keep the centrifuge flask at 4°C for 12−16 h.

   During the autoclaving process, the PEG−NaCl solution may separate into two phases. Let it cool and then shake it; the phase separation is reversible.

5. After centrifugation (8000 × *g*, 40 min, 4°C), collect the phage pellet, resuspending it in 3 mL TBS (50 mM Tris—HCl, 150 mM NaCl, pH 7.5) by shaking at 150 rpm, for 30 min and 37°C. Transfer it to 1.5-mL microtubes and centrifuge the phage solution 10,000 × *g* for 15 min.
6. Proceed with titer determination and store the supernatant at 4°C in TBS containing 0.02% NaN_3 for subsequent rounds of panning.

8.4.4 Phage Titration

Titration is the procedure used to obtain the phage concentration, i.e., to determine the number of infectious particles per milliliter of stock solution. In fact, this information is necessary to know the volume of phage stock to be used in the next panning.

Also important is the evaluation of output—input phage ratio after each round of panning to determine the phage recovery efficiency. A gradual increase of output—input phage ratio between pannings is the first evidence of a successful screening.

Titering phages is also convenient because the individual colonies obtained from plates after each panning could be immediately assayed against the antibody or ligand.

Protocol 5.

1. Pick a single colony from an agar plate and inoculate into 10 mL LB medium without antibiotics, as described earlier. Incubate at 37°C, at 225 rpm overnight.
2. Add 5 mL from the preculture in a 500-mL Erlenmeyer flask containing prewarmed 100 mL of LB. Incubate the flask at 37°C, shaking at 225 rpm, and continue culturing until the culture reaches 1.8 at 600 nm. After that, incubate the culture for 30 min, shaking at 50 rpm at 37°C to pili formation.
3. For titration of eluted (output) phages, prepare 100-fold serial dilution as following:
   a. Add 10 μL eluted phage to 990 μL LB in a 1.5-mL microtube (10^{-2} dilution).
   b. Add 10 μL of 10^{-2} dilution to 990 μL LB in a 1.5-mL microtube (10^{-4} dilution).
   c. Add 10 μL of 10^{-4} dilution to 990 μL LB in a 1.5-mL microtube (10^{-6} dilution).
4. For titration of amplified (input) phages, prepare a 100-fold serial dilution as described, including 10^{-8}, 10^{-10}, and 10^{-12} dilutions.
5. Transfer 200-μL K91 cells from an exponentially growing culture to autoclaved 1.5-mL microtubes and add 10 μL of phage dilutions. To bacterial cells add an aliquot from 10^{-4} and 10^{-6} dilutions of output phages and 10^{-8}, 10^{-10}, and 10^{-12} dilutions of input phages, respectively (Fig. 8.2). Mix well by carefully pipetting up and down. Incubate at 37°C for 15 min without shaking and for 15 more min of shaking at 225 rpm at 37°C;
6. Prewarm freshly prepared 100-mm LB agar plates containing 20 μg/mL tetracycline at 37°C for 2 h. Label one plate per sample, including one for uninfected K91 cells used as a control. After overnight incubation, the control plate containing only K91 *E. coli* and dilution medium should have no plaques.
7. Gently add 200 μL of each infected K91 cell onto the prewarmed plates and spread them until the plate is completely dry. Invert and place the plate overnight in a 37°C incubator to allow colonies to grow and be isolated.

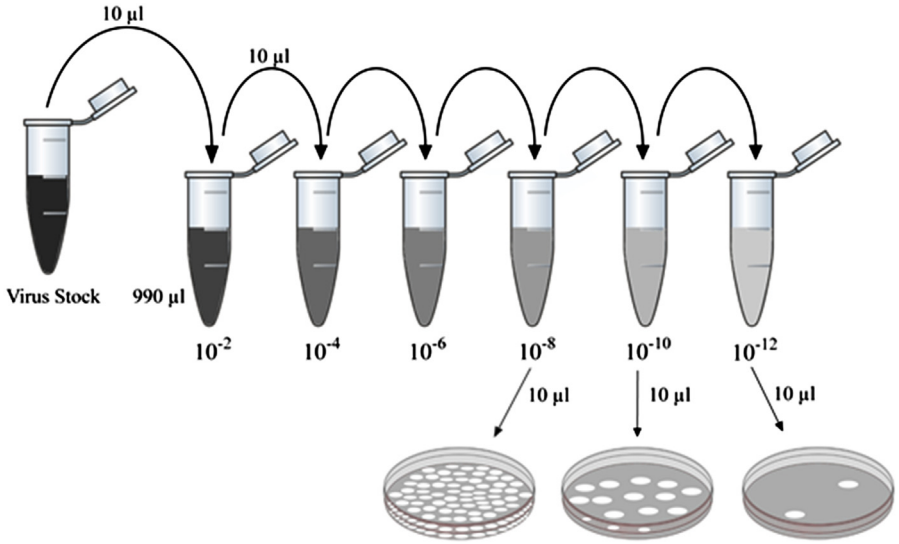

FIGURE 8.2 Procedure to determine the phage stock titer. A series of 100-fold dilutions is obtained by putting 10 µL of the stock solution into 990 µL of LB medium (10^{-2}). Each subsequent dilution is made from the previous dilution, as illustrated. After that, 10 µL of each dilution is incubated with host cells, which are then spread onto LB agar plates.

8. Observe plates and determine the titer in transducing units per mL (TU/mL) according the equation:

$$\text{TU/mL} = \text{number of colonies} \times \text{fold dilution} \times 100$$

For example:

$$50 \times 10^{8} \times 100 = 5.10^{11}\,\text{TU/mL}$$

The multiplication factor 100 is used to obtain the phage concentration per milliliter once 10 µL corresponds to the volume of phages used to infect K91 host cells.

Generally, it is recommended to select the plate with a number of colonies around 100 to determine the titer with respect to minimizing the statistical counting error [37]. If it is not possible, i.e., if all plates have fewer colonies than expected, an independent determination is recommended for each sample, and then it is best to take the average value from it to obtain the sample titer [38].

9. Based on the measurements published by Day and Wiserman [39], another possibility is to determine the phage concentration (phage particles per milliliter) by absorbance at 269 and 320 nm [40] according to the following formula:

$$\text{Particles/mL} = (A_{269} - A_{320}) \times \left(6.10^{16}\right)/(\text{DNA bases in the phage genome})$$

8.4.5 Cloning

Individual colonies derived from titering plates of the final eluate, generally the third one, or output phages after each panning round should be cloned, propagated for further analysis, and/or saved in sterile 96-well culture plates in medium containing 20% glycerol at −80°C.

Protocol 6.

1. Fill the wells of two microplates with 200 μL LB medium containing 20 μg/mL tetracycline and inoculate each with a single colony from the agar plate using toothpicks or sterile pipette tips.
2. Cover the microplates with a polystyrene lid and incubate at 37°C overnight using an orbital shaker at 225 rpm.
3. The next day, spin one of the plates in a centrifuge equipped with a microplate adaptor at 1500 × *g* for 5 min and collect the supernatants to subject them to an enzyme-linked immunosorbent assay (ELISA) analysis.
4. To save the clones or to perform reanalysis at a later time, add an appropriate amount of glycerol to the second microplate to get a final concentration of 20% and, for high viability, store at −80°C.

8.5 Phage Enzyme-Linked Immunosorbent Assay

It is possible to use several ELISA configurations to select phages according their reactivity to antibody or target. Here, the interaction between the phage-displayed peptide and its target is evaluated by coating a microplate with the antibodies initially used as targets in the rounds of panning.

Protocol 7.

1. Coat 96-well microtiter plate overnight at 4°C with 10 μg/mL antibody in 100 μL of 0.1 M carbonate buffer, pH 8.6.
2. The next day, wash the plate with PBS-T three times.
3. Add 150 μL of 5% skim milk in PBS-T to each well and incubate the plate at 37°C for 1 h.
4. After washing the plate three times with PBS-T, add 100 μL of each phage supernatant isolated (Protocol 6) diluted 1:2 in PBS-T containing 2% skim milk and incubate at 37°C for 2 h.

   As negative and positive controls use 100 μL of wild-type phage supernatant and the native antigen, respectively, in concentrations previously established.

5. Wash the plate three times with PBS-T.
6. To detect phage binding, add to each well 100 μL of anti M13/fd bacteriophage antibody—horseradish peroxidase diluted in PBS-T containing 2% skim milk according to the manufacturer's recommendations, and incubate the plate at 37°C for 1 h.

7. Wash the plate five times with PBS-T. Add to each well 100 μL of a substrate–chromogen solution containing 0.02% (v/v) hydrogen peroxide and 0.2 mg/mL *o*-phenylenediamine in 0.1 M citrate-phosphate buffer, pH 5.0, and incubate for 15 min at room temperature.

After incubation, if the microplate is read immediately, the color development should be measured spectrophotometrically at the wavelength of 450 nm. On the other hand if a stopping agent is add to each well, for example, 20 μL of 2 M H_2SO_4, the orange color is measurable at 492 nm.

8. Save all binding clones by using 20% glycerol as described in Protocol 6. Amplify them to isolate single-stranded DNA (ssDNA) and use the primer 5′-TCG GCA AGC TCT TTT AGG-3′ for sequencing.

To analyze phage-displayed peptides by sequencing, a high concentration of ssDNA may be necessary. Sometimes the procedure of amplification does not result in enough material. For this reason it is recommended to prepare ssDNA from PEG–NaCl-precipitated phages.

8.6 Characterization of Peptide Sequences

In dealing with tropical neglected diseases, one of the most important applications of the phage display is to identify mimotopes with effective antigen-like characteristics such that the original antigen can be replaced in diagnostic assays without losing sensitivity and specificity [41].

Besides antigenicity, if phage-displayed peptides are selected for binding to pathogen-specific antibodies capable of performing important immunologic roles such as neutralization [9], activation of the classical complement pathway [42], and antibody-dependent cellular cytotoxicity [43], and are able to induce response immune, they may prove to be good vaccine candidates.

Interest in identifying new diagnostic targets using the phage-display technique is based on the fact that most functional antibodies recognize conformational antigenic determinants [44]. In addition, the technical procedures of selecting immunodominant epitopes of the etiologic agent direct them to immunoglobulins to obtain a more specific and sensitive diagnostic test.

Use of the ELISA, as described earlier, is an initial trial to validate the interaction between phage-displayed peptides and antibodies used initially as targets. This immunochemical assay determines the relative affinity between the phage-presented peptide and the antibody without considering whether this interaction depends on the peptide conformation on the phage surface [18].

The chemical synthesis of the peptides (mimotopes) on the cellulose membrane (SPOT-synthesis) [31] may be an effective approach to confirm antigen–antibody reactivity. This technique allows straightforward localization and the precise identification of each key

residue essential for recognition [32]. In addition to being a method based on the same procedure used in conventional chemical synthesis on resin, the cost per peptide sequence corresponds to less than 1% of a soluble synthetic peptide [45], not to mention the possibility of regenerating and reprobing the membrane several times with excellent reproducibility.

SPOT technology is a parallel chemical synthesis of peptides [32] on small circular regions on a cellulose membrane, in which protected and activated amino acids are spotted positionally addressed, forming a pattern of small spots.

On each spot a different peptide is synthesized. Although longer peptides may be prepared, a peptide length between 12 and 15 amino acids is commonly used.

Once a mimotope has been identified by phage display, assayed by ELISA and its sequence is known (Fig. 8.3A), it is possible to determine the contribution of individual amino acid residues to binding energy of the antigen–antibody interactions. It is necessary to prepare a series of alanine analogs of the epitope (alanine scanning) (Fig. 8.3B) and probe them with antibody [32,46]. In peptides with alanine in their original sequence, this residue can be substituted by serine.

Comparing the reaction of the antibody against the wild type and an alanine-scanning mutant peptide, it is possible to indicate, according to the decrease, or sometimes the lack of reactivity, which amino acid residue is crucial to mimotope–antibody interactions (Fig. 8.3C).

In Fig. 8.4A, four phage display–derived-peptides corresponding to mimotopes of *T. saginata* obtained by competitive elution [14] were synthesized on a cellulose

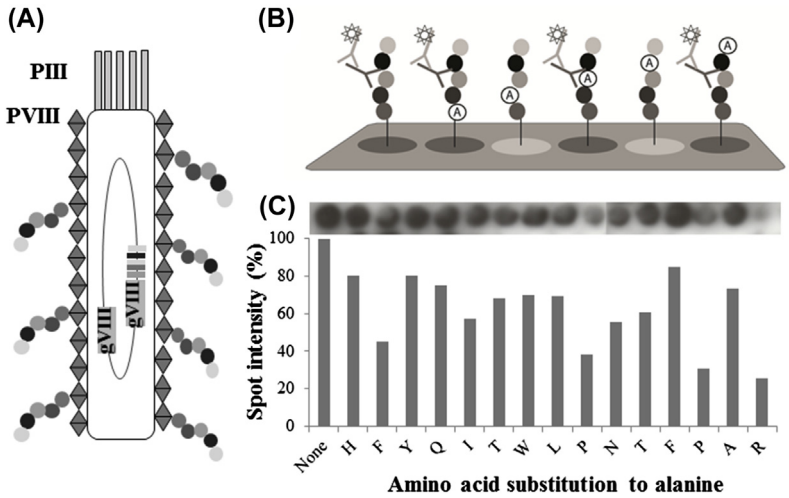

FIGURE 8.3 Alanine scanning. (A) Illustration of a filamentous phage that displays peptides at the N-terminus of the pVIII coat protein. (B) Representation of the reaction between a wild-type peptide and analogs with alanine replacement with a specific antibody revealed with an enzyme-labeled secondary antibody and enhanced chemiluminescence detection on an SPOT membrane. (C) Determination of which residues are important to the antigen–antibody interaction. Each bar represents the reactivity of a serine or alanine-mutated peptide. Reactivity of the wild-type peptide (*first bar*) was considered 100%.

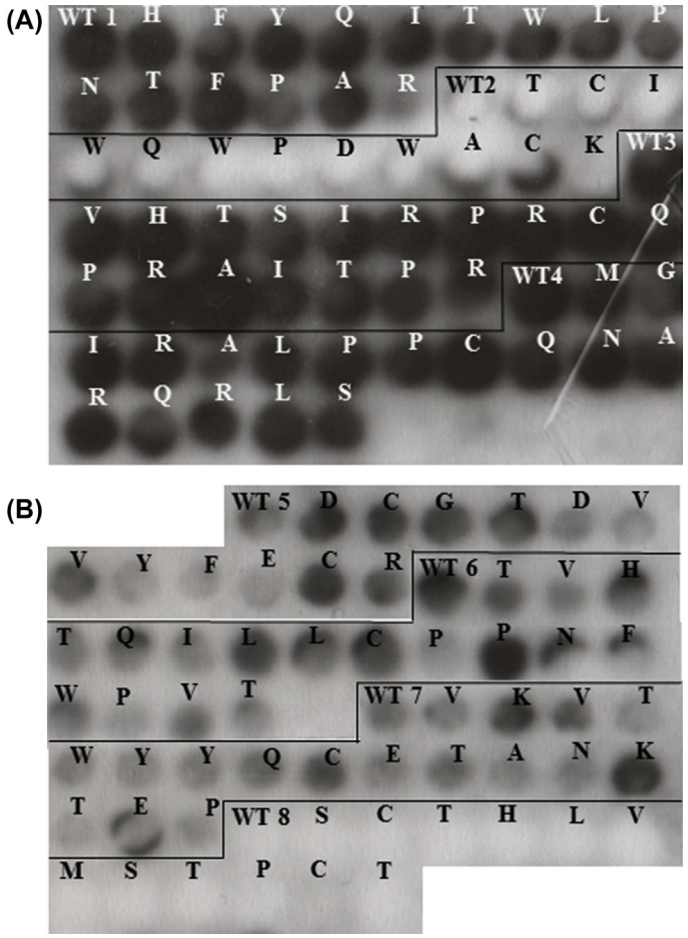

FIGURE 8.4 Comparison of anti *Taenia saginata* antibody reaction and eight cellulose-bound phage-displayed peptides, obtained after competitive (A) and acid elution (B). The wild-type peptide (WT) is synthesized in the first spot of the sequence following by its analogs with alanine replacement. The amino acid residues substituted by alanine (or serine) are indicated on the top of each spot.

membrane in which an alanine was used to replace each amino acid residue sequentially. The same procedure was performed with *T. saginata* mimotope sequences (Fig. 8.4B) eluted in a nonspecific manner, i.e., through a change of pH.

There was a significant difference between the antigenic reactivity of cellulose-bound phage-displayed peptides from specific and nonspecific elution when the membranes were probed with a positive bovine serum pool under the same conditions and at the same time. These data reinforce the concept that the competitive elution is a better strategy to affinity select specific ligands.

Although the specific elution is the best alternative, both strategies present similar results regarding the absence of reactivity in some synthetic peptides spotted on the

Table 8.1 Peptide (Pep) Sequences Selected by Specific and Nonspecific Elution

Clone	Amino Acid Sequence	Residues (*n*)	Elution
Pep 1	H F Y Q I T W L P N T F P A R	15	Competitive elution [14]
Pep 2	T C I W Q W P D W A C K	12	
Pep 3	V H T S I R P R C Q P R A I T P R	17	
Pep 4	M G I R A L P P C Q N A R Q R L S	17	
Pep 5	D C G T D V V Y F E C R	12	Acid elution
Pep 6	T V H T Q I L L C P P N F W P V T	17	
Pep 7	V K V T W Y Y Q C E T A N K T E P	17	
Pep 8	S C T H L V M S T P C T	12	

membrane, such as peptides 2 and 8 (Fig. 8.4). Considering that both peptides present two cysteines and that no treatment on the membrane was done to obtain cysteine-bridged peptides [32], one hypothesis is that the conformation of these peptides is probably essential for recognition by antibodies, and this characteristic is preserved only when they are exposed on the surface of the phage.

Curiously, no consensus sequences have been selected between the phage-displayed peptides (Table 8.1). Only Pep 1 (HFYQITWL**PNTF**PAR) [14], eluted by competition with *T. saginata* crude antigen, presented some homology with a highly conserved sequence from GK-1 (GYYYPSD**PNTF**YAPPYQ) [47], a peptide from KETc7, a protein found in the Taeniidae family [48] with immunogenic [49] and antigenic characteristics [50] against cysticercosis caused by *Taenia solium*.

Regarding neglected tropical diseases, phage display technology integrated with other technologies such as SPOT synthesis may eventually increase the possibility of obtaining high-affinity ligands so as to contribute to the identification of molecules suitable for diagnosis or to the rational design of mimotope-based vaccines.

8.7 Conclusion and Perspectives

Thirty years ago, George P. Smith described the phage display technique without realizing the impact that it would have on the generation of monoclonal antibodies, drug development, protein engineering, cancer therapy, design of vaccines, and the diagnosis of several diseases. Considered an inexpensive, simple, and resourceful technique, phage display, which is aligned to the chemical synthesis of mimotope/epitope sequences, has been consolidating as a technology of great importance in the development of alternative diagnostic methods mainly with regard to neglected tropical diseases.

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9 Systems and Synthetic Biology Applied to Health

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9.1 Systems Biology: The Dawn of a Holistic Vision in Biomedicine

The concept of systems biology, which has its roots in the first studies of enzyme kinetics at the beginning of the 20th century, has been used extensively in the life sciences only starting from the beginning of the current century. Modern systems biology can be seen through a variety of aspects, (not comprehensively) encompassing:

1. a field of study that focuses on the complex interactions among biological systems components;
2. a paradigm antithetical to reductionism (integrating instead of reducing, and observing the whole instead of single parts);
3. a research protocol, i.e., a recursive sequence of steps that includes hypothesis and modeling, experimental validation, quantitative description, improved hypothesis and modeling, and so on; and
4. a socioscientific phenomenon that regards the strategy devoted to pursuing the integration of massive, heterogeneous data as coming from different experimental sources and different methodologies and instrumentation, as well as people from disparate scientific backgrounds [1].

Typically the main reason referred to as when taking into account a "system" is that "the whole is greater than the sum of the parts." The interaction of the system's parts enables peculiar properties or functions to emerge that would not been expected from the single elements each on their own. Such emergent properties arise out of "more elementary" entities, and yet are "novel" or "irreducible" with respect to them. This irreducible nature makes emergent properties not easily predictable; in other words, there is no linear or proportional input–output relation.

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Through this perspective, systems biology is a holistic approach (from the Greek word ὅλος meaning "all," "entire," or "whole"), according to the concept that the parts of a given system are intimately interconnected, and that they are explicable only by reference to the whole. This view differs from reductionism, i.e., the description and analysis of a (complex) phenomenon in terms of its simplest or fundamental elements. It is to be stressed that reductionism has been and still is fundamental to an understanding of the nature of biological constituents. However, with modern high-throughput (e.g., next-generation sequencing) and computational technologies, there is the chance to move on, and to understand and reconstruct the single parts into the whole.

In modern science systems, biology can be considered an integrated approach that aims for:

1. the comprehension of the *structure* of the system, that can be real (e.g., network of neurons interconnected through synapses) as well as more abstract (e.g., metabolic and cellular signaling networks, genetic regulation networks);
2. the comprehension of the *dynamics* of the system by means of qualitative and quantitative analysis (kinetics) of fluxes and flows among the system's elements, and related mathematical modeling;
3. the comprehension of *system control* and its regulation procedures, i.e., the principles that drive the dynamics; and
4. the comprehension of the *original design* of the system and the principles of self-organization (the "instruction manual" that you need to put all of the parts together).

9.2 Approaches to Systems Biology

Because it is a wide concept, cataloging all approaches related to systems biology comprehensively is not a trivial task. Virtually, any (recursive) workflow involving the extensive use of mathematical and computational methods for hypothesis drawing and predictions based on experimental data and able to be validated can be classified as a systems biology approach. This section is not the place for a complete review of such approaches, so it will briefly introduce some of the main techniques and processes. For a wider essay with a focus on drug development see Ref. [2].

Integrating and analyzing high-throughput data, from multiple experimental sources, gathered using different "omics" technology platforms using informatics (combined computational, statistical, and mathematical) tools and techniques is considered a main systems biology approach. Through this perspective, the states of genes, proteins, and posttranslational modifications, for example, can be assessed in a large-scale systemic fashion (working all together) to understand how they are regulated, expressed, or modified during a specific process (physiologic function, disease, etc.) toward the generation of a testable hypothesis about the components involved (signaling pathways or set of genes) or the discovery of a process or disease markers.

To give an idea of the complexity of biological systems, a group composed of member with different expertise and 15 different laboratories studied together how a nonpathogenic bacterium as *Bacillus subtilis* completely changed its metabolism when growing in different nutrient sources as glucose or malate. More than 30 different conditions were analyzed and different methods were developed to better understand the metabolic networks involved in the bacteria adaptation [3,4]. This kind of approach was extremely important in the sense that it creates several computational tools to lead with data complexity and at the same time opened up the perspective to apply the same approach for understanding pathogenic organisms and how they are involved in disease processes.

Computational and mathematical modeling of single or multiple biological scales (from the gene level to the whole organism), as well as of pathophysiologic processes and biological modules using existing information (e.g., databases, literature) aims to generate predictions for target selection (prioritization of genes or proteins) for clinical guidance or the design of clinical trials. Network biology and agent-based simulation, for example, can be attributed to this branch.

Finally, ex vivo human cell–based systems, i.e., combination of human cells, can be used to screen emergent and complex systemic behavior in response to pharmacologic treatment or other perturbations.

9.3 The Network: A New, Powerful Paradigm in Biology

Networks describe how things connect and interact, graphically representing players (*nodes* and *vertices*) and their relationships (*links* and *edges*) in a mathematically tractable object called a *graph*. Lying among the main approaches that fall under the wider systems biology's umbrella, the network paradigm and graph theory is a specific branch of mathematics that is extensively used in many fields going well beyond the biological field, such as social, engineering and informational sciences [5]. Indeed, network science is a broad interdisciplinary academic field that has practical applications in the study of several types of complex networks, e.g., telecommunications networks, computer networks, biological networks, cognitive/semantic networks, and social networks, a phenomenon also testified to by the flourishing of dedicated institutes and conferences.

In biomedicine, the network abstraction enables complex biological systems to be considered as a whole, as well as to undergo mathematical analysis and quantification, aiming to discover major systemic properties and provide an accurate and analytic view at a glance of actors and their relational linkages and functions. Indeed, the cooperation of biological systems is possible through the organization and coordination of the many different elements and processes of the organism. In this view, the network approach highlights how the characteristics and behavior of single elements influence the network structure (i.e., the *topology*), as well as how the network structure impinges upon processes spreading over the system (i.e., the network dynamics), or how perturbations may affect network performance. In this regard, the network abstraction represents a useful functional view that can complement analyzes and approaches from molecular biology and from many different "omics" [6].

9.4 Network Measures for Biological Systems

Several measures and parameters can be used to analyze network structure, component relevance, key nodes, general efficiency, etc. A few are briefly mentioned here referring to more complete works in the field [7,8].

The *degree* of a node is the number of edges linked to it. In the paradigmatic case of protein–protein interactions (PPIs), the degree is the number of *physical interactions* that a specific protein shows with other proteins. In other types of network, e.g., in social networks, in which the nodes are persons, the degree can be the number of her or his friends and acquaintances.

The *path length* is the number of nodes that need to be crossed when going from one node to another. Many different paths can be chosen to reach one node from another, but the *shortest path length* is particularly significant: when averaged on all node couples, it provides a rough measure of the network navigability.

The average number of neighbors of a node in the network (sometimes referred to as *connectivity*) shows how densely the network is populated with links and may also be represented by means of a normalized version of this parameter, i.e., the network density (a value between 0 and 1).

Of particular interest in biology, some *centrality* measures account for interesting biological characteristics and behaviors. For example, the *betweenness centrality* of a node attests to the grade of control this node exerts over the interactions of other nodes in the network, and its biological relevance has been demonstrated in cancer as well as other biological fields [9].

9.5 Types of Biological Networks

Because of the power of such network representation, diverse biological networks have been figured out and analyzed with a certain degree of success. Networks can be scanned in a single picture and in a graph-theoretic framework for all known events, processes, and associations related to the system. A noncomprehensive list of the most important biological network types is provided next.

PPI networks are among the most well-known and used network representations in molecular biology. PPI networks are built by connecting two or more proteins in the presence of intentional physical contacts established between them. PPI networks, also referred to as *interactomes*, are a powerful tool to visualize, analyze, and understand the key nodes, structure, and dynamics of numerous physiologic and pathologic processes, and provide a way to integrate data of different nature.

Intracellular signaling networks represent molecular and soluble signals generally going from the cell surface to the cell nucleus (pathways) through a branched cascade of several different events such as PPIs, phosphorylation reactions, and metabolic processes.

Transcription factor regulatory networks are built by analyzing bound DNA elements within the gene proximal regulatory regions. The aim is to map the combinatorial

cross-regulation of several hundreds of sequence-specific transcription factors that eventually define regulatory networks controlling cellular identity, features, and function.

Protein phosphorylation networks represent phosphorylation events such as different phosphorylated forms, phosphorylation state−specific PPIs, and kinase−substrate relationships.

Metabolic networks represent sets of metabolic and physical processes that determine the physiologic and biochemical properties of a cell, such as interactions between enzymes and metabolites. Metabolic networks, now mostly available online by mean of dedicated databases such as KEGG, among others, are powerful tools for studying and modeling metab*olism*.

Genetic regulatory networks (GRNs) show interactions between usually large numbers of genes, which involve downstream-regulated genes as well as their regulators. As for other types of biological networks, GRNs are used to visualize and analyze the regulatory relationships.

Co-expression networks depict genetic maps in which genes are connected if their transcripts are co-regulated.

Finally, *disease networks* map pathologies and disease genes linked by known disorder−gene associations, indicating common origins of many diseases.

9.6 Networks Studies Applied to Understand Key Molecules and Disease Processes

With the explosion of high-throughput technologies, the integration of data coming from the various "omics" (genomics, transcriptomics, glycomics, proteomics, and metabolomics, among others) represents a powerful strategy aimed to reconstruct and analyze complex multidimensional interactions, and subsequently to enable deeper understanding.

Nevertheless, data integration is never a trivial task, and issues coming from the lack of standardization or shared biological identities [10], just to mention a few, need to be faced to overcome major hurdles in integrative workflows. In this perspective, the PPI network framework represents an assessed integrative approach that has been used in computational biology to understand disease pathogenesis, prioritize cancer-associated genes, and predict functional linkages among genes, among many other examples [11−13]. Here are two examples of such integrative procedure used to frame in a systemic landscape a paradigmatic autoimmune disease [rheumatoid arthritis (RA)] and a major inflammatory signaling pathway [nuclear factor-κB (NF-κB)].

9.7 Rheumatoid Arthritis

RA is a paradigmatic autoimmune and autoinflammatory disease, and its complex etiology makes the effective use of current therapies challenging, also in terms of

the difficulties in controlling side effects and adverse events in the numerous districts involved [14].

With the intentions of collecting available molecular knowledge into a single picture and of providing a wider framework to estimate the systemic impact of therapeutic targets on the numerous biochemical compartments implicated in RA, some of us compiled and analyzed a multi-omic data map that assembles genomic, epigenomic, transcriptomic, posttranscriptomic, proteomic, and (for the first time in a systemic manner) host-microbiome interface data [15] (Fig. 9.1).

Thirteen different sources (online databases and literature screening) supplied all data sets that enlisted molecules experimentally associated to RA (*core* data set, 377 proteins from manual curation of literature sources), and additional molecules and pathways strongly yet not explicitly associated to RA (*extended* data set, 4709 proteins). The core set constitutes a more specific RA map, whereas its extension offers a more systemic and practically usable map, notably in terms of the significance of the statistics run on the extended map. Such comprehensive systemic information was integrated at the functional level of PPIs to obtain the multi-omic landscape map of the disease, finally undergoing mathematical analysis and pharmacologic treatment simulation.

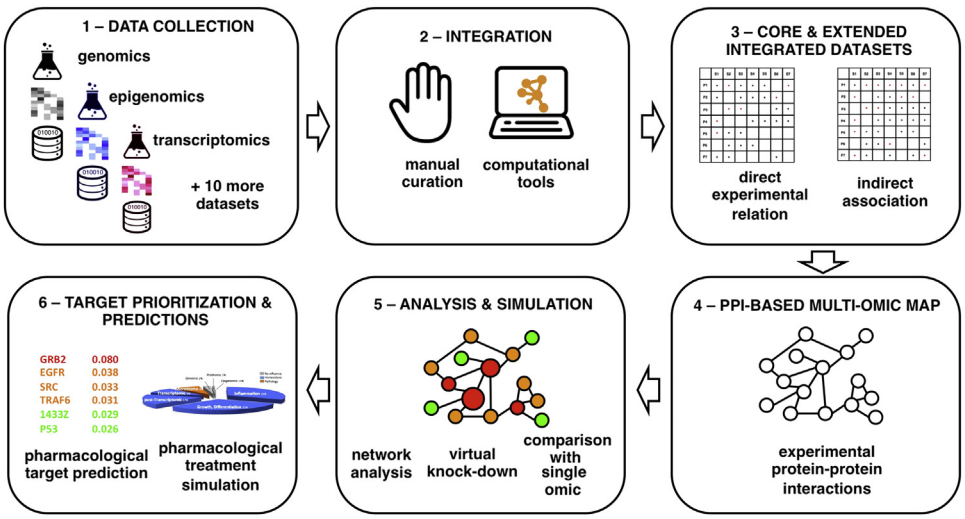

FIGURE 9.1 RA is a systemic, inflammatory disease with unclear etiology. A systems biology, multiple "omic" approach has been used to help disentangle its multifactorial causation. A large number of data from "omics" approaches, including genomics, epigenomics, transcriptomics, posttranscriptomics, proteomics, and host–microbiome interfaces to gut intestinal bacteria metagenomics (1) have been integrated using computational tools and manual curation (2) to obtain two main data sets, one specifically related to experimental evidences, and its extension, able to offer a more systemic view (3). From these sets, protein–protein interaction–based RA maps have been derived (4) and undergone network analysis and simulation (5). Hypotheses and predictions about candidate pharmacologic targets and the outcome of existing medication use from such a mathematical and computational approach are provided, ready to be tested, and validated in a laboratory environment (6).

The topological analysis evidenced, among other results, the extraordinary relevance of the mediator growth factor receptor-bound protein 2 (GRB2) and the notable network role of interleukin-1 receptor associated kinase-4 (IRAK4) (Fig. 9.1).

GRB2 is an effective target and mediator in several oncopathologies (mainly leukemia) and a crucial player in the host−microbiome interaction of *Helicobacter pylori* and *Prevotella intermedia*, besides being considered a marker of RA in synoviocytes. Interestingly, a member of the *Prevotella* genus (*Prevotella copri*) was associated to RA [16] as a specific marker of the gut intestinal microbiome dysbiosis that goes along the disease. From the network perspective, this seemingly dispersed knowledge converged in a connected map setting up a robust rationale for accounting GRB2 as a target for RA.

IRAK4 has a critical role in initiating response to foreign pathogens; because of this it was presented to the American College of Rheumatology as a potential treatment for RA, but according to the network perspective, this choice comes with a warning. Indeed, the multi-omic map evidenced that whereas a regression of some aspects of the disease was observed, the control on IRAK4 (by IRAK4 inhibitors) also exerts a significant influence on the response to pathogens, with an impact on immune-specific dendritic cells of patients with RA [17], an event that may severely limit the competent innate host response in case of bacterial infections.

In this work novel information was gathered by condensing knowledge from approximately 4000 selected molecules and about 15 public databases, with the aim of drawing hypotheses in support of basic research and further clinical practice. The work focusing on two major areas of application: (1) support in identifying novel drug targets, and (2) support in identifying potential contraindication to novel therapies.

9.8 The Nuclear Factor-κB Pathway Interactome

It is progressively acknowledged that the number of elements that impinge upon phenotypic outcomes of signal transduction pathways may be higher than that taken into consideration from canonical representations [18], and that pathways may work as larger “mega-networks” rather than as linear cascades [19]. Such considerations pushed some of us to compile a larger reference map of NF-κB, a protein complex that promotes both induction and repression of more than 400 genes. In mammalian cells, there are five NF-κB family members, and different NF-κB complexes can be formed from their homo- and hetero-dimers. The combinatorial control and signaling cross-talk of the NF-κB pathway are intricate, and limited analyses to the canonical NF-κB pathway elements may turn out to be insufficient in uncovering key mechanisms or actors in the regulation of this system.

With this perspective, data from multiple existing sources were integrated and analyzed under a network approach to chart the NF-κB interactome map. This type of comprehensive approach can provide a deeper understanding about how specific stimuli trigger particular subsets of NF-κB target genes or about the regulatory hierarchy that rules the selection of target gene expression.

To compile the NF-κB map, multiple data sources, i.e., manually collected data from literature, binary PPI data, protein annotation data, and NF-κB downstream gene data, were mined and integrated into a single picture. Three different sets of proteins were compiled, showing evidence of involvement in the upstream regulation process of NF-κB, and one set of downstream genes and related proteins, whose expression appears to be regulated by NF-κB. Starting from these sets, three NF-κB interactomes were derived by using PPI data from multiple databases and then checked for experimental evidence of physical interactions. Interactomes then underwent mathematical analysis (centrality measures computation) and results were interpreted through a biological perspective. Confirmation of known key elements in feedback loops suggested biological insights about other high-ranking (high betweenness centrality) proteins that were not reported in the literature as crucial regulatory components.

This systems-oriented map may have helped improve the understanding of one of the most complex signaling networks and highlighted the impact of single elements, their feedback, and cross-talk regulations on cellular processes. Besides confirming previous studies and insights, this interactome mapping emphasized that the number of elements impinging upon the outcome of NF-κB pathways is much higher than that usually taken under consideration, pushing for a rethinking of canonical pathway representations.

9.9 Introduction to Mathematical Modeling

As discussed previously, systems biology is an active area of research that exploits the availability of large biological experimental data sets and the power of modern computers. Mathematical modeling of biological phenomena constitutes a way to analyze a system by breaking it down into parts and studying their dynamics. It is a reductionist approach. The systems biology viewpoint is to reassemble the constituents and evaluate the mutual influence of the various components through a holistic view. This task is accomplished by means of both classical mathematical methodologies such as differential equations and computational tools developed after the advent of automatic calculus.

Mathematics is a language. It allows the expression of the relationships among entities entitled in a phenomenon. In addition, it does so in a logical and precise way. The description might be wrong but the way to progress from the assumptions to the conclusions is exact.

The most difficult part in writing a mathematical model is not to derive the conclusions but to describe the system and formulate the problem. So, if it cannot provide sure answers to the desired questions, how can it be useful? It depends on how well the problem is formulated. In other words, what are the assumptions that can be done to make the system conceptually manageable? There is no other way to describe real life than to simplify it; therefore a mathematical description has to be based on choices taken to include what is relevant to the purpose of understanding a certain aspect of the system and to leave out what is not.

The usefulness of describing nature by mathematical equations lies in the process of studying the phenomenon: that is, in the fact of reasoning about something by using the rigid logic of mathematics. The formulation of the problem itself becomes a way to see the system through a different perspective, which forces researchers to note otherwise elusive things. Once formulated, the mathematical (and now computational) model is a formidable instrument to confirm or reject hypotheses about how things really work, and/or to find points of intervention to control the system under investigation.

Mathematics has been "the" language to describe physical phenomena from the very beginning of the scientific revolution of the 17th century, but only relatively a short time ago its attention was turned to biological phenomena. Throughout the centuries biology has shown a higher resilience to mathematical investigation compared with physical sciences because of the enormous complexity of living matter compared with nonliving things: a complexity stemming from the redundancy, multifactorial, and dynamism of the mechanisms governing living things [1].

This complexity originates mainly from the distributed/systemic characteristic of the phenomena and from the enormous variety of components involved, ranging from molecules and cells to organs and organisms.

One of the first biomathematical models, which dates back to the beginning of the 20th century, is the Lotka–Volterra system of nonlinear differential equations, which describes the dynamics of two species, in which one is the predator and the other the prey [20]. This model has inspired many other variants and generalizations that have proven useful in predicting the temporal evolution of two or more interacting species in generic ecosystems under disparate constraints (e.g., availability of resources or landscape restrictions). The model formulation describing, for example, the relationship between foxes (F) that predate rabbits (R) looks like:

$$dR/dt = a\,R - b\,F\,R$$

$$dF/dt = c\,F\,R - d\,F$$

The sense of these sentences in mathematical language is that there is an interdependence between the time variation of the prey and that of the foxes. The fate of the two species is joined because there cannot be foxes without rabbits and the prosperity of the rabbit species depends on the abundance of foxes. The model assumes unconstrained access to food resources for the rabbits. Those, two lines can be "spelled out" as follows. Rabbits grow at a rate and are killed by the foxes at a rate that is proportional to the fox population (bF). Foxes grow proportionally to the food availability (cR) and die by aging at a constant rate (d). Rabbits also die, but for them it is a growth rate greater than the death rate that is assumed.

Models of this type are constituents of much more complex descriptions of biochemical reactions such as the Belousov–Zhabotinsky (BZ) oscillating chemical reaction, often referred to as a paradigmatic example of nonequilibrium dynamics characterized by the propagation of waves in a reactive medium. There are various forms

of BZ reactions, each using a different chemical "recipe." One of the simplest is based on a mix of hypobromous acid, bromide, cerium-4, bromite, and a general organic species. In its simple form it may be comprehended in terms of the following three concurrent and competing processes [21]: (1) reduction of bromate to bromine; (2) introduction of hypobromous acid as a reducing agent for bromate; and (3) reduction of the catalyst formed from the previous processes. The chemical formulation would be:

$$A + Y \rightarrow X + P,$$

$$X + Y \rightarrow 2P,$$

$$A + X \rightarrow 2X + 2Z,$$

$$2X \rightarrow A + P,$$

$$B + Z \rightarrow \mathrm{h}Y + Q,$$

where the variables represent the concentrations of the specific reactants and h and Q are constants. In the classical mathematical formalism of ordinary differential equations, the system would look like:

$$dX/dt = AY - XY + AX - 2X2,$$

$$dZ/dt = AY - XY + hBZ,$$

$$dZ/dt = 2AX - BZ.$$

Oscillating patterns characterize the temporal evolution of this mathematical system, whereas traveling (in space) waves would emerge in the spatially extended version of the differential system, therefore written by means of partial differential equations.

These examples are meant to help trace a line between reality and mathematics whereby the obtained models become toy systems in the hands of the researcher who can then play to understand and discover. Those systems are much simpler than reality for the purpose of keeping the complexity manageable. Systems biology, however, points to a unified vision in which components are brought together to connect, interact, and compete. The latest anticipation in the field of mathematical modeling is to build on existing models by creating tools, standards, and theoretical frameworks to unwind the task of linking models together. This job often entails bridging different physical scales (i.e., time and length), leading to the complex domain of multiscale biomodeling [22].

The advent of digital computers has given the means of stretching the reach of classical mathematical models that otherwise would have been strongly limited by the availability of "analytical" solutions of the model systems. In simple language, once formulated in differential equation form expressing the dynamical evolution of quantities such as concentrations or cells counts, a system of differential equation needs to be

mathematically disentangled to obtain the "solution": that is, a formula explicitly stating the relationship between the quantity and the variable "time." This is not an easy task, and most mathematical models do not yet have an explicit solution. Computers detour this venture by digitally iterating rules that match the passage of infinitesimally small intervals of time, eventually uncovering the relationship between the variable and time. Actual desktop computers can handle thousands of differential equations in a matter of seconds. That is a powerful tool in the hands of a systems biologist.

9.10 From Classical Modeling to Microscopic Simulations

Modeling by differential equations is not the only method. There is a "new kind of science" that is gaining consensus in the theoretical biology domain [23]. This "new science" has different names. It is discrete mathematics in the computer and adheres to a simple idea: to represent reality by enacting its simple constituents, which are particles, cells, molecules, and agents, whatever individual entity composes a system. A system of cooperating cells is therefore not described by means of lumped variables such as those expressing the foxes and the rabbits in the differential equation example given earlier, but by means of a number of unique representations of single foxes and rabbits. In the digital world of a computer, this designates an individual portion of memory in which to annotate the different characteristics of the agents; this is instrumental to represent the processes carefully, resulting in the overall dynamics at a population level. In the example, not all foxes are equal; some are bigger and stronger, eat more, and run faster, and thus predate more. Likewise rabbits are not all nothing-more-than runners; some may be more inclined to hide or are less risk-takers than others. This modeling paradigm that here we call microscopic simulation or microsimulation, gives a rationale to those details that makes life so diverse and complex. It is a computational paradigm rather than a mathematical one in the classical sense, and it exists because the rules that describe how the entities behave and interact among them and with the environment are effortlessly executed in the machines.

Stanislaw Ulam and John von Neumann originally conceived the microsimulation concept in the 1940s while they were contemporaries at Los Alamos National Laboratory and were interested in laying the theoretical foundation of self-replicating machines. Although some researchers studied microsimulation throughout the 1950s and 1960s, it was not until the 1970s and John H. Conway's Game of Life that interest in the subject expanded beyond academia. The Game of Life is the most famous of a larger class of models. It takes the name of cellular automata (CA) and consists of a checkerboard on which cells live and die. The dynamic evolution of the combined fate of the living "cells" is governed by simple logical (and local!) rules. Because the entities are stored in binary digits and the rules, no matter how complex, are coded as Boolean functions (i.e., transformations of bits), it is not surprising to realize that CAs are worthy children of the computer age.

9.11 Microscopic Simulation of the Immune System

Microscopic simulations provide researchers with a tool to study complex biological occurrences. They represent the agents and dynamical rules and then set the systems free to evolve over time through agent interactions, with a minimum of a priori assumptions about the dynamic itself. Macroscopic system behavior is then observed as an emergent property. The richness of details one can take into account in microscopic CA-like simulations makes this methodology appealing for the description of biological systems whose behavior and heterogeneity of interacting components are not safely reducible to some stylized or simple mechanism.

Paradigmatic of such complex phenomena is the immune system, which has been studied for a long time by means of detailed simulations [24]. This model originates from a couple of ideas. The first is to use discrete entities to represent the population of lymphocytes, other immune cells, and antigens such as bacteria or viruses. The second is to use binary strings to represent molecular binding sites so that a digital match (computer scientists call it the Hamming distance) can identify the degree of molecular binding affinity. This microsimulation model includes both innate and adaptive immunity. The adaptive branch of the immune system is further specified in both cellular and humoral immunity. The whole adaptive immunity model is bolted on the clonal selection theory of the Nobel laureate F. M. Burnet [25], developed on the tracks first highlighted by P. Ehrlich at the end of the 19th century. To have a fully equipped clonal selection process as a response to an antigenic insult, the model has to be equipped with the concept of specificity. Here is where the mentioned binary string and affinity comes into play: More lymphocyte clones of different specificity are represented (it is a bit-string polyclonal model, as opposed to monoclonal models in which only a single population of genetically identical lymphocytes is represented). Major classes of cells of the lymphoid lineage (lymphocytes T-helper and cytotoxic, lymphocytes B- and deriving antibody-producing plasma cells, and natural killer cells) and some of the myeloid lineage (macrophages and dendritic cells) are represented in the model. Other working immunology-grounded assumptions are hematopoiesis; lymphocytes maturation, and thymic selection of T-cells; Hayflick limit in the number of duplications; aging and memory of past infections; hypermutation of antibodies; bystander effect; cell activation and anergy; cell interaction and cooperation; and antigen digestion and presentation. Each of these subjects identifies a component or a process of the immune system and is implemented by algorithmic rules whose complexity sets it apart from what could be reasonably be manageable with differential equations.

The microsimulation modeling paradigm allows a step further in the representation of the immunologic specificity. For example, it was implemented a novel approach in which the lymphocyte receptors as well as antigenic binding regions are not binary strings but the molecular primary structure [26]. This modification is a great step toward realism but it carries a number of complications and requires a number of strong

assumptions that, it is hoped, in a not so distant future will be removed by adopting new more reliable techniques.

The whole method relies on techniques developed in immunoinformatics, a new experimental and theoretical discipline emerging from growing knowledge gathered for decades in experimental immunology and immunogenomics [27]. In immunology, what is of utmost importance is to "predict" which part of the antigen will constitute an immunogenic epitope; broadly speaking, there are two ways of doing it. The first is to simulate the chemical–physical interactions between peptides and major histocompatibility complex (MHC) molecules. The second is to resort to machine learning and statistical methods to extract and generalize information from available experimental data of, for instance, MHC–peptide sequences [28].

This simulation framework test hypotheses or elucidate causal relationships among the various subsystem components actually fulfilling the core requirements of the systems biology approach. For example, a basic immunological question is the contribution of the humoral and the cellular branch of immunity in viral infections by selectively tune processes pertaining to viral evolution [29]. Or to investigate the role of MHC haplotype heterozygosity and homozygosity with respect to the influenza virus to show that there is an advantage to heterozygosity (Fig. 9.2) [26]. Furthermore, one can compare vaccine formulations to elucidate the protective role of the immune memory

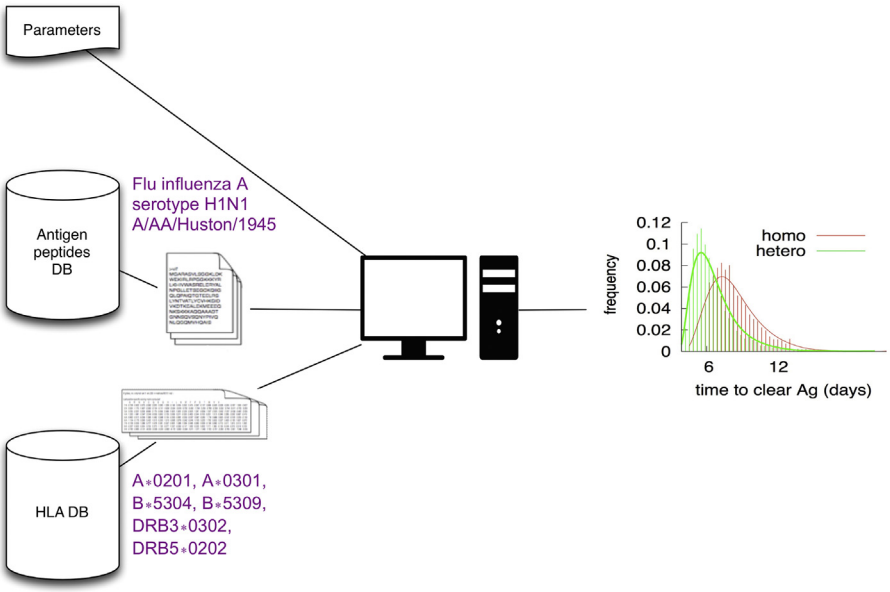

FIGURE 9.2 Mathematical models transform consolidated knowledge coming, for instance from experiments, into new knowledge by enacting hypothetical conditions through numerical calculations. The advantage to human leukocyte antigen (HLA) haplotype heterozygosity was demonstrated (A*0201, A*0301, B*5304, B*5309, DRB3*0302, and DRB5*0202) with respect to homozygosity (A*0201, B*5304, and DRB3*0302) when simulating infection by flu influenza A serotype H1N1 (genome ID: HU13275) [26]. DB, database and Ag, antigen.

elicited by priming with either influenza vaccines or influenza infection. In this specific example, reported in Castiglione [30], a number of computer experiments were performed to test the level of influenza-specific $CD4^+$ T-cell cross-reactivity resulting from various vaccination protocols consisting of priming with the split (i.e., an approximation of the vaccine consisting of the 11 proteins of the virus at equal concentrations) A-Brisbane-59−2007 H1N1 influenza A virus vaccine followed by various boosts such as the hemagglutinin molecule of virus H1N1 influenza A California-04-2009 alone or a set of molecules from the swine-origin influenza virus vaccine consisting of molecules of the A-California-04-2009 virus. Other protocols were included in this comparison, including a simulated version of a "live" attenuated virus (i.e., encompassing the major steps of the lifecycle of the virus) consisting of the total 11 molecules of the A-Brisbane-59−2007 H1N1 influenza A virus. The resulting dynamics has proven to be in qualitative and to a certain extent quantitative agreement with previous ex vivo studies. In particular, the level of immunoglobulin G simulated by the computer model was in agreement with previous experimental results [30].

Complete control over the setup of virtual experiments and deep analysis of the results of such a detailed model allowed the effect of different rules to be calculated for each coding for a biological hypothesis, idea, or immunologic mechanism. To mention a few, it is possible to simulate the consequences of varying the vaccine dose or vaccination schedule, its formulation (soluble antigen, complex and conjugate, virus-like particle, the effect of using adjuvant, etc.), and the difference between injecting a compound or the single components separately, among others.

9.12 Basic Aspects of Synthetic Biology

The "omics" technology and system biology approaches have been used extensively to describe complex biological networks through a deep characterization of molecular components or biological parts that compose the organisms (i.e., genes, different RNA families, proteins, and metabolites) and their interactions, as discussed earlier using networks. The massive knowledge acquired may be used to design or redesign new genetic circuits and biological parts with several applications to biotechnology. These purpose-driven approaches leading to fabrication of biological components and systems that do not already exist in the natural world are named synthetic biology. Considering biological organisms to be programmable manufacturing systems, synthetic biologists use engineering concepts to supplied genetic instructions allowing execution of the desired function. These concepts are based on modularized propriety of biological parts that can be combined in different ways and will then work as intended.

Work involving synthetic biology is highly dependent on a set of well-characterized biological parts. These biological parts are DNA sequences that encode proteins as enzymes, transporters, or regulatory elements as promoters, noncoding RNAs, and different binding sites for several elements. Because of the acid nucleic nature of

biological parts, the design and build of engineering biologically based devices and systems is closely linked to DNA editing involving classical molecular biology experiments as gene synthesis, polymerase chain reaction (PCR), digestion with restriction enzyme, and cloning and assembly of biological parts that will produce the desired function. Because a range of vectors and protocols can be used to isolate and stock these biological parts, combination of components is usually laborious, expensive, and slow work. A stimulating challenge is standardization of biological parts libraries allowing their use by many research groups to build different synthetic devices. In 2002, Knight [31] developed the concept of BioBrick standardization of biological genetic parts. Each BioBrick contacting the functional sequence between a prefix composed of restriction site to enzymes *Eco*RI, *Not*I, and *Xba*I and a suffix containing site to enzymes *Spe*I, *Not*I, and *Pst*I. Two genetic parts may be connected through a ligation link among compatible overhangs from the first BioBrick, previously digested by *Spe*I and a second BioBrick cut by *Xba*I creating an uncuttable junction (Fig. 9.3A). This protocol allows easy assembly with a controlled number and order of BioBricks.

BioBricks are suitable for several applications, but the system often fails in the construction of protein fusions owing to the production of a sequence that encodes an in-frame stop codon. Other approaches have been proposed to solve this issue [32,33]. BglBricks (Fig. 9.3B) is one option based on the presence of the *Bgl*II enzyme site as a prefix and *Bam*HI as a suffix that when linked produces a scar sequence encoding a dipeptide glycine and serine [32]. Therefore BblBricks are suitable to build synthetic devices with protein fusion. An alternative methodology regardless of the restriction endonucleases frequently used to join biological parts is the Gibson assembly approach (Fig. 9.3C). DNA sequences that will be connected using this approach should have an overlapping region that after digestion with a 5′-exonuclease will result in single-stranded regions. These single-stranded cohesive ends overlapping regions of different fragments can anneal. The DNA polymerase extends paired overlapping regions and the DNA ligase seals nicks in the assembled DNA. Gibson assembly was used to produce the first genome composed exclusively of chemically synthesized DNA [34].

After suitable biological parts are selected to produce the desired result, they generally are inserted inside a *chassis*, where regulatory sequences will exert their effects and protein-coding sequences will be transcribed and translated. A chassis is a cell that may harness its own biochemistry to produce the intended work, and thus organism models with well-established DNA manipulation protocols are often used [35]. Moreover, availability of a complete genome sequence is desirable to accelerate research using the selected organism. The most common chasses used in laboratories are bacteria as *Escherichia coli* and *Bacillus subtilis* [35], but these chasses may not be feasible for the production of compounds, proteins, or synthetic circuits depending on specific eukaryotic aspects. Among eukaryotic chassis, yeast, is often chosen owing to rapid growth and easy genetic manipulation [36,37]. Mammalian cells represented by lineages such as Chinese hamster ovary and HeLa derived from human cervical cancer cells are also used mainly for potential applications that require

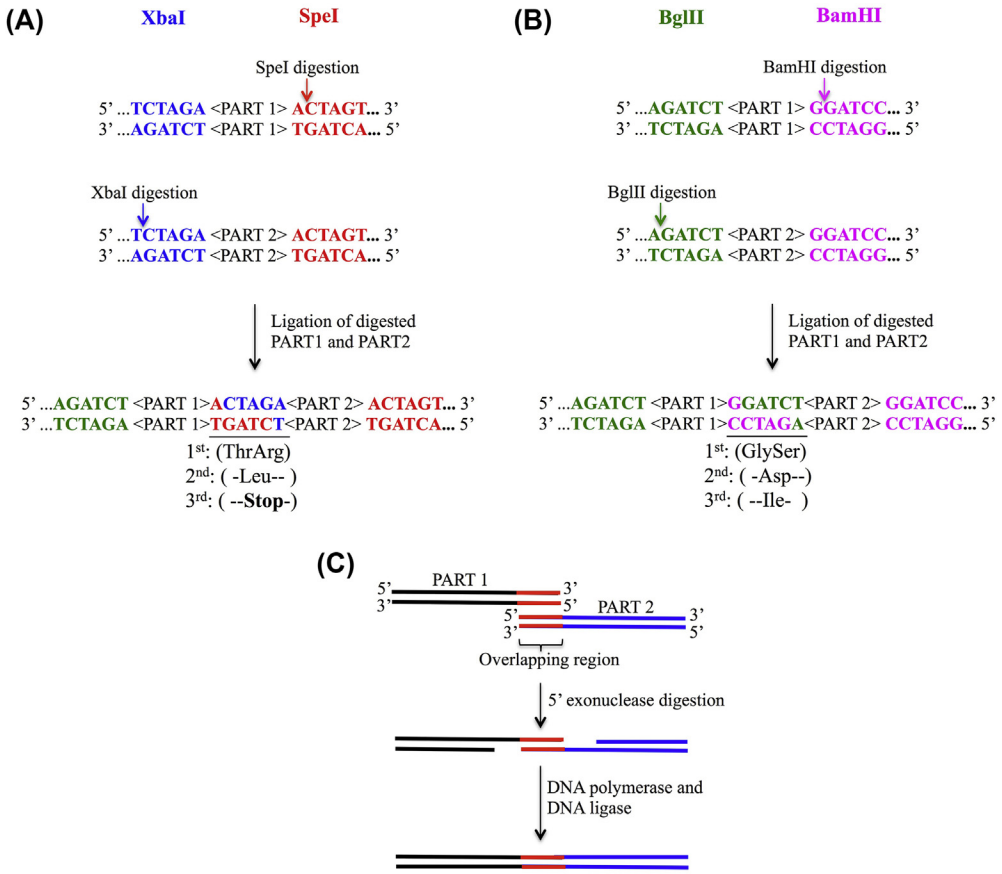

FIGURE 9.3 Approaches to biological parts assembly. (A) BioBrick system. Each biological part is between *Xba*I (*blue letters—dark gray in print version*) and *Spe*I (*red letters—gray in print version*) endonuclease sites. Parts can be fixedly connected by DNA ligase when the first part is digested by *Spe*I enzyme and the second part is cleaved by *Xba*I enzyme, forming cohesive ends that after ligation form a scar sequence (underscore region) not accessible to the same enzymes, allowing large construction containing several parts. Analysis of three possible reading frames (first, second, and third labels) of the produced scar sequence shows a stop codon in the third frame. (B) BglBrick system. The *Xba*I and *Spe*I sites were replaced by *Bgl*II (*green letters—gray in print version*) and *Bam*HI (*pink letters—light gray in print version*), respectively, that produce a scar sequence without a stop codon to all possible reading frames. (C) Gibson assembly. Synthetic DNA containing overlapping regions are digested by 5′ exonuclease, allowing annealing between cohesive single-strand ends from different fragments. After PCR extension using DNA polymerase, the fragments are joined using DNA ligase.

mammalian characteristics [38] as well as implants in humans or animal models [39]. Besides living chasses, cell-free devices based on cell extracts or in vitro transcription and translation are emerging as amenable and safety systems and are ideal for producing toxic or inhibitory products for living chasses [35,40–42].

Methods for genome editing of chasses have experienced significant breakthroughs during through the discovery and technical improvement of transcriptional

activator-like effector (TALE) nuclease, zinc finger nuclease (ZFN), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9) systems. TALE are DNA-binding proteins first identified in *Xanthomonas* plant pathogens that mimic eukaryotic transcription factors regulating plant genes during pathogenesis [43,44]. Chimeric proteins obtained by fusion of a TALE repeat array with a nonspecific *Fok*I nuclease have the ability to cleave genomic DNA from living cells (Fig. 9.4A). Other chimeric fusion of protein containing zinc finger domain previously known by DNA binding ability, with *Fok*I nuclease originated in ZFN systems was used for genome editing [45,46] and allowed specific double breaking of genomic DNA (Fig. 9.4B). CRISPR together with Cas9 was initially identified to participate in the adaptive immune system of several bacteria and archaea against bacteriophages and other foreign nucleic acids [47] and has been optimized to work in different cells such as bacteria, yeast, protozoa, plant and mammalian lineages [48–50]. The CRISPR/Cas9 system uses guide RNA that binds to target genome sequences, initiating a double-strand break after a protospacer-associated motif represented by the NGG trinucleotide sequence (Fig. 9.4C). All of

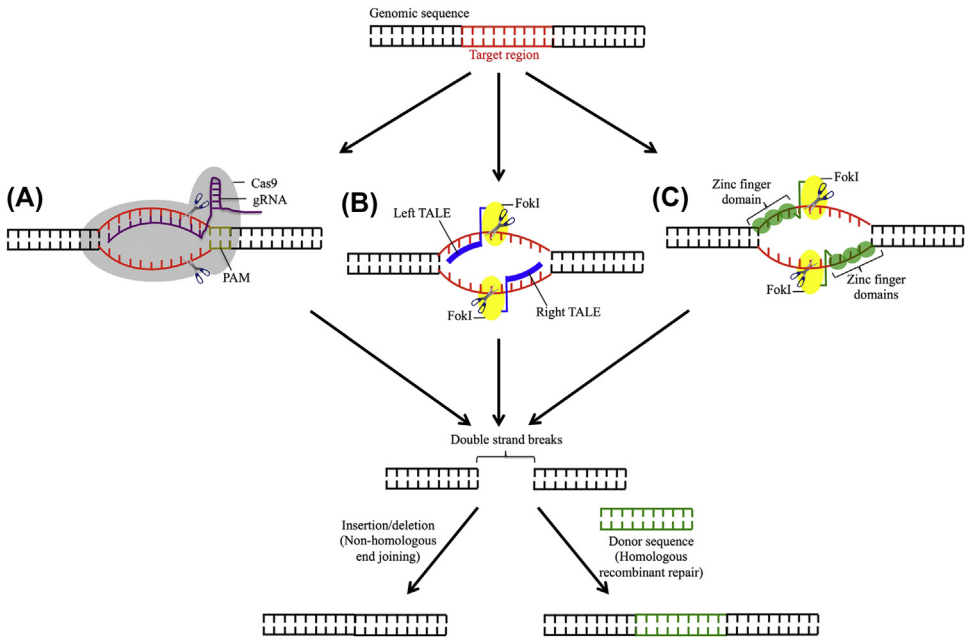

FIGURE 9.4 Genome editing tools. Double-strand break of target region in genome sequence can be performed: (A) CRISPR/Cas9 system has a guide RNA (gRNA) that directs CRISPR-associated nuclease 9 (Cas9) to the target site and the cleavage occurs before a protospace adjacent motif (PAM) composed of NGG trinucleotides; (B) TALE system that directs a nonspecific *Fok*I enzyme to target site of each strand; (C) ZFN system uses zinc finger DNA-binding function that also directs *Fok*I to the target site of each genomic strand. Double-strand breaks can be repaired by nonhomologous end joining or homologous recombination that inserts DNA fragments from the donor sequence with overhanging ends with the cleaved regions.

these systems allow the degradation of specific DNA fragments or can be replaced by recombination to insert single and large nucleotide alterations [45,46,50–52].

Intermediate work in synthetic biology based on the design of new genetic circuits to control some system propriety involves the concept of logic gates well-established in electrical engineering [53–55]. Logic gates are implemented as a Boolean function that can assume only an ON or OFF stage in a determined moment, and the output of a system depends on the combination of its inputs (Fig. 9.5A and B). For instance, AND logic gates of a hypothetical system that receives two inputs will produce the output only when both inputs are present (Fig. 9.5B). On the other hand, OR or NOR logic gates will produce the output when at least one input or no one is present, respectively. Moreover, simple logic gates can be combined, resulting in highly complex control of the system [56]. From the biological point of view, logic gates may be built using transcriptional or posttranscriptional control stimulated or inhibited by different elements such as RNA, proteins, metabolites, light, pH, and heat that control a determined output such as a

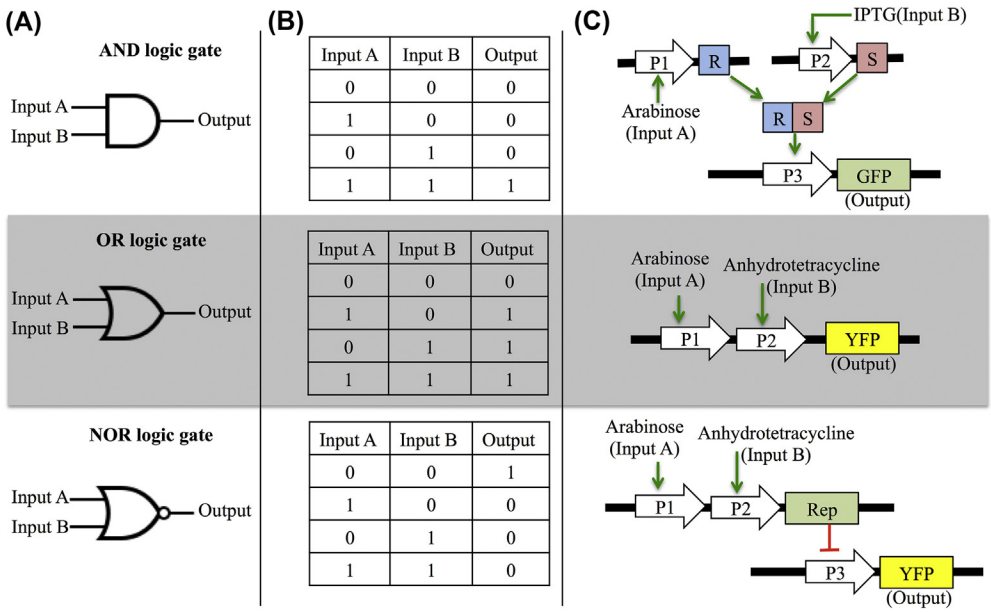

FIGURE 9.5 Genetic circuits based on AND, OR, or NOR logic gates with two inputs. (A) Schematic representation. (B) Expected logic outputs based on the presence (1) or absence (0) of each input. (C) Synthetic biological circuit example. In synthetic biological AND circuit [63], green fluorescence protein (GFP) production (output) is controlled by a promoter that produces the protein after binding of a heterodimeric complex (RS). The R complex component is stimulated by the presence of arabinose (input A) and the S subunit is produced under isopropyl β-D-1-thiogalactopyranoside (IPTG) control (input B), resulting in output production only when both inputs are present. To the OR logic gate [64], YFP production (output) is under the control of two independent promoters stimulated by arabinose (input A) and anhydrotetracycline (input B), respectively. Only one input or both are sufficient to trigger YFP production. The NOR logic gate [64] is represented by a repressor component independently stimulated by arabinose (input A) or anhydrotetracycline (input A). The repressor inhibits production of YFP (output) and this protein is only produced in the absence of both inputs.

protein, metabolic reaction, or photon emission (Fig. 9.5C) [57]. A toggle switch composed of two repressors and two constitutive promoters arranged in a mutually inhibitory network and developed in *E. coli* showed interesting properties for biotechnological application such as bistability [58]. In addition to switches, synthetic biological oscillators have been developed [59] and several mechanisms for transcriptional or translate control of synthetic circuits such as riboswitches [60,61] have enabled significant advances in synthetic biology. Eventual problems in synthetic genetic circuits are associated with the production of transcripts even in the absence of an activating signal, and they often needs computational and experimental optimization [62].

Especially in human and animal health, synthetic biology is a useful tool for improving diagnosis, treatment, and preventive health care. The next sections provide examples of applications of synthetic biology approaches for the development of improved diagnostic devices and optimized drug delivery systems and biological chasses for drugs and other compound production (Fig. 9.6). Furthermore, the application of synthetic biology to prevent disease will be discussed based on vaccine system design and genetic correction by genome editing. Although the potential of synthetic biology application is widely discussed and recognized, the field is just emerging and the vast majority of published works have only presented proof of concept results.

9.13 Synthetic Biology and Disease Diagnosis

An ideal biomarker for disease diagnosis should have high sensitivity and specificity to identify patients affected by the disease compared with healthy individuals or patients with other diseases. An advantage of using synthetic biological devices to diagnosis disease is the ability of these systems to identify different targets and integrate signals produced by each target in a more specific response. In addition, the synthetic device may be optimized to amplify the signal response, making it more sensitive even at low target levels. Disease diagnosis based on multiple targets uses logic gates concepts to detect different elements (compounds) in a sample; only when all target elements are present, will the output be detectable [65–67]. For instance, a switch that allows production of two stable states implemented in *E. coli* was used to evaluate environmental conditions of murine gut and correctly discriminated mice previously treated with anhydrotetracycline and untreated animals [68]. However, biosensors containing synthetic genetic circuits that work as logic gates can present a number of problems such as low sensibility and signal-to-noise ratio, and thus they should be optimized through analysis of thresholding, digitalization, and amplification of biologically relevant signals [69].

The versatility of synthetic biological devices to indicate the presence of liver metastasis commonly associated with more advanced stages of certain types of cancer including colorectal, breast, and pancreatic [70,71] was demonstrated using an engineered *E. coli* strain [72]. Gene encoding lacZ enzyme was inserted in *E. coli* Nissle 1917 strain and administrated orally to mice with liver metastatic tumors. Owing to the

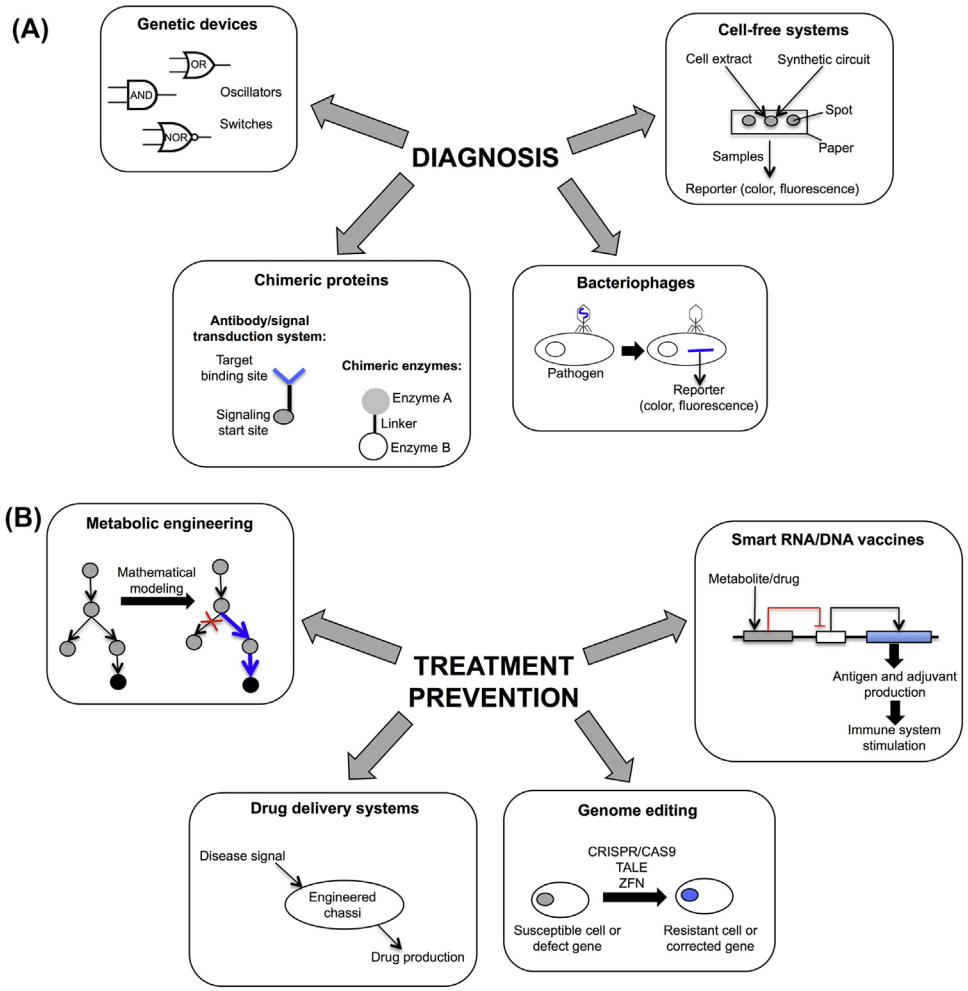

FIGURE 9.6 Current health-based biological synthetic devices. (A) Disease diagnostic tools. (B) Synthetic systems applied to disease, treatment, and prevention.

natural and selective propensity of this bacteria strain to colonize tumor tissues, it proliferates within 24 h after reaching liver metastatic tumors, resulting in a high level of lacZ secretion that can be detected in urine samples by a colorimetric test. Furthermore, another synthetic genetic circuit was built to identify microRNAs of specific cancer cells and resulted in apoptosis as a reporter response by an RNA interference mechanism [73]. The synthetic circuit was transiently expressed within a HeLa cervical cancer cell line and normal cells leading to apoptosis only in cancer cells.

In addition to synthetic genetic circuits, synthetic biology may be applied to produce chimeric proteins to increase the efficiency of traditional diagnostic approaches. This

approach is applied but not limited to immunodiagnostic methods based on the detection of specific antibodies. Protein engineering applied to the production of synthetic chimeras composed of different epitopes sequences may contribute to increase the specificity and sensibility of immunodiagnostic assays [74,75]. This approach was validated for Lyme disease caused by *Borrelia burgdorferi* using a chimeric protein that reached 98% sensitivity and 100% specificity for disease diagnosis [76].

Synthetic biology also provided significant advances for the detection of human bacterial pathogens using engineered bacteriophages [77,78]. Bacteriophages are viruses with the ability specifically to infect and replicate inside target bacteria by injecting their acid nucleic content that incorporates into the bacterial genome or remains a stable episome replicating with their host. Determined environmental and biological conditions lead to the production of viral proteins by bacterial transcription and translation machinery, resulting in a large quantity of new viral complete particles that are released after disruption of the host cell in a process called the lytic cycle [79,80]. Heterologous genes associated with the different report system were added to some bacteriophages that specifically infect human clinical bacterial pathogens such as *Mycobacterium tuberculosis* [81–83], *Yersinia pestis* [78,84], and *Bacillus anthracis* [85], allowing identification of these pathogens in samples such as blood, serum, processed sputum, nasal swab, and laboratory cultures. A report system used to detect the pathogens was based on the addition of a the gene encoding luciferase enzyme that uses endogenous and exogenous substrate and cofactors to produce light that can be detected by different devices [86–89]. Moreover, systems based on the detection of specific fluorescence produced by the exogenous gene encoding proteins such as green fluorescent protein and yellow fluorescent protein were also developed [90]. Strong bacterial promoters were inserted downstream of both reporter genes in the altered phage genomes, contributing to the expression of the high level of reporter genes in the early stage of infection. The current reference standard of diagnosis of these clinical pathogens requires slow cultivation that needs a specific laboratory structure. The synthetic device based on bacteriophages may be used and provides fast and accurate results owing to the short time of the bacteriophage lytic cycle and the specificity of different phages for each pathogen, respectively. Another important advantage is that the engineered bacteriophage may also be used quickly to detect food and waterborne pathogens in samples of water and food that will be consumed by the population [91].

Because the clinical use of cell-based synthetic devices has been hampered owing to feasibility and biosafety issues, another promising approach based on synthetic biology to diagnosis disease has been developed in cell-free systems [41,92]. Most early work aimed to develop cell-free systems used fresh or frozen cell extracts containing all necessary biological machinery and liposome technology to assemble artificial cells [40,93–96]. Pardee and collaborators developed a versatile and stable cell-free synthetic gene network system embedded onto paper that could easily be distributed and stored as well as activated by adding water [42]. This technology was used as a glucose and mRNA strain-specific Ebola virus sensor.

9.14 Synthetic Biology and Disease Therapy

Several approaches have been described using synthetic biological devices to treat disease including metabolic engineering of different organisms to produce therapeutic compounds at a high yield [97−100], design smart drug delivery systems [39,101,102]and edit the genome of host cells, targeting pathogens [103,104]. Metabolic engineering has allowed the production of natural drugs that are not viable by chemical synthesis owing to their structural complexity and that are present in low levels at their original source. One of the most successful cases of metabolic engineering has been applied for the production of artemisinin, an antimalarial drug previously isolated from medicinal plant *Artemisia annua* [105]. The artemisinin biosynthetic pathway was extensively studied [106,107] and the heterologous expression of key enzymes for drug biosynthesis from mevalonate and deoxyxylulose 5-phosphate pathways was performed in *E. coli* [98] and yeast [99] chasses, resulting in high-titer production of artemisinin. Moreover, the original plant source was optimized by genetic engineering to overexpress its own genes evolving in artemisinin biosynthesis as well as heterologous genes that contribute to the production of high levels of the drug [108,109]. The genes were modified or inserted using an *Agrobacterium* transformation system that uses the ability of bacteria from the genus *Agrobacterium* to infect plant cells and integrate regions from large tumor-inducing and rhizogenic plasmid into the plant nuclear genome [110]. Another example of metabolic engineering of *E. coli* in drug production is the isoprenoid pathway optimization for the overproduction of taxol [100], a potent anticancer drug first isolated from the conifer plant *Taxus brevifolia*. Production of drugs using metabolic engineered microorganisms is described in Ref. [111].

Exciting advances in synthetic biology have allowed the designer of complex circuits to control the production of drugs and therapeutic products in the host cells when required owing to pathologic events. These systems are usually responsible for small molecules or others stimulator signals that trigger the production of therapeutic components. The microencapsulated immortal human HeLa cell line containing a synthetic genetic circuit that secrets urate oxidase was built to recognize increases in uric acid levels in the bloodstream [39]. This system was validated through peritoneal implantation in mouse models exhibiting pathologic urate levels and resulted in a decrease in uric acid crystal deposits that may compromise renal function with valuable potential for application in patients with tumor lysis syndrome and gout. Promising results have also been obtained with a synthetic multifunctional mammalian pH sensor that was able to identify increases in diabetic ketoacidosis levels in mice and produced insulin until homeostasis recuperation [101]. Unlike responses to endogenous metabolites, glycemic control caused by type II diabetes in a mouse model was also reached using a synthetic genetic device that produced glucagon-like peptide 1 when stimulated with an external blue light [102]. Long-time durability, the possibility of pathology control in the early stage of disease, and better fine-tuning of therapy to disease progression and reduction are advantages of a self-sufficient control system compared with classical therapy.

Genome editing technologies have also been used as potential therapeutics through modifying human cells to avoid the progression of disease. The most successful example is the alteration of the genome of target cells from patients infected with HIV, to produce resistant lineages that are refractory to virus entry [103,104,112]. In these works, key regions from different host receptors necessary for virus invasion, such as CCR5, CXCR4 from T-cells, or hematopoietic stem cells, were mutated by site-directed technology such as ZNF, TALE, or CRISPR/Cas9, and virus-resistant cells were transplanted back into the patients or humanized mouse models [112].

9.15 Synthetic Biology and Disease Prevention

Synthetic biology has contributed to disease prevention mainly through designer or regulatory devices for DNA or RNA vaccines [113–115] and genetic correction by genome-editing methodologies [116,117]. The first approach is based on modulation of the immune system to early recognition and elimination of infecting pathogens or malignant cells in the early stages of disease establishment. The use of DNA as a vaccine was established in the early 1990s through the direct introduction of a plasmid or viral vectors containing the DNA sequence encoding antigens with the ability to trigger a protective immune response [118,119]. On the other hand, direct vaccination with mRNA has also been used to provide an antigen source for adaptive immunity, simultaneously stimulating innate immunity mediated by interaction with Toll-like receptor 7 [120]. DNA or RNA vaccines have several advantages compared with traditional approaches, including an increase in stimulation of both B- and T-cell response, vaccine stability, and low-cost, large-scale manufacture [121]. The rational modification of a synthetic Gag protein in DNA vaccine against HIV type 1 by an increase in CG content and optimization of codons that increase more than 10 times the antigen expression in target mammalian cells enhanced the immunogenicity and efficacy of the vaccine [122]. Synthetic biology has also contributed to an increase in the yield of antigens produced by heterologous expression through optimization of selected chasses as well as mRNA structures and gene sequences based on codon use of producing organisms [113]. Furthermore, protein engineering has contributed to the production of chimeric proteins generating multivalent vaccines that are safer and more efficient [114,123,124].

Synthetic biology has been applied to disease prevention using genome-editing tools to correct genetic alterations that could develop metabolic and genetic syndromes. For instance, the CRISP/Cas9 system has applied as proof of concept for functional genome repair of stem [116] and embryonic [48,117] mammalian cells, including human cells. Intestinal stem cells with mutated transmembrane conductor receptor recovered from patients with cystic fibrosis were rescued through homologous recombination directed by the CRISP/Cas9 system, resulting in fully functional alleles [116]. Mouse zygotes with the mutated Crygc gene associated with dominant cataract disorder were also submitted to genome correction, resulting in progeny with a repaired Crygc allele, and were disease-free [117]. The estimated efficiency in rescuing

the mutated β-globin gene that caused β-thalassemia in tripronuclear human zygotes was approximately 25%, and the author observed off-target cleavage along unexpected sites including exonic regions of C1QC and transthyretin genes [48]. Therefore potential problems observed in all works as the absence of complete understanding of the molecular mechanism, evaluation of stability, and off-target effects should be addressed before clinical application approval.

Fig. 9.6 shows the tools, molecules, devices, and systems produced by synthetic biology that are important in diagnosis and therapeutics.

9.16 Biosafety and Ethical Aspects Surrounding Synthetic Biology

Although the initial results from applying synthetic biology to the development of new health solutions are extremely exciting, studies are in the early stages and many issues of biosafety and ethics should be addressed before clinical use. Regarding safety, it is difficult to assess the risks of extensively genetically modified organisms. To deal with these problems, a group from Harvard Medical School developed a bacteria strain that depends on a specific amino acid, which does not occur in nature, to grow up. In this way, this bacterium only grows up in a laboratory under specific conditions [125].

The efficacy of such synthetic systems needs to be thoroughly tested to ensure the beneficial effects and track possible unexpected problems including side effects. The stability of construction is another important aspect associated with new technologies that should be validated to guarantee the quality of a synthetic biological product. Furthermore, necessary equipment to stock and maintain the viability of synthetic devices should be at hand. Efficient discard protocols of live synthetic systems should be available to avoid environmental contamination and reduce the risks of unexpected effects during interaction with other biotic elements. All of these issues depend on an elaborate training program for synthetic biologists and others involved, and rigorous standardization of protocols to assess quality.

These issues may become more significant with the progress of synthetic biology [126].

9.17 Conclusion and Future Perspectives

Systems biology and synthetic biology are emerging disciplines that have a high impact on several areas of bioscience, especially for the understanding, diagnosis, and treatment of diseases. A deep understanding of biological systems is far from being achieved and for a better understanding of a system as a whole, several challenges should be overcome: (1) the quality and standardization of “omics” data; (2) multisource data integration; (3) the development of methods to evaluate the dynamics of a system; (4) adaptation of computational tools to become more friendly; and (5) a decrease in the time to process data [127].

Once these challenges are overcome, systems biology approaches will hold the promise of substantially improving the current state of the art in medicine by providing the ability to distinguish among multiple disease states and to enable identification of the molecular causes underlying diseases [127]. This is particular important for multifactorial diseases such as cancer [128–130], obesity [131], RA [132], and cardiac disease [127], among several others.

In addition, with the advance of technology regarding DNA sequencing and synthesis and DNA manipulation such as Gibson assembly and TALEN, ZFN, and CRISPR/Cas9 tools, the understanding and treatment of diseases will completely change. DNA sequencing and synthesis is an easy, fast, and cheap method that allows rapid design, fabrication, and testing of synthetic systems. The Gibson assembly method allows a synthetic cell to be built [34] and has an impact in the construction of synthetic influenza vaccine viruses for rapid response to pandemics [133]. There is now a great potential for creating new vaccines using synthetic genomes. Nucleases associated with zinc finger proteins have affected the treatment and cure of diseases such as HIV [103,112]. With the advent of CRISPR/Cas9, one can imagine a series of applications to treat diseases such as Yao and Chen summarized regarding cancer therapy [134]. This deep understanding not only of clinical symptoms but also of the molecular components and how they interact to result in a specific disease is a prerequisite for the development of approaches to personalized medicine.

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10

Noncoding RNAs as Critical Players in Regulatory Accuracy, Redox Signaling, and Immune Cell Functions

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10.1 Noncoding RNA Molecules: From Evolution to Human Disease Therapy

The use of genome-wide approaches and the large output of genome sequencing technologies have revealed that the mammalian transcriptome is much more complex than initially thought because it includes a large number of small noncoding RNAs (sncRNAs) and long noncoding RNAs (lncRNAs) [1,2]. As the genome sequence analysis of diverse organisms was accomplished, no direct correlation was found between genome size or the number of protein-coding genes and the complexity of the organism, even when the generation of protein diversity by alternative splicing and post-translational regulation was taken into account [3]. On the other hand, it was established that 75% of the human genome is transcribed into RNA, whereas only 3% is transcribed into mRNAs [4–6], which clearly shows that the number of noncoding RNAs (ncRNAs) is probably much higher than that of genes coding for proteins. This fact, together with growing data regarding ncRNAs, suggests that organism complexity better correlates with the percentage of each genome that is transcribed into these molecules [7].

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Supporting this view is the fact that most ncRNAs display complex patterns of expression during differentiation and development, as well as tissue-specific expression.

A great number of different classes of ncRNAs, based on their length, biogenesis pathways, features, and functions have been reported in the literature [8,9]. However, three classes of sncRNAs have been the most studied, specifically short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). The siRNAs and miRNAs are small RNAs about 20−30 nucleotides (nt) long that are produced from double-stranded RNA (dsRNA) precursors that can be exogenously introduced into, or formed in cells by, gene transcription of both sense and antisense DNA strands, transcription of pseudogenes, and inverted repeats. These ncRNAs are involved in mechanisms related to the silencing of specific genes by mRNA degradation and/or translational repression, and also to cell/genome defense against virus, mobile repetitive DNA sequences, retro-elements, and transposons [10].

The less characterized class of sncRNAs is the piRNAs, small RNAs of about 24−31 nt in length, that have been essentially detected in germline cells of mammals, fish, and *Drosophila melanogaster* [11,12]. These sncRNAs have important roles in germline development by suppressing transposon activity. In fact, disruption of the piRNA biogenesis pathway is associated with increased transposon expression, and in mouse and fish it causes germline-specific cell death and sterility [13]. In *Drosophila* heat shock protein 90 kDa (Hsp90), PIWI and Hop form a protein complex that seems to be required to prevent phenotypic variation despite genotypic variation and environmental influences (canalization) [14]. Hsp90 seems to be able to avoid phenotypic variation by preventing the mutagenic activity of transposons, probably because it is a capacitor [15,16]. Thus piRNA pathway−dependent mechanisms may have been critical during evolution in the establishment of developmental robustness.

The ability of piRNAs to protect genomes against parasitic nucleic acids and the importance of this protection for development seem to have been established early in evolution because protozoa ciliates have a mechanism that resembles that of piRNAs. In these organisms the polyploid macronucleus (the somatic nucleus) maintains the vegetative growth of the cell. This nucleus develops from the diploid micronucleus (the germline nucleus) by complex programmed genome reorganization after sexual conjugation. This process requires chromosome fragmentation and elimination of germline-specific sequences (internal eliminated sequences, transposons, and other repeated sequences). This occurs by a mechanism that compares the germline and somatic genomes followed by a genomic subtraction between meiosis-specific germline sncRNAs (small RNAs that resemble piRNAs) and longer noncoding transcripts from the maternal somatic genome [17]. Consequently, an epigenetic memory of rearrangement patterns is preserved through sexual generations. This evidence associated with the involvement of piRNAs in developmental robustness strongly supports the role of piRNAs in the ancestral development of complex organisms.

Besides sncRNAs, a large percentage of the transcriptomes consists of ncRNA transcripts that are longer than −200 nt in length and are designated as lncRNAs [2,18,19]. The estimated number of gene members integrating this class of ncRNAs ranges from 10,000

to >200,000 [21]. These molecules are transcribed from diverse origins such as intergenic regions and promoter regions but can also be interleaved, overlapping, or antisense to genes encoding proteins [22]. Transcribed pseudogenes can also be an origin for lncRNAs [23]. Similar to primary transcripts produced by transcription of genes encoding proteins, lncRNAs are processed acquiring a 5′ cap and a poly $(A)^+$ tail and can undergo alternative splicing and RNA editing [24,25]. In past years a wide variety of regulatory functions have been ascribed to lncRNAs [26] such as epigenetic regulation and transcriptional and posttranscriptional control including translation regulation. These regulatory events take place during both developmental and differentiation processes, which make lncRNAs active players in dosage compensation (X chromosome inactivation), genomic imprinting, cell differentiation, and organogenesis. It is also clear that the number of lncRNA species increases in genomes of developmentally complex organisms, which strongly suggests that these RNA molecules were probably critical in the evolution of multicellular organisms [7]. The biological importance of lncRNAs is also supported by the fact that they display complex patterns of expression and different subcellular localizations.

Interestingly it was shown that some lncRNAs can be transcribed from mitochondrial genomes [27]. Taking into account the assumed endosymbiont origin of mitochondria, these results suggest that ncRNAs may also exist and have important regulatory roles in prokaryotes. In fact, the transcriptomes of distinct species of bacteria as different as, e.g., *Helicobacter pylori*, *Bacillus subtilis*, *Escherichia coli*, and *Mycoplasma pneumoniae* contain antisense RNA (asRNA) transcripts for a high percentage of genes. The sizes of asRNAs are diverse, ranging from 100 to 300 nt (e.g., SymR [28], GadY [29], and SyR7 [30], to 700−3500 nt) [31]. Interestingly, asRNAs share functional similarities with eukaryotic *trans*-acting regulatory RNAs: They are involved in the regulation of transcription termination, degradation of protein-coding RNAs, control of translation, transcriptional interference, and enhanced stability of their respective target transcripts [31]. On the other hand, bacterial sncRNAs (sRNAs) have been identified, e.g., in *E. coli* and *Salmonella typhimurium*. These sncRNAs are usually smaller than 300 nt and are able to pair with mRNAs, leading to the activation or inhibition of mRNA translation (RNAIII) [32], mRNA stabilization (GadY) [29], or mRNA degradation (RyhB) [33]. Although many of these mechanisms are distinct from those operating in eukaryotic cells, many also require cooperation between RNAs and protein partners [34].

Consequently, ncRNAs seem to have been evolutionarily important for the establishment of complex metazoa, but growing data concerning these molecules also suggest that they were already present and performing biological roles before the split of eukaryotes and prokaryotes. These facts refurbish discussion regarding the hypothesis of a primordial "RNA world" that proposes that the first organisms could rely only on RNA molecules, and only later on was the evolution of a more complex system based on proteins established [35,36]. Interestingly, data concerning bacterial riboswitches shows that similar to sRNAs, these RNA structures, located within the 5′-untranslated region (UTR) of mRNAs that regulate gene expression at the level of transcription, translation, or splicing [37,38], can also act in *trans* [39]. Moreover, specific ligands are able to change the riboswitch conformation, and in certain cases it was proposed that mRNA

decay mediated by the riboswitch could be a target to RNase E. If this is the mechanism and the regulator riboswitch can control gene expression precisely by modulating the RNase cleavage site, this would be additional support for the "RNA first" model [34].

On the other hand, several data point to the fact that, at least for some ncRNAs such as XIST, H-19, and the brain-specific Human Accelerated RNA [40], sequence conservation interspecies is not observed. Also a group of brain miRNAs found in humans and chimpanzees is not conserved away from primates, and some seem to be species-specific, which suggests a recent origin [41]. This evidence has been used to support the theory that miRNA relative nonconservation suggests that metazoan ncRNAs are a relatively recent evolutionary macro event and not vestiges of the old RNA world [42]. Alternatively, this can indicate that evolution of miRNAs is still occurring, and that along with ancient, highly conserved miRNAs, there are a number of emerging miRNAs.

Beyond this discussion remains the fact that new data strongly support the idea that RNAs evolve together with proteins and DNA, constituting a complex system of information in prokaryotic and eukaryotic genomes. In fact, the progressive characterization of these molecules has been having a considerable impact on our view of pathophysiologic states, thus providing new insights into the molecular basis of several diseases. It was shown that human α-thalassemia may be caused by the up-regulation and translocation of an antisense long noncoding transcript to nearby the α-globin gene. This asRNA is responsible for the dense methylation of a CpG island involved in the transcriptional regulation of the α-globin gene that becomes silenced during development, subsequently causing α-thalassemia [43]. Also, single-nucleotide polymorphism associated with an increased risk for autoimmune thyroid disease (AITD) simultaneous occurs in the 3′-UTR of the zinc finger gene *ZFAT* (a gene in the AITD susceptibility region) and in the promoter region of the small antisense transcript of ZFAT, with a critical role in B-cell function by inhibiting the expression of the antisense transcript, which in turn causes increased ZFAT mRNA stability [44]. These examples are merely illustrative, because the implication of ncRNAs in human diseases is continuously growing. For example, lncRNAs have been associated with distinct cancers [45,46], neurologic diseases [45,47], several heritable syndromes (e.g., hemolysis, elevated liver enzymes, and low platelets; syndrome congenital skeletal malformation brachydactyly type E) [48], facioscapulohumeral muscular dystrophy [49], and schizophrenia 2 [50]. Because in most of these cases lncRNAs have been associated with the diseases through abnormal expression patterns, they may become important for diagnosis as biomarkers.

Also, the involvement of miRNAs in regulating early embryonic development, neuronal, muscle and lymphocyte development, and their functional association with various stem cell populations, in the context of neural function and cardiovascular biology, in metabolism and aging, and during cancer, positioned them as critical molecules in maintaining homeostasis [51]. In fact, results coming from gain- and loss-of-function studies have revealed prominent roles for miRNAs: for example, in cardiovascular disorders including myocardial infarction, cardiac hypertrophy, heart failure, angiogenesis, vascular stenosis, and fibrosis [52–57]. In this context, ncRNAs are

emerging as "hopeful" molecules attracting attention not only for basic research but also for biomarker discovery and therapeutic applications that have been explored with the development of nanotechnologies [8,45,52,58]. These potential therapeutic applications of ncRNAs have been reinforced by the accomplishment of successful phase I and phase II clinical trials of Santaris Pharma's anti-miR against miR-122, miravirsen, for the therapy of patients infected with the hepatitis C virus (HCV) [52]. Also, the first cancer-targeted miRNA drug, MRX34, a liposome-based miR-34 mimic, entered in phase I clinical trials in April 2013 for patients with advanced hepatocellular carcinoma [8,59]. On the other hand, attention is also focused on lncRNAs by designing inhibitors of the natural antisense transcript (NAT) class of lncRNAs, known as antagoNAT oligonucleotides, and exploring their potential therapeutic application. The success of the first clinical data showing the safety and efficacy of an anti-miR supports the pursuit of ncRNAs as a new class of drug targets [45].

In this chapter we will give an overview of the biogenesis pathways of miRNA, piRNA, and siRNA, as well as lncRNAs, focusing on the miRNA and lncRNAs gene expression regulatory roles specifically in redox signaling and immune cell functions [60,61]. We will also explore the interconnection between cellular redox homeostasis, immune system function, and the complex networks of ncRNA actions.

10.2 Biogenesis Pathways of Noncoding RNAs: An Overview

10.2.1 Small Noncoding RNAs

Small ncRNAs such as miRNAs, siRNAs, and piRNAs, are produced by different biogenesis pathways and regulatory mechanisms, but they share the common feature of using nucleotide sequence complementarity to identify target genes and regulate their expression.

10.2.1.1 Short Interfering RNAs and MicroRNAs

Both siRNAs and miRNAs originate in the cleavage of dsRNA precursors. Regarding siRNAs, long dsRNAs can be produced from transposons, replicating virus, or endogenous genes that produce transcripts that are able to base pair, or when dsRNA is introduced experimentally in cells triggering gene silencing by a process designated by RNA interference (RNAi). These long dsRNA molecules are then processed by the Dicer ribonuclease, a member of the ribonuclease III (RNase III) family that originates small RNA duplexes with 2-nt overhangs at their 3′ ends and phosphate groups at their 5′ ends [62].

miRNAs are single-stranded RNAs conserved throughout the phylogenetic tree and their exact number is far from being estimated because identification of miRNAs occurs continuously. For example, the latest release (version June 21, 2014) of the miRBase database (http://www.mirbase.org), a primary miRNA sequence repository, has 28,465 entries that result in 35,828 mature miRNAs from 223 species including animals, plants, unicellular algae, and viruses [63]. Contrary to siRNAs, these sncRNAs are generated

from endogenous transcripts that are transcribed by RNA polymerase II, although some have been described as products of RNA polymerase III [64], in long dsRNA precursor transcripts (possessing several hundreds to thousands of nucleotides) designated by primary miRNA (pri-miRNA) that are capped and polyadenylated [9,65]. miRNAs were shown to be encoded within the cellular genome in three main ways: as unique genes, as intronic sequences within protein-coding genes, or as polycistronic miRNAs/single transcript encoding multiple miRNAs [66]. As a consequence of these encoding possibilities, their regulation can be subject to different mechanisms through alternative processing or miRNA processing.

The pri-miRNA precursors are processed by the ribonuclease (RNase) III Drosha-DGCR8/Pasha nuclear complex that originates smaller precursor miRNAs of 60−100 nt that possess a hairpin structure. These precursor miRNAs (pre-miRNAs) are transported to the cytoplasm through nuclear pores by the system of exportin5-RanGTP [67]. There, they are further cleaved by the Dicer enzyme into approximately 22-nt double-stranded mature miRNAs (miRNA−miRNA* duplexes, where miRNA was assumed to be the antisense, or guide/mature strand and miRNA* the sense, or passenger strand). Also, it has been generally presumed that the passenger strand was degraded. However, this view is now questioned by evidence showing that functional mature sequences can be derived from both arms of the hairpin [68,69]. Moreover, a shift between both arms of the precursor to produce functional mature microRNAs may occur between species or may be tissue- or developmental stage−specific [70].

As mentioned, miRNAs may derive from short intronic hairpins. These are termed “mirtrons” and are generated by an alternative, noncanonical biogenesis pathway that was identified both in vertebrates and invertebrates [71−73]. In this case, precursor miRNA (pre-miRNA)-like hairpins are produced by the action of the splicing machinery and the lariat-de-branching enzyme. This pathway converges with the canonical one in the export of mirtrons to the cytoplasm where both types of hairpins are going to be processed by Dicer into double-stranded miRNAs. Then, it is supposed that helicases separate both miRNAs strands and the mature strand is loaded into the RNA-induced silencing complex (RISC) a hetero-oligomeric complex integrating an Argonaute (AGO) protein, Dicer, and a dsRNA-binding protein (the TRBP in humans). Only one strand associates with the AGO protein, becoming the guide strand; the nonguide strand is cleaved during loading of mature miRNA strand into RISC [74,65]. The strand with the less stably paired 5′ end is preferentially chosen to be loaded into the AGO proteins [75].

AGO proteins are the direct binding partners of small RNAs and are a highly conserved protein family found from humans to Archaea [74]. They have evolved into specialized clades (or subfamilies) [76]: the AGO subfamily or AGO clade, the first member was identified in *Arabidopsis thaliana* ARGONAUTE 1 (AGO1); the PIWI subfamily first identified in *D. melanogaster P*-element-induced wimpy testis (PIWI); and the WAGO subfamily (worm-specific AGO) of specific proteins of *Caenorhabditis elegans*. In humans, the AGO clade and PIWI clade are composed of four members each [77]. RISC-specific functions depend on each AGO protein, and probably on other proteins that are able

to recognize it and integrate the complex. Thus AGO1 and AGO2 (AGO2-RISCs are functionally distinct, silencing different types of target RNAs by different mechanisms [78,79]. The sorting of different ncRNAs into different RISC complexes implies the selection of individual AGO proteins, and this selection seems to rely on the intrinsic RNA duplex structure. For example, in the case of duplexes displaying unpaired central regions, as is generally observed in the case of miRNAs, the small RNA is routed to AGO1. When the complementarity between the miRNA bound to AGO1 and the target RNA is high, this causes miRNA tailing and 3′- to 5′-trimming. If the duplex displays perfect base pairing, as in the case of most siRNAs, it is driven to AGO2 [79,80]. The discrimination between AGO1 and AGO2 also seems to depend on the action of Hen1, an enzyme that adds the 2′-*O*-methyl group at the 3′ ends of small RNAs bound to AGO2, but not those bound to AGO1 [81,82]. This methyl group is known to block tailing and trimming of the miRNA.

A second, non-Dicer−dependent pathway of miRNA biogenesis has been discovered [83,84]. This alternative pathway relies on AGO2 to mediate final processing of the miRNA. Indeed, AGO2-mediated cleavage of pre-miRNAs, followed by uridylation and trimming, generates functional miRNAs independent of Dicer. Also, here the secondary structure of the pre-miRNA seems to be important in determining the pathway of biogenesis, with distinct loop and hairpin structures determining whether the miRNA precursor is cleaved by Dicer or AGO2. Of the miRNAs processed by this pathway, miR-451 is the best characterized so far, and it contains a small, distinctive loop structure [83].

Interestingly, loading of the double-stranded siRNA or miRNA to the AGO protein requires interaction with a complex containing the molecular chaperones heat shock cognate protein 70 kDa and Hsp90, which maintains the AGO protein in a conformation that allows loading of the RNA duplex [85−87].

Finally, the guide-strand drives the RISC complex toward the target RNA that has a nucleotide sequence complementary to that of siRNA or miRNA (for miRNAs, this so-called “seed” sequence or short sequence at nt 2−8 on the 5′ end of the miRNA typically binds to the 3′-UTRs of their target mRNAs), and after pairing this is degraded or its translation is abolished [10,81,88]. It is estimated that approximately 85% of miRNA-mediated regulation in mammals occurs at the level of mRNA decay [89].

The target mRNA choice seems to require additional factors that interact with Dicer. In *Drosophila*, the Loqs-PB Dicer-partner cleaves pre-miR-307a, generating a longer miRNA isoform with a distinct seed sequence and target specificity [90]. In mammals, the mammalian TRBP homologue also interacts with Dicer to cleave pre-miR-132 generating a longer miRNA and consequently targets different mRNA molecules [91]. In *Schizosaccharomyces pombe*, AGO1 protein also seems to be associated with transcriptional silencing by inducing heterochromatin formation because it is a component of the RNA-induced transcriptional silencing complex [92]. Besides AGO1, this complex contains Chp1 (H3K9- binding protein) a poorly characterized protein Tas3, and siRNAs originated from centromeric repeat sequences [93], and is involved in recruitment of methyltransferases to specific genomic regions, where it methylates histone H3 at lysine

9 (H3K9). These data suggest the existence of cross-talk between siRNAs and chromatin remodeling [88] that does not seem to be a yeast specific characteristic because RNAi has also been linked to transcriptional silencing in humans [94].

10.2.2 The PIWI-Interacting RNAs

The piRNAs are the most recently described sncRNAs and the least characterized. piRNAs have been described to have important roles in transposon silencing, epigenetics, and posttranscriptional regulation of gene expression [81]. They differ from siRNAs and miRNAs because their biogenesis does not involve the transcription of long dsRNA and does not require Dicer activity and they interact with the specific clade of AGO proteins: the PIWI proteins [PIWI, Aubergine (Aub), and AGO3] [74]. Also, unlike siRNAs and miRNAs, which are expressed in most cells and tissue types, piRNAs are preferentially expressed in germline cells, as observed in mammals, zebrafish, and *D. melanogaster* [12,13].

piRNAs were originally described during studies aimed at clarifying how the tandemly repeated *Stellate* (*Ste*) gene was silenced in the *Drosophila* male germline. This is a critical event for spermatogenesis progression and its failing causes male infertility. The authors observed that *Ste* silencing depended on a Y-chromosomal locus (designated Suppressor of Stellate) that has been shown to contain tandem repeats with complementary sequences to the *Ste* gene [95–97]. The further cloning and characterization of abundant piRNAs in the *Drosophila* male and female germline revealed that they include a previously discovered class of small ncRNAs called repeat-associated siRNAs [11] and are derived largely from retrotransposons and other repetitive sequence elements that thus could not be assigned to specific chromosomal loci [98–100]. However some piRNAs can also map to specific genomic loci such as in *Drosophila* and mammals where they are found as clusters in large pericentromeric or subtelomeric domains, generally spanning from 50 to 100 kb and enriched in numerous transposable DNA elements and their fragments [100]. Other piRNAs are transcribed from 3′-UTRs of protein-coding genes and dispersed euchromatin copies of transposable elements [100,101].

The main function of the PIWI–piRNA pathway in the germline has been suggested to be the transposon suppression. This not only because piRNAs map largely to transposable elements but also because PIWI loss-of-function mutations or depletion lead to drastically increased levels of transposons that are accompanied by severe infertility phenotypes [95,102–107]. In addition, mutations in *Drosophila* piRNA biogenesis factors result in disruption of transposon silence, causing DNA breaks to accumulate during female germline development and leading to defects in the posterior and dorsoventral axis [100,108–110]. However further studies suggested that observed developmental defects are indeed a consequence of DNA damage resulting from transposon derepression [111]. These data support the idea that piRNAs are required for the integrity of germline cell genomes protecting them from damaging transposons. Strikingly, if new transposons are actively introduced into piRNA clusters, and if they are hereditable by

progeny, novel piRNAs (able to control the new transposons) will be produced showing that mechanisms that drive adaptation to transposon invasion may be mediated by the piRNAs pathway [100,112]. Other studies showed that a subset of piRNA genes is implicated in the telomere protection complex assembly [111].

The somatic functions of PIWI have long emerged since their discovery. PIWIs are expressed in diverse cell types ranging from naive pluripotent stem cells to differentiated somatic cells, with most somatic expression related to various totipotent and pluripotent stem cells [113]. The expression of PIWIs in mammalian somatic tissues implies the existence of somatic piRNAs. In fact, the analysis of high-throughput small RNA sequencing data in *Drosophila*, mouse, and rhesus macaque samples showed that piRNAs are abundant in other tissues such as in the germline [22].

The piRNA biogenesis pathway is still poorly understood, although it is well accepted that there are two different mechanisms. In the first case, a single-stranded RNA precursor is processed in pre-piRNAs (primary piRNA biogenesis) that are preferentially cleaved at U residues. Those pre-piRNAs are then loaded onto PIWI proteins and are subsequently 3′ trimmed and methylated [100,114]. Therefore they commonly have a uridine at the 5′ end and a 5′-monophosphate. At the 3′ end, piRNAs have a 2′-*O*-methyl nucleotide modification, a feature that they share with the plant miRNAs but not with mammalian miRNAs [81,115]. This process finally originates mature piRNA-induced silencing complexes (piRISCs) [100,114,116,117]. In the second case, piRISCs that possess an active Slicer activity can trigger secondary piRNA biogenesis. In the presence of corresponding sense and antisense precursor RNAs, PIWI and/or Aub directs the slicing of sense strand transposon transcripts [100,118]. The sliced sense strands are then bound by AGO3, and this complex directs the slicing of antisense transposon transcripts [100,114,119], generating its 5′ end (ping-pong amplification cycle) [100,118]. The two piRNAs engaged in ping-pong have opposite orientations and exhibit a characteristic 10-nt 5′ overlap (ping-pong signature) [100,118].

The mechanism that allows the production of piRNAs from a single-strand precursor is still a matter of debate [120]. In fact, until recently there were no clues about how precursor piRNA transcripts were selected from the global pool of transcripts to be driven to the subsequent steps of the biogenesis pathways. However, the discovery that the *Drosophila* protein Rhino, a homologue of the heterochromatin protein 1 (HP1), generally accepted to be involved in transcriptional silencing [121], binds to many piRNA clusters and is required for piRNA transcription [112], brought new insights into the transport of precursor piRNA transcripts. In *S. pombe* HP1 binds to H3K9me3 histones in heterochromatin and is also an RNA-binding protein that is able to accept newly synthesized transcripts from its bound loci and to transport them to sites where they will be degraded [122]. Therefore, it was proposed that this protein will be an excellent candidate to route precursor piRNA transcripts for subsequent processing steps occurring at specific cellular sites such as an electron-dense granular material found between the nucleus and the cytoplasm at the nuclear pores of germ cell nuclei (nuage) [123,124].

Supporting this idea is the fact that Rhino was found to colocalize with the DEAD box protein UAP56 at nuclear speckles bound to piRNA precursors.

The next step of piRNAs biogenesis is the 5′ end formation. Although some controversy surrounds the protein Zucchini (Zuc) concerning its enzymatic activity, resolution of the crystal structure of mouse and *Drosophila* Zuc clearly supports the nuclease activity of the protein [125–127]. Moreover, Zuc is able to catalyze the hydrolysis of single-stranded DNA and RNA *in vitro* [126,127]. These facts, together with the observation that Zuc mutants induce the up-regulation of two telomere-specific transposable elements (Het-A and Tart) and the expression of Stellate protein in the *Drosophila* germline, events associated with the inability to produce piRNAs [128], strongly suggest that Zuc is a key protein for the cleavage of the single-stranded RNA precursor in the piRNA pathway. Consequently, Zuc is a strong candidate to be the 5′ nuclease involved in primary piRNA biogenesis. However, proof of this involvement requires additional experimental support because *in vitro* Zuc does not seem to specifically generate fragments with a 5′-U base, which is the feature of primary piRNAs, and also mice depleted in Zuc still have low amounts of processed piRNAs [120].

The following step of piRNAs biogenesis requires the loading of piRNAs onto PIWI (PIWI, Aub, and AGO3) proteins just before the generation of piRNAs mature 3′ end. Detailed sequence analysis of the piRNAs associated with the PIWI subfamily [100,118] in the *Drosophila* female germline showed that the most abundant piRNAs are mainly generated from the antisense strand of retrotransposons, and these preferentially associate with PIWI and Aub proteins [100,118]. On the other hand, those present in the single major somatic cluster are mainly originated from the sense strand and are associated with AGO3. Similar to what was observed for the loading of siRNAs into AGO proteins, which requires the chaperone system Hsp90-Hsp70, it was shown in *Drosophila* that loading of piRNAs onto PIWI proteins also involves the evolutionarily conserved chaperone Shutdown (Shu) protein [129]. The Shu chaperone belongs to the immunophilin class, harbors a peptidyl-prolyl-*cis*/*trans*-isomerase domain (PPIase) and a TPR domain [130], and collaborates with Hsp90 at the loading step of piRNAs onto PIWI proteins. Finally the 3′ end of piRNAs should be trimmed but the responsible enzyme was not yet identified. piRNAs are heterogeneous in length and show greater variability at the 3′ end than at the 5′ end, which suggests the activity of a 3–5′ exonuclease. As mentioned, 3′ maturation requires methylation that seems to protect piRNAs against nontemplated uridylation and destabilization [120]. Hen1 is also the methylase that adds a methyl group to the piRNA at the 2′OH position of the 3′ end base [82]. This methylation seems to be a common feature to sncRNAs that base pair to target RNA molecules, i.e., miRNAs and siRNAs [131,132].

The role of PIWI–piRNAs pathways has been extended to numerous somatic functions. Indeed this pathway is important not only for transposon silencing but also genome rearrangement and epigenetic programming during development, with biological roles in stem cell function in regeneration, memory, and possibly cancer [113,133].

In *Drosophila* development the role of piRNAs is clear during maternal-to-zygotic transition, because it was shown that the expression of a piRNA cluster was required [134] to promote degradation of maternal mRNAs such as the embryonic posterior morphogen Nanos (Nos). piRNAs and their associated proteins are involved in recruiting the CCR4 deadenylation complex to specific Nos mRNAs, thus promoting their decay. If Nos mRNA is not degraded, head development defects are observed in the *Drosophila* embryo. Because the expressed piRNA cluster is transcribed from transposable elements, this has led to the suggestion of a close relationship between transposable elements and the regulation of gene expression during development [134]. Another example of the participation of piRNAs in development was revealed by deep sequencing of extracted RNA from the mouse hippocampus. piRNAs are necessary for spine morphogenesis because antisense suppression of this piRNA in hippocampus causes a decrease in the dendrite spine area [135].

Growing evidence also indicates that PIWI and piRNAs have a crucial part in epigenetic regulation through alterations in methylation patterns. Interestingly, in *Drosophila* ovarian somatic cells piRNAs mediate the inhibition of transcription of hundreds of transposon copies by promoting methylation of H3K9 on transposons and their genomic surroundings [136]. Moreover, in *Drosophila* the somatic silencing of the transposable elements depends on maternally transmitted piRNAs from the *tirant* retrotransposon locus, which correlates with an increase in histone H3K9 trimethylation of the active *tirant* element [137]. Also, in the *Drosophila* early embryo it was observed that piRNAs were implicated in heterochromatin formation and that this was necessary for heterochromatin establishment in nongonadal somatic cells, with an impact in the phenotype of the adult [138]. In addition, in Aplysia sensory neurons, a PIWI−piRNA complex is involved, in a serotonin-dependent way, in repression of the transcriptional repressor of memory CREB2, because it is involved in the methylation of a conserved CpG island in the CREB2 promoter [139]. Therefore, the role of the PIWI−piRNA pathway is extended to modifications in the gene expression patterns of neurons involved in long-term memory storage. The complexity of the regulatory networks involving the PIWI−piRNA pathway is also evident because they also enhance transcriptional activation [140]. Participation of the PIWI−piRNA pathway in *de novo* methylation is not restricted to invertebrates, because it was also found in mammalian genomes, as in the case of the differentially methylated region of the imprinted mouse *Rasgrf1* locus [141].

PIWI-piRNA pathways were also shown to have an important role in stem cell biology. Planarians are flatworms able to regenerate the whole body from a small part because they possess a population of stem cells called neoblasts that have the ability to originate all somatic and germ cells [142,143]. Remarkably, in planaria depletion of smedwi-2 and smedwi-3 proteins, which are members of the PIWI/AGO family, cause a decrease in piRNA levels and impair regeneration [144,145]. These results indicate that smedwi-2 and -3 are required for proper function of neoblasts, e.g., in replacing aged tissue.

PIWI proteins have also been implicated in regeneration processes. It was shown that the colonial ascidian tunicate *Botrylloides leachi* (sea squirt urochordate) can regenerate functional adults from microscopic vasculature fragments. This regeneration seems to rely on cells that contour the internal vasculature epithelium of blood vessels after an insult and express *de novo* PIWI [146,147]. After PIWI expression these cells are activated, change morphology, and migrate to places of the vasculature lumen, where they proliferate and differentiate, regenerating a functional organism. If the PIWI synthesis is inhibited, these cells are unable to proliferate or differentiate and subsequent regeneration does not occur [146,147].

There is also growing evidence associating the expression of PIWI with cancer cells. For example, in *Drosophila* it was shown that mutations in PIWI delay tumor growth in a sensitized *lethal [3] malignant brain tumor* [*l(3)mbt*]-mutant background [148]. In addition, it was observed that other genes encoding members of the piRNA pathways are induced in *l(3)mbt* tumors. It was also suggested that PIWI is expressed in response to an increase in transposon activity in cancer cells, but additional experiments are required to clarify these issues. However, it is possible that, with better understanding of the role of the PIWI-piRNA pathway in cancer biology, new avenues may be open to cancer therapy.

The role of PIWIs and piRNAs in the development of evolution has been mentioned in this chapter. In fact, during evolution, piRNAs may have been important components of a mechanism that guaranteed correct genomic rearrangements during macronucleus development (the nuclei that is transcriptionally active and the somatic line in ciliated ancestral unicellular organisms), but they might also act as capacitors with a critical role in regulating phenotypic variation and morphologic evolution by controlling how and when genetic variation is expressed. Together they seem to be involved in maintaining balance between the generation of "genomic variability" and "genomic stability," which is also linked to integrity. Therefore, they may have been critical for the sustained evolution of genomes allowing the establishment of complexity integrating environmental information. In this view, it is tempting to speculate that these two features may also be related to their involvement in stem cell maintenance and self-renewal, and hypothetically in cancer regulation.

10.2.3 Long Noncoding RNAs

Through the extensive analysis of data obtained from complementary DNA libraries and RNA sequencing data by high-throughput transcriptome projects, it became clear that the mammalian genome produces a large transcriptome of lncRNA (defined as ncRNA $>$ 200 nt in length). However lncRNA's biological importance is just emerging because only a small percentage of lncRNA has been functionally characterized [7,149]. Growing interest in its biological role has been strongly stimulated by evidence that connects them to several human diseases [45,46,48,49].

Although a comprehensive classification of these molecules is still missing and some effort has been made to establish comprehensive conceptual guidelines [150], lncRNA's

transcriptional origins are commonly used for this purpose. Therefore lncRNA has been designated as promoter-associated long RNAs [25], NATs or opposite-strand transcripts [151], long intergenic ncRNA (lincRNA) [152], and enhancer associated RNAs (eRNAs) [153,154]. This criterion sometimes creates noise because often one lncRNA can be classified into more than one class. For example, intergenic RNAs can also be found in introns contributing to a significant fraction of ncRNAs in mammalian cells, and therefore the term "lincRNA" is inadequate [155].

The international FANTOM Consortium demonstrated that at least 25–40% of mammalian protein-coding genes display overlapping transcription [156,157]. In fact, lncRNAs can be transcribed as RNA molecules that display a primary nucleotide sequence that is total or partially complementary, and then able to pair to RNA transcripts with known function; therefore, they are designated NATs [158].

NATs can be designated as *cis*-NATs in cases in which they are originated by transcription of the antisense strand of a given genomic locus. The *cis*-NAT sequence will be complementary to that of the sense transcript of the same locus. Alternatively, they can be transcribed from different genomic loci and may have only partially complementarity with the sense counterpart designated as *trans*-NATs [159]. Thus, in the same cell a pair of sense-antisense transcripts of a genomic locus can be found, and they may have different degrees of overlapping. There are examples in which these pairs of sense–antisense RNAs overlap by their 5′ regions (5′ to 5′), by their 3′ regions (3′ to 3′), or do not overlap or fully overlap (in this last case, we have two overlap genes) [26].

An extensive analysis of human lncRNAs produced by the GENCODE consortium, composed of 9277 manually annotated genes producing 14,880 transcripts [19], revealed a set of features of lncRNAs: (1) they seem to be synthesized and processed by pathways resembling those of genes encoding proteins; (2) they tend to be transcripts that possess two exons; (3) they are expressed at levels in general lower than those of genes encoding proteins presenting patterns of expression that are more tissue-specific; (4) they preferentially localize at the chromatin and nucleus although there are cytoplasmic pools; and (5) some seem to be able to be processed into small RNAs. Sequence analysis of lncRNA suggests that they do not possess open-reading frames with better features than those found at random [19].

Moreover, it seems that compared with gene-encoding proteins, lncRNAs are subjected to a lower selective pressure but higher than neutrally evolving sequences (ancestral repeat sequences). The promoter region of lncRNAs is the region under a higher selective pressure with levels of conservation equivalent to the promoter region of gene-encoding proteins [19,152,153,160,161]. This may reflect the expression specificity that these molecules seem to possess; on the other hand, it may be the operative mechanism used by these lncRNAs. It is conceivable that their function may be conserved although their molecular mechanism of action does not require extensive sequence conservation.

The roles of lncRNAs have been ascribed to their ability to interact with proteins and protein complexes originating ribonucleoprotein complexes that participate in a variety of

different steps of gene expression regulation [18,20], and to establish base pair interactions with DNA and RNA that can mediate the role of the referred ribonucleoprotein complexes. These abilities are shared with small ncRNAs, as for example miRNAs and small nucleolar RNAs and other ribonucleoprotein complexes. Nevertheless lncRNAs have striking distinctive features because they seem to be able to adopt complex secondary structures and other high-order structures that create a new level of information for interaction and recognition, and which cannot be found among sncRNAs [7,18,20]. These assumptions have led to the proposal of regulatory models in which, by the ability of changing their secondary structure, lncRNAs will be sensors of signals. The lncRNA's structure changes will affect its ability to be recognized and interact with a partner or to be processed. In both cases, structure changes will allow the fast and focused transduction of information [42]. Therefore, attention should be paid to how secondary structures are conserved throughout interspecies lncRNAs. Interestingly, in these models lncRNAs display mechanisms of action with similarities to those of bacteria riboswitches [37,38].

In this scenario it has been proposed that because of their ability to pair with nucleic acids, lncRNAs could trap specific sites of transcription factors, facilitate interactions between distinct proteins by acting as scaffolds, recruit and guide proteins (i.e., those involved in chromatin remodeling), etc. [18,20,42,162].

The documented diversity of roles of lncRNAs in gene expression regulation will be analyzed and discussed later this chapter.

10.3 MicroRNAs: Key Posttranscriptional Regulators of Gene Expression

Since their initial discovery as regulators of larval development in nematodes [163], miRNAs have been increasingly involved in regulating important cellular processes of multicellular eukaryotes, including cell proliferation, development, differentiation, apoptosis, and oncogenic transformation [164].

miRNAs mediate the repression of target gene expression, acting as sequence-specificity guides for the RNAi machinery. They are estimated to affect most mammalian coding genes [165]. The growing list is curated in the miRBase, which currently accounts for approximately 1881 miRNA species in the human genome and 1193 in the mouse genome [63] that regulate 60% of all protein-coding genes [81]. Because most mRNA targets contain multiple miRNA binding sites, each miRNA can regulate multiple genes. As such, deregulated miRNA levels can perturb the expression of many genes and may be responsible for the onset of several diseases, some of which are addressed in this chapter.

10.3.1 MicroRNA-Mediated Regulation of Gene Expression

As mentioned, it has become increasingly clear that the major mechanism by which miRNAs function is at the mRNA transcriptional level rather than the translational level [89].

mRNA decay is the most frequent miRNA-mediated transcriptional inhibition mechanism used by miRNAs to inhibit target gene expression, but they can also promote direct mRNA cleavage and chromatin reorganization, resulting in decreased mRNA levels. miRNA-mediated mRNA decay can occur via deadenylation, decapping or 5′ to 3′ degradation of the mRNA [166]. mRNA cleavage is a rare miRNA-mediated repressive mechanism in animals but it is frequent in plants and normally occurs when there is full complementarity between miRNA and its mRNA target [167]. miRNAs also have the capacity to reorganize chromatin by increasing methylation of the targeted mRNA promoters, thereby inhibiting their expression [168]. Interestingly, the repressed mRNAs, AGO proteins, and miRNAs frequently accumulate in processing bodies, which are cytoplasmic structures enriched in the mRNA degradation machinery but in which the translational machinery is normally absent [169].

The second mechanism of miRNA-mediated activity includes repression of translation initiation and/or elongation, premature termination, and nascent polypeptide degradation. Endogenous let-7 micro-ribonucleoproteins (miRNPs) or the tethering of AGO proteins to reporter mRNAs in human cells inhibit m(7)G-cap–dependent translation initiation, which suggests that miRNPs interfere with the recognition of the cap [170]. In addition miRNA-repressed mRNAs contain 40S but not 60S components, which indicates that miRNAs repress translation initiation by preventing the 60S subunit from joining miRNA-targeted mRNAs [171]. The fact that repressed mRNA targets seem to be associated with polyribosomes points to the fact that miRNAs can also repress translation at the elongation step [172–174]. Silencing by miRNAs can also occur before completion of the nascent polypeptide chain, causing a decrease in translational read through at a stop codon, with ribosomes on repressed mRNAs dissociating more rapidly after a block of initiation of translation than those of control mRNAs [174]. These observations suggest a role for miRNAs in ribosome drop-off–mediated repression.

Curiously, some studies provide exceptional evidence for miRNA-mediated transcriptional [175] and translational [176] activation. For example, miRNA-373 was shown to induce expression of genes with complementary promoter sequences [175], whereas miRNA-10a can bind to the 5′-UTR of ribosomal protein mRNAs and enhance their translation [177]. Furthermore, several studies have demonstrated that miRNAs and their associated complexes (microRNPs) can elicit both stimulation of gene expression and the typical repressive roles [178,179].

10.3.2 Impact of MicroRNAs in Immune Cell Development and Functions

The immune system is one of the best, if not the best, characterized developmental systems in mammals. Briefly, this system is generated from hematopoietic stem cells (HSCs) through a series of ordered events of lineage commitment, differentiation, proliferation, and cell migration. This includes developmental programs of ordered T-cell receptor (TCR) and immunoglobulin gene segments that are included on each

lymphocyte to form a single antigen receptor (TCR in the case of T cells and BCR in the case of B cells). It also includes differentiation of the cells into distinct cellular subsets with distinct effector functions, and innate and adaptive responses to antigens. Cell interactions between lymphocytes of different subsets and between lymphocytes and other cells of the hematopoietic system, such as dendritic cells, have a critical role and may be accompanied by the generation of immunologic memory. The surface markers of immune cell subsets and of signaling pathways as well as the transcriptional programs underlying developmental progression, cellular selection, and cell migration are extremely well characterized.

Given the considerable knowledge about the functions of immune cells, it was surprising to find that there was an additional unrecognized layer involved in control of the development and physiology of this system that is mediated by miRNAs.

After an initial demonstration that overexpression of an miRNA in HSCs affected B lymphopoiesis *in vivo* upon HSC transplantation [180], it became apparent from experiments in which Dicer was conditionally inactivated in T or B lymphocytes of the mouse that miRNAs are critical for lymphocyte development and differentiation.

A deficiency in miRNAs was first suggested to affect B-cell development by the conditional deletion of AGO2 in hematopoietic cells, which resulted in partial deficiency in miRNAs and compromised development of B and erythroid cells [181]. When Dicer was deleted in the B-cell lineage from the earliest stage of B-cell development, an almost complete block at the proto pre-B transition resulted, which at least partly resulted from apoptosis of Dicer-deficient pre-B cells [182].

In the case of T-cell development, deletion of Dicer in immature thymocytes led to a 10-fold reduction in total thymocyte numbers, an effect that was not as drastic on the periphery [183]. Also, there were major differences in T-cell differentiation in Dicer-deficient mice, which showed a major failure to repress interferon-gamma (IFN-gamma) expression [184]. Ultimately, disruption of this balance between miRNA expression patterns and cytokine production leads to exacerbated inflammation and autoimmunity. In fact, in mice deficient for Drosha or Dicer specifically in the regulatory T-cell population, there is a loss of regulatory T cell–mediated suppressive activity, which results in the development of fatal autoimmunity [185,186]. Also, the absence of miRNAs throughout the T-cell compartment results in spontaneous inflammatory disease [187].

Whereas these examples clearly show the global importance of miRNAs in the immune system, unique spatial and temporal expression patterns in the hematopoietic lineages suggest multiple roles for miRNA in immune cell differentiation and functions. The importance of individual miRNAs became apparent in 2007, when it was observed that the genetic inactivation of miR-155 affected a large spectrum of immune reactions ranging from cytokine production by T and B cells to antigen presentation by dendritic cells and the germinal center B-cell response [188,189]. Since then, many other miRNAs were implicated in the regulation of immune cell function, including early hematopoietic cell development and myeloid and lymphoid

cell development and function. Individual miRNAs have been shown to repress the expression of HSC-relevant genes and affect the production of hematopoietic progenitor and lineage-positive cells. For example, miR-221 and miR-222 regulate KIT expression, which is thought to affect stem cell homeostasis [190]. miR-223 is a myeloid-specific miRNA that promotes monocyte differentiation by repressing nuclear factor I/A production [191]. It also represses C/EBPβ and myeloid ELF1-like factor 2C (MEF2C) expression to modulate neutrophil differentiation and proliferation [192]. Expression profiling showed that stimulation of monocytes with lipopolysaccharide (LPS) induced the expression of miR-132, miR-146, and miR-155 [193,194]. miR-146 targets Traf6 and Irak1, components of the Toll-like receptor (TLR) signaling pathway that is activated by LPS, which suggests a negative feedback loop [194].

In T cells, microRNAs were shown to be more relevant during the early thymic differentiation rather than at later differentiation stages and during peripheral homeostasis, which are both largely miRNA-independent. During thymic development, T-cell survival and selection are influenced by the miR-17-92 cluster [which targets mRNAs encoding BCL-2—interacting mediator of cell death (BIM)], and phosphatase and tensin homologue (PTEN), and miR-181a (which targets mRNAs encoding phosphatases such as dual-specificity protein phosphatase 5 (DUSP5), DUSP6, SH2-domain—containing protein tyrosine phosphatase 2, and protein tyrosine phosphatase, nonreceptor type 22 (PTPN22), controlling TCR signaling thresholds in T lymphocytes [195]. On the periphery, mature T-cell differentiation is modulated, among others, by miR-326, which promotes skewing toward TH17 cells by targeting the mRNA encoding ETS1 [196] and miR-155, which promotes skewing toward T-helper 1 (Th1) cells by repressing macrophage-activating factor or Treg survival by repressing the expression of suppressor of cytokine signaling 1 [197].

Taking these findings together, miRNA control has emerged as a general regulatory mechanism in immune development and immune responses. In the next section, we provide updated information on mechanisms employed by miRNAs to regulate immune functions.

10.3.3 Mechanisms by Which miRNAs Exert Their Function in the Immune System

Based on their characteristics, such as on the combined protein—miRNA gene expression program, miRNAs are able to confer unique properties to a gene regulatory pathway in immune cells [198,199]. miRNAs are particularly important for regulating cellular responses that are time dependent. In fact miRNAs are thought to act on a shorter time scale than protein transcriptional repressors. For example, in inflammation there is an induction of both miRNAs and transcriptional factors, but because miRNAs do not require translation or translocation back into the nucleus to begin repressing their targets, they become active more quickly [198]. This may indeed be important in innate

responses in which a short time frame is critical to begin the fight against pathogenic organisms. On the other hand, miRNAs may act more slowly than protein factors that could directly target and inactivate miRNA targets [199]. Because miRNAs act at the RNA level, a protein that has been already produced and is active will remain active until miRNA action down-regulates the amount of new protein being produced. For example, miR-21 causes delayed inhibition of programmed cell death 4, a proinflammatory protein, following induction by nuclear factor (NF)-κB [200].

Another important notion is that although the mechanism of action of miRNAs is the same in different cells, they exert distinctive functions in different cell types. This is mainly because each cell type has a specific transcriptional program that limits the number of relevant targets for a given miRNA.

Also relevant is the fact that miRNA inhibition of target mRNAs does not lead to a complete knockdown of the respective protein. Indeed, unlike transcription factors that act as "switches," miRNAs are "fine-tuners" of gene expression, which is of extreme importance for the immune cells that can respond quickly to small changes in gene expression. This "fine-tuning," however, can still be modulated. In fact, distinct cellular concentrations of a given miRNA can dictate a different protein output of its target genes, as demonstrated in a study in which the interaction of miR-150 and its target c-Myb was addressed quantitatively [201]. When miR-150 was ectopically expressed at graded concentrations in lymphocyte progenitors *in vivo*, dose-dependent down-regulation of c-Myb protein levels was observed, with a 35% reduction caused by the highest concentration of the transgenic miRNA. There was a concomitant dose-dependent developmental block at the pro- to pre-B transition, which was proven to be mainly caused by the reduction in c-Myb protein levels [201].

Finally, miRNAs can regulate immune cell responses by modulating negative regulators, controlling distinct nonsynergistic regulatory pathways, or even controlling simultaneously functionally interrelated pathways. For example, by down-regulating several negative regulators of TCR signaling (PTPN22, DUSP5, and DUSP6), miR-181 changes the outcome of signaling triggered by TCR engagement by peptide–major histocompatibility complexes (MHCs): double-positive thymocytes (expressing high levels of miR-181), but not mature T cells, respond to self-antigens and are positively or negatively selected [195]. miR-155 is an example of a miRNA regulating distinct, non-synergistic pathways, involved in controlling the generation of class-switched B cells and c-*myc*–immunoglobulin H translocations. miR-155 down-regulates AID, an enzyme essential for class switch recombination, and the transcription factor Pu.1, which counteracts the differentiation of class-switched cells [202]. The net effect, possibly involving additional miR-155 targets, is the promotion of this process. As for c-*myc*–immunoglobulin H translocations, miR-155 down-regulates AID and other target genes, which synergize with AID in mediating these events [203]. A final example is that of the miR-17-92 cluster, which regulates components of functionally interrelated pathways by targeting PTEN (an inhibitor of the PI3K pathway, which promotes cell cycle progression), BIM (a proapoptotic factor), p21 (an inhibitor of the G1/S cell-cycle transition),

and Rbl2 (a member of the Rb family of pocket proteins that negatively regulate cell cycle), and E2F1, E2F2, and E2F3. The final outcome is cell proliferation, which correlates with the fact that miR-17-92 is highly expressed in proliferating progenitor cells during lymphocyte development, decreasing two- to threefold upon maturation [204,205].

Although there is still a lot to uncover regarding miRNA-mediated regulation, these small noncoding molecules are unequivocal key players in immune cell regulation.

10.3.4 MicroRNA-Mediated Regulation of Cellular Redox State

Cellular redox state is another important process regulated by miRNAs and its misregulation is frequently associated with immune-mediated responses such as inflammation, as further explored next.

Cells form reactive oxygen species (ROS) such as superoxide radical $\left(O_2^-\right)$ or hydrogen peroxide (H_2O_2) and reactive nitrogen species such as nitric oxide (NO) or peroxynitrite ($ONOO^-$). There is increasing evidence that ROS such as H_2O_2 have a key role in the redox regulation of protein activity and signaling pathways through the use of protein thiols and disulfides as functional switches. Therefore, cells keep a tight control of ROS steady-state levels through antioxidants to prevent molecular oxidative damages and disruption of redox signaling and control, i.e., oxidative stress.

Work from several laboratories has shown that miRNAs can modulate the intracellular and extracellular redox state. In particular, the expression of antioxidant enzymes such as catalase and superoxide dismutase (SOD) and also nitric oxide synthase, which are responsible for the synthesis of NO, is controlled by miRs (Fig. 10.1).

In human retinal pigment epithelial cells (ARPE-19), a sublethal H_2O_2 concentration (200 μM) up-regulates the expression of miR-30b, a member of the miR-30 family, and leads to a decrease in catalase expression at both the transcript and protein levels. miR-30b antisense (antagomiR) not only suppressed the miR-30b mimics-mediated inhibition of catalase gene expression but also dramatically increased the expression of catalase, confirming that miR-30b regulates catalase expression [206]. Suppression of miR-551b also increased catalase expression and decreased ROS levels in a dose-dependent manner in human lung cancer cell lines with apoptosis resistance (A549-AR and H460-AR cells) that express low levels of catalase compared with wild-type cells [207]. Because inhibition of miR-551b in A549-AR cells increased catalase 3′-UTR luciferase reporter activity and the reporter activity was higher in A549 WT cells that express lower levels of miR-551b, miR-551b probably binds directly to catalase 3′-UTR to suppress catalase expression.

There is also evidence that miRs regulate SOD. In fact, cells overexpressing miR-21 have unchanged SOD1 levels but reduced SOD2 and SOD3 levels [208,209]. The 3′-UTR of SOD3, the extracellular SOD, represents an authentic miR-21 binding site and SOD3 is a direct target of miR-21. However, SOD2 expression is not regulated directly by miR-21 but indirectly through tumor necrosis factor-α (TNF-α). TNF-α is a direct target

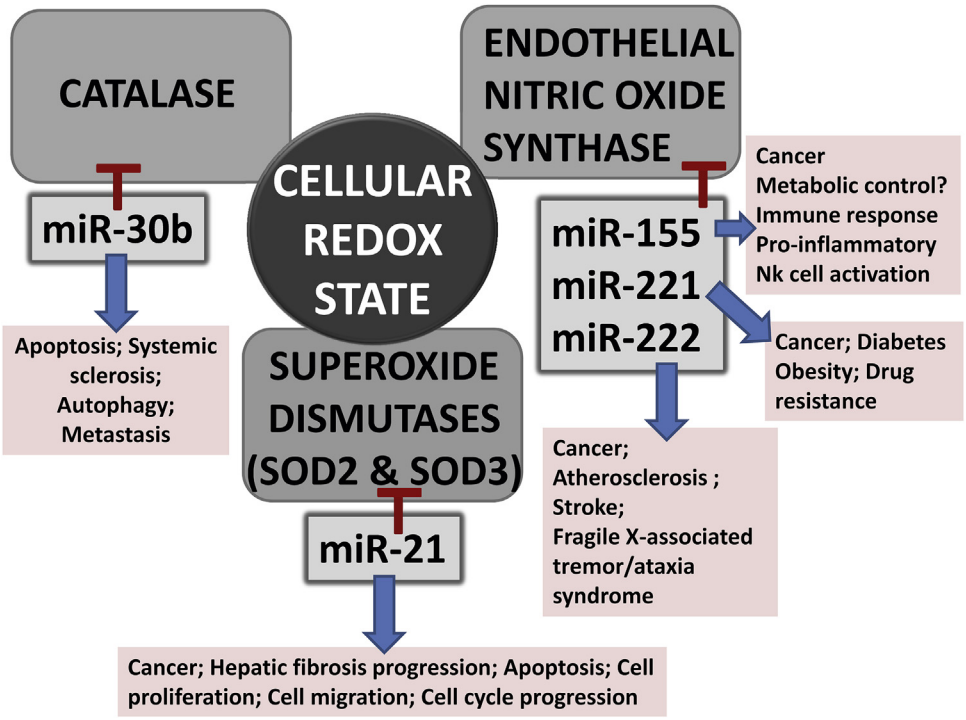

FIGURE 10.1 Regulation of cellular redox state by microRNAs (miRNAs). Control of the redox state of the cell through regulation of major antioxidant enzymes catalase, superoxide dismutase (SOD), and the nitric oxide synthase responsible for the synthesis of nitric oxide. The main cellular roles and physiopathologic states that are mediated by these miRNAs are also indicated. *Data were collected from http://www.ncbi.nlm.nih.gov/geneID (miR21, ID: 406,991; miR30b, ID: 407,030; miR-155, ID:406,947; miR-221, ID:407,006; and miR-222, ID:407,007).*

of miR-21 and its levels are decreased in cells overexpressing miR-21. A consequence of miR-21–induced changes in SOD2 and SOD3 expression levels is that overexpression of miR-21 in the human bronchial epithelial cell line NL20 leads to increased superoxide (O_2^-) levels and reduced H_2O_2 levels. A study also showed that in primary cortical neurons overexpression of miR-424 decreases cellular injury induced by 200 μM H_2O_2 for 1 h through a mechanism involving increased SOD2 activity [210].

A role for miRNAs in regulating endothelial nitric oxide synthase (eNOS) has long been suspected since the knocking down of Dicer, increased eNOS expression [211]. In addition, miR-221 and miR-222 mimics partially reverse the increase in eNOS elicited by Dicer down-regulation, although neither of these miRNAs directly targets the eNOS mRNA. It was shown that miR-155 directly targets eNOS mRNA by binding to its 3′-UTR to decrease enzyme expression and NO production [212]. Proinflammatory cytokines such as TNF-α, interleukin (IL)-1, interferon-gamma, and bacterial LPS increase miR-155 expression in human endothelial cells, most probably via AP-1 and NF-κB [212]. Conversely, inhibition of miR-155 partially prevents the TNF-α–induced decrease in

eNOS expression [212]. It was also shown that miRNA-24 overexpression in human umbilical vein endothelial cells regulates eNOS expression by leading to the inhibition of its expression [213].

Whereas miRNAs function as regulators of stress-related factors, the reverse is also true, i.e., stress conditions can regulate miRNA expression. Indeed, together with inflammatory signals, these are part of a growing list of factors that modulate miRNA expression, as detailed in the next section.

10.3.5 Modulation of MicroRNA Expression in Immune Cells and During Cellular Stress Responses

miRNAs are mostly regulated during their biogenesis at the transcription and processing levels, and there is also evidence that some of these processes are influenced by immune challenge, inflammation, and other forms of cellular stress.

The expression of miRNAs is initially controlled at the transcriptional level by transcription factors that act in specific cell types during development or in response to environmental challenges, as further detailed in Sections 10.3.5.1–10.3.5.3.

The next layer of regulation is at the posttranscriptional level, affecting processing and including various stages of regulation from the initial primary transcript to the delivery of mature, single-stranded miRNAs to their target mRNAs. Regulators of miRNA processing bind to the stem or loop regions of miRNA precursors and influence their processing by Drosha and/or Dicer. Adenosine deaminase acting on RNA is an example of an RNA-modifying enzyme that is up-regulated during inflammation and can introduce mutations in double-stranded miRNA precursors, thereby changing the targeting specificity of the miRNA [214]. After being transported to the cytoplasm, some pre-miRNAs are posttranscriptionally modified by terminal uridyl transferase 4 (TUT4), which mediates uridylation of pre-let-7 miRNA upon recruitment of Lin28 [215,216]. This modification prevents maturation of pre-let-7 miRNA, thus inhibiting its function.

In addition to transcriptional and posttranscriptional regulation of miRNAs, there is evidence suggesting that under some conditions, such as after the onset of cellular stress, miRNAs were associated with stress granules [217]. This suggests that miRNAs can also be regulated by subcellular localization. However, further investigation is required to understand the role of these organelles in miRNA biology.

Finally, epigenetic mechanisms also seem to act on miRNA expression. A genomic analysis of miRNA sequences has revealed that CpG islands are present both upstream and downstream of miRNAs coding sequences. A significant portion of these CpG islands (40%) was shown to overlap predicted transcription start sites [218]. In addition, DNA hypermethylation and histone modifications of miRNA genomic regions have been associated with altered miRNA activities with a potential oncogenic role [219], which opens an unexplored area regarding the epigenetic regulation of miRNA gene expression in normal tissues, tumor development, and progression.

10.3.5.1 Immune Signals Influencing MicroRNA Expression

The transcription of certain miRNAs found in immune cells, called immuno-miRs [220], was shown to be modulated in response to inflammatory stimuli and proinflammatory cytokines. For example, miR-155 and miR-146a transcription is up-regulated in response to inflammatory stimuli such as TLR ligands or proinflammatory cytokines [193,194]. TGF-β is a potent immunosuppressive cytokine implicated in the inhibition of T-cell activation and the down-regulation of proinflammatory cytokines. It was shown to specifically affect the expression of several miRNAs which themselves have immunomodulatory functions [221–223]. TGF-β promotes the induction of miR-155 expression levels in human intestinal $CD4^+$ lamina propria T cells, which in turn targets Itk (a key signaling molecule downstream of the TCR), leading to decreased expression of IL-2 and impaired T-cell activation [223]. In human $CD8^+$ T cells, TGF-β was shown to induce the three members of miR-23a cluster (miR-23a, -27a, and -24), which exhibited immunomodulatory functions [222]. miR-27a and miR-24 targeted the proinflammatory cytokine IFN-gamma, whereas miR-23a targeted CD107a, an essential component of the cytotoxic granule machinery [222].

In addition to these specific roles, TGF-β exerts pleiotropic effects on the miRNome because its downstream signaling effector proteins, the Smad protein family, have been shown to have an impact on the generation of miRNAs at the pri- to pre-miRNA processing step performed by the Drosha microprocessor complex [224,225]. In fact, Smad proteins activated by TGF-β signaling bind to a consensus sequence found within the stem region of numerous pri-miRNAs. Both Smads and p53 associate with Drosha via p68 and increase the processing of their specific target pri-miRs [224,225]. KH-type splicing regulatory protein binds sequences in the loop region of several miRNA precursors and recruits Drosha and Dicer to the pri- and pre-miRNA, respectively, enhancing their processing [226]. In contrast, Lin28 represses both Drosha and Dicer processing of most let-7 family members by binding conserved sequences in their precursor loops. This mechanism involves Lin28 recruitment of TUT4 to pre-let-7, causing it to be oligo-uridylated and degraded [227].

Cytokines and immune factors other than TGF-β were shown to influence miRNA expression. Interleukin-2, primarily produced by antigen-activated T cells, was actually the first cytokine shown to have an effect on the miRNA repertoire. Together with TCR activation induced miR-182 expression, targeting the transcription factor FOXO-1, a negative regulator of T-cell proliferation [228].

These examples highlight an important layer of immune-mediated miRNA regulation and the transcriptional networks associated with it. In the next sections we will focus on stress-related factors that also affect the different stages of miRNA biogenesis.

10.3.5.2 Modulation of MicroRNA Biogenesis by Hydrogen Peroxide

As described earlier (Section 10.3.4), miRNAs can regulate the cellular redox state, but the reverse is also true: stress conditions can affect miRNA levels. In this section we will explore how stress factors, namely hydrogen peroxide (H_2O_2), regulate miRNA biogenesis.

Why focus our attention in H_2O_2? The biological effects of ROS depend on their concentration, with high levels being responsible for toxic effects, whereas low to moderate levels mediate important molecular pathways. Among ROS, H_2O_2 is one of the most abundant species whose biological effects have been characterized. The specificity of H_2O_2 biological effects results from activation of sensor proteins with diverse reactivity toward H_2O_2 and from localized production of H_2O_2 that activates signaling pathways in localized cellular spots. Its targets include signaling enzymes such as phosphatases [229], kinases [230], and transcription factors [231] and, as reviewed here, ncRNAs.

Biogenesis of miRNA is down-regulated by H_2O_2 by several potential mechanisms. As described in Section 10.2.1.1, pri-miRNAs are cleaved by Drosha to generate pre-miRNAs in the nucleus [232]. Phosphorylation of Drosha at Ser300 or Ser302 by glycogen synthase kinase-3β (GSK-3β) is required for Drosha nuclear localization [233,234]. GSK-3β is inhibited by phosphorylation of Ser9 catalyzed by AKT kinase [235], and so by activating the PI3K–AKT pathway H_2O_2 indirectly inhibits GSK-3β [236,237]. Thus, we may speculate that H_2O_2 may inhibit the nuclear localization of Drosha (Fig. 10.2).

In addition, DiGeorge critical region-8 (DGCR8), which forms a complex with Drosha to cleave primary miRNA, may also indirectly be modulated by H_2O_2, because the binding of ferric heme enhances its activity [238]. H_2O_2 may down-regulate the cellular level of ferric heme by (1) activating the gene expression of heme oxygenase 1 [239], the enzyme responsible for converting heme to biliverdin, carbon monoxide, and ferrous ions, and by (2) inhibiting the first enzyme of the heme biosynthetic pathway, δ-aminolevulinate synthase-2 (Fig. 10.2), with an inhibition constant measured *in vitro* of $K_i = 5\ \mu M$ [240]. Heme oxygenase 1 is under the control of NRF2, a redox-sensitive transcription factor that is activated by H_2O_2 [241]; such control may have a significant impact on miRNA abundance because heme oxygenase modulates miRNA by a multitude of mechanisms other than heme availability [242].

After their formation by the Drosha–DGCR8 complex, pre-miRNAs are subsequently exported into the cytoplasm where they are further processed by Dicer [232], which is down-regulated by H_2O_2. In primary cerebromicrovascular endothelial cells derived from young rats (3 months old), treatment with 0.1 μM H_2O_2 decreased Dicer mRNA expression by 40% after 24 h [243]. Interestingly there was a decrease in Dicer mRNA with age that was reversed by overexpressing catalase [243]. In 3T3-F442A preadipocytes, rapid decreases of Dicer mRNA (90 min) were observed for higher H_2O_2 concentrations. However, delivery of H_2O_2 as continuous production by glucose oxidase yielded biphasic effects, because increased Dicer mRNA levels were observed for lower H_2O_2 production rates, whereas higher rates of H_2O_2 production decreased Dicer mRNA levels [244]. Dicer protein levels were nearly abolished 24 h after treatment with 250 μM H_2O_2 for 15 min in human JAR trophoblast cells (Fig. 10.2) [245]. The mechanism underlying H_2O_2-mediated Dicer inhibition is unknown, but it has been suggested that is mediated by the let-7 family of microRNAs [246], which are known to mediate suppression of Dicer (Fig. 10.2) [247,248] and are up-regulated by H_2O_2 [249].

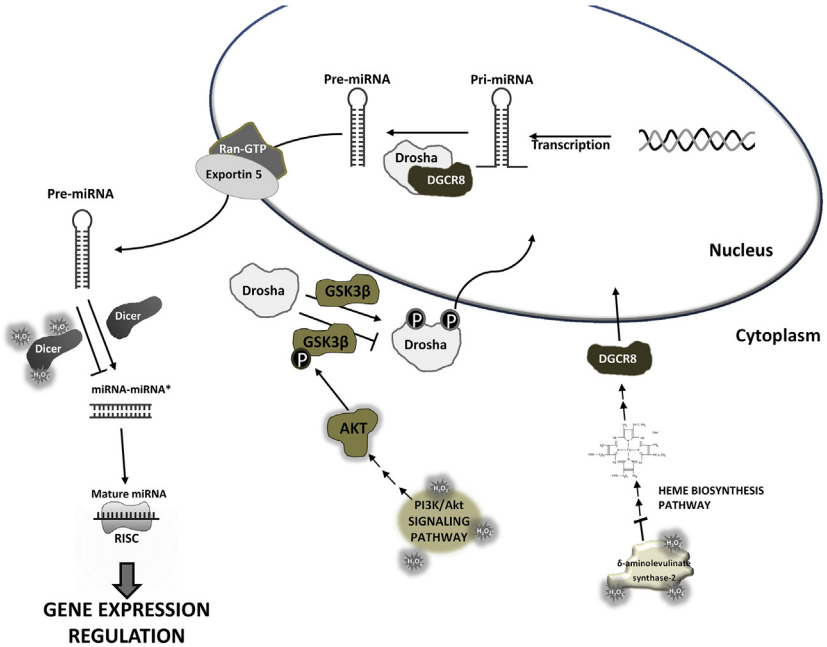

FIGURE 10.2 Modulation of microRNA (miRNA) biogenesis by H_2O_2. Biogenesis of miRNA is down-regulated by H_2O_2 at critical steps of the pathway. Drosha nuclear translocation: phosphorylation (P) of Drosha by glycogen synthase kinase 3β (GSK3β), which is downstream of the PI3K−AKT pathway is regulated by H_2O_2. Biosynthesis of DiGeorge critical region-8 (DGCR8): binding of ferric heme enhances its activity and H_2O_2 by activating gene expression of heme oxygenase 1 may lead to increased degradation of heme, thus reducing its availability. H_2O_2 inhibits δ-aminolevulinate synthase-2, the first enzyme of the heme pathway, which consequently down-regulates heme synthesis. Finally, Dicer, a member of the ribonuclease III family, which creates small RNA duplexes with 2-nucleotide overhangs at their 3′ ends and phosphate groups at their 5′ ends, is down-regulated by H_2O_2 (see text for references). *Pre-microRNA*, precursor miRNA; *Pri-microRNA*, primary miRNA; *RISC*, RNA-induced silencing complex.

10.3.5.3 Regulation of Specific MicroRNAS by Hydrogen Peroxide

In addition to a role as a general inhibitor of miRNA biogenesis, H_2O_2 is also responsible for regulating particular miRNAs that regulate specific cellular responses (Table 10.1). This suggests that H_2O_2 mediates several steps of miRNA processing and that miRNAs might be important downstream effectors of the redox signaling events. The mechanism by which H_2O_2 regulates the expression of specific miRNA is still largely unknown. Table 10.1 shows the H_2O_2-regulated miRNA for which the H_2O_2 dose and the recipient cell type are known. These data suggest that cell type, H_2O_2 concentration, and exposure time are key factors in regulating miRNA expression and might account for the differences observed in the H_2O_2-dependent regulation of particular miRNAs. Nevertheless, some miRNAs such as miRNA-1, miRNA-21, and miRNA-200c, were similarly regulated by H_2O_2 despite the different cellular contexts and H_2O_2 doses.

Concerning miRNA-1, it is involved in myocyte progenitor determination during development [264,265] and is up-regulated in patients with coronary artery disease [266].

Table 10.1 Hydrogen Peroxide Regulates microRNA Expression

miRNA	Target	Hydrogen Peroxide	MicroRNA (miRNA) Effect	Cell Type	References
miRNA-1	Insulin-like growth factor-1 (untranslated region)	Up-regulated 200 μM (16 h)	Proapoptotic	Induced pluripotent cells (induced from normal human foreskin fibroblast, SCRC-1041)	250
		Up-regulated 100–400 μM (6 h)	Proapoptotic (by Akt phosphoinhibition)	Cardiomyoblasts	251
miRNA-145	Aortic expression of contractile proteins (e.g., myocardin)	Up-regulated 300 μM (12 h)		Aortic smooth muscle cells	252
	BCL2/adenovirus E1B 19-KD protein-interacting protein 3	Down-regulated 50 μM (0.5–8 h)	Antiapoptotic	Cardiomyocytes (neonatal rat ventricle myocytes)	253
miR-466h-5p		Up-regulated 1–5 mM	Proapoptotic role	B/CMBA.Ov (mouse cells)	254
miRNA-30b	Catalase	Up-regulated 200 μM	Retinal pigmented epithelial oxidative stress	ARPE-19	206
miRNA-34a	sirt1	Up-regulated 200–500 μM	Proapoptotic role	Rat cardiomyocytes	255
miRNA-302	ARID4a and CCL5	Down-regulation 200 μM	Progression to s-phase (cell cycle)	AG01522 normal human fibroblasts	256
miRNA-21	Maspin and programmed cell death 4	Up-regulated depending on p47phox expression	Protumorigenic effect	Prostate cancer (PC-3M-MM2)	257
	?Unknown target	Up-regulation 800 μM (1 h)		RAW 264.7 (macrophages)	258
miRNA-21, let-7 family (and others array)	Programmed cell death 4	Up-regulated 25–200 μM (6 h)		Rat aortic smooth muscle cells (primary cultures)	249

Continued

Table 10.1 Hydrogen Peroxide Regulates microRNA Expression—cont'd

miRNA	Target	Hydrogen Peroxide	MicroRNA (miRNA) Effect	Cell Type	References
miR-27a, miR27b, miR-29b, miR-24-2,	?, Unknown target	Down-regulation 800 μM (1 h)	Proinflammatory	RAW 264.7 (macrophages)	258
miRNA-200c (and others s miR-200 family miRNA- 200a 200b, 141, 429)	ZEB1	Up-regulated 200 μM	Proapoptotic role	Myoblast c2c12 human umbilical vein endothelial cells (HUVEC)	259
miR-9, miR-200c, miR-708, miR-377, miR-26b, miR-296, miR-369, miR-32, miR-1956, miR-1190, miR-135b and miR-201		Up-regulated 200 μM (6 h)		Primary hippocampal neuron cultures from SAMR1 mice	260
mir-291a, miR-190b, miR-297c, miR-713 and miR-470		Down-regulated 200 μM (6 h)		Primary hippocampal neuron cultures from SAMR1 mice	261
miR-126		Down-regulated 50–200 μM	Increase survival	HUVEC line CRL-1730	262
miR-214		Up-regulated 30–100 μM (6 h)	Proapoptotic role	Primary neonatal rat cardiac ventricular myocytes	263
miR-92a	SIRT1, KLF2, and KLF4	Up-regulated	Proinflammatory	Cultured endothelial cells	264

In the presence of H_2O_2 (100–400 μM), miRNA-1 is up-regulated in both iPS cells and cardiomyoblasts, promoting apoptosis and decreasing insulin-like growth factor-1 (IGF-1) signaling activation [250]. The promoter region of miR-1 contains two potential binding sites for Foxo3 that regulate the expression of this miR in myocytes (C2C12) and neonatal cardiomyocytes [267]. Foxo3 is a transcription factor that mediates the oxidative stress response and might be involved in H_2O_2-dependent up-regulation of miR-1. IGF-1 and the IGF-1 receptor, which are key regulators of myocyte function and survival, were identified as targets of miRNA-1, which indicates a potential mechanism for H_2O_2-induced apoptosis [267,251].

miR-21 is known as an oncomiR because its overexpression in multiple human cancers targets tumor-supressor genes expression [268]. miR-21 is up-regulated in several cell types by extracellular H_2O_2 (25–800 μM) or indirectly by NADPH oxidase (NOX) overexpression. Increased expression of miR-21 in prostate cancer cells depends on the high levels of ROS produced by NOX; the activation of Akt is important for this induction [257]. Besides cancer, miR-21 is also involved in cardiac disease because it increases the ERK–MAP kinase activation pathway in cardiac fibroblasts by targeting SPRY1, a potent inhibitor of the Ras–MEK–ERK pathway [55].

miR-200c is up-regulated by H_2O_2 (200 μM) in myoblasts (C2C12), endothelial cells (HUVEC), and primary hippocampal neurons [259]. The miR-200 family targets ZEB1 and ZEB2, which are transcriptional repressors of E-cadherin promoting the mesenchymal-to-epithelial transition [269]. In endothelial cells, the down-regulation of ZEB1 owing to oxidative stress also increases apoptosis and senescence [259].

H_2O_2, among other stress stimuli, was shown to up-regulate miR-92 expression via Sterol regulatory element-binding protein 2n (SREBP2) transactivation in endothelial cells [263]. As a consequence of this activation, the expression of antiinflammatory genes, including SIRT1, KLF2, and KLF4 decreases, resulting in increased inflammasome and impaired eNOS-NO bioavailability [263]. Thus, oxidative stress–induced miR-92a increases endothelial innate immunity and the SREBP2-miR-92a–inflammasome may be a crucial pathway linking oxidative stress, inflammation, and endothelial dysfunction.

ROS, and H_2O_2 in particular, are produced by macrophages for microbial killing but also have an important effect in regulating macrophage activity in inflammation and antigen presentation [270]. The regulation of miR expression is an important mechanism by which H_2O_2 fine-tunes macrophage function [237]. This is the case of miR27b, which is down-regulated in the presence of H_2O_2, leading to an increase in NF-κB signaling activation [258].

These last examples clearly show that miRNAs can promote cross-talk between oxidative stress and immune functions, which is further explored in the next section.

10.3.6 MicroRNAs in the Crossroad Between Immune Functions and Oxidative Stress

Here we focus on the role of miRNAs as regulators of oxidative stress and immune responses and how misregulation of these mechanisms can cause disease. The correlation

between inflammatory and immune cells (such as mast cells, neutrophils, leukocytes, macrophages, monocytes, eosinophils, dendritic cells, phagocytes, and natural killer cells) and oxidative stress is well known. The generation of ROS, including H_2O_2, occurs in persistently inflamed tissues and also in cardiovascular disease and aging [271]. In extreme cases these can cause DNA damage and contribute to carcinogenesis by activating oncogenes and/or inactivating tumor suppressor genes [272]. The loss-of-function (inactivation) of tumor suppressor genes and the gain-of-function (activation) of oncogenes often cooperate to induce inflammation-driven carcinogenesis [273]. Next we will address the action of specific miRNAs on molecules involved in oxidative stress and immune dysfunction.

10.3.6.1 Toll-Like Receptor Pathways and Reactive Oxygen Species Activation in Inflammatory Responses

TLRs are an evolutionarily conserved receptor family that regulate antimicrobial host defense in plants, invertebrates, and mammals [271]. They recognize bacterial compounds and activate intracellular signaling pathways resulting in inflammatory responses. There is evidence that TLRs such as TLR-4 can mediate ROS activation of NF-κB. It is known that, on the one hand, H_2O_2 can trigger nuclear translocation of NF-κB, and on the other hand, agents that stimulate NF-κB activation increase the generation of intracellular ROS [274]. In agreement with this, several antioxidants prevent NF-κB nuclear translocation and activation, which suggests that NF-κB activation is induced when intracellular ROS generation is increased (Fig. 10.3).

A study by Asehnoune et al. [275] addressed the mechanisms by which ROS modulate the transcriptional activity of NF-κB in response to TLR-4–dependent signaling. Antioxidant treatment inhibited LPS-stimulated production of inflammatory cytokines and activation of the kinases (IKK-a and IKK-b) and IRAKs in neutrophiles. These results indicated that TRL-4–mediated signaling is oxidant-dependent and that ROS can modulate NF-κB–dependent transcription through their involvement in early TLR-4–mediated cellular responses [275]. The specific role of miRNAs in ROS production in the context of TLR activation is not known. However, several miRNAs are involved in the negative regulation of innate immune and inflammatory responses, which target genes involved in TLR pathways (Fig. 10.3) [276,190]. It has been reported that the TLR-4 ligand, LPS, increased the expression of miR-146a, miR-155, and miR-132 in monocytes [194]. Ligands for TLR2, TLR-4, and TLR-5 also induce miR-146a/b expression. Importantly, TRAF6 and IRAK-1, which are the key molecules in TLR-mediated NF-κB activation, are miR-146 targets [194], which suggests that miR-146a may function as a negative feedback regulator of TLR-mediated signaling in innate immune and inflammatory responses (Fig. 10.3).

miR-155 expression can also be stimulated by ligands for TLR3 and TRL4 and also cytokines such as IFN-beta and IFN-gamma via TNF-α autocrine signaling [277]. miR-155 targets FADD, IKK-, and receptor-interacting serine/threonine-protein kinase-1 mRNA [278], which suggests that miR-155 is a component of the innate immune response to inflammatory stimulators that enhances TNF-α translation.

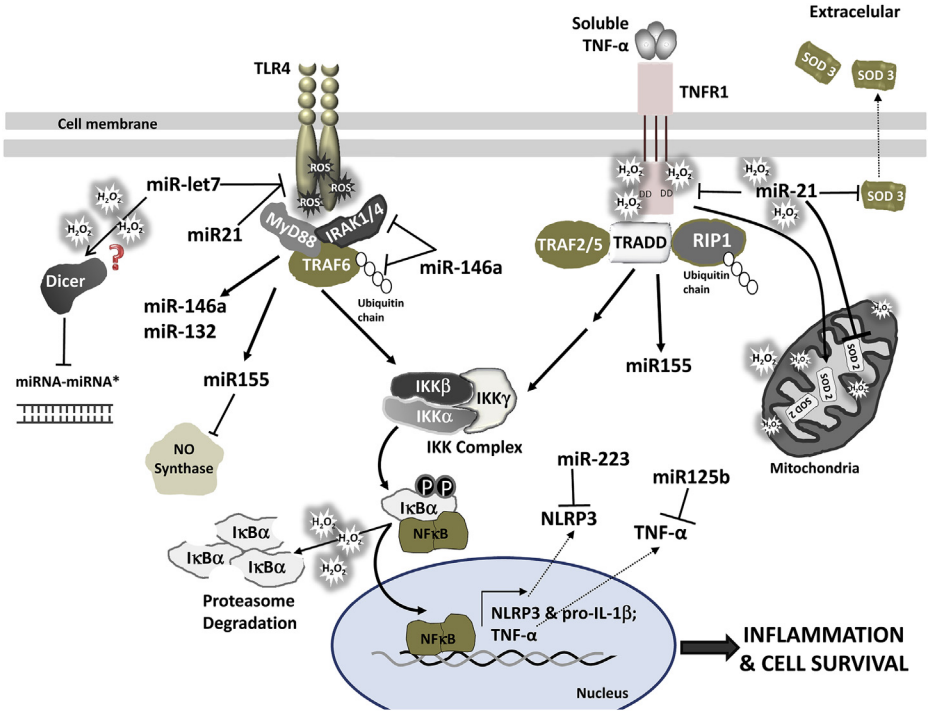

FIGURE 10.3 Complex regulatory role of miRNAs in the network of innate immune and inflammatory responses mediated by tumor necrosis factor receptors (TNFR) and Toll-like receptors (TLR) involving the nuclear factor κB (NF-κB) activation pathways and reactive oxygen species (ROS) signaling. TLR and TNFR can mediate ROS activation of NF-κB. Activation of the TNFR or the TLR-4 receptors induces the assembly of signaling complexes that promote activation of the Iκα and Iκβ kinase (IKK) complex. These signaling complexes are composed of myeloid differentiation primary response gene 88 (MyD88), interleukin-1 receptor-associated kinase 1 and 4 (IRAK1/4) and TNFR-associated factor 6 (TRAF6) for TLR-4 receptor, and by TNFR type 1-associated DEATH domain protein (TRADD), receptor-interacting serine/threonine-protein kinase 1 (RIP1), and TNFR-associated factors 2 and 5 (TRAF 2/5) for TNFR. Assembly of these complexes results in the phosphorylation (*black/white P*) and subsequent degradation by the proteasome of Iκβ and in the NF-κB translocation into the nucleus, where it activates the target genes. The recruitment and activation of the IKK complex depend on polyubiquitination of RIP1 and TRAF6, respectively. These signaling pathways are regulated by miRNAs. The TLR-4 ligand, lipopolysaccharide, increases the expression of miR-146a, miR-155, and miR-132. miR-155 expression can also be stimulated by cytokines such as interferon (IFN)-β and IFN-γ via TNF-α autocrine signaling. TRAF6 and IRAK-1 are miR-146 targets, which suggests that miR-146 may function as a negative feedback regulator of TLR-mediated signaling in innate immune and inflammatory responses. miR-125b inhibits TNF-α production, whereas NLRP3 is negatively regulated by miR-223. miR-let7 and miR-21 target TLR-4 mRNA at the posttranscriptional level. Many of these miRNAs are also involved in the cellular redox state, such as miR-21, which targets mRNAs of mitochondrial superoxide dismutase (SOD2) and extracellular superoxide dismutase 3 (SOD3), and in turn is up-regulated in several cell types by extracellular H_2O_2 or indirectly by NADPH oxidase (NOX) overexpression. miR-155 that down-regulates endothelial nitric oxide synthase (NO Synthase). It has been suggested that Dicer inhibition by H_2O_2 is mediated by the let-7 family of miRNAs that are known to suppress the role of Dicer and are up-regulated by H_2O_2 (see text for references).

LPS, the TLR-4 ligand, was shown to repress miR-125b expression. Because this miRNA targets TNF-α, its suppression leads to increased production of TNF-α during macrophage inflammatory responses [278]. TLR-4 itself can be a target of another miRNA: miR-let-7. In fact, it was observed that overexpression of miR-let-7 significantly decreased TLR-4 expression, and stimulation with *Cryptosporidium parvum* decreased miR-let-7 in a MyD88/NF-κB -dependent manner, while increasing expression of TLR-4 [279].

Upon NF-κB activation by TLR-mediated ROS induction, several genes are up-regulated, including the Nod-like receptor protein NLRP3 and pro-IL-1β [280]. The protein NLRP3 has emerged as a central regulator in the inflammatory process and is implicated directly in hereditary cryopyrinopathies and indirectly in diseases such as gout, type 2 diabetes, and atherosclerosis. Upon activation, NLRP3 forms a so-called inflammasome complex with the adaptor molecule ASC, which controls the activation of caspase-1, the enzyme that processes the immature form of the proinflammatory cytokine IL-1β into the active protein. An alternative priming mechanism has been put forward that results in the posttranslational activation of NLRP3 by deubiquitination and requires TLR-induced ROS [281]. Importantly, NLRP3 regulation seems to depend on a particular miRNA, miR-223, which has been identified as a negative regulator of NLRP3 [282,283] (Fig. 10.3). Interestingly, miR-223−deficient mice exhibit phenotypes consistent with deregulated NLRP3, such as neutrophilia, spontaneous lung inflammation, and increased susceptibility to endotoxin challenge [284]. ROS can induce TLRs, which in turn activate downstream molecules such as NF-κB or NLRP3, involved in inflammatory processes that can themselves be subject to regulation by miRNAs.

10.3.6.2 Reactive Oxygen Species, Aging, and Immune Senescence

The immune system is essential to combat pathogenic stress. Age-associated immune dysfunction is also known as “immune senescence” and results from cooperative as well as antagonistic changes in multiple pathways. The mechanisms that mostly contribute to senescence are thymic involution, oxidative stress, proteostasis, telomere attrition, DNA damage signaling, epigenetic alterations, increased inflammation, and transcriptional alterations.

Regarding oxidative stress, it remains unclear whether increased ROS levels, for example, are the cause or consequence of aging. However it is becoming increasingly clear that not all ROS are deleterious. For example, data show that hematopoietic stem cells and early progenitors contain lower levels of ROS than their more differentiated progeny and that these may be critical in maintaining their stem cell potential [285].

Regarding ROS action, it appears that at small levels of H_2O_2, the main ROS present *in vivo*, and also O_2^-, have a key role in cellular signaling whereas at higher ROS levels deleterious effects occur [231,286,287].

Specifically in T cells, redox unbalance creating oxidative stress have a significant role in the induction of lowered T-cell responsiveness. This ROS-mediated response is observed in both aging and chronic pathologic conditions [288]. Inhibition of T-cell

proliferative response and activation has also been observed as a consequence of oxidative stress-mediated depletion of intracellular stores of glutathione [289,290]. In addition, redox conditions strongly influence $CD4^+$ T-helper cell polarization, skewing them toward a more Th2 type than a Th1 type, similar to that observed during aging [291].

In the context of immune senescence there are some reports of miRNA-mediated regulation of the generation of memory cells, inflammatory gene inductions, chromatin remodeling, and MHC expression implicating a role of miRNA in regulating immune decline in aging [292,293]. Also, the miR-17−92 cluster (further discussed subsequently) has been implicated in aging in various human cell types, including $CD8^+$ T-cell subtypes [294].

It still remains unclear which specific miRNAs regulate the various pathways, including those regulating ROS signaling and aging, to understand better how senescence is regulated at the immune system level.

10.3.6.3 Reactive Oxygen Species, Inflammation, and Cancer

As referred to earlier, immune cells present in inflamed tissue can generate ROS, which ultimately may lead to DNA damage and can contribute to carcinogenesis. Indeed, loss-of-function mutations of tumor suppressor genes and the gain-of-function of oncogenes often cooperate to induce inflammation-driven carcinogenesis [273]. Under oxidative stress conditions, caused by accumulation of ROS during chronic inflammation, for example, DNA strand brakes, base modifications, or illicit cross-links can occur. These can lead to replication errors and the subsequent genomic instability might be associated with tumor initiation [295,296]. Also, chronic exposure to UV radiation can lead to inflammatory tissue damage and ROS generation [297], which might cause *Ras* oncogene activation and p53 tumor suppressor gene inactivation, thus contributing to skin carcinogenesis [298]. Thus, chronic inflammation can lead to the generation of cellular ROS; or oxidative stress resulting from an imbalanced cellular redox regulation or exposure to environmental stress can induce tissue inflammation locally, both of which contribute to carcinogenesis.

Although there is no direct evidence of miRNAs linking ROS production, inflammation, and cancer, several miRNAs were shown to have significant roles in inflammation-associated carcinogenesis [299]. For example, when the proinflammatory cytokine IL-6 is stably overexpressed it causes induction of miR-let-7a, causing the constitutive phosphorylation of STAT3 in malignant cholangiocytes [300]. By contrast, IL-6 overexpression in malignant cholangiocytes repressed the expression of miR-370, miR-148, and miR-152, leading to induction of DNMT-1 and subsequent inactivation of methylation-sensitive tumor suppressor genes such as Rassf1a and p16INK4a [300,301]. Thus, by activating STAT3, IL-6 serves as a bridge between inflammation and cancer. Interestingly, miR-155, which is overexpressed in many human cancers, is inversely correlated at the expression level with SOCS1, an endogenous inhibitor of the IL-6-STAT3 signaling pathway, in human breast cells [302]. In fact, when miR-155 is overexpressed in breast cancer cells, the JAK-STAT3 signaling pathway is activated, an effect similar to the incubation of these cells

with IL-6 or LPS [302]. Furthermore, diffuse large B-cell lymphomas displayed increased levels of miR-155, which correlated with down-regulation of the TNF-α–induced expression of the inositol phosphatase SHIP1. Treatment with TNF-α antagonists such as infliximab diminished miR-155 expression, restoring the level of SHIP1 and reducing cell proliferation [303].

It appears that several miRNAs that are regulated by cytokines can function as a powerful link between inflammation and cancer. Given that some cytokines can modulate enzymes involved in the production of ROS (e.g., TNF-α can decrease eNOS expression) [212], it is possible that miRNAs controlling the production of these cytokines in a tumor context might alter the oxidative status of a tumor microenvironment.

Overall, these small ncRNAs, the miRNAs, have been increasingly implicated in several human disorders and are starting to be used as biomarkers in several immune and stress-related dysfunctions. Increasing knowledge of their regulation, mechanism of action, and targets will benefit their use as therapeutic agents in clinical settings, as further discussed in Section 10.5.

10.4 Long Noncoding RNAs: How Far Are Their Strategies to Regulate Gene Expression?

In past years we have seen the discovery of a variety of functions for lncRNAs covering almost all aspects of gene expression regulation ranging from epigenetic to translational, including transcriptional and posttranscriptional control. Although the illustrative examples are growing throughout the phylogenetic tree, the molecular mechanisms underlying these functions are still poorly understood. However, it is assumed that through their ability to pair with nucleic acids, and owing to their complex secondary structures, these molecules are able to fine-tune regulatory proteins to target gene promoter regions, GpC regulatory islands, mRNAs, and other proteins, creating scaffolds for interactions.

10.4.1 Chromatin Remodeling Complexes and Epigenetics

The lncRNA "*Xist*" was one of the first examples showing that in nuclei, lncRNAs are involved in chromatin modulation by recruiting histone modifiers and chromatin remodeling complexes [26]. *Xist* acts in *cis* and promotes chromosome X inactivation by forming transcriptionally inactive heterochromatin. This lncRNA recruits and binds the Polycomb repressive complex 2 (PRC2) together with H2K27 to the inactive X chromosome [304]. This regulation is complex because not only do independent PRC2 pathways seem to occur [7] but *Xist* is also positively and negatively regulated by other lncRNAs such as *Tsix*. *Tsix* inhibits *Xist* transcription by promoting epigenetic alterations in *Xist* promoter [305]. Other examples of lncRNAs, such as Air, Kcnq1ot1, Mira, and HOTAIR [306–310], which are able to induce chromatin imprinting by recruiting chromatin remodeling complexes, have been described. These new examples showed that lncRNAs

regulate gene expression by acting in *cis* and/or *trans*, which means that they are able to repress or induce gene expression that are near their transcription sites and/or at independent loci, respectively. For example, the histone H3K4 methyltransferase MLL1 complex is recruited by the lncRNA (Mistral-Mira) and the interaction of MLL1 with chromatin-associated Mira triggers dynamic changes in chromosome conformation that mediate activation of *Hoxa6* and *Hoxa7* transcription, which culminates in transcriptional activation of genes involved stem cell differentiation [308,309]. Another example is that of HOTAIR, a *trans*-acting lncRNA that serves as a scaffold for two histone modification complexes binding to PRC2 and to LSD1 (in complex with CoREST/REST). This lncRNA coordinates the targeting of PRC2 and LSD1 to chromatin for coupled histone H3 at lysine 27 methylation and lysine 4 demethylation, leading to subsequent gene silencing [310]. Transcription of the lncRNA ANRIL is induced by transcription factor E2F1 in an ataxia-telangiectasia mutated−dependent manner after DNA damage. The high levels of ANRIL prevent the expression of INK4a, INK4b, and ARF tumor suppressor genes at the end of DNA damage response [311]. Also, the chromobox 7 (CBX7) of PRC1 binds to ANRIL, and both CBX7 and ANRIL repress the INK4b/ARF/INK4a locus and control senescence by H3K27 methylation [312].

The involvement of lncRNAs as a chromatin modulator in organ development is illustrated by the role of two lncRNAs involved in mouse cardiac development designated by braveheart (*Bvht*; also known as *Gm20748*) [313]) and *Foxf1* adjacent noncoding developmental regulatory RNA (*Fendrr*) [314]. Both *Bvht and Fendrr* interact with critical factors required for epigenetic regulation, namely, PRC2 and Trithorax protein group−MLL1 complexes; this strongly supports the idea that they are involved in cardiac commitment and correct heart development, respectively [313,314].

The use of co-immunoprecipitation and RNAi assays revealed that other members of the class of long intergenic noncoding RNAs (lincRNAs), that range in size from about 300 nt to several thousands of nucleotides, associated with chromatin-modifying complexes to specific genomic loci regulating gene expression [315]. Moreover, the ncRNA promoter-associated RNA (pRNA) is complementary to the rDNA promoter and mediates *de novo* CpG methylation of rRNA genes (rDNA). In fact, pRNA interacts with the target site of the transcription factor TTF-I, originating a DNA−RNA triplex that is specifically recognized by the DNA methyltransferase DNMT3b [316].

10.4.2 Long Noncoding RNA Structural Labels and Partners of Transcription Factors

Besides their ability to bind and recruit complexes involved in chromatin remodeling, lncRNAs are able to regulate gene-encoding proteins by interacting with their promoter regions (pRNAs). This is based on their capacity to base pair with DNA, probably creating local recognizable structures, “labels,” that are able to recruit, allow recognition, and promote interaction with transcription regulatory proteins. lncRNAs may also modulate transcription factors and/or establish bridges between them and co-regulators working

as scaffolds. For example, the lncRNA *PANDA* is transcribed from the *CDKN1A* gene promoter (encoding a cell-cycle inhibitor) and is induced by DNA damage in a p53-dependent manner [317]. Interestingly, *PANDA* interacts with the transcription factor NF-YA, inhibiting its ability to up-regulate proapoptotic genes. Therefore, whereas *CDKN1A* mediates cell-cycle arrest, *PANDA* promotes cell survival by inhibiting the induction of apoptotic genes. There is also evidence that this link is exploited by tumor cells. The regulatory network of lncRNAs in response to DNA damage and involving p53 seems to be complex. In fact, Huarte et al. [318] described an lncRNA named lincRNA-p21 that is transcribed from a locus 15 kb upstream of *CDKN1A* that is induced by p53 and mediates p53-dependent gene repression. Therefore, the upstream regulatory region of the *CDKN1A* gene regulates expression of multiple coding and noncoding transcripts involved in the response to DNA damage. Another example comes from the studies of Zhu et al. that showed that the lncRNA ANCR, required to maintain the undifferentiated cell state within the epidermis, is able to associate with the enhancer of zeste homolog 2. This association results in inhibition of the expression of the transcription factor Runx2, which is required for osteoblast differentiation [319].

Activation of gene transcription through cross-talks between lncRNAs and transcription factors is also mediated by the ncRNA Evf-2 that forms a stable complex *in vivo* with the homeobox transcription factor Dlx2, activating its activity and with an important role in forebrain development and neurogenesis [320]. This type of interaction is also relevant in the role of several lncRNAs associated with pluripotency or specific differentiation events [321]. In fact, some lncRNAs are induced upon the reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) [322]. Illustrating this is the case of the lincRNA -RoR (Regulator of Reprogramming), which is regularly enriched in human iPSCs regardless of their origins and regulates the reprogramming that leads to pluripotent stem cells. [322]. Many of these ncRNAs are coexpressed with transcription factors known to be involved in regulating pluripotency. Indeed, experimental data show that lincRNAs are direct regulatory targets of several pluripotency-associated transcription factors (including Oct4, Sox2, Nanog, cMyc, nMyc, Klf4, Zfx, Smad, and Tcf3) [323]. The depletion of these lncRNAs from the cells, by performing loss-of-function assays, has consequences for gene expression that are comparable to the knocking down of embryonic stem cell regulators [323]. These authors observed that the knockdown of many lincRNAs causes the exit from the pluripotent state or, alternatively, the up-regulation of pathways of commitment programs [323].

Despite available experimental data, the full scenario of how lncRNAs regulate transcription at the promoter level is far from being complete because many pieces of information are still missing from the puzzle. For example, experimental data show that enhancers can also be transcribed originating enhancer-noncoding transcripts (eRNAs) that probably have regulatory roles [324,325]. Moreover, long (antisense) pRNAs have the potential to form double-stranded molecules that can be processed into endo-siRNAs. These endo-siRNAs are able to be pair to the promoters, inducing transcriptional gene silencing [326–328] or activation [175,176,329] in a way similar to short pRNAs [22].

The regulatory activity of lncRNAs is not limited to transcriptional factors and co-regulators but also extends to the general transcription factors and to polymerase II. For example, a lncRNA that is transcribed from an upstream minor promoter of the dihydrofolate reductase (DHFR) gene mediates the regulation of basal transcription by forming a stable RNA–DNA triplex within the major promoter of the DHFR gene, preventing the binding of TFIIB, a component of the transcription initiation complex [330].

10.4.3 Long Noncoding RNAs: Linking Chromatin Remodeling and Transcription Regulation

lncRNAs are also able to combine regulatory mechanisms coordinating the activity of transcription factors, co-regulators, and chromatin remodeling complexes. The RNA-binding protein TLS is a critical transcriptional regulatory sensor of DNA damage signals that is allosterically regulated by RNA binding. Ligation of the protein to RNA causes the shift from an inactive to an active conformation that in turn allows the binding and inhibition of the CBP/p300 histone acetyltransferase activities on the repressed cyclin D1 (*CCND1*) gene [171]. Recruitment of TLS to the promoter of cyclin D1 is directed by low–copy number $NcRNA_{CCND1}$ transcripts that therefore negatively regulate *CCND1* gene transcription. In the central nervous system, where lncRNAs are considerable abundant, they have been associated with neurogenesis and the evolution of the complexity of brain. For example, a microarray analysis for lncRNAs in human embryonic cells (hESCs) differentiating into neurons showed that some hESC-specific lncRNAs were involved in pluripotency maintenance [331]. Knockdown studies showed that depletion of these lncRNAs inhibited neurogenesis, and immunoprecipitation studies revealed interaction with the transcription factor SOX2 and the SUZ12 component of the PRC2 in undifferentiated hESCs [331].

10.4.4 Long Noncoding RNAs and Alternative Splicing

Several examples also illustrate that even alternative splicing is a regulatory step under the control of lncRNAs. Indeed it was shown that long noncoding antisense RNAs may base pair with their sense RNAs, an event that will hide the splice sites and have an impact on which splice variant will be produced. In fact, the antisense transcript RevErbAα regulates the thyroid hormone receptor α gene (TRα) splicing controlling the balance between the TRα1 and TRα2 mRNA levels [332]. lncRNAs with snoRNA (Small nucleolar RNA) ends corresponding to positions of intronic snoRNA were also described [333]. In fact, during exonucleolytic trimming, the sequences between the snoRNAs are not degraded, leading to the accumulation of lncRNAs flanked by snoRNA sequences but lacking 5′ caps and 3′ poly(A) tails. These sno-lncRNAs associate strongly with Fox family splicing regulators and produce changes in splicing patterns. Furthermore, these sno-lncRNAs map to a genomic region that is deleted in patients with Prader–Willi syndrome, which strongly suggests an association of these sno-lncRNAs with the disease [333]. Supporting these observations are data comparing transcriptomes of human, rhesus, and mouse that demonstrating that

the expression of sno-lncRNAs is species-specific and that their processing is closely linked to alternative splicing of their parent genes [334].

lncRNA interact with multiple proteins including members of the importin-β superfamily, and regulate the nuclear trafficking nuclear factor of activated T cells [335]. The abundant 4 kb ncRNA, named nuclear-enriched autosomal transcript 1 (NEAT1), localizes at "paraspeckles" [336] and contributes to the formation of these dynamic structures of the interchromatin space that are implicated in mRNA nuclear retaining [336]. Paraspeckles have been proposed to regulate transcription (for example, via the sequestration of key transcription factors) [337,338], control alternative splicing patterns [339], and modulate the DNA damage response [340].

10.4.5 Long Noncoding RNA Mediators of Messenger RNA Stability and Translation

By base pairing with their sense messenger RNAs (mRNAs), long noncoding antisense RNAs are also able to control the stability of their counterpart mRNA, creating a scaffold to recruit proteins involved in RNA degradation, such as Staufen 1 (STAU1), a protein that binds to double-stranded mRNA and induces its decay [341]. In the case of the 3.7 kb lncRNA, terminal differentiation-induced ncRNA (TINCR), it directly binds to the STAU1 protein creating the TINCR–STAU1 complex that mediates the stabilization of epidermis differentiation mRNAs, such as keratin 80. TINCR is required for high levels of key differentiation genes, many of which are mutated in human skin diseases, including *FLG*, *LOR*, *ALOXE3*, *ALOX12B*, *ABCA12*, *CASP14*, and *ELOVL3* [342]. TINCR–mRNA interaction occurs through a 25-nt "TINCR box" motif that is strongly enriched in the interacting mRNAs [342]. Another example is that of the lncRNA named β-site APP-cleaving enzyme 1-antisense (BACE1-AS) that base pairs with the mRNA (BACE1) encoding the β-secretase an enzyme responsible for β-amyloid production, stabilizing it [343]. These examples show that the role of lncRNAs in regulating mRNA stability is also complex because they have the ability to increase or decrease the stability of the target coding RNAs.

Formation of the sense–antisense duplex complexes may also be involved in regulating protein synthesis by regulating translation, as in the case of PU.1 mRNA that encodes the transcription factor PU.1, an important regulator of hematopoiesis. In this case the translation of PU.1 is negatively regulated by the noncoding antisense polyadenylated transcript that exists at low levels but with a longer half-life than that of the sense PU.1 transcript [344]. The suppressor tumor protein p53 pathway is also negatively regulated by the lncRNA co-repressor 1 (Trp53cor1; also known as lincRNA-p21) [345]. The lincRNA-p21 associates with the ubiquitous ribonucleioprotein HuR that regulates cell proliferation, survival, carcinogenesis, and the stress and immune responses. HuR performs these functions mainly by associating with subsets of mRNAs and by increasing their stability and/or modulating their translation [346,347]. In HeLa cells, low HuR levels cause lincRNA-p21 accumulation that increases its association with *JUNB*

and *CTNNB1* mRNAs and causes the inhibition of their translation. High HuR levels decreased lincRNA-p21 levels, which in turn derepressed JunB and β-catenin translation, increasing the levels of these proteins [345]. Similarly, the lncRNA urothelial carcinoma-associated 1 (UCA1) interacts with hnRNP I, forming a functional ribonucleoprotein complex that increases UCA1 stability. Interestingly, hnRNP I enhances the translation of p27 by interacting with the 5′-UTR of p27 mRNAs, an event that is competitively inhibited by the presence of UCA1. This observation correlates with the fact that UCA1 has an oncogenic role in breast cancer [348].

Another mechanism that most probably underlies lncRNA's roles regulating mRNA stability is based on its potential to form siRNAs that will be involved in mRNA decay. This mechanism involves the formation of a sense−antisense pair of transcripts able to pair up and then be processed into siRNAs. These pairs can be created directly from the same loci (*cis*-NATs) or from different loci (*trans*-NATs). Interestingly, in rice a small number of pseudogenes are transcribed originating antisense transcripts that after pairing with the coding gene or a paralogous pseudogene transcript are processed into siRNAs [349]. Similarly, in mammalians pseudogene transcripts can be processed into siRNAs with the capacity to suppress gene expression in mouse oocytes [350,141]. Consequently, NATs may regulate gene expression through a mechanism similar to that of miRNAs and siRNAs, a hypothesis supported by several examples. During spermatogenesis, a complex process that is accompanied by microtubule cytoskeleton remodeling, it was observed that the gene encoding TBCA, a protein that binds to β-tubulin and is involved in the folding and dimerization of new tubulin heterodimers (the building blocks of microtubules), is regulated by a *Tbca* pseudogene that is transcribed in both directions [351]. During testis maturation, *Tbca* pseudogene levels decrease, whereas those of the *Tbca* mRNA progressively increase, which suggests that this *Tbca* lncRNA is required to maintain the undifferentiated state of spermatids. Another example comes from the regulation of the gene codifying for nitric oxide synthase (NOS2A) that is regulated by its nonsense counterpart. Similarly to the pair anti-*Tbca*/*Tbca*, anti-NOS2A RNA and the NOS2A mRNA have reverse patterns of expression in undifferentiated hESCs and in hESCs differentiating into neurogenic precursors [158].

Interestingly, NATs may be interlinked with miRNAs and siRNAs through the regulatory molecular mechanisms involved in gene expression regulation but they may also be components of the regulatory networks that control miRNAs and siRNA, creating a multilayer regulatory system. For example, lncRNAs may compete with coding mRNAs for specific miRNAs, binding and sequestering them acting as "miRNA sponges" [352]. Thus these lncRNAs have been designated competing endogenous RNAs (ceRNAs). In myogenesis lncRNA, muscle differentiation 1 (linc-MD1) was one of the first lncRNAs to be identified [353] but more have already been described [7]. Indeed, linc-MD1 regulates the time of muscle differentiation by acting as a ceRNA in mouse and human myoblasts [353]. linc-MD1 seems to operate by acting as a "sponge" of miR-133, regulating the transcription factors MAML1 and MEF2C involved in activating muscle-specific gene expression. Interestingly, linc-MD1 levels are strongly reduced in muscle cells of patients

with Duchenne muscular dystrophy [353]. In hESC self-renewal, lincRNA-RoR regulates a core of pluripotency transcription factors (Oct4, Nanog, and Sox2) by linking them to the regulatory network of miRNAs by behaving as an endogenous miRNA sponge [354].

These various examples clearly show that lncRNAs display a vast repertoire of strategies to regulate protein-encoding genes. A central role of these molecules is their involvement in regulating gene expression during development by interacting with ubiquitous regulatory proteins to form cell type−specific RNA−protein complexes that coordinate cell type−specific gene expression programs. The number of examples of lncRNAs involved in regulating development and differentiation is continuously growing, which indicates that lncRNAs has a broad role in development. Examples such as lincRNA-EPS, which is involved in erythroid terminal differentiation [355], and lncRNAs, which participates in regulating adipogenesis, support this idea [356]. Also, as detailed in the next section, lncRNAs have been shown to have increasingly important roles in immune cell differentiation and function.

Interestingly, lncRNAs seem to be active, in general, in the cell undifferentiated states repressing target genes whose expression is critical for cell fate. In these processes they work as integrative molecules concentrating and distributing and/or combining signals among chromatin-modifying complexes, transcriptional, splicing and translational regulatory molecules, and also factors regulating mRNA transport and stability, therefore decreasing transduction noise.

10.4.6 Role of Long Noncoding RNAs in the Immune System

Although miRNAs are extremely abundant and regulate many aspects of immune cell differentiation and function (Section 10.3.2), lncRNAs, which, as mentioned earlier, have mainly been studied in the context of genomic imprinting, pluripotency, and cell differentiation control and chromosome dynamics, are emerging as important regulators of immune cell differentiation and activation. Interestingly, the expression profiles and molecular and biological functions of lncRNAs involved in all of these processes (including regulation of immune cell function) have been compiled and integrated in the NONCODE database (http://www.noncode.orghttp://www.noncode.org) that includes the first integrated collection of expression and functional lncRNA data obtained from reannotated microarray studies [357]. Several genome-wide studies have revealed that immune cells express large numbers of lncRNAs. For example, $CD8^+$ T cells express many hundreds of lncRNAs, 96 of which are specific to lymphocytes and are dynamically regulated during differentiation or activation (29 of transcripts were specific for $CD8^+$ T cells, 21 of the ncRNAs were significantly modulated during memory T-cell differentiation, 81 were during effector cell activation, and 4 were significantly regulated during both transitions) [358]. Interestingly, some of these overlap either with immunologically important protein-coding genes or with miRNAs and siRNAs, which suggests that proteins and different functional RNA species might regulate lncRNA functions.

Unique signatures of lncRNAs expression were also observed in response to severe acute respiratory virus or influenza virus [359], many of which were regulated downstream

of type I IFN signaling. Rhabdomyosarcoma cells are also reported to express lncRNAs when they are infected with enterovirus 71 [360]. $CD11c^+$ dendritic cells also express a large number of lnRNAs when stimulated with LPS, an activator of TLR-4 signaling [152]. The lnc-IL7R that overlaps with the 3′-UTR region of the human IL-7 receptor α-subunit gene (IL7R), is also involved in inflammatory response. This lncRNA was able to decrease the LPS-induced inflammatory response, as observed by the increased expression of LPS-induced E-selectin, VCAM-1, IL-6, and IL-8 in lnc-IL7R knockdown cells [361].

It was also shown that lncRNAs can be important regulators of LPS-induced innate immune response through TLR-4 signaling in bone marrow−derived macrophages [362]. As reviewed in Pagani et al., $CD4^+$ T cells are reported to express many lncRNAs [363].

The molecular functions of some of the lncRNAs expressed in these immunologic contexts are beginning to be explored. They were shown to be important to both adaptive and innate immunity. The next two examples refer to lncRNAs acting in acquired immunity. The lncRNA Theiler's Murine Encephalitis Virus Possible Gene1 (*Tmevpg1*) also named NEttoie Theiler's Pas Salmonella (*NeST*), initially proposed to control Theiler virus persistence in mice [364], is the first identified enhancer lncRNA of the immune system to regulate expression of a master cytokine such as IFN-gamma [364−366]. Located downstream from the IFNG gene and transcribed from the antisense strand relative to IFNG, *NeST* acts as an enhancer-like lncRNA by enhancing transcription of INF-gamma. Earlier studies demonstrated that transcription of *Tmevpg1*/*NeST* was Th1 selective and dependent on Stat4 and T-bet, transcription factors involved in Th1 cell differentiation [364]. *NeST* RNA binds WDR5, which alters histone 3 lysine 4 trimethylation at the IFN-gamma locus. Consequently, both IFN-gamma RNA and IFN-gamma protein levels are increased in activated $CD8^+$ T cells [365]. In Th2 cells, lincR-Ccr2-5′AS is transcribed in the direction opposite the chemokine Ccr2 and is located between Ccr2 and Ccr3 genes [367]. Silencing of lincR-Ccr2-5′AS leads to lower expression of the neighboring Ccr1, Ccr2, Ccr3, and Ccr5 genes. These chemokines are required for trafficking of Th2 cells to the lungs, and knockdown of lincR-Ccr2-5′ AS also reduced migration of Th2 cells to the lung.

lncRNAs also have relevant roles in innate immunity [368]. TNF-α was shown to regulate many lncRNA in murine fibroblasts, including 54 pseudogene lnRNAs, several of which exhibited a selective expression in response to specific cytokines and microbial components in an NF-κB−dependent manner. One of them is Lethe, which interacts with the NF-κB subunit RelA to inhibit RelA DNA binding and target gene activation [369]. Another lncRNA exerting its functions in innate cells is KIR, an antisense lncRNA expressed in progenitor or pluripotent cell lines that when overexpressed in NK cells causes decreased expression of the KIR protein-coding gene [370]. KIR antisense lncRNA overlaps with KIR-coding exons 1 and 2, as well as a proximal promoter upstream of KIR. Transcription of KIR antisense lncRNA appears to be regulated by myeloid zinc finger 1, which leads to silencing of KIR through an unknown mechanism. Also it was observed that during innate activation of THP1 macrophages, a group of 159 lincRNAs was

differentially expressed. In this group, linc1992/THRIL was shown to regulate the transcription of TNF-α gene by binding to its promoter by forming a complex with a heterogeneous nuclear ribonucleoprotein L. Moreover, expression of linc1992/THRIL was correlated with the severity of symptoms in patients with Kawasaki disease, an acute inflammatory disease of childhood [371].

HOTAIRM1 is an lncRNA specifically expressed in myeloid cells and appears to have an important role during granulocyte maturation [372]. It is antisense to the HOXA genes and is transcribed in NB4 promyelocytic leukemia cells. When these are activated by all-*trans* retinoic acid, which induces granulocyte maturation, HOTAIRM1 is required for the expression of HOXA1, HOXA4, CD11b, and CD18.

A study demonstrated that lincRNA-Cox2 is an lncRNA significantly induced upon TLR2 engagement by a synthetic bacterial lipoprotein (Pam3CSK4) [373]. Hundreds of immune genes were shown to be both positively and negatively regulated by lincRNA-Cox2 during an innate immune response. Many of the target genes are repressed (including chemokines, chemokine receptors, and IFN-stimulated genes) at the transcriptional level and are mediated by an RNP complex consisting of lincRNA-Cox2 and hnRNPA/B or hnRNPA2/B1.

Interestingly, NEAT has two isoforms, 3.7 kb NEAT1 and 23 kb NEAT2/MALAT-1, that do not share significant homology with each other but are conserved within the mammalian lineage, which suggests a functional role for these lncRNAs [374]. As mentioned, NEAT2 is a component of nuclear speckles [316]. Also, this variant was induced by a TLR3 ligand (polyI:C), infection with herpes simplex virus-1(HSV-1), and the measles virus (MV). Moreover, the paraspeckle protein, SFPQ, binds to the IL-8 promoter blocking its expression. In cells stimulated by PolyI:C or infected with HSV-1 and MV, this regulator SFPQ protein is displaced to the NEAT1 promoter, which allows formation of the paraspeckles and the activation of IL-8 [337].

This evidence strongly indicates that lncRNAs regulate several molecular functions in the immune system.

10.4.7 Long Noncoding RNAs as Sensors of Environmental Conditions: Role in Stress Response

It is possible to envisage a system in which environmental and stress conditions can affect development throughout the establishment of a web of lncRNAs regulating gene expression in coordination with distinct regulatory proteins. In fact, data suggest that they also seem to be sensors for environmental conditions mediating cellular response, for example, to stress conditions. In the plant *Arabidopsis*, winter cold induces the synthesis of a sense transcript from flowering locus c (*FLC*) called *COLD-ASSISTED INTRONIC NON-CODING RNA* (*COLDAIR*), which has similarities with lncRNAs by directly interacting with the PRC2 complex and targets it to *FLC*, establishing stable repressive chromatin at *FLC* and an epigenetic memory [375]. In fact, in cold weather the H3 at *FLC* is methylated, which inhibits the expression of genes required to trigger

flowering. The locus *FLC* is also differentially regulated by an antisense transcript lncRNA, called COLD-INDUCED LONG ANTISENSE INTRAGENIC RNA (COOLAIR). In fact, changes in the abundance, splicing, and polyadenylation of this lncRNA are observed in response to different environmental conditions and are associated with altered chromatin regulation and differential *FLC* expression [376]. In mammals there is not yet much information relative to the role of lncRNAs, but this is probably because only a few studies have been performed. For example, the lncRNA Uchl1 is involved in the translation enhancement rate of the ubiquitin carboxy terminal hydrolase L1 (UCHL1) mRNA by promoting its association with polysomes [377]. The inhibition of mTOR activity by stress causes the repression of cap-dependent translation. Under these conditions, *Uchl1-as1* is transported from the nucleus to the cytoplasm, where it can base pair with the *Uchl1* mRNA and stimulate its cap-independent translation. The UCHL1 protein is a specific neuronal protein involved in the rapamycin-neuroprotective function associated with neurodegenerative diseases. Also, Adapt33/gadd7 is an lncRNA associated with polysomes in hamster cells, which is involved in the stress response to many agents including the adaptive response to hydrogen peroxide [378–381].

The impact of lncRNAs in regulating gene expression is vast, but in the future, new roles of lncRNAs may arise because some contain open-reading frames with the potential to produce small peptides that may have roles *in vivo*. If this will be true we probably have only a faint image of their evolution, functions, and molecular mechanisms of action.

10.5 Final Considerations

10.5.1 Are Noncoding RNAs the Desired Miracle Molecules in Human Disease Therapeutics?

From what was analyzed in this chapter, there is no doubt that ncRNAs have vast widespread regulatory activity in different steps of gene expression throughout the phylogenetic tree, creating various regulatory layers with an impact on development, differentiation, the maintenance of genome integrity, and homeostasis. Moreover, they are able to establish an incredible dynamic meshwork with regulatory proteins to sense and signal from internal and external environments, and improve the accuracy of signaling pathways by decreasing noise and by increasing the speed of responses. These and some other ncRNA features render these molecules appealing for diagnosis (as biological markers) and as therapeutic targets, and strong efforts have already been made to that purpose. In fact, an excellent review described and discussed the potential role of ncRNAs as noninvasive biomarkers and already explored RNA therapeutic drugs under study for the treatment of several diseases such as neurodegenerative diseases, myopathies, and cancer, as well as difficulties and future challenges [8]. Regarding miRNAs, because they are stable molecules and easily identified in body fluids, they may

constitute excellent specific biomarkers; experimental data already support this view in the case of lung cancer, colorectal cancer, and diabetes [382]. There are also examples showing the existence of correlations between circulating miRNA levels and the response to anticancer agents. In this case, this type of assay may be used to follow the effectiveness or resistance of certain therapeutic molecules [383], which also opens doors for personalized medicine.

On the other hand, numerous strategies have been used to regulate the levels of critical miRNAs, taking advantage of their structures and biogenesis pathways. Three main approaches have been blockage/loss-of-function and down-regulating or up-regulating their expression [8,384]. The strategies are diverse; for example, misexpressed miRNAs may be trapped using artificially designed target RNAs that compete with endogenous targets (miR sponges), sequester them, and abolish their functions. For example, small antisense oligonucleotides or locked nucleic acids can be designed to antagonize specific miRNAs [384]. miRNAs function can also be suppressed by decreasing their levels, by promoting degradation (siRNAs, ribozymes, and deoxyribozymes). In all of these approaches there will be an increase in the expression levels of the protein-encoding genes normally down-regulated by the miRNA. Alternatively, their function can be blocked by using small molecule inhibitors of specific miRNAs [385]. The interaction of small molecules and miRNAs was termed "small molecule compounds" (SMIR) by Melo and Calin et al. [386].

Similarly, antagomirs, which are cholesterol-conjugated synthetic RNAs with a 2′-*O*-methyl linkage and phosphorothioate modification that are complementary to the full sequence of the targeted miRNA, can pair with the miRNA, blocking its function.

Another strategy is to restore the correct levels and function of the ncRNAs, e.g., using small molecules (nonspecific induction of miRNA expression), employing double-stranded synthetic RNAs that mimic endogenous miRNAs (miRNA mimics) or using miRNA expression vectors [387]. Most of these distinct methodologies are already being used in preclinical studies and some are currently in phase I and IIa [8]. Indeed, consulting the ClinicalTrials.gov website (US National Institutes of Health; http://www.clinicaltrials.gov), we find 272 ongoing clinical studies (June 2015) focused on the role of miRNAs that clearly showed developing efforts to explore these molecules clinically. Most of these studies involve the role of miRNAs in cancer, but many other human diseases are under evaluation, such as neurodegenerative diseases (e.g., Alzheimer), amyotrophic lateral sclerosis, autism spectrum disorder syndrome, coronary heart disease, multiple sclerosis, diabetes, Duchenne muscular dystrophy and a few dealing with immunologic problems at the level of allergic diseases.

Regarding lncRNAs, many studies using conventional siRNA treatment to down-regulate lncRNAs have oncogenic roles, because many of these ncRNAs have modified patterns of expression in different types of cancer cells. Another approach is to deplete lncRNAs using antisense oligonucleotides, as well as ribozymes or deoxyribozymes, which use different interaction mechanisms with the target lncRNAs. The use of synthetic RNA molecules that form hairpin structures that mimic lncRNAs is also an alternative to

removing specific lncRNAs. Finally, by using single-stranded oligonucleotides that are able to inhibit the formation of sense—anti-sense RNA pairs through competition, leading in general to an increase in the mRNA counterpart levels, it is possible to increase the encoded protein levels directly [388]. Therefore this is one of the few ways to increase the synthesis of a specific protein directly, an output difficult to achieve in general with other conventional drug designs [389]. Experimental data illustrating this come from an assay in which the continuous infusion of an oligonucleotide with a phosphorothioate backbone targeting cytosolic Cu,Zn-superoxide dismutase (SOD1) was injected into the right lateral ventricle of rats [390]. In the brain and spinal cord of these rats, decreases in SOD1 mRNA and protein levels were observed. Interestingly, amyotrophic lateral sclerosis, a progressive neurodegenerative disease, is characterized by mutated forms of SOD1; currently there is no helpful treatment [47].

Combined strategies have also been used and are promising. Studies *in vitro* have shown that combined treatment with BCL-2 siRNA and miR-15a synergistically improved methotrexate-induced apoptosis of Raji cells [391,367].

Many of these strategies clearly have advantages and are extremely promising compared with traditional therapies, but as expected, some limitations can also be described. In most cases the use of oligonucleotides as therapeutic agents requires complex formulation and delivery. However, the development of nanotechnologies and strong investments in discovering and using new materials, nanoparticles, and alternative drug delivery strategies will probably allow most difficulties to be to overcome eventually. Moreover, each strategy has weak spots [392]. For example, one disadvantage that needs to be taken into consideration is that SMIRs can have off-target effects in both the tissue of interest and throughout the body and are more complex to design compared with oligonucleotides. In the case of sponges, they have considerably low binding affinities at the concentrations required to trap miRNAs effectively. Therefore, they should be used in excess, which can increase the risk of unwanted toxicities. Another obstacle is the difficulty of assessing the efficacy of a given anti-miR treatment because it was observed that in certain cases, anti-miRs do not always reduce the corresponding miRNA levels, but instead abolish their role by creating a highly stable heteroduplex, as in the case of miravirsen and miR-122 [224]. Because Northern blot and polymerase chain reaction are the preferred techniques to assess anti-miR effects, in this case the effect of the anti-miR is not correctly followed. Moreover, there are no data about the potential cellular toxicity of the continuous accumulation of these complexes *in vivo*. Attention should also be given to innate immune responses. For example, the evaluation of a high-profile study using siRNAs targeted to vascular endothelial growth factor (VEGF) showed that an off-target immune response was indeed responsible for the observed suppression of VEGF [393].

In addition, miRNAs have multiple mRNA targets, many of which are involved in a specific pathway or in redundant pathways, which can be an advantage. Although this can useful, it deserves attention because monitoring anti-miR effects may require a more massive analysis of targets by using, for example, expression array analyses, to have a

complete scenario of the impact of the treatment. Attention should also be given to the fact that many more systematic studies on biogenesis and the molecular mechanism of the roles of ncRNA are required.

In fact, despite remarkable multidisciplinary development in the miRNA field, it is still difficult to predict the phenotypic consequences of manipulating miRNAs *in vivo*. Many of these difficulties are still rooted in our lack of knowledge of ncRNA biology. For example, processing of an miRNA results in an intermediate duplex of two potentially mature products that derive from the two arms (5′ and 3′) of the precursor hairpin [70]. Moreover, data showed that both precursor arms may create two functionally mature microRNAs. Thus alternative mature products produced from the same precursor microRNAs will have different targeting properties and therefore different biological functions, which should be kept in mind in a therapeutic strategy. Furthermore, by using 20-*O*-methyl oligonucleotides complementary to conserved terminal loops of the corresponding pri-miRNAs, Michlewski et al. [394] showed that these oligomers were able to block their processing efficiently. They also demonstrated that mutations in the terminal loop of pri-miR-18a that do not affect the structural architecture of the stem abolish its efficient processing. Thus they proposed the existence of auxiliary factors that bind to conserved terminal loops. No clues are available concerning the behavior of these factors under therapeutic strategies. Also, although miRNAs frequently have hundreds of conserved targets, most miRNA knockouts lack obvious effects on viability, fertility, visible morphology, and behavior [395–397], and in certain cases there are substantial discrepancies between knockouts and inhibitor-based methods [398]. Data are also available showing that manipulation of the levels and functions of a given ncRNA may be adequate for a given signaling pathway but cause problems in another in which this ncRNA participates. For example, antagomirs to let-7 and miR-122 have potential therapeutic applications for metabolic disorders and/or HCV [8], but these sncRNAs also have tumor-suppressor functions. That many miRNAs have greater effects in certain environmental backgrounds [399] highlights the need for genetic and environmental interaction studies. Therefore, much remains to be understood about how miRNAs are participating in biological pathways. Of crucial future importance will be efforts to further understand basic cellular disease mechanisms based on ncRNA networks. In *Drosophila* there is growing evidence suggesting that miRNA gain-of-function may be a much more frequent cause of disease than miRNA loss-of-function [400,401], which should be kept in mind and explored. Finally, it is starting to be well-accepted that miRNAs are modulators of protein-coding gene targets by fine-tuning their expressions rather than by causing drastic inhibition. Although this might be extremely important for specific cellular processes, it could also create background noise in therapeutic strategies.

The challenge of establishing ncRNAs as new therapeutics requires better knowledge of their molecular mechanisms and functions *in vivo* and overcoming biological barriers (e.g., intravascular barriers, immune cells, receptor-mediated endocytosis and endosomes), to clearly determine the side effects and find approaches for controlled delivery and sustained release.

10.5.2 Noncoding RNAs and the Evolution of Regulatory Molecular Mechanism Accuracy: The Development of Multicellularity and Plasticity

Our view of RNA molecules has rapidly changed in the past decade. Essentially they have transformed from intermediate molecules in the information flux between DNA and proteins to ubiquitous active players in regulating gene expression from prokaryotes to eukaryotes. In the past years it also became clear that they have roles by being true partners of proteins to control gene expression at different levels of regulation, not only cooperating with them, but also creating structural environments that recruit, position, and allow or create interactions among proteins. Therefore, their ability to pair up with RNA or DNA allows the construction of a new level of information still based on nucleotide sequence that is used to mediate more accurate recognitions and interactions. This idea could have been anticipated much earlier if we had paid more attention to the role of RNAs in ribonucleoprotein complexes such as the spliceosome, telomerase, or even ribosomes. Without ribosomal RNAs, the peptidyl transferase activity of the ribosomes will be compromised as well as their decoding functions and specific tRNA acceptance [402]. This probably means that complex networks between proteins and RNAs were established early in evolution and have contributed to several basic processes progressively acquired accuracy, culminating in protein synthesis. In most cases in which a cellular mechanism requires precise recognition and precise interaction, RNA molecules are involved as key players or assist in the process. The prominent role of all ncRNAs in development and differentiation programs may also reflect this feature. From an evolutionary point of view, it seems that the "invention" of proteins such as telomerases was a critical step in establishing accurate spatial and temporal regulatory processes that probably allowed the evolution of eukaryotic complexity and later, the appearance of multicellularity. For example RNA editing [403], a mechanism based on protein or protein−RNA complexes responsible for the RNA editing reaction, requires a "guide RNA" molecule which, through base-pairing with the target RNA molecule, determines the editing site. By this mechanism an mRNA sequence may be posttranscriptionally altered, and consequently a new protein will be synthesized and information variability is generated. In ciliates, the development of the somatic line from the germline, a process that requires complex genomic rearrangements and processes, is essentially based on a mechanism that requires RNA pairing involving small RNAs that resemble piRNAs [17] and guides the process of rearrangements. This mechanism keeps an epigenetic memory throughout different generations. In fact, piRNAs have been associated with developmental robustness and the maintenance of genomic integrity against transposons and other parasitic nucleic acids, two events that are probably related. It would be interesting to analyze the precise regulation of these processes under challenging environmental conditions. In fact, ncRNAs are also emerging as interfaces between environmental conditions and epigenetic programs and/or the regulation of gene expression. It is expected that in these complex regulatory

networks several hubs exist. These may be composed of ncRNAs and proteins or, alternatively, only proteins or ncRNAs, which will also allow the rapid and better integration of different environmental signals. Experimental efforts to identify these critical molecules will be welcome.

This scenario is rapidly evolving in plants, where numerous studies have shown the importance of ncRNAs in response to environmental stress conditions such as temperature, heavy metals, or oxidative stress caused by H_2O_2 [404]. A study by Zhang et al. [405], based on expressed sequence tags (EST) analyses in *Arabidopsis*, showed that 25.8% of ESTs containing miRNAs were found in stress-induced plant tissues. Rice seedlings growing under exposure to H_2O_2 show complex patterns of expression of miRNAs, where some are up-regulated and others down-regulated. In these studies the validated targets of the H_2O_2-responsive miRNAs were demonstrated to have important roles in transcriptional regulation, nutrient transport, auxin homeostasis, cell proliferation, and programmed cell death [406]. This picture resembles that observed in animal cells, in which examples of the involvement of ncRNAs in stress response are also starting to emerge.

In the view of an "RNA world hypothesis," it is tempting to speculate that one of the first roles of RNA was to maintain the viability and integrity of "cell precursors" defending them from destructive invader molecules (stress agents), keeping at same time the fidelity of the first ongoing molecular mechanisms. Although these mechanisms could then be explored later and developed into gene regulation, the ancestral defense functions of ncRNAs would be present and operating in modern cells.

During evolution, some of the stress molecules, in particular H_2O_2, became signaling molecules requiring cells to evolve mechanisms to distinguish responses to toxic H_2O_2 from those to signaling H_2O_2. In fact, it is now well accepted that H_2O_2 is a key regulatory molecule involved in biological processes as diverse as immune cell activation, vascular remodeling, proliferation, survival, migration, adhesion, cell polarity, apoptosis, and senescence [286,407–410], by regulating different levels of gene expression from transcription factors to molecules involved in posttranscriptional regulation [231,286]. The attractive question to put forward is whether ncRNAs were involved in establishing H_2O_2 as a prominent regulator in multicellular organisms. In these organisms distinct types of cells are exposed to a new environment and information is mainly received via receptors in the plasma membrane. Indeed, ncRNAs seem to be able to modulate the levels of H_2O_2 by regulating the expression of catalase and SODs, which would support an affirmative answer. However this answer might be too simplistic because H_2O_2 also regulates miRNA biogenesis. On the other hand, it is known that H_2O_2 regulates several transcription factors through redox-sensitive cysteine residues at their DNA-binding sites, such as NF-κB, AP-1, HIF-1α, and p53 [231]. Conformational changes resulting from oxidation of amino acid residues have also been implicated in regulating proteins such as TRX, IB, RAS, and Akt [378]. H_2O_2 can also modulate protein function by changing the oligomerization state of proteins or their ability to interact with protein partners, as in the case of the transcription factor NRF2 [231]. Consequently, it is

tempting to suggest that H_2O_2 and certain ncRNAs could be partners directly collaborating in regulating certain signaling pathways exploring together protein conformation alterations and interactions. If this is true, this type of cross-talk between H_2O_2 and ncRNAs would have contributed to establish H_2O_2 as a regulator molecule.

Finally, it would also be desirable to investigate further how far different classes of ncRNAs (sncRNA and lncRNA) interact with each other, not only at the level of their biogenesis pathways but also at the functional level. Such valuable information would help to understand some puzzling results and could be directly translated to new therapeutic strategies. This information is missed to a certain extent because the different classes of ncRNAs have essentially been investigated separately and in most cases at uneven levels.

This is especially true for the studies of ncRNAs in the immune system, in which miRNAs have been thoroughly investigated but lncRNAs are just beginning to be functionally characterized. One possible explanation could be that, unlike miRNAs, many lncRNAs appear to be expressed at low levels in immune cells [373]. However, these low levels might be enough to produce strong phenotypes, as in the case of *NeST*, which can regulate the expression of INF-gamma and susceptibility to a viral and bacterial pathogen by $CD8^+$ T cells [366]. Also, unlike most miRNAs, many lncRNAs are not conserved between human and mouse [19], which makes cross-species studies more difficult. In fact, a future challenge would be to determine whether this species-specificity would be responsible for major differences between the mouse and human immune systems. Indeed, identification of these differences might be relevant to determine, in general, the relevance of ncRNAs to the complexity of across species, because these differences are not reflected in their genome sizes.

Importantly, many cells of the immune system, including T cells ($CD4^+$ T cells and $\gamma\delta$ T cells [411]) are endowed with the feature of plasticity, which implies that a given effector phenotype can revert rapidly to another differentiation state. This allows the immune system to adapt quickly to the many challenges it faces. Understanding how the network of coding and ncRNAs acts to regulate this plasticity in a coordinated fashion is of major importance in immunology and could ultimately have an impact on the development on novel therapeutic targets for immune-mediated diseases.

We can envisage that the ability of ncRNAs to allow cells to cope with stress conditions, in the context of multicellularity, was explored and contributed to set H_2O_2 as a regulatory molecule but also to allow specific cell types to respond to environmental signals by changing their differentiated state to a new one more adapted to deal with the initial challenge, i.e., by exhibiting plastic behavior. In this view we can assume the capacity of ncRNAs to protect cells against destructive molecules as one of their primitive features that was progressively redefined in the course of evolution, helping organisms to attain higher regulatory complexity levels.

In the field of ncRNAs a fantastic development has occurred in past years but, despite the large bulk of information that is available, we are still missing important data regarding the complexity and mechanisms underlying the establishment of regulatory

networks between RNAs and proteins. Therefore, efforts should continue to be made because it is expected that this knowledge may soon be directly translated to medicine, improving human health and even other areas such as agriculture by producing stress-tolerant plants through manipulating ncRNAs [404]. Hence, these evolutionary-old molecules that were critical in the emergence and complexity of life will continue to surprise us with their biological roles, which will probably render them important biotechnological tools.

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11

Implications of Substrate Topographic Surface on Tissue Engineering

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11.1 Biomimetic Constructs for Tissue Engineering

"Tissue engineering is a multi-disciplinary field where biology, biochemistry, physiology, physics, and chemistry combine to produce material science modeling samples to be used in tissue engineering towards the development of biological substitutes that restore, maintain, or improve tissue function" [1,2]. The use of scaffolds on tissue engineering is based on temporary three-dimensional supports, which is accomplished with the extracellular matrix (ECM), allowing the proliferation and communication of host cells harvested from natural tissue in vitro and in vivo. Within this context scaffolds can have the following roles: maintaining cell viability and growth, permitting cell signaling and communication, and allowing cells to reach their target site (e.g., bone repair, damage nerves, wound healing, burned skin) as would cells from their original niche site. Also, scaffolds are supposed to supplement or replace missing, compromised, or injured *tissue* or organs to keep their normal function [3]. Actually the tissue engineer field has been progressed from this simple concept of scaffolds as a bioinvisible, inert, inoffensive, and long-lived biomaterial. Currently, the principle is that scaffolds should confer biofunctionality, along with biodegradability over time, allowing replacement of biomimetic material with the original tissue.

Scaffolds are expected to have more than functionally durable and stability. Dynamic cell-control signaling must be integrated into these biomimetic materials, getting into their temporary structure and improving their homing tissue. In order to acquire improved properties (e.g., durability, flexibility, physical functions, communication), scaffolds must incorporate biological functions and improve the cellular control

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conditions. [4]. Furthermore, for clinical applications of this new concept of scaffolds, it will be necessary for them to allow cell−seed accomplished with nanomaterials complexed with drugs or growth factors able to be made as a drug and biomolecules carriers [5−8]. Diverse stem cells origins from adult or embryonic tissues and induced pluripotent stem cells are being evaluated as technologies for cell therapy (allogeneic, xenogeneic, or autologous) [9]. However the production or acquisition of cells alone is only part of the answer. Scaffolds must ensure a three-dimensional (3D) structure that allows cells to grow, communicate, differentiate, migrate, and produce an ECM substituting the transplanted material with functional native tissue in solid implants [10]. It is mainly related to mammalian connective tissue that often undergoes repair with scarring rather than functional regeneration [10,11].

In other words, scaffolds need to be constructed based on cues that allow cell functions enabling tissue remodeling rather than just providing single signaling molecules (e.g., growth factors, cytokines, or pharmacologic agents) to induce tissue reconstruction [10,12]. Depending on the type of tissue that needs to be repaired or replaced, these molecules can be of different natures. Tissues can be classified as highly cellular or noncellular tissues (e.g., secretory, metabolic, barrier). For the first type, cell-to-cell contact dominates control: for example, cell signaling, communication (e.g., extracellular signals induce transcription factors activation and consequently gene expression) [9,13−18], cell adhesion, and cell perfusion and nutrient consumption (e.g., gases such as CO_2, O_2, and nutrients as glucose) are supported. In the case of noncellular or poorly cellular tissues such as connective and contractile tissues, properties to make scaffolds biomimetic materials are related to their 3D topographic and directional cues, which means their mechanical and substrate characteristics, along with external mechanical loading and neurovascular innervation.

Biomimesis is related to the criteria of spatial dimensions that a scaffold can form. That is the essence of 3D growth rather than a flat culture surface. Signals attached to substrate can act through all three spatial axes of the cell, which allows them to communicate within all directions to generate new information, resembling to that of original tissues. This new information can offer stochastic chemical reactions, spatially polarized or homogeneous signaling networks [19−21], with different cells communicating with each other in spatiotemporal domain, in contrast to 2D monolayer cultures in which cues have polarized directional, with just a two-coverage, one “dorsal” fluid−covered surface and a “ventral” plastic−attached surface. Obviously, nonepithelial cells such as fibroblasts or other stromal cells are not appropriate for this system.

Three-Dimensional scaffolds can be replaced by normal tissue depending on its properties like complaisance, perfusion (pore size) or degradation rate. Based on that, Scaffolds can be divided into (1) synthetic polymer based, especially polylactide/glycolic acids [2,22] and (2) bioartificial natural polymers such as collagen/collagen−glycosaminoglycan (e.g., Integra) sponges [23], collagen gels [24], and fibrin [25]; in addition, a wide range of possibilities can be classified.

This is a key division relating to the question of scaffold degradation. When soft fibrous tissues are replaced with 3D engineered tissue, the initial scaffold must be replaced with the production of new tissue from the host cells. Otherwise, nondegradable mineral-based scaffolds for bone repair are more appropriate. This level of control is almost impossible for conventional biodegradable polymers, because of the diversity of physicochemical conditions at any given implant, pathology, or injury site. This is because of the action of hydrolyzation/dissolution to be *independent* of cell activity. In scaffolds constructed with native proteins aggregated from the cellular matrix, they are susceptible to being cleaved, consumed, and replaced through the metabolism of ECM proteases (meaning biological turnover and remodeling) and tend to be replaced by cell action as new matrix is produced. However, for conventional polymers this is not possible because they disappear too rapidly, providing poor support; or more commonly they disappear too slowly, inhibiting or counteracting cell-based renewal [10]. However, using synthetic polymers or their blends, and synthetic and natural polymers blends with precisely controllable degradation rates (Table 11.1) can solve this problem [26].

Questions have been posed about how much or how little scaffold design must have similarity with the host tissue to be classified as biomimetic. Nowadays, tissue engineering success is achieved considering composition and structure, which is much simpler than using native tissue, and it is possible to manipulate cells and biomaterials in vitro [27]. An example is the use of bioprosthetic heart valves resembling bovine pericardium. Even lacking the trilayered structure of native leaflets, these artificial valves yield adequate function [27]. Considering the large range of tissues to mimic, we can consider extreme types of scaffold designs, such as those that are cell-rich and matrix-rich [28]. Naturally, there are many intermediate types, which can be achieved by remodeling their own format into another over time. Thus it is possible to have 3D scaffolds structured for adherent cell masses using a cell-rich construct [29,30]. Those scaffolds allow soft tissue and organs (e.g., intestine, lung) and full stromal differentiation and matrix deposition resembling their embryonic tissue templates and with little connective tissue content. Otherwise, scaffolds that require significant mechanical functions as support and/or contraction force guides (such as bone tissue, nerves, and blood vessels) use matrix-rich constructs.

Considering how long grafts have been studied, more experience and sensibility were achieved that actually occurs in natural tissue development and wound repair. Although occasionally function must be restored immediately before new tissue formation for tissue repair to cover a skin wound or to repair a ruptured tendon, during tissue repair these scaffolds must create conditions to restore by tissue native function through the regenerative process (for example, the restoration of nerve function or integration of a bone graft). Thus an implantable device requires an initial design that mimics the structure and function of the native tissues forming an initial repair template while providing a reconstitution in situ by its environment for final structures and functions.

Table 11.1 Synthetic Polymers Characteristics, Advantages and Disadvantages

		Characteristics	Advantages	Disadvantages	Applications
Polymers	Polyglycolic acid (PGA)	Highly crystalline, hydrophilic, byproduct is glycolic acid	• Biocompatible and biodegradable • Bioabsorption (2–4 weeks) • Electrospinning yields diameters ~200 nm • Good choice for high strength and elasticity and fast-degrading material	• Fast degradation causes pH change • Tissue may require buffering capacity	• Bone tissue strengthening • Cardiac grafts • Collagen and cellular interaction • Differentiation with mesenchymal stem cells
	Polylactic acid (PLA)	Hydrophobic, lower melting temperature, byproduct is lactic acid	• Biocompatible and biodegradable • Bioabsorption (30 weeks) • Good choice for drug delivery owing to predictable degradation	• Larger-diameter fibers ~ microscale	
	Polydioxanone (PDO)	Highly crystalline	• Biocompatible and biodegradable • Degradation rate between PGA-PLA • Shape memory • Excellent flexibility • Modulus comparable collagen and elastin • Good source for future vascular grafts	• Lack of knot retention • Lack of adaptability to develop tissue	
	Polycaprolactone (PCL)	Semicrystalline properties, easily copolymerized, byproduct is caproic acid	• Biocompatible and biodegradable • Inexpensive • Highly elastic • Slow degradation (1–2 years) • Good choice for human mesenchymal stem cell seeding to induce differentiation	• No shape retention (highly elastic)	

Blends	PGA-PLA (PLGA)	• 75% PLA—25% PGA • 50% PLA—50% PGA • Blended PLA and PGA together in HFP at ratios of 100:0, 75:25, 50:50, 25:75	• Hydrophilicity proportional to composition of copolymer • Degradation rate proportional to composition of copolymer		• Cardiac tissue in mice for tissue regeneration • Individual cardiomyocytes attachment at seeding • Scaffold loaded with antibiotics for wound healing • Mechanical properties such as tangential modulus, peak stress, and strain to failure, of copolymers and blends appear to be controlled by fiber/polymer composition
	PGA-PCL	Not wide variance in fiber diameters and is assumed that individual polymers become entangled in regular pattern dictated by their chemistry and concentration In addition, owing to long degradation time of PCL (typically 1—2 years), it would be reasonable to expect that blend would degrade slower than PGA alone	• PGA: high stress tolerance • PCL: highly elastic • Optimal combination PCL-PGA: 1/3 • Longer degradation time ~3 months (PCL: 2 years; PGA: 2—4 weeks)		Blend seems well-suited to tissue engineering applications in which high elasticity is driving factor
	PLA-PCL	PLA-CL (PLCL) can be produced at various weight percents from methylene chloride at 25°C. The 70:30 ratio was hard solid, 50:50 ratio was elastomer, and 30:70 ratio was gummy solid consisting of fused mesh. The two ratios that resulted in fibrous scaffolds, 70:30 and 50:50, were determined to have Young's modulus of 14.2 and 0.8 MPa, respectively [136]	• Greater elasticity than PGA + PCL • Similar tensile strength to PLA • ~5% addition of PCL increased strain by eightfold • Overall best synthetic extracellular matrix (ECM) for cardiac applications	• Decreasing PLA + PCL ratios decreases strain capacity, optimized at 95:5	An engineered vascular graft must be strong enough to accommodate large pressure increase while having enough elasticity to expand to accept large bolus of blood passively and contract to push blood downstream Furthermore, smooth muscle and endothelial cells were seeded on these scaffolds and cultured for 7 days, with favorable results

Continued

Table 11.1 Synthetic Polymers Characteristics, Advantages and Disadvantages—cont'd

		Characteristics	Advantages	Disadvantages	Applications
	PDO-PCL	Fibers ranged from 940 nm for pure 100 mg/mL PDO to 1.5 μm for 70:30 ratio of 100 mg/mL PDO:500 mg/mL PCL	• PCL high elasticity • PDO shape memory	• Lower tensile capacity than PDO • Lower elasticity than PDO • Larger fiber diameter	
Natural polymers: no controllable degradation rates	Elastin	The most linearly elastic biosolid known. It is a highly insoluble, hydrophobic protein Electrospun solubilized elastin (bovine neck ligament) at concentrations ranging from 200 to 333 mg/mL in HFP, resulting in the creation of scaffold of flat, ribbon-like fibers	• Linearly elastic biosolid • Insoluble and hydrophobic • Critical role in shape and energy recovery for organs	• Less elastic than native elastin • Needs to be combined with PDO to increase tensile strength • Fiber ~300 nm (not as small as PDO ~180 nm) • Varying diameter	It constitutes large proportion of walls of arteries and veins to provide elastic recoil and prevent dissipation of transmitted pulsatile energy into heat. It is also a large component of ligaments and lung parenchyma owing to its elastic properties. The skin contains thin strands of elastin that help keep it taut and smooth
	Gelatin collagen	Gelatin (denatured collagen)	• Biocompatible and biodegradable • Inexpensive	• Quick to dissolve	Has been used for many years as vascular prosthetic sealant, carrier for drug delivery, and dressing for wounds. Through electrospinning, has gained interest as tissue engineering scaffold
	Fibrillar collagen	The electrospun collagen solutions ranged in concentration from 0.03 to 0.10 g/mL of HFP resulting in mats/scaffolds composed of 100 nm to 5 μm diameter fibers Electrospun calfskin type I collagen revealed the 67 nm banding that is	• Fibril-forming (types I, II, III) • Most abundant natural polymers in body • Important role in ECM • Type I: principal structure in ECM • Type II: pore size and fiber diameter easily controlled • Type III: still under investigation	Typical procedures used to isolate and reprocess this natural scaffolding into engineered material may compromise many of its biological and structural properties	Fibril-forming collagens, types I, II, and III, are most abundant proteins (natural polymers) in the body, are found throughout the interstitial, and provide the overall structural integrity and strength to tissues. More important, the collagen structure (ECM) provides cells with appropriate

		characteristic of native collagen Hence, an electrospun collagen mat may be a truly biomimicking scaffold, since sub-micron diameter fibers possessing the natural collagen ultrastructure can be created			biological space for embryologic development, organogenesis, cell growth, and wound repair
Collagen blends	Fibrinogen	• Low concentration produced fibers within range of fibrinogen fibers in plasma clots (80, 310, 700 nm) • Stress capacity comparable to collagen (80–100 MPa) • Electrospun fibrinogen scaffolds can be significantly enhanced, with no loss of bioactivity, by blending the natural polymer with PDO	• High surface area to volume ratio: Increases area available for clot formation • Translates into relative surface area to volume ratio of 1300 cm^2/cm^3 or relative surface area to weight ratio of 4.1 m^2/g		
Synthetic and natural polymer compilation		• PGA, PLA and PLGA most commonly used • PDO most similar to elastin collagen blend (limited by shape memory) • PCL most elastic and mixed frequently with other materials • Provide nanoscale physical features • Collagen type I and III + PDO: best possible match for blood vessels	(Elastin and PDO) dynamic compliance measurements produced values ranging from 1.2% to 5.6%/100 mmHg for set of three different mean arterial pressures, with 50:50 ratio closely mimicking compliance of native femoral artery Hydrated PDO–collagen samples were able to sustain mean peak stresses between 4.6 and 6.7 MPa, mean strain to failure values between 56.5% and 186.4%, and	Scaffolds were electrospun in following blends [PDO –collagen (by wt%)]: 100:0, 90:10, 80:20, and 70:30; when mixture of collagen types I and III was used, it was 50:50 blend of collagens. Scaffold fiber diameters for blends ranged from 210 to 340 nm, but increase in percentage of collagen did not have significant effect on mean fiber diameter	Different ratios of elastin and PDO (100:0–50:50 PDO:elastin by volume) create bioactive scaffolds that remain mechanically viable Mechanical properties of electrospun PDO–collagen blended vascular prosthetics fall within ranges of corresponding values for

Continued

Table 11.1 Synthetic Polymers Characteristics, Advantages and Disadvantages—cont'd

	Characteristics	Advantages	Disadvantages	Applications
	• Indication that blends of PDO and collagen may match mechanical and morphological requirements of a blood vessel's microenvironment (similar to PDO section)	mean tangential moduli between 7.6 and 18.0 MPa Hydrated electrospun PDO—collagen structures achieved modulus between maximal modulus of elastin and minimal modulus of collagen. Peak stress exhibited by electrospun PDO—collagen is within range of elastin and near lower limit of collagen. Percent strain at break range of electrospun PDO—collagen spans both ranges of elastin and collagen and exceeds upper limit of collagen	(although addition of collagen to PDO decreased fiber diameter compared with PDO alone)	traditional vascular materials. Human dermal fibroblasts seeded onto PDO—collagen type III scaffolds displayed favorable cellular interactions; cells migrated into thickness of scaffolds containing collagen but merely migrated on seeded surface of 100% PDO scaffold

HFP, Hexafluoroisopropanol (1,1,1,3,3,3-Hexafluoro-2-propanol)

From I.K. Kwon, S. Kidoaki, T. Matsuda, Electrospun nano-to microfiber fabrics made of biodegradable copolyesters: structural characteristics, mechanical properties and cell adhesion potential, Biomaterials 26 (2005) 3929—3939.

Increasing discussion led to certain basic elements that biomimetic constructs must possess:

1. it should be 3D,
2. it should be rich in matrix that is able to undergo cellular remodeling, and
3. it should have built-in physicochemical signals to direct cell behavior.

Essentially, such constructs must be susceptible to cell-mediated remodeling after implantation. During cellular remodeling the actual ECM environment rapidly becomes protein-based, mainly collagen. In this chapter we will focus on how it is important for 3D biomimetic collagen scaffolds, which are developed to be used for tissue engineering, to resemble ECM. Also, we will discuss carbon nanotube uses for tissue replacement and organizational levels of scaffold structure considering biomimetic elements of nanoscale, microscale, and macroscale.

11.1.1 Collagen-Based Biomimetic Constructs

Collagen is encoded by a large gene family that is subject to strict patterns of temporal regulation. Collagen proteins form an intricate network with themselves and with other extracellular cell matrix proteins, providing a huge range of structures and functions and supporting the *body's structures* and binding or connecting all *tissues*. These complex fibrous protein networks have the main role of maintaining the structural and biology integrity of ECM for both soft and hard connective tissues. These interconnected networks also undergo constant and dynamic remodeling of tissue structure [31]. Furthermore, the ECM participates in cellular signaling and communication through adhesion receptors and its own cleaved proteins (matricellular signaling), which presents and regulates growth factors and cytokines to cells (matricrine signaling) [32]. ECM transduces hemodynamic forces and resists traction forces produced by cells (mechanical signaling) (Fig. 11.1) that occur during development, repair, or natural conditions.

A total of 28 different molecular structures of collagen family have been identified, each of which contains at least one triple-helical domain formed from three α-chains [31]. The triple helix is composed of three polypeptide chains, each with the repeating triplet Gly-X-Y, in which X and Y are frequently proline (approximately 22% occurrence of each in type I collagen) and 4-hydroxyproline, respectively. Those triple helices can be constituted of homotrimers or heterotrimers with various α-chain combinations. This huge variety in collagen type may be due to the splicing process, the addition of glycosyl and hydroxyl groups, the presence of other nonhelical domains, or how they assemble, determining their function [33]. Within the huge varieties of the collagen family, some types are more probable to occur as I, II, III, V, and XI, characterized as fibril-forming collagens assembled into highly organized supramolecular fibrils. The most abundant (about 70% of collagen, which constitutes

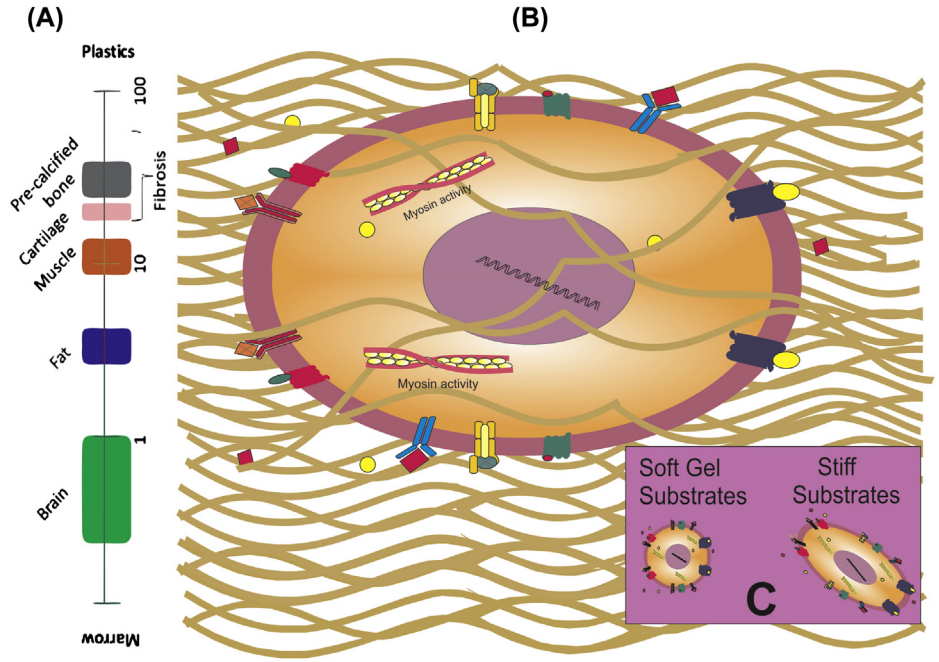

FIGURE 11.1 (A) Scale of tissue elasticity ranging from softest (brain) to stiffest (bone). (B) Signals from growth factors bound to the ECM affect cell function by mediating gene expression through various kinases. (C) Cells cultured on gels that mimic a soft tissue environment anchor less strongly to the substrate than do cells cultured on gels that mimic a stiff tissue environment. (Shown in green (light gray in print versions)) It is possible to see focal adhesions. (Shown in red (gray in print versions)) The actin cytoskeleton is presented. *Modified from D.E. Discher, D.J. Mooney, P.W. Zandstra, Growth factors, matrices, and forces combine and control stem cells, Science 324 (2009) 1673–1677, AAAS.*

mammal tissues) is fibrillar type I collagen, which has the key property of possessing distinct morphologies for different tissues performing different functions depending on its own interactions. Bone tissue is the association of inorganic crystals (hydroxyapatite) providing rigidly and shock-resistant bone tissue with a high Young's modulus [34]. Otherwise in tendons collagen acts like an elastomer, possessing low rigidity and high deformation, allowing rupture, whereas in cornea it has optical properties such as transparency [31]. Type IV collagen is the main nonfibrillar collagens; it is the major component of the basal lamina that forms a *sheet-like* structure, integrating other ECM fibrous (e.g., laminin). There are also more types of collagens including types IX, XII, XIV, XVI, XIX, XX, XXI, and XXII, localized on the surface of collagen fibrils depending on the tissue location. All of these collagens are fibrils that are associated with the fibrous collagens constituting interrupting triple helices. In various connective tissues, microfibrillar collagen type VI generates fine filaments producing a distinct network, whereas other types such as X and VIII, which possess a short chain, form hexagonal networks [35].

11.1.2 Collagen Used as a Biomaterial

Use of collagen as a biomaterial dates back to ancient Egypt, when sutures were made from animal sinew. It is possible that collagen was used even earlier in the first century AD in both Greece and India. During this time collagen may have been used for plastic surgery to repair mutilations caused by battle and punishment [36]. In India a medical encyclopedia by Sushruta recorded a procedure to repair the cut-off noses of patients using a prosthetic nose. This prosthesis was made using skin graft from the patient's cheek [36]. This may be the first record of a prosthetic autograft, the ancient use of biomaterials for tissue engineering. Also, during the Second World War, the first group to report the use of collagen sutures and sheets was that of Professor F.O. Schmitt, who used it to cover denuded areas of skin [37]. A decade later, with great success, Rosenberg's group used modified bovine blood vessels as heterologous grafts for the first time. Many preclinical and clinical studies confirmed the efficiency of this graft [38]. Since these successful medical applications, many others also used biomaterials, e.g., in cardiovascular, plastic, or neurosurgery as injectable fillers for cosmetic surgery including as commercial products (e.g., Zyplast, Zyderm, Contigen), wound dressings [39], and tubes for nerve, skin repair, and blood vessels (e.g., Integra, Apligraf) [40]. Collagen has low antigenicity owing to its preserved amino acid sequence across species, which explains why it can be used in varied application; as a collagen it is an ECM protein and its breakdown products are not harmful, which allows the use of collagen from both porcine and bovine sources for clinical application. It is also possible to reduce the immunogenicity of implanted collagen by using a subproduct of collagen called atelocollagen. Its structure is the nonhelical telopeptide extremity of the collagen molecule [41]. Because collagen was used in medicine it was proof that was safe and biocompatible for tissue engineering applications.

The use of collagen as a construct in tissue engineering can be divided into two general strategies: (1) a *top-down* or decomposition approach, in which the system is essentially broken down, gaining insight into its compositional subsystems. In our concept, ECM from connective tissue is composed mainly with collagen. All cell content is removed in a reducing process; e.g., when collagen is subtract from cadavers or animals, allowing construction of an architecture that resembles that required by tissue; (2) a *bottom-up* approach usually meaning synthesis, when collagen molecular blocks are built, assembled into a scaffold. Therefore, whereas the *top-down* approach reduces the complexity of native tissues, breaking them down, the *bottom-up* approach tends to increases the complexity of the system, assembling the isolated collagen into building blocks for tissue engineering.

To reduce systems, a *top-down* approach commonly uses acidic and basic solutions that can be hypotonic and hypertonic, alcohols, detergents, and enzymes to promote decellularization of the tissue, thus obtaining new scaffolds. The origin material can be obtained from humans, rats, bovines, and porcines allowing the production of constructs for tissue regeneration. The detergent sodium dodecyl sulfate produced blastema

rings obtained from rabbit's pinna colonization [42], and another detergent, Triton X 100, made it possible to reconstruct heart valves [43].

Through the *bottom-up* approach, diverse biomimetic materials together with inorganic, metal, other ECM proteins (e.g., elastin), or nanomaterials (e.g., carbon nanotubes), to mention just a few examples, have been used to fabricate constructs that are used to regenerate tissue. It is commonly used for skin, muscle and bone regeneration and nerve repair, among others [4,34,44–48].

Advantages of using native ECM for grafts are that in vivo cells need integrated physical and chemical communication between themselves and the ECM. In fact, foreign materials do not interact directly with living cells, but upon contact with soluble matrix proteins such as fibronectin, vitronectin, and fibrinogen, they readily attach to the adsorbed layer of proteins in biological fluids [49]. All this joined with physiologic fluids in vivo or culture medium in vitro will affect cellular interaction [50], allowing an ECM 3D structure to be maintained [50,51]. Disadvantages of using "ready-made" ECM as grafts include its density and its relatively impervious structure. It can inhibit cells to repopulate tremendously in the scaffold, slowing process and becoming uncontrollable. Also, a foreign transplant can become immunogenic or promote infection because of residual antigens in animal tissues or even cryptic infectious materials. In addition, it is particularly difficult to remove such macromolecular and cellular elements throughout the dense 3D structure without disrupting it. This is why integrated cellular/ECM scaffolds that are simpler in terms of their composition and architecture represent the most promising emerging technologies for regenerative repair [10].

A huge diversity of work with porous and sponges collagen scaffolds (a pseudo-3D structure) has been conducted because these provide a useful substrate for the bulk delivery of cells [52,53]. Their major application is in tissues that do not need fibers to be aligned, such as in skin replacement. Otherwise, collagen hydrogen represents a 3D scaffold comparable to nanoscale fibers [24,25,54] and their primary use has been as a substrate for the growth and delivery of cells [55], because they are easily seeded interstitially within the fibril network. Other properties of collagen hydrogels include their ability to retain water; their capacity to maintain cells in a stretched position because of their tenacity; their condition to have nanoporosity and microporosity, allowing seeded cells to grow and arrange in 3D; their potential to be biodegradable, creating space for new cells; and their interconnectivity by pores that allowing nutrients and gases flow freely in and around the growing cell mass [56].

The development of the carbon nanotube–collagen (CNT-Col) gel fabrication process was critically influenced by the realization that the excess fluid present in untreated collagen gels is a result of casting. CNT-Col hydrogels rapidly produce dense fibrillar collagen sheets (100–200 μm thick) with a tissue-like architecture, strong mechanical properties (greater than those of native tissue collagen), and biomimetic function (e.g., supporting high cell viability) [34,47]. This process is versatile in terms of the volume, density, and shapes that can be produced (e.g., sheets can be layered or rolled), which creates the possibility of fabricating a wide range of final scaffold structures. Indeed,

several studies have demonstrated the suitability of CNT-Col scaffolds in engineering biomimetic tissue constructs such as skin, bladder, muscle, blood vessels, bone, and cornea.

11.2 How Scaffold Is Structurally Organized

The regeneration and production of functional tissue are the main goals of tissue engineering. The human body develops from a single diploid cell called a zygote and contains an estimated 85 trillion cells by adulthood, of which more than 150 billion turn over every day. Those trillions of cells are divided into about 260 different phenotypes, many of which go on to proliferate and differentiate and can self-assemble into complex and integrated systems of tissues and organs over time and space [56]. Artificial biomaterial scaffolds are designed to support cell and tissue growth, aiming to recreate the complexity and, nanoscale, microscale, and mesoscale details observed in real organs at the level of the cell–matrix interaction [46,57–60]. All this complex structure is related to living organs, with specific functions resembling the level of organization of the target tissue, also providing the correct morphology of the cell-carrying template.

However, we can distinguish among nanoscale, microscale, and macroscale structural elements, which are part of the structure of constructs, improving cell capacity in regulating tissue/scaffold functions and their biofunctionality [12,47]. These characteristics have profound effects on cell physiology and communication, and also influence cell shape and gene expression, which are related to cell growth and the maintenance of original host cells [61]. In this section we present some major levels of organization of native tissue and give some examples of how to build biomimetic constructs incorporating such elements.

11.2.1 Nanoscale and Microscale Scaffold Structure of Biomimetic Elements

The ECM's fibers at nanoscale and microscale reveal details not found on artificial materials. Many different fibers with distinct roles participate in the ECM's structure, providing suitable conditions for cells to form complex and different tissues such as brain, heart, liver, and bone. A major property of eukaryotic cells is that their proteins from the cytoskeleton, such as actomyosin, are capable of modifying cell shape according to signaling molecules and the environment. There are many more cytoskeletal proteins from cells that interact with ECM proteins. All of these cell–ECM interacting proteins have a key role as a substrate for cellular adhesion and can drive cell signaling and differentiation. Molecules such as collagen, laminin, fibronectin, integrins, and various proteoglycans are part of the ECM [32]. Some of these proteins are part of cell secretion in response to stress on the cytoskeleton. Interaction between the cytoskeleton and the ECM is important in fibrosis, wound healing, tumor growth (metastasis), and connective tissue development [32,62,63]. ECM proteins present multiple motifs that

have as their target cell receptors. In mammals, over 24 different integrin heterodimers have been described and they recognize motifs such as Arg-Gly-Asp within proteins of the ECM such as fibronectin, osteopontin, and vitronectin [64,65]. When bound, integrins activate a cascade of intracellular signaling pathways, leading to changes in gene expression and affecting most aspects of cell behavior. An important property of integrins is their connection to form focal adhesions. In addition, integrin distribution, structure, and function in native tissue with 3D architecture are distinct from those in 2D culture [66,67].

In bone, for example, the 67-nm-wide periodic spacing observed in type I collagen is essential for the proper assembly of ions leading to the nucleation of hydroxyapatite crystals. At a higher scale, 50- to 500-nm-diameter collagen fiber of the ECM surrounds, interacts with, and signals osteoblasts, whereas osteocytes rely on microscopic 1- to 5-μm-diameter canaliculi for their viability, communication, and proper function. Likewise, multiscale topographic characteristics of the ECM of all tissues have a key role in structure and signaling. Examples include the diverse porosity and topography of the epithelial basement membrane effects on cell polarity and migration during embryogenesis [68,69] and contact guidance provided by Schwann cells surrounded by basal lamina that guide axon growth in peripheral nerve regeneration [70].

Cells are sensitive not only to ECM adhesion but also to its rigidity and elasticity. It was observed that there is an inverse correlation between matrix density or rigidity and cell migration. Discher and collaborators demonstrated the importance of matrix elasticity on stem cell fate [71–73]. Depending on the elasticity of the surface on which the mesenchymal stem cells (MSCs) were grown, they could differentiate into lineages that corresponded to the stiffness of the native environment that was resembled (Fig. 11.1).

For example, MSCs cultured on soft gels (approximately 0.1 to 1 kPa) to mimic brain elasticity developed a neuronal morphology, with filopodia branching and spreading including expressing genes related to neuronal differentiation pattern. Furthermore, medium-stiffness gels (8–17 kPa), which mimic striated muscle elasticity, promoted differentiation to myogenic cells, and gels with the highest stiffness (25–40 kPa), to mimic bone elasticity, enhanced osteogenic differentiation.

Increasing matrix stiffness disrupts cell morphology and leads to increased proliferation. As shown in Fig. 11.1B, increasing ECM rigidity elevates the activity of the Ras homolog gene family member A, which subsequently induces cytoskeletal tension, decreases cell–cell contact, disrupts cell polarity, and increases the growth rate.

Furthermore, other architectural features of the ECM are important concerning cell behavior. For example, because cell metabolism occurs by consuming nutrients, it is necessary to remove their degradation residues, which corresponds to the diffusion of the system. Diffusion properties of extension and nature can be largely affected by the porosity and permeability of the ECM, which have a direct influence on cell processes. All of these characteristics demonstrate that physical cues are as important as chemical ones in many aspects of tissue engineering [10,11,74,75]. Unfortunately, the signaling mechanism and the way it works related to material-based signals are not yet clear.

This topology-dependent phenomenon is referred to as "ECM dimensionality sensing." Migrating cell morphology and directional persistence are markedly altered in 3D culture systems compared with standard 2D cultures [69,76,77].

A distinct effect between 2D and 3D cell culture ECM is that in the first, when the dorsal cell surface, which is in contact only with culture liquid, is substituted with another substrate (e.g., synthetic or even collagen gels), it is possible to modify the cell morphology and turn the cell physiology to migrating cells [78,79]. Also it causes profound effects on the cell cytoskeleton [80] and dynamics [81] resembling that of cells culture in 3D culture [82]. A 3D environment also influences solute *diffusion* and binds many effector *proteins*, *ensuring* cell viability and optimal function [56]. The scaffold architecture also affects cell attachment and spreading. Cell interaction with fiber-based scaffolds occurs within microscale architectures that flatten and spread, resembling that of the cell culture on flat surfaces, regarded as a 2D culture (Fig. 11.2). It is possible to create a *pseudo* third dimension in the system by causing modifications or disturbances during scaffold production, incorporating some pores, grooves, or channels with approximately the same cellular dimensions (Fig. 11.2). For example, a large pore size can create a pseudo-3D surface, which presents cells on flat surfaces, but distributed

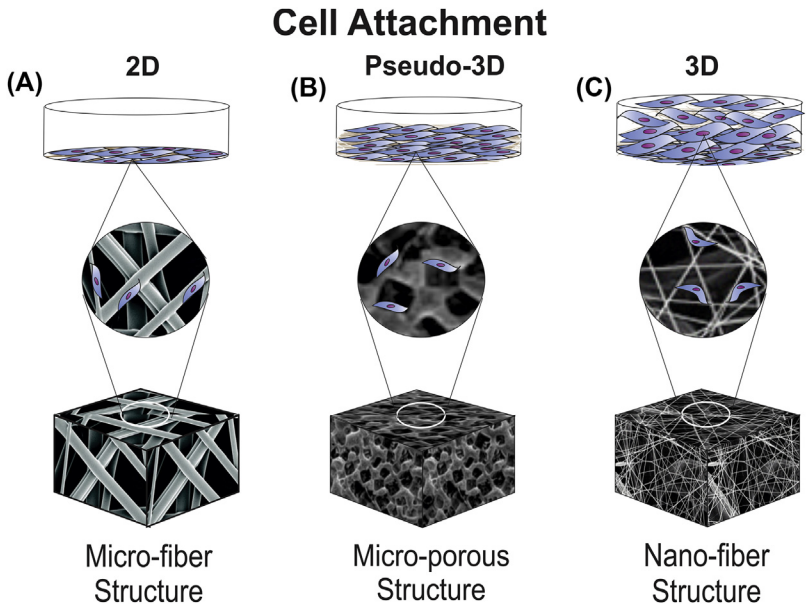

FIGURE 11.2 Schematic demonstration of cell attachment on 2D, pseudo-3D, and 3D surfaces. (A) Cells binding to scaffolds with fiber-based microscale architectures flatten and spread as if cultured on flat 2D surfaces. These cells have a polarized relationship, with one side of the cell attached to the scaffold and the other exposed to physiologic media. (B) Scaffolds containing pores, grooves, and channels of cellular dimensions provide a pseudothird dimension in the system because cells are presented with a series of effectively flat surfaces, but in all three planes. (C) Scaffolds with nanoscale architectures provide a third dimension and have larger surface areas to adsorb proteins, with many more binding sites to cell membrane receptors, allowing cell attachment to the substrate through most of their membrane surface.

throughout an 3D plan. On the other hand, in 3D cultures, the truly biomimetic scaffold, all cell surface is able to be attached to the construct surface. This is what happens with constructs nanoscale structures that have large surface area to volume ratios absorbing proteins, and allow more sites with which ECM receptors can bind [12,83,84]. That why stem cells and more specialized cells (e.g., neural, osteoblast, smooth muscle) have a greater proliferation rate on nanofiber-based than non−fiber-based scaffolds [83]. However, the concept of 3D cell attachment is not as simple as that because each side of the cell in 3D scaffolds has different possibilities for attachment. Even when cells can attach on their 360 degree surface, the 3D cell attachment modifies cell behavior through migration and differentiation and their relationship with neighboring cells as contact and communication. In addition, asymmetries in the scaffold structure can promote local distinct attachment.

The diverse nature of tissue architecture requires that different microenvironments be provided for repair or regeneration. This principle can be demonstrated in the use of scaffolds with tissue-specific pore sizes of different lengths according to tissue rigidity and elasticity. This was demonstrated for in vitro bone tissue regeneration that required pore sizes ranging from 100 to 350 μm, as well as 40−100 μm for osteoid ingrowth, and 20 μm for the ingrowth of hepatocytes [85,86]. Adult mammalian skin could be cultivated within a construct with a pore size of 20−125 μm and for liver regeneration with 45−150 μm [87]. Research has described ideal pore sizes to improve cell physiology (proliferation, differentiation, migration, matrix production, and nutrient and gas diffusion) [2,22,23] using random, nondirectional materials [88,89]. It was demonstrated that fibroporous meshes made of small-diameter fibers are less susceptible to encapsulation than are other architectures [90−92]. Many studies also described how surface charge can modify vessel ingrowth into small-fiber, fibrous encapsulation, fiber porous, the number of cells within a scaffold, and material meshes. Meshes electrospun from negatively charged polyurethane with defined fiber diameters of about 5 μm and a mean fiber spacing of 65 μm may facilitate vessel ingrowth into fibroporous mesh biomaterials have been described [93,94]. Thus the use of small-fiber, fibroporous meshes combined with surface coatings of an appropriate surface charge might facilitate specific cell nuclei densities and vessel ingrowth responses without fibrous capsule formation [94].

Other research has shown that the pore size of a construct allows vascularization of macroporous biomimetic materials in vivo [95]. Blood vessels can grow ideally within pore sizes greater than 400 μm. A pore size smaller than 400 μm of diameter will restrain the blood vessels growth, which generates vessels with small diameter. [95]. Also many studies have been conducted to describe the relationships among distinct characteristics such as porosity, pore size, and shape, and how are they interconnected, all of which are a macroporous biomimetic material for tissue ingrowth [96−99]. Among those parameters, pore size is receiving the most attention. Generally (although it is an open question), the pore size is 50−100 μm, and if they are interconnect with a larger pathway greater that 20 μm it is possible to obtain blood vessels enabling cell invasion with a greater biological response [100−102]. The first studies demonstrated that small

pores induce faster tissue ingrowth and more tissue formation [103,104], although later studies demonstrated faster bone ingrowth with larger pores [98,105–108]. Someone proposed an explanation for this apparent controversy: "smaller pores expose a much larger surface to the invading tissue elements and thus favor deposition." If we consider each of these conditions on the porous structure of constructs, it is necessary to evaluate three properties: the number of pores on surfaces (porosity), how much these pores are interconnected and how great this pathway is (interconnection size), and how great the macropore is. All of this has a direct or indirect influence on tissue ingrowth. However there is no common point of view to explain the adequate macropore size for tissue ingrowth, probably because there is no efficiently controlled production of the porous surface on the constructs. Moreover, when someone varies one parameter, e.g., the pore size, other parameters are also varied, e.g., the porosity or the size of pore interconnections [95]. Also, the charge surface, elasticity and hardness can control cell differentiation and proliferation, which can take more variables to determine the properties of pore sizes. It will be enlightening if new research takes into account the charge surface, elasticity and hardness, and porosity and interconnections to evaluate tissue ingrowth including blood vessel formation.

In tissue the physiologic conditions, cell distribution, cell survival, migration, proliferation, and differentiation are ensured by ideal uniform distribution, high porosity, and pore interconnectivity [47]. However, such criteria can be successfully determined only at early culture stages when cells occupy the pores on scaffolds and fill them with ECM constituents that limit cell perfusion. This will promote a diverse randomly distribution of cells and ECM network, enabling more identical spatial and random distribution of the pores. In addition, this is not a good biomimetic template for spatial organization during normal development, and in healthy tissue, these structures resemble scar tissue that has disoriented fibers and a disoriented structure [10,11]. More than the scale of topography (nanoscale or microscale), the presence of ridges, grooves, pockets, and planes that design the pattern of the substrate, together with its symmetry independent of the underlying material's chemistry [109], contribute to modulate cell behavior [110].

For example, microposts have been used to study cell adhesion [111–113] and stimulate mesenchymal stem cell growth [114,115]. Microchannels have been used to guide cell morphology and migration of a large variety of cells [116] and microholes have been used to enhance osteogenesis [117,118] and cell differentiation [119], study cytoskeletal morphology [120], or create platforms for the controlled growth of undifferentiated embryonic stem cells [121,122]. Other types of microgeometries have been used to optimize nonviral transfection systems [123] or investigate signal transduction in cell migration [124].

Controlled cellular alignment has a crucial role in the microarchitecture of many human tissues, dictating their biological and mechanical function. Furthermore, fiber alignment directly determines tissues' mechanical properties [125] and provides a dynamic topographic cue to resident cells [126,127].

11.2.2 Biomimetic Elements of Macroscale Scaffold Structure

Many tissue-engineering approaches must supply a scaffold that immediately mimics the functional architecture of the damaged tissue. For example, heart valve replacements need to be functional from the start, as opposed to bone repair sites that could be supported and immobilized until functionally mature. Engineering homogeneous tissues that can mimic the gross architecture of an organ is easily acquired by scaffold construction using native (e.g., collagen, fibrin, hyaluronic acid, or other ECM matrix constituents) or synthetic (e.g., polylactic acid, caprolactone, or others polymeric gels) biomaterials that function as integral units providing a 3D support to cultured cells [27]. Another method that carries the potential for engineering composite tissue structures with macro/microscale heterogeneity (e.g., osteochondral, nerve fascia, and vascular wall structures) is that of sheet-based engineering such as layer-by-layer tissue reconstruction using either cell sheets [128] or biomaterial sheets [129].

Cell-sheet engineering approaches imply the use of temperature-responsive polymer culture surfaces in which cultured cells can be retrieved or harvested as intact sheets by nondestructive (protease-free) ways [29,128]. However, the use of cell-sheet approaches for tissue engineering is not suitable for the generation of matrix-rich, cell-sparse tissues such as skin, tendon, bone, or cartilage, unlike the scaffold-based approach, because cell sheets contain relatively little ECM [128]. In addition, it is also difficult to fabricate a 3D architecture similar to that found in cardiac tissue and liver. To overcome these difficulties, is possible to layer individual cell sheets producing millimeter-thick tissues and integrate multilayer cell sheets with other biomaterial sheet scaffolds that can provide structural support to the construct.

Undoubtedly, the optimal approach to successful large-construct fabrication must rely on engineering angiogenesis by promoting the new formation of a functional microvascular network that can be integrated with other scaffold components, supporting their viability. It is important, however, that scaffold structures be engineered around the establishment of angiogenesis.

As previously discussed, Nano- and Microtopography is important for cell-surface interactions. The 3D spatial organization of sheet-based scaffolds can assume more than one form depending on the tissue guidance systems, even if it be more flexible than fifiber-based guidance. In this case of guidance directed by micrometer diameter fibrils, the cells motility and migration are constrained to only one plane at a time. The organization can limit any changes in guidance direction through consistency and range of orientation of the scaffold fibers, as well as by the efficiency of cell guidance. In contrast, interface guidance, acquired with 3D scaffolds, is an example of single-plane constraint. In this case, the restriction of motion is in only one plane, with movement permissible in the other two planes between adjacent sheets (within the interface). However, free cell motion between the layers or interfaces and along the length of the construct is possible, with cells following the interface [87]. Whereas constraint is achieved entirely by the density of the substrate (in this case, collagen), migrant cells gradually degrade it. This

emphasizes the importance of temporary, early cues to guidance [87]. This form of guidance resembles that of connective tissues present in sliding surfaces between organs and conductive tissues, such as nerves and blood vessels [130–132]. This biomimetic approach, which can control tissue structure such as cell guidance, represents a potential method for improving scaffold biofunctionality by interacting between sheet/layer 3D spatial organization and perfusion of resident cells within the participating layers [133].

11.3 Effects of Scaffold Physical Structure on Cell Behavior

11.3.1 Effects of Scaffold Topography on Cell Function

Cell function can be regulated by a nonbiological and noninvasive method termed engineering scaffold topography, which acts as an extracellular physical milieu without involving biomolecules. However, this method can provide a biomimetic cell-stimulating signal, because cells in vivo have contacts at the mesoscale structure imitating natural tissue structures, unlike smooth interfaces. We can classify topography with regard to its scale and pattern. At the basement membrane level, composition includes pits, pores, protrusions, striations, particulates, and fibers. These are structures of nanoscale dimension (5–200 nm), which may be more biomimetic in affecting cell behavior than microtopography [134]. Regarding topography patterns, cell functions such as differentiation and proliferation were evaluated, and can be affected through cell alignment along anisotropic topographies represented by grooves and ridges, or isotropic topographies, in which protrusions or pits are randomly or evenly distributed [110]. Beyond the topography classification on scale and pattern, it is important to distinct whether the patterned substrate is fabricated to allow interstitial or superficial cell seeding: in other words, to have two or three dimensions, because it is imperative regarding stromal cells that react with surface topography in different ways compared with epithelial cells. Although 3D structures resemble tissue structures with much more reliability, the role of topography in cell function regulation has been studied mainly on 2D substrates [110]. Although 2D surfaces are valuable tools for studying basic cellular response to nanotopography, translation of these findings toward clinical application will require 3D structures.

11.4 Conclusions and Perspectives

In this chapter, the influence of different scaffolds on cell behavior and function has been described, as well as their organizational levels (nano, micro, and macro scaffold structure). These topics are closely related because the main goal when developing scaffolds for tissue engineering is to mimic, with greater similarity, the structure of the tissue's ECM. To do this, a biomimetic scaffold has been developed through different methods. A large number of reports has covered the cell's reaction on 2D and 3D

substrates with different topographies. The cell–surface interaction and the cell's migration, proliferation, and differentiation in response to a specific nanotopography have also received attention. It is expected that this chapter will enlighten readers about the importance of scale and topography of scaffolds resembling the ECM structure in regenerative medicine, especially when it comes to their use in tissue engineering.

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12

Tissue Engineering: The Use of Stem Cells in Regenerative Medicine

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12.1 Introduction

12.1.1 Regenerative Medicine and Tissue Engineering

Regenerative medicine is a multidisciplinary field that includes tissue engineering. Its main objective is to propose innovative ways to keep body tissues and organs working properly, promoting health, especially when some kind of impairment occurs in them [1]. An example is tissue loss after an accident, which requires assistance to achieve tissue regeneration to regain normal function (Fig. 12.1). Tissue engineering offers an alternative by creating structures that mimic normal tissue. Tissue engineering not only regenerates injured or diseased tissue but contributes to factors that can dictate cell fate and searches for new diagnotic technology [2].

SCs are important tools to provide substitute tissue. That is why modern tissue engineering history merges with the history of SCs. Although we can consider tissue engineering history to have begun in the 17th century (when grafts were used to repair bones in Amsterdam) [3] and to have evolved until hemopoietic cell transplantation became successful in the late 1950s [4], the foundation of modern tissue engineering was established only after 1998, when SC lines became available [5]. From that moment, evolution in the field of regenerative medicine became increasingly fast and effective. To develop a tissue engineering strategy, it is essential to pay attention to four important components [6,7]:

1. Cells: they must be able to reestablish the tissue's function. For instance, if bone tissue and SCs will be used, they must be able to differentiate into bone cells.

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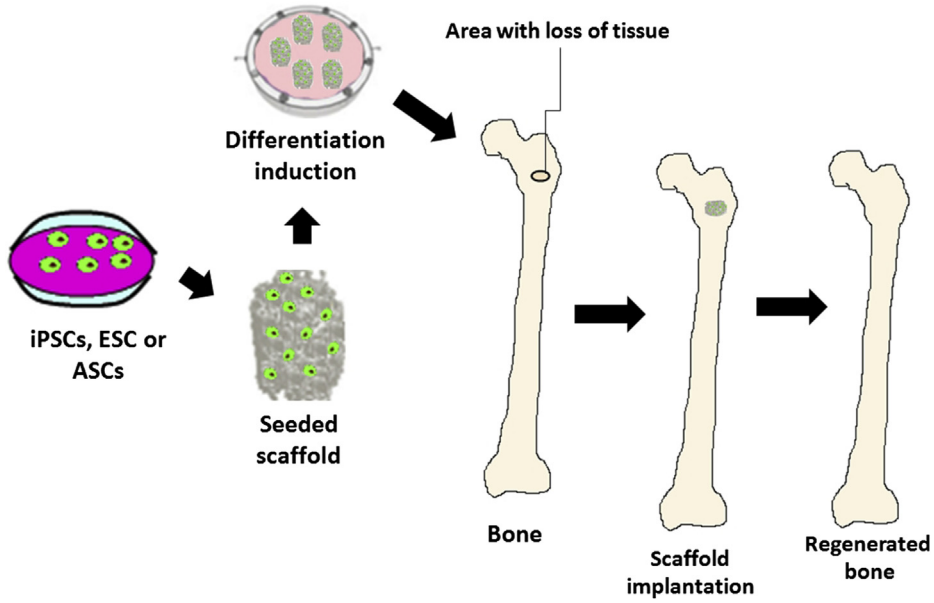

FIGURE 12.1 Stem cells (SCs) and tissue engineering. Stem cells can be used to promote tissue regeneration. *ASC*, adult SCs; *ESC*, embryonic SCs; *iPSC*, induced pluripotent SCs.

2. Culture medium: it should contain all necessary cytokines and growth factors to support cell differentiation into desirable cells, to proliferate and grow, and also to keep the desirable cells alive, performing their expected activities in that kind of tissue.
3. The porous matrix: it is necessary for the porous matrix to mimic the tissue extracellular matrix, in which cells can infiltrate to regenerate the injured tissue. It must have desirable physical and chemical properties and be made of biocompatible, bioreabsorbable, and biodegradable materials, all of which support cell growth.
4. The bioreactor: the other three components are placed into this one, which should offer ideal conditions for cell multiplication and correct displacement into the scaffold, by mimicking physiologic conditions.

In this chapter we will focus attention on briefly exploring SC types that can be used in tissue engineering to regenerate tissue.

12.1.2 Stem Cells

SCs are unspecialized cells that can differentiate into various cell types in addition to having the ability to proliferate to self-renew, maintaining the SCs population [8]. According to their source, SCs can be classified as embryonic SCs (ESCs) or adult SCs (ASCs). ESCs are not used as much owing to ethical concerns regarding their obtainment. An important technique was also developed to convert normal cells into SCs to generate induced pluripotent SCs (iPSCs).

12.1.2.1 Embryonic Stem Cells

During the vertebrate's embryo development there is a stage called blastocyst that contains a region called inner cell mass (ICM). The ICM is the source of ESCs that are pluripotent [9]. They were first obtained in 1981 from mice [9], and only 17 years later from humans [10].

ESCs can differentiate into cells from ectoderm, endoderm, or mesoderm because they are pluripotent. Thus if the intention is to regenerate cardiac tissue, ESCs can be induced to differentiate into cardiomyocytes [11]. Nevertheless if the stimuli that trigger differentiation are not present, ESCs also have the ability to maintain themselves undifferentiated for years [12].

ESCs can originate from different cell types under specific stimulations. The same sample of human ESC can be stimulated to differentiate into neural tissue, cartilage, liver, and blood vessels. The condition that dictates the different fates of cells can be the different three-dimensional structure of scaffolds [13].

In some countries the use of ESCs in research is prohibited because there is an ethical impasse arising from religious concerns that using these cells in research means killing humans. This idea comes from the fact that to obtain ESCs it is necessary to fertilize a human oocyte with human sperm, cultivated it until it reaches the blastocyst stage, and then human development needs to be interrupted to obtain ESC from the ICM (Fig. 12.2).

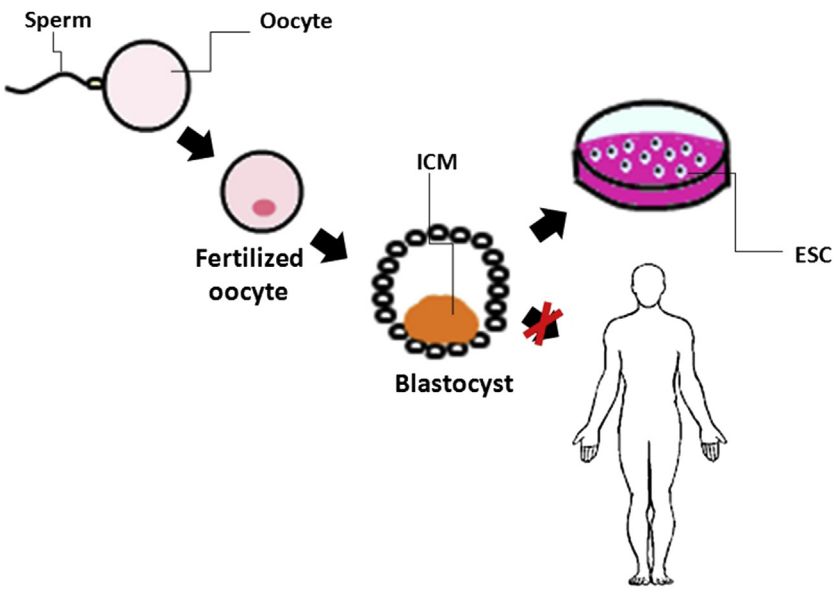

FIGURE 12.2 Embryonic stem call (ESC) obtainment. The ESC can be obtained from the inner cell mass (ICM); however, inducing fertilization to achieve the blastocyst stage and obtain ESCs means interrupting the natural development of the fertilized oocyte into a new human.

12.1.2.2 Adult Stem Cells

ASCs are an alternative to using ESCs in tissue engineering because their isolation does not require the destruction of an embryo. ASCs can be found in differentiated tissues present in the adult body. Their main role is to maintain tissues' condition in the case of injury or damage caused by sickness, promoting tissue repair [14].

ASCs have important characteristics that make them suitable for regenerative medicine. They are easy to cultivate in vitro and possess a high rate of proliferation. However, ASCs and ESCs are not the same. For example, ASCs are multipotent and not pluripotent as are ESCs. That limits the application of ASCs because the differentiation of these cells is already committed to specific cell lines. For example, hematopoietic SC (the first ASC to be studied [15]) can be found in bone marrow, umbilical cord, and placenta blood, and originates in all blood cells but not neurons.

Another important ASC is multipotent SCs derived from adipose tissue [16], which can be isolated from lipoaspirate obtained from plastic surgery.

ASCs have been used successfully to treat leukemia [17] and hepatic cirrhosis [18], for example, and to repair cartilage defects [19,20]. Studies continue to be published in this field; to cite an example, the potential of resident adult renal stem/progenitor cells is under investigation with the aim to treat acute renal injury [21].

12.1.2.3 Induced Pluripotent Stem Cells

iPSCs can also be obtained from adults, but this requires reprogramming of a common differentiated cell to an ESC-like state. These cells were first obtained from mouse somatic cells in the beginning of the 21st century [22]. iPSCs have a molecular signature and morphology that resembles ESCs, and as with ESCs, they can be directed to differentiate into a cell type of interest.

Reprogramming can be achieved, for example, by providing the sequences encoding the four protein factors to the cell: the products of genes *OCT4*, *SOX2*, *NANOG*, and *LIN28* [23]. The sequences can be delivered using adenovirus [24] and bacterial artificial chromosomes [25] or the recombinant proteins can be provided to the cells [26].

New protocols to generate iPSCs continue to be developed [27,28], always with the intention of developing an easy, inexpensive, and efficient methodology. Once iPSCs are obtained, it is possible, as with ESCs, to induce their differentiation into a desirable cell type. De Peppo and coworkers, for example, used iPSCs to achieve bone regeneration [29].

12.2 Stem Cell Isolation and Differentiation

12.2.1 Isolation of Adipose-Derived Stem Cells

The isolation of adipose-derived SCs can be performed using as a start material adipose tissue removed during plastic surgery by liposuction aspiration. This protocol was used by Bunnell and coworkers [30] and it should be performed a maximum of 24 h after obtaining the adipose tissue.

First, wash the tissue sample extensively with phosphate-buffered saline (PBS) containing 5% penicillin–streptomycin to remove the debris. Place the sample on a sterile tissue culture plate with 0.075% collagenase type I in PBS containing 2% penicillin–streptomycin aiming for tissue digestion. Pipette the sample up and down several times to facilitate the process. Incubate the sample for 30 min at 37°C, 5% CO_2.

Neutralize collagenase type I activity by adding 5 mL α–minimal essential medium (MEM) containing 20% heat-inactivated fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA). Pipette the sample up and down to disintegrate aggregates of the adipose tissue. Transfer the sample to a 50-mL tube and centrifuge the sample at 2000 rpm for 5 min, avoiding solid aggregates.

Take the samples out of the centrifuge and shake it vigorously to disrupt the pellet thoroughly and mix the cells. Repeat the centrifugation step. Aspirate all of the collagenase solution above the pellet without disturbing the cells. Resuspend the pellet in 1 mL lysis buffer and incubate it for 10 min on ice. Wash with 20 mL PBS/2% penicillin–streptomycin and centrifuge it at 2000 rpm for 5 min. Remove the supernatant and resuspend the cell pellet in 3 mL stromal medium (alpha-MEM; Mediatech, Herndon, VA) supplemented with 20% FBS, 1% L-glutamine (Mediatech), and 1% penicillin–streptomycin (Mediatech). Filter the cell suspension through a 70-μm cell strainer. Wash the cell strainer with an additional 2 mL stromal medium. Plate the sample containing the cells in a lysine-coated culture plate and incubate it at 37°C, 5% CO_2.

12.2.2 Osteogenic Differentiation

The following protocol for in vitro osteogenic differentiation was developed by Pourebrahim and coworkers [31]. It is recommended that the adipose-derived SCs be in passage 3 culture for use.

Seed 5×10^6 ASCs in 3 mm × 3 mm × 3 mm hydroxyapatite (HA)/β-tricalcium phosphate (β-TCP) scaffold (Ceraform, Teknimed, France): 60% HA and 40% β-TCP with a mean pore size of 200–800 mm. The medium should be an osteogenic one, containing 50μg/mL ascorbic acid 2-phosphate (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA), and 0.1 mM dexamethasone (Sigma, USA). Incubate the seeded scaffold in osteogenic medium at 37°C and 5% CO_2 for 21 days.

12.3 Cells Maintenance and Characterization

12.3.1 Adipose-Derived Stem Cells

Adipose-derived SCs isolated from adipose tissue can be identified by some characteristics such as adherence to cell culture plates after isolation, the ability of the cell to differentiate into various lineages, and the expression of specific markers [32]. The last is the least laborious one and it uses a flow cytometer.

Cells can be stained simultaneously with phycoerythrin conjugated monoclonal antibody to CD44 (ab58754; ABCAM Antibodies, Cambridge Science Park, UK), and

fluorescein isothiocyanate-conjugated monoclonal antibody to CD90 (ab22541; ABCAM Antibodies) before analyses through flow cytometry [31].

Adipose-derived SCs also express a high level of markers CD49d, CD105, CD13, and CD7 and can maintain this high expression for at least 3 months and seven passages of in vitro culture [33].

The capacity to differentiate into various lineages requires more time and effort. Adipose-derived SCs can be stimulated to differentiate, for example, into adipogenic cell types (culture medium supplemented with 0.5 mM isobutylmethylxanthine, 50 μM indomethacin, and 0.5 μM dexamethasone with a change of medium every 3 days until mature adipocytes are obtained), osteogenic cell types (culture medium supplemented with 1 nM dexamethasone, 2 mM β-glycerolphosphate, and 50 μM ascorbate-2-phosphate, with a replacement of medium every 2–3 days), chondrogenic cell types [high-glucose Dulbecco's MEM supplemented with 500 ng/mL bone morphogenic protein-6, 10 ng/mL transforming growth factor-β3, 10^{-7} M dexamethasone, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL proline, 100 μg/mL pyruvate, and 50 mg/mL insulin, human transferrin, and selenous acid and premix (Becton Dickinson: 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin, 5.35 mg/mL linoleic acid, with a replacement of medium every 2–3 days)], and neural cell types [culturing in neurobasal medium supplemented with B27 (1:50), 20 ng/mL basic fibroblastic growth factor, and 20 ng/mL epidermal growth factor for 4–7 days] [30].

12.3.2 Generated Bone Cells

Bone cells generated inside the scaffold can have characterization performed in vitro and function accessed in vivo. Among analyses that can be performed to characterize bone cells from in vitro or in vivo samples are the determination of osteopontin concentration, alkaline phosphatase activity, calcium content, gene expression profile, and histologic examination (with or without antibodies).

12.3.2.1 Determination of Osteopontin Concentration, Calcium Content, and Alkaline Phosphatase Activity

Osteopontin is a protein with important roles in bone biomineralization [34] and remodeling [35]. Its concentration can be determined by using the culture medium present in in vitro cell culture through the human osteopontin enzyme-linked immunosorbent assay kit [36].

Cells in culture or from in vivo samples can be used to determine the calcium content; trichloroacetic acid in water [0.15 mL of 5% (vol/vol)] can be added to them for 30 min under repeated pipetting. Then the supernatants can be submitted to calcium (CPC) Liquicolor assay [36].

Alkaline phosphatase is an enzyme largely present and active in bone tissue, and its increased activity is observed during bone formation. The seeded scaffold can be exposed, on ice, to lysis buffer [PBS, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium

deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate, 0.1 mg/mL phenylmethylsulfonylfluoride, and 0.3% (vol/vol) aprotinin] until its disintegration. After centrifugation, the supernatant can be incubated at 37°C until the development of yellow color, in alkaline buffer and nitrophenyl-phosphate substrate solution. At this stage the reaction is stopped with 0.5 M NaOH. Enzyme activity can be accessed by absorbance reading at 405 nm and comparison with a standard curve may be obtained from *p*-nitrophenol solutions of known concentrations [36].

12.3.2.2 Histologic Analyses (With or Without Antibodies)

Histologic analyses to evaluate bone regeneration can be performed using biopsies of bone obtained from animal submitted to in vivo seeded scaffold transplantation. The samples can be fixed in 10% buffered formalin and decalcified in formic acid and sodium citrate. After they are washed, submitted to dehydration, cleared, included in paraffin, and processed, they can be stained using Masson trichrome. Histomorphometric analysis can be performed with measurements of the newly formed bone through automated image analysis software (IHMMA, Version 1, Sbmu, Iran) [31].

Inclusion in paraffin is also useful for immunohistochemical analyses, and methylmethacrylate inclusion may be used for other histologic analyses.

Staining with hematoxylin–eosin and Masson trichrome procedures, it is possible to observe collagen generation (positive blue areas); staining the samples with Goldner, osteoid formation can be accessed (positive red areas) [36].

In histologic analyses using antibodies, it is possible to detect the presence of, for example, osteopontin, bone sialoprotein, and osteocalcin.

12.3.2.3 Gene Expression Analyses

The analysis can be performed after RNA extraction and synthesis of complementary DNA strands. The hybrid DNA–RNA can be submitted to real-time polymerase chain reaction to quantify transcript levels of alkaline phosphatase, osteocalcin, collagen I, and osteopontin, for example, in relation to a housekeeping gene of preference such as glyceraldehyde 3-phosphate dehydrogenase [29,31].

RNA can also be used to generate a double-stranded labeled template (start material for microarray analyses) with the aim to access the expression rate of genes involved in osteogenesis [29].

12.4 Conclusions and Perspectives

SCs possess a large array of clinical applications owing to their large capacity to differentiate into different cell types according to the scientific challenge. Regenerative medicine has developed with SC technologies and made new strategies possible to surpass the barrier of immunologic rejection in transplants from a donor that is not the receptor. It is already possible to use SCs and it a common procedure for a person to transform this cell in vitro to a cell of interest inside a scaffold and to use the result to

transplant it back to that person as a new tissue derived from his or her own cells [37]. Adipose-derived SCs, for example, are being used to repair defects in soft tissues [38,39] and to generate bone tissue as mentioned in the protocol section [31].

New scaffolds are being developed from biomaterials [40] and commercial options are available. It is also possible to use as scaffold decellularized extracellular matrix [41] originated from an intact organ that undergoes several washings, leaving only the extracellular matrix. Growing organs in vitro and ex vivo is now also a reality [42]. However, there are still some limitations to developing blood capillaries in the tissue graft (especially in the inside part of the engineered tissues), and the clinical applications of laboratory-made organs remain a distant prospect.

Another application for SCs lies in the diagnostic field, because some conditions result in an increase in SCs in bloodstream, which can be used as a biomarker. For example, after myocardial infarction, patients have circulating SCs in the peripheral blood; thus these cells may be an important marker for risk evaluation in patients with cardiac disease [43]. Patients affected by osteoporosis possess mesenchymal SCs circulating as a consequence of the impairment of osteoblast differentiation [44]. Despite all of the progress made in the field of SC, further research is required before they are safe enough to be applied largely to strategies in humans.

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13

Tissue Engineering and Regenerative Medicine Solutions for the Abdominal Organs

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13.1 Introduction

In recent decades, organ transplantation technology and techniques have progressed substantially, resulting in its emergence as the reference standard treatment for all diseases leading to irreversible organ failure [1]. Over 100,000 solid organ transplants are performed every year worldwide including approximately 70,000 kidneys and 20,000 livers [2]. Although success rates have improve dramatically over the years, the challenges of donor organ supply and complications related to immunosuppression remain chief obstacles in the way of further progress for the treatment of irreversible end organ damage. End-stage renal disease, for example, represents an extraordinary public health burden worldwide, and has contributed to the kidney's being the most commonly transplanted organ. In 2014, however, about 15,700 people received kidney transplants while over 100,000 candidates remained on the waiting list at the end of the year owing to the limited supply [3,4]. Those candidates who do receive organ transplantation can develop secondary neoplasms or other adverse sequelae associated with prolonged immunosuppression.

Developing methods in tissue engineering and regenerative medicine (TERM) have shown great promise for addressing these two challenges hampering contemporary transplantation. Investigators within TERM and its subdisciplines seek to restore function by facilitating the transition from a diseased state to a healthy one in tissues that might not perform this task spontaneously or sufficiently [5]. This is commonly accomplished in experimental and clinical settings via the therapeutic administration of cellular material, naturally derived or synthetic biomaterials, or a combination of the two [6]. One way in which biomaterials are often employed are as geospatially appropriate

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"scaffolds" upon which to seed cells or to allow the migration and proliferation of cells in vivo as a way to replace damaged tissue.

Significant interest has been growing in the cell-scaffold method, particularly for its use in abdominal organs, many of which are solid, complex, and modular. Given the dramatic success of surgical transplantation for the treatment of diseases afflicting the abdominal organs, researchers have sought to employ TERM methodologies to bioengineer functional organs or organ-shaped scaffolds ex vivo for subsequent implantation into the patient. Indeed, it has been recognized that transplantation historically shares many of the same guiding principles with the more recently developed TERM disciplines [7]. For example, the idea of replacing diseased tissue by the direct administration of bioactive cellular material is certainly applicable to both. By seeding the appropriate cellular material onto a biocompatible organ-shaped scaffold, an investigator can bioengineer a functional organ for use as a replacement [8].

A new technology under investigation and experimental characterization employs whole-organ decellularization to generate a native three-dimensional extracellular matrix (ECM) scaffold that preserves native tissue architecture, including intricate vascular networks and structural components [9]. It has long been understood that cells require supporting structures and cues to grow, expand, and exert function [10]. In fact, biomaterial scaffolds are employed for this very purpose. Nevertheless, ECM, the defining feature of connective tissue, constitutes the supporting structure in vivo and is specialized to carry out this function, making it the optimal choice. Its interaction and cooperation with adjacent cells is absolutely necessary for physiologic functioning across the board [11]. Tissue decellularization, achieved via perfusion of detergent through an organ's vasculature, leaves behind ECM scaffolds that can be used as the ideal biomaterial structure destined for recellularization [12]. With regard to the state of the art of abdominal organ engineering, as will be discussed in subsequent sections, both experimental and clinical investigations have been extremely promising [13].

Whether by direct healing via administration of cellular material and biomaterials or the ex vivo fabrication of whole organs for subsequent transplantation, although it still very early in its development, TERM is not limited by an organ shortage because cell cultures can be expanded in the laboratory and biomaterials can be manufactured without donor morbidity or mortality. Furthermore, the use of autologous cells would overcome the need for brutal immunosuppressive regimes that carry significant risks for the patient [14]. The aim of this chapter is to review the most important developments in TERM as they pertain to each abdominal organ.

13.2 Cell Types in Organ Bioengineering

TERM approaches to organ bioengineering typically involve cellular material either alone or in conjunction with a supporting scaffold. Determining the proper cell type most suitable for regeneration and/or seeding onto a scaffold remains a chief objective for each abdominal organ system. Experimental and clinical investigations have

commonly involved fully differentiated adult cells, organ-specific progenitor cells, or pluripotent stem cells [15].

Adult somatic cells hold the advantage of being well-defined and readily isolated. However, compared with progenitor and stem cells, their lifespan and regenerative potential are limited. Furthermore, they lose function and differentiability when removed from the native environment. Their use is confined to the specific organ from which they are obtained. For example, cultured hepatocytes or tubular cells can be used only for liver scaffolds or renal scaffolds, respectively, and they would likely be minimally proliferative. Stem cells, however, possess the notable capacity of self-renewal and differentiability. Their applicability has a broader range than do adult somatic cells. Within the category of stem cells, the two types most commonly used are adult stem cells and embryonic stem cells (ESCs) [16].

ESCs are pluripotent stem cells derived from the inner cell mass of the embryo during the blastocyst stage. As hinted at by their purpose in the developing organism, they hold the ability to differentiate into any cell type when induced via specific environmental stimuli. Furthermore, they can self-renew indefinitely [17]. Researchers have successfully induced their differentiation into cell types from all three germ layers in vitro including cardiac cells [18], endothelial cells [19], neurons [20], insulin-producing cells [21], and renal tubular cells [22]. In the context of abdominal bioengineering, the use of ESCs would find wide applicability for experimental and clinical purposes. Nevertheless their use is significantly limited by ethical issues [23] along with potential teratogenicity [24]. Induced pluripotent stem cells (iPSCs) are generated via special reprogramming of adult cells resulting in their dedifferentiation into pluripotent stem cells. This process does not involve manipulation or destruction of primordial embryos, thus avoiding the ethical obstacles posed by ESCs. They have been derived from several cell types in experimental investigations, including human keratinocytes [25] and umbilical cord blood mononuclear cells [26].

Adult stem cells, however, benefit from wide availability in the adult organism and the relative ease of acquisition. They are thought to perform a major role in cellular repopulation and the replenishment process over time [27]. Indeed, stem cells residing in anatomical "niches" are responsible for the continual replacement of damaged or dead tissue compartments in response to microenvironmental cues [28]. Wound healing and bone remodeling are familiar examples of tissue systems with high turnover. Tissue systems with recognized niche populations of stem cells include skin [29], fat [30], intestine [31], and kidney [32], among others. Unlike somatic cells, they can be induced to differentiate along lineages other than the organ system from which they are derived. For example, fat stem cells have been successfully differentiated into cartilage cells, hematopoietic cells, neurons, bone cells, and skeletal muscle cells [33]. Nevertheless their differentiation potential is generally recognized to be limited to the germ layer from which they are derived, unlike the pluripotency of the ESC.

Adult cells harvested for regenerative purposes can be autologous, i.e., acquired from the patient. Autologous cells are beneficial because they are shielded from the recipient patient's immune system, because they would not be recognized as nonself. Given their

ability to circumvent rejection, autologous cells represent the most desirable cell type in TERM investigations. Allogeneic cells are another option; acquired from another individual, their use is complicated by potential immunogenicity [34]. Heterologous cells derived from nonhuman organisms (e.g., porcine) represent another option under investigation. Ideally, cells used for TERM purposes would be expanded ex vivo and either (1) introduced directly into the patient to repair the damaged organ system, or (2) seeded onto a supporting scaffold for eventual reimplantation into the patient.

13.3 Gastrointestinal Tract

The intestinal and bowel conduits are complex, hollow structures involved in nutrient passage and absorption, digestion, excretion, and the innate immune system. The human tract undergoes continuous turnover and renewal throughout life, a process driven by the activity of resident stem cells [35]. This preexisting process of regeneration and renewal underscores the unique applicability of methods in regenerative medicine for bowel engineering. Taking into consideration the microanatomical and functional characteristics of the organ, the endeavor would aim toward regeneration of smooth muscle, specialized neuronal tissue, and a mucosal-epithelial bilayer. Correspondingly, one of the chief obstacles is the functional regeneration of diverse motility patterns, because motility is essential to proper intestinal functioning. Nevertheless, preliminary investigations have been promising [36].

The stomach has a major role in the gastrointestinal tract as a storage reservoir and digesting organ. Methods in tissue engineering could find applicability in cases involving gastrectomy, peptic ulcer disease, caustic ingestion, or trauma. Simple approaches have involved the use of collagen scaffolds to patch stomach wall defects [37]. This approach produced an intact mucosal layer with proton pumping cells but failed to recapture motility functioning when challenged with acetylcholine. An affiliated group later attempted again to employ collagen scaffolds but soaked them in autologous blood and bone marrow aspirate [38]. These collagen constructs were sutured over 5-cm circular defects of the stomach wall in canine models. The regenerated stomach retained native tensile strength and histologically showed villous mucosa and underlying dense connective tissue. Nevertheless the authors concluded that their engineered construct, although of improved biocompatibility, was still short of a functional gastrointestinal segment.

Speer et al. isolated organoid units from mouse glandular stomach and seeded them onto biodegradable scaffolds composed of polyglycolic acid (PGA) coated with poly-L-lactic acid (PLA) and type I collagen [39]. The seeded scaffolds were implanted into the omentum of adult mice and harvested at designated times for analysis. The constructs were found to grow and proliferate as expanding spheres with simple columnar epithelium organized into gastric glands and an adjacent muscularis. Mucous, enteroendocrine, chief, and parietal cells were all expressed in the regenerated epithelium. Maemura et al. employed biodegradable polymers seeded with stomach epithelial

organoid units obtained from neonatal rats in various investigations [40]. They replaced recipient rat stomachs with tissue-engineered stomachs and discovered no evidence of stenosis or obstruction at the sites of anastomoses. Furthermore, histologic evaluation demonstrated well-developed vascularized tissue and stratified smooth muscle layers [41]. They also used the seeded scaffolds to patch gastric wall defects in rat models, with considerable success [42].

The small intestine is the primary segment for nutrient absorption within the gastrointestinal tract. This process depends on the integrity of microvilli structures that line the intestinal epithelium. Early regenerative investigations employed autologous tissue patches to repair defects in the intestinal wall. Later studies underscored the potential use of absorbable biomaterials as a patch-scaffold to facilitate tissue ingrowth [43]. Commonly used biomaterials have been collagen scaffolds, PLA, and PGA, among others. Fibrin hydrogels demonstrated sufficient mechanical rigidity to allow self-organization of circular sphincteric and intestinal smooth muscles, even in humans [44,45].

The advent of organ decellularization allows for the development of natural scaffolding material with an intact ECM, preserving the structural and biochemical properties necessary for tissue growth while obviating the need for biocompatible polymers. Notably, the ECM preserves the villus-crypt architecture and vasculature that supports intestinal epithelial regeneration [46].

The Vacanti group employed intestinal organoid units acquired from the small intestine and remodeled them on biocompatible matrices [47]. These implants were able to rescue morbidity associated with massive bowel resection in rat models. Chen et al. implanted ECM-derived submucosal tubes in canine models with small bowel resections without seeding material, to ascertain the regenerative potential [48]. The investigation used four dogs and concluded that this method was not feasible, although patch repair showed the potential for wall defect regeneration.

Numerous studies reported the use of bone marrow–derived mesenchymal stem cells (MSCs) for the regeneration of intestinal tissue. Hori et al. were able to regenerate intestinal segments by seeding MSCs onto collagen scaffolds [49]. Their constructs showed a transient distribution of α-smooth muscle actin-positive cells but failed to regenerate a muscle layer, which is essential for peristalsis. ESCs and iPSCs have also been used to regenerate functional or semifunctional intestinal tissue, under proper induction by growth factors [50,51]. Watson et al. reported on the generation of human intestinal organoids procured in vitro from human ESCs or pluripotent stem cells that were able to engraft and vascularize in mouse models [52]. In vivo transplantation resulted in the marked expansion and maturation of differentiated epithelium and mesenchyme and also demonstrated digestive functions.

Although clinical translation has not yet proven feasible, findings and ongoing studies are showing great promise. Given the limited results of employing cells or biodegradable scaffolds alone, adult or stem cell seeding onto ECM-based scaffolds will likely achieve optimal results in future investigations. Identifying the most suitable cell type remains a

primary challenge for future studies, along with establishing a scaffold that recaptures the microenvironmental cues of native intestine.

13.4 Bladder

The architecture simplicity of the hollow bladder along with its relatively straightforward singular functions (i.e., storage and passage) renders it particularly amenable to bioengineering solutions. Furthermore, the limitations of commonly employed detubularized bowel segments for bladder replacement underscore the advantages of a tissue engineering approach [53]. The application of regenerative medicine technologies for bladder repair has mostly involved two basic approaches: (1) a cell-free, scaffold-only approach; and (2) scaffolds seeded with cells. The scaffold-only strategy employs a biocompatible and geometrically appropriate matrix to create an environment permissive of cellular infiltration and proliferation, which constitute the regenerative process. The cell-scaffold approach uses uroepithelial cells, smooth muscle cells, and/or other cell types and seeds them onto biocompatible scaffolds to bioengineer a bladder construct ex vivo for the purpose of eventual implantation. In theory, the latter approach is advantageous owing to the preparation of a more fully differentiated and phenotypically mature construct before implantation. In this way, it more closely resembles contemporary methods in surgical transplantation.

Both naturally derived materials such as collagen and alginate and synthetic polymers such as PGA and PLA have been employed as bladder wall substitutes in both experimental and clinical investigations. Naturally derived materials such as collagen have the advantage of enhanced biocompatibility, whereas synthetic materials have the benefit of large-scale manufacture with closer control over microstructural and mechanical properties. Both have been confirmed as being compatible with uroepithelial and smooth muscle tissue in experimental settings [54]. Decellularized scaffolds such as intestinal submucosa and acellular bladder matrices have also been employed in experimental investigations [55,56]. Nevertheless, these approaches are limited by the absence of cellular material, which can result in scarring, reduced reservoir volume, and graft contraction [57]. Furthermore, studies suggest that this approach does not adequately regenerate the muscular compartment, resulting in suboptimal elasticity and contractility [58].

More recent investigations suggest that the use of autologous cells seeded on bladder-shaped biomaterial scaffolds is the best strategy in bladder engineering [59]. Atala et al. reported the first human clinical study of cystoplasty using cell-seeded collagen scaffolds, with dramatic success [60]. Seven patients needing cystoplasty were implanted with bladders manufactured using autologous urothelial and muscle cells expanded in culture and seeded on bladder-shaped scaffolds. Follow-up examination (mean, 46 months) showed increased compliance, decreased end-filling pressures, increased capacities, and longer dry periods over time. Raghavan et al. obtained a full-thickness

bladder biopsy from a 17-year-old with neurogenic bladder caused by an iatrogenic spinal injury [61]. Urothelial and smooth muscle cells were isolated from the biopsy, expanded in vitro, and seeded onto a biodegradable scaffold, which was allowed to mature. This "neobladder construct" was then implanted surgically to augment bladder capacity and wrapped with omentum to provide neurovascular support. No follow-up has yet been reported.

The most suitable cell type for reseeding in bladder engineering is still under investigation, even with the encouraging results using smooth muscle and urothelial cells. Of course, autologous cells are highly advantageous because of their lack of immunogenicity [62]. Urothelial progenitor cells concentrated in the bladder neck and trigone area represent a strong candidate for the source of autologous material [63]. Amniotic fluid–derived [64], bone marrow–derived [65], and adipose-derived stem cells [66] are other potential autologous options because they have all have been shown to differentiate into bladder tissue under proper guidance.

There has been rapid growth in bladder engineering technology over the past several years [67]. Indeed, clinical trials are finishing up or are ongoing [68]. With proof-of-concept more than established, investigators must now focus on optimizing protocols while clinical trials lend increasing credibility to the technology.

13.5 Kidney

The kidney is a complex solid organ with important roles in endocrine, metabolic, and immunologic homeostasis. Specifically, the kidney functions to filter the blood and to concentrate urine with toxic metabolic waste products, which highlights the crucial role of the kidney. Currently, dialysis and transplantation represent the reference standard treatment modalities for chronic kidney disease. However, dialysis does not cure kidney damage; it assumes a portion of the kidney's functions, particularly filtration. As such, dialysis leaves much to be desired. With the growing worldwide burden of hypertension and diabetes, the prevalence of chronic kidney disease is reaching epidemic proportions [69,70] and the need for functional kidneys is rising correspondingly.

The translational success of TERM strategies for the replacement of renal function depends on a better understanding of the native repair and regenerative processes occurring in vivo at the cellular and molecular levels [71,72]. Because of the proportionally increased metabolic demand of the kidney and its waste and toxin filtration function, renal tubular cells are constantly under the threat of acute injury and oxidative stress. For this reason, these cells contain unique regenerative abilities when damaged. In fact, researchers have observed surviving renal tubular cells giving rise to a new population of the cells after physiologic kidney damage [73–75]. Nagaike et al. observed that unilateral nephrectomy induces mitogenesis and hypertrophy in the contralateral kidney [76]. Cochrane et al. induced cortical tubular cell atrophy, tubular dilation, and interstitial macrophage infiltration via ureteral obstruction in a murine model of renal

injury. They showed that reversal of the obstruction induced a rapid process of reconstruction and interstitial matrix expansion that ultimately restored the glomerular filtration rate [77]. However, continuous and supraphysiologic damage characteristic of chronic kidney disease overpowers the regenerative properties of these cells. Furthermore, the growth of new nephrons, i.e., frank nephrogenesis, has not been shown to occur [78].

Researchers have thus explored the potential of cell therapy to restore kidney function in the face of widespread damage. Cell-based approaches seek to achieve kidney repair and regeneration in situ upon therapeutic administration. They are based on the observation that exogenously supplied cells stimulate the repair and proliferative process [79]. Progenitor cells harvested from the proximal tubules, glomerulus, peritubules, and papillae have all demonstrated some level of therapeutic capacity [80,81]. Stem cells obtained from urine have also shown some potential to reverse kidney damage and aid in the repair process [82].

Recruitment of bone marrow−derived MSC, a type of adult stem cell, to renal tubular damage sites has enhanced regenerative outcomes [83]. It is thought that MSCs stimulate the release of more proliferative mediators from native cells to enhance their own regenerative process. MSCs can also secrete specific chemokines, cytokines, and growth factors to promote growth and cellular protection from further damage. Using the nephrotoxic drug cisplatin to induce acute kidney damage in mice models, researchers have looked at MSC-induced proliferation and regeneration of epithelial cells in damaged renal tubules [84]. Although the use of MSCs is promising for acute kidney injury, there is a paucity of current literature regarding their use in models of chronic kidney disease, which hosts a different set of challenges and conditions. A study using rat models found that chronic kidney disease leads to premature senescence of MSCs and inhibits their typical regenerative potential, potentially limiting their use [85].

Enhanced understanding of the regenerative properties of renal cells has led to another avenue for the treatment of kidney damage: the use of embryonic kidney tissue. These primordial cells have been shown to integrate within adult organ systems, richly vascularize, and form new, mature nephrons (i.e., frank nephrogenesis) [86,87]. Ureteric bud and metanephric mesenchyme cultures have shown, though the inherent ability of mesonephric duct tissue, the ability to form collecting ducts through tubulogenesis and epithelization. The in vitro tissue was implanted into mice models and survived for over 5 weeks, demonstrating glomerular vascularization in vivo and thereby pointing to the therapeutic potential of these primordial tissues [88]. Imberti et al. implanted renal primordia under the kidney capsule of male rats with kidney injury [89]. The grafts went on to develop glomeruli and tubuli that filtered blood and produced urine in cyst-like structures. Furthermore, they initiated a process of regeneration in host tissue segments indicated by increased cell proliferation and vessel growth.

Researchers have explored the potential to bioengineer kidneys de novo for eventual implantation into patients, harnessing the advantages of surgical transplantation. The most promising strategy under investigation has been the cell-scaffold approach. Native

kidneys are stripped of cellular material using detergents while preserving the ECM upon which to seed new cellular material. ECM scaffolds produced from animal and human kidneys have been shown to retain their innate biomolecular and biophysical properties along with their external and internal three-dimensional architecture [90]. Upon seeding with cells, signaling cytokines and growth factors from the retained ECM can guide cell differentiation toward organ-specific phenotypes and promote vasculogenesis necessary for full integration into the host.

Bonandrini et al. used ESCs to recellularize rat whole-kidney scaffolds by perfusing them through the renal artery along with circulating cell medium [91]. The investigators observed the loss of pluripotency and the shift toward mesoendodermal lineage, supporting the idea that decellularized kidneys can undergo rapid recellularization of vascular structures and glomeruli and induce differentiation along appropriate pathways. Nakayama et al. decellularized both the kidneys and lungs of rhesus monkeys and reseeded them with undifferentiated ESCs [92]. Expression levels of tubule markers and other kidney genes were higher in cells cultured on kidney ECM compared with those on lung ECM, which lent evidence to the idea that ECM guides differentiation selectively.

Our group successfully decellularized porcine kidneys and implanted them into porcine hosts with evidence of preserved functionality [93]. Moving to more clinically relevant models, a source of human kidneys available for bioengineering investigations was identified: organs originally intended for transplant purposes but discarded because of functional or anatomical anomalies (approximately 2600 kidneys annually in the United States) [94]. Upon detergent-perfusion, the successful production of viable ECM scaffolds using discarded kidneys was confirmed [95]. In a later study, amniotic fluid–derived stem cells were seeded on discarded kidney scaffolds to assess the potential to recellularize [96]. The cells attached, proliferated, and furthermore synthesized and secreted various chemokines and growth factors involved in angiogenesis and matrix remodeling including vascular endothelial growth factor, transforming growth factor-α, interleukin-8, metalloproteinase-2, and tissue inhibitor of metalloproteinase-2. Furthermore the cells were found to express early kidney developmental markers after 2 weeks of culture on the ECM, suggesting regeneration.

Both cell therapy and cell-scaffold strategies demonstrated potential for the treatment of kidney disease, which is currently limited to dialysis and transplantation. Further studies are needed to determine the best cell type for renal regeneration: adult stem cells, progenitor cells, or frank stem cells.

13.6 Pancreas

The pancreas is functionally composed of two parts, the exocrine pancreas and endocrine pancreas. Despite deriving from the same embryologic cells, these components are structurally different and are important for different physiologic systems. Although it accounts for 90–95% of the pancreas mass, the exocrine pancreas is not essential for

survival owing to exogenous pancreatic digestive enzyme replacement therapy [97]. In contrast, the endocrine pancreas accounts for only roughly 2% of the pancreas mass while serving a vital physiologic role. The endocrine pancreas is not localized to a specific region of the pancreas but is interspersed around islets of Langerhans (highly vascularized centers) over the entire tissue. There are five main types of endocrine pancreatic cells (alpha, beta, delta, gamma, and epsilon) but the beta cell has a particularly vital role in glucose homeostasis and energy metabolism, thus drawing the most attention for bioengineering research and treatments with regard to endocrine pancreatic deficiencies.

One of the most common pathologic endocrine deficiencies is type 1 diabetes mellitus, in which beta cells are destroyed by the host's own immune system. About 1 in 300 individuals in the United States is diagnosed with type 1 diabetes mellitus by age 18 years [98]. Therapies for type 1 diabetes mellitus focus on the supplementation of exogenous insulin that is normally produced by beta cells. However, this method requires strict patient compliance and adequate patient education. Innovations have removed the need for conscious dosing. Although exogenous insulin therapy is effective at preventing acute decompensation, fewer than 40% achieve and maintain therapeutic targets [99]. The most commonly accepted treatment regimens lower the incidence of diabetic emergency. However, they require lifelong pharmacologic intervention without the possibility of remission. Beta-cell replacement, whether by whole pancreas or individual islet cell transplantation, is currently the only method to restore long-term, stable euglycemia in type 1 patients with diabetes.

Pancreatic transplantation is an invasive option that provides the patient an opportunity to be free from strict glycemic control by way of exogenous insulin. However, it is typically offered to adults only in conjunction with kidney transplants because of the high risk of surgical complications and lifetime of immunosuppressant medications [100]. Nevertheless recipients exhibit greater improvements in the micro- and macrovascular complications of diabetes while enjoying a great quality of life. The 5-year organ survival rate for pancreatic transplantations is roughly 50% [101].

An alternative solution involves the ectopic implantation of purified islet cells and simultaneous depletion of T-cells to recapture beta cell function while suppressing the autoimmune component of the disease [102,103]. Human islet cell purification and implantation have the same 5-year insulin independence rate as pancreatic transplantation but it has been found have a lower risk of complication and represents a less invasive solution [104].

Contemporary investigations employ TERM solutions to treat the disease without the need for lifelong pharmacologic intervention. One of the most heavily researched areas involves the induction of beta cell regeneration within host islets. There is a current debate in the research community regarding the source material of endogenous beta cell regeneration: It is still unknown whether existing beta cells or resident stem cells are primarily responsible [105,106]. Insulin-producing cells derived from ESCs are physiologically closer to beta cells than any adult stem cell–based beta cell. The conversion of

human ESCs toward beta cell phenotypes has been achieved through the initial differentiation into endodermal cells that can be further differentiated into insulin-producing endoderm derivatives [107,108].

To avoid the controversy of ESC procurement, advancements in beta cell replacements have turned toward iPSCs. Through the use of retroviruses to manipulate gene expression, adult somatic cells can be reprogrammed and individualized to specific patients. In animal models, experimental therapy has already demonstrated long-term correction of hyperglycemia [109]. Although promising, the technology surrounding iPSCs still faces obstacles posed by premature senescence, subchromosomal abnormalities, and destruction of patient-derived cells caused by autoimmune phenomena.

The recellularization of ECM-based scaffolds has also been under investigation regarding the de novo fabrication of pancreas organs. It has been observed that islets cultured in vitro on ECM increase the longevity of insulin production in response to glucose [110], likely owing to natural growth factors within the ECM that direct cell lines toward beta cells. In addition, studies have hinted at the potential use of xenographic transplants. Sani et al. demonstrated that a porcine pancreas could be decellularized while maintaining ECM composition and patent vasculature, and can subsequently be seeded with human amniotic fluid–derived stem cells [111]. The seeded islets demonstrate the ability to support normal pancreatic function by showing an increased metabolic rate as well as insulin secretion compared with isolated islets. Goh et al. successfully decellularized mouse pancreata and recellularized them with acinar and beta cell lines [112]. They showed successful engraftment within the three-dimensional decellularized pancreas along with strong up-regulation of insulin gene expression. Their findings supported the utility of whole-pancreas ECM for enhancing pancreatic cell functionality. Other investigators have reported similar findings [110].

Whereas all of these technologies may restore the functionality of destroyed islets, the primary pathology of type 1 diabetes is autoimmune, which will remain a persistent obstacle if left unaddressed. Future strategies must incorporate adjuvant immunomodulation or autologous tissue into current technologies to terminate the chronic disease state and preserve endocrine pancreas function.

13.7 Liver

The liver has an innate capability to regenerate beyond the potential of other abdominal organs. In the event of injury to less than 70% of its total, the liver can fully regenerate within 6 months of the trauma [113]. However, this occurs through cellular hyperplasia of the residual liver and is not considered true regeneration [114]. Instead there is an observed compensatory enlargement of the remnant liver to meet the functional demands of the body. These properties of the liver make it uniquely amenable to methods in abdominal organ bioengineering. Current investigations involve two distinct regenerative pathways. One, similar to approaches in other organ systems, involves the use of

synthetic scaffolds or decellularizing an existing liver. These scaffolds would then be reseeded with host cells and allowed to regrow either in vivo or in vitro. Another option is to manipulate the existing cellular physiology of liver hepatocytes to induce liver regeneration, eliminating the need for a scaffold because the host will be able to use existing liver tissue.

Research has focused on the use of decellularization and recellularization to manufacture livers with healthy, disease-free tissue [115,116]. For liver regeneration to become clinically relevant, more studies must be performed to demonstrate:

1. that whole liver can be decellularized and that this can be applicable to human liver,
2. that human hepatocytes can be seeded and maintain viability on a decellularized scaffold, and
3. that the regenerated liver cells can match the prior functioning of the native liver in terms of protein synthesis and breakdown, detoxification, and bilirubin metabolism.

Studies have demonstrated a technique in which the murine liver can be successfully converted into an acellular scaffold by applying sodium dodecyl sulfate detergents that remove cellular material and debris while retaining the proper collagen structure, laminin basement membranes, and the vascular network [115]. In the same study, the scaffold had enough structural integrity to withstand cannulation and perfusion of the reseeding cells. A follow-up study illustrated the ability of the perfused cells to leave the vasculature and distribute among the matrix [117]. Using electron microscopy, rodent hepatocytes were able to migrate to decellularized sinusoidal spaces and display increased levels of urea synthesis and select drug metabolites compared with the sandwich hepatocyte cultures. This is particularly important because the liver requires a wide range of enzymes, cofactors, serum proteins, and acute phase reactants to be fully functional. This graft was able to withstand transplantation for 8 hours in a rat host before harvesting, which showed that the hepatocytes retained their morphology, position, and integrity.

Yagi et al. reported on the successful decellularization of porcine livers, thereby producing human-sized ECM scaffolds that were capable of supporting hepatocyte engraftment and reorganization in three dimensions [118]. Baptista et al. [13] also illustrated that it is possible to decellularize whole liver (in mice, rats, ferrets, rabbits, and adult pigs) and that they can be successfully seeded with human hepatocyte progenitors. This study used the inferior vena cava for decellularization and the portal vein as well as the vena cava for the perfusion of cells. Immunohistochemical staining suggested an architectural blueprint dictated by the acellular scaffold. Discrete populations of differing cells were found throughout the matrix, which suggested that the retention of glycosaminoglycans and collagen structure provides the necessary environmental signaling to regulate cell differentiation and dictates the fate of the progenitor hepatic and endothelial cells. Wang et al. [119] demonstrated that progenitor cells seeded onto a scaffold could differentiate into hepatocytes and cholangiocytes, illustrating that the

scaffold could dictate the differentiation toward distinct cell lineages. Porcine liver has been successfully decellularized and repopulated with human cells [120]. Analysis revealed that the cells were able to differentiate into mature hepatocytes while maintaining active metabolism, the ability to withstand physiologic shear stress from blood flow, and the ability to synthesize albumin. Implanted liver ECM has even been shown to induce hepatic regeneration via hepatocyte proliferation, which further illustrates the critical relationship between ECM and cells.

Observations on the natural regenerative capacity of injured livers led to the use of progenitor liver cells for bioengineering research. As mentioned, cellular hyperplasia replaces the lost volume after liver amputation. Current studies attempt to enhance the innate regenerative ability of the liver through terminal differentiation via resident bone marrow–derived or liver progenitor cells [121–123]. Infusing autologous bone marrow cells into patients with decompensated liver cirrhosis has already been demonstrated to be safe and in some cases clinically beneficial [124,125]. Another avenue is the use of cytokine granulocyte colony-stimulating factor (G-CSF) to activate hematopoietic stem cell differentiation and mobilization while increasing the ability of resident progenitor cells to respond to injury [126]. Stem cells isolated from the peripheral bloodstream of patients with liver disease infused with G-CSF were isolated and infused back into the patients. These patients showed significant improvements in liver enzyme levels as well as the serum bilirubin compared with the control group [127,128].

Despite these findings, researchers are unable to determine the specific progenitor cell type that actually aids the regeneration process. Current targets include the bone marrow–derived MSCs [129,130] and hematopoietic stem cells [131–133], whereas fetal liver progenitor cells also remain a possibility [134]. Fetal hepatoblasts have been shown to have the ability to differentiate mature hepatic phenotypes when seeded on decellularized scaffolds [135]; however these cells are not common in adult liver. More studies are required to determine the cell types needed to repopulate the scaffold to form a functional liver. Furthermore, liver viability and functionality need to be confirmed after implantation.

13.8 The Challenge of Immunosuppression

One of the pillars of modern bioengineering and transplant research is the desire to achieve durable immune tolerance. Current organ transplants require long-term treatment with brutal immunosuppressive protocols, which target an array of molecular sites to inhibit the communication between antigen presenting cells and T-cell receptors [136]. Long-term use of these drugs leads to comorbidities and complications that extend beyond the physiologic arena serviced by the transplanted organ. These effects include drug-specific toxicities, metabolic complications, and secondary malignancy, among others [137,138]. In fact, 40% of kidney transplant recipients have an increased risk of secondary malignancies compared with the age-matched nonimmunosuppressed population after 20 years of chronic immunosuppressant use. In the United States alone,

there are over 1000 individuals living with an intestinal transplant [139], approximately 14,000 with a pancreatic transplant [140], 11,000 with a lung transplant [141], over 27,000 with a heart transplant [142], over 59,000 with a liver transplant [143], and almost 200,000 with a kidney transplant [144]. This totals over 300,000 transplant recipients needing long-term immunosuppression and/or tolerance monitoring.

After anoxia, the leading cause of mortality in kidney transplant recipients is cardiovascular disease resulting from the cardiotoxicity of the long-term immunosuppressive treatments [145,146]. Thus there is a strong need to balance the safety risks of high immunosuppressive therapy with graft efficacy. TERM investigations may provide innovative treatment solutions that can eliminate the need for antirejection therapy.

To ensure the graft survival and functionality, adherence to long-term immunosuppression is vital. The costs of immunosuppressive agents and overall antirejection therapy costs create an immense financial burden, which makes it a leading cause of nonadherence to the therapy [147]. At risk, patients may be faced with the decision between affording their medications and maintaining an adequate livelihood. In these cases, patients are at risk for a lower quality of life owing to the cost of medication, increased morbidity as a result of the medications, graft failure because of insufficient adherence to the medications, and death as a result of any of these conditions. Pressure to engineer innovative tactics to minimize costs and improve long-term recipient outcomes stems from the current high costs associated with transplant maintenance, organ procurement, and transplantation. Immune tolerance afforded by TERM would serve to reduce the cost and amount of immunosuppression required by transplant recipients, thereby improving quality of life on multiple fronts.

Durable tolerance can be achieved by one of two avenues: increased genetic compatibility of the graft and recipient host or better immunologic tolerance of the recipient. These states are difficult to arrange and the latter usually requires immunosuppression. Liver transplantation has seen limited success as a result of fulfilling extensive criteria in both the donor and the recipient [148], although this has been less accessible in other organ systems. Notably, up to a third of monozygotic twin organ transplants require long-term immunosuppressant therapy as a result of slight genetic variance, which suggests that rejection extends beyond human leukocyte antigen matching [149]. To optimize TERM solutions and guide the use of exogenous cellular material, increased understanding of the intricate factors that dictate tolerance of foreign bodies by the host immune system will be required.

The field of bioengineering hollow organs such as the bladder and blood vessel from autologous cells has already successfully transplanted hollow organs without the need for immunosuppression [150,151]. Approximately 200 patients have undergone successful transplantation of bioengineered grafts (author's calculation), which surpasses the number of hollow organ transplant recipients successfully removed from of long-term immunosuppression. These results are an early indicator of future advancements in the biofabrication of complex organs that eliminate the morbidities associated with current antirejection therapy.

13.9 Conclusions and Perspectives

Large-scale clinical translation of abdominal organ bioengineering remains a distant possibility, because substantial work in the laboratory remains. Nevertheless the chief obstacles posed by current medical and surgical modalities (particularly transplantation) are concerning and have created a pocket of necessity within which TERM investigations are taking place. The use of autologous cellular material has the potential to obviate the need for lifelong antirejection therapies. Furthermore, de novo organ fabrication using cell-scaffold technology could theoretically provide a limitless supply of transplantable organs for patients on the waiting list, thus circumventing the challenge of organ shortage.

Further research is needed to identify the most suitable cell type for regenerative investigations. Although adult somatic cells specific to the organ system being treated have shown promise, stem cells have the advantage of being multipotent and renewable, which are particularly attractive qualities in the context of TERM. However, the abundance of subcategories and subniches of stem cells add more complexity to identifying the best candidate. Furthermore, the risks associated with their use need to be further characterized before they can be used safely in patients on a large scale.

The immunogenicity of decellularized scaffolds is another issue that needs to be assessed. Although their tolerability has been recognized both empirically and theoretically, suboptimal decellularization protocols can leave behind residual antigen activity, triggering an immune response in the host.

Investigators must also seek to better understand the interaction between ECM and cells both endogenous and exogenous. The improved knowledge base would likely result in improved cellular engraftment, migration, and differentiation within the acellular matrix. The source of this understanding could well lie in developmental biology and organogenesis, in which ECM-guided differentiation is paramount.

Although TERM solutions for abdominal organ engineering show great promise, current researchers must also keep an eye toward research funding limitations and the role of commercial entities in bringing their innovations from bench to bedside. With these last obstacles addressed, advances in abdominal organ bioengineering may well bring about a paradigm shift for the treatment of end-stage organ diseases.

Abbreviations

TERM	Tissue engineering and regenerative medicine
ECM	Extracellular matrix
ESC	Embryonic stem cell
IPSC	Induced pluripotent stem cell
MSC	Mesenchymal stem cell

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14

Mesenchymal Stem Cells Seeded on Biofunctionalized Scaffold for Tissue Engineering

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14.1 Introduction

Regenerative medicine has been looking for new approaches and among these, biomaterials studies have emerged in recent years and have been developing and manipulating molecules, cells, tissues, and organs grown in laboratories to replace human body parts. Many such materials have been used for this purpose, be they synthetic or biological, to provide new medical devices.

In general, engineering technology involves the selection of an appropriate substrate to sustain and promote the growth of a particular injured tissue. Tissue engineering technology offers enhanced recovery of injured tissues and organs.

Previous works have demonstrated that pluripotent stem cells can be generated from somatic cells as mesenchymal stem cells (MSCs) from diverse origins. In addition, they can be induced from somatic cells known as induced pluripotent stem cells (iPSCs), via overexpression of key transcription factors, creating a new cell reprogramming method [1–3]. In addition, the substrate is key for the cells' proliferation and plays an important role in this way.

Three-dimensional (3D) substrates can be prepared based on the tissue of interest. Several types of scaffolds are known: nondegradable synthetic polymers; degradable synthetic polymers; nonhuman collagen gel, without pores; nonhuman collagen gel, with human collagen tissue pores; and decellularized tissue, for example, amniotic or cadaverous tissue [4,5].

The cell microenvironment is mainly composed of soluble growth factors, cell–matrix interactions, and cell–cell interactions. In natural tissues, the extracellular matrix composition, cell density, and physiological properties are often nonhomogeneous. The great challenge of tissue engineering is the distribution of cells throughout tissue-engineered scaffolds as a biomimetic material that is as close as possible to the

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natural tissue, and it should be analogous to the extracellular matrix (ECM), providing the same function as the tissue [6].

Cell deposition is mainly determined by local wall shear stress, which, in turn, is strongly influenced by the architecture of the scaffold pore network. The deposition rate of suspended cells on a surface depends on many parameters such as advective transport and diffusivity, colloidal interactions, concentration of ligands and receptor binding strength, bond-forming kinetics, and available surface area [7–11].

14.2 Pluripotency of Stem Cells

Extensive clinical experiments with stem cells, particularly in the treatment of oncohematological diseases, opened up the possibility of trying stem cells to treat non-hematopoietic diseases. MSCs subsequently emerged as a promising source for the regeneration and repair of various tissues in the treatment of a range of diseases, because of their presence in all derived mesenchymal tissues in adult solid organs as well as in mesoderm from embryonic tissue. This, along with their pluripotency and the fact that they were easily obtainable, meant that MSCs represented an important source for studies in regenerative medicine [12].

Pluripotency is defined as the ability of a cell to differentiate into any of three germ layers. Pluripotency is a natural property of MSCs from both adult and embryonic tissues and of others that have been shown to be capable of differentiating into various types of mesoderm-derived cells as well as ectoderm cells, such as skeletal muscle cells, neurons, cardiomyocytes, osteocytes, chondrocytes, and endoderm cells. Nevertheless, the differentiation could be (1) spontaneous, (2) in the niche where the cells were transplanted into the tissue, or (3) induced by the medium conditions, the substrate, or gene transfection, as known for iPSCs. As MSCs are the majority of isolated cells and their expansion will be carried out under good manufacturing practice (GMP) conditions for the development of cellular therapy, this chapter emphasizes their characteristics, potential, and details, for example, the neurospheres (precursor of neurons) obtained by substrate interactions on a biofunctionalized scaffold.

14.2.1 Mesenchymal Stem Cells

Originating from the embryonic mesoderm, MSCs persist in adult organisms, promoting the maintenance and replacement of various cell types that regenerate, particularly where the tissue was injured [13–20].

These cells were isolated ex vivo first by Friedenstein et al. [21] from the bone marrow of *Rattus norvegicus*. The authors reported having obtained a very abundant cell fraction that was not phagocytic and not hematopoietic, with a fibroblastoid aspect and the property of adhesion to plastic (the substrate) [21,22]. The last property is a characteristic that was described for the first time and that usually permits their isolation.

In subsequent years, the same group also showed that this cell fraction showed in vitro clonogenic and differentiation ability to form structures compatible with the bone,

cartilage, fat, muscle, connective, and fibrous aspects of tissue [23,24]. Only in the early 1990s was the term "mesenchymal stem cell" used to designate this cell fraction [25].

In the following 2 decades, MSCs became significantly more frequent in publications on stem cells. Showing the possibility of their isolation from a diverse number of tissues other than the bone marrow, such as placenta, umbilical cord blood, Wharton's jelly, amniotic fluid, amnion, fallopian tubes, peripheral venous blood, adipose tissue, liver, thymus, spleen, parathyroid, pancreas, lung, dental pulp, periosteum, dermis, and skeletal muscle [20,26–35].

The diversity of potential sources for the isolation of MSCs showed significant differences between these cells. Of the reported differences, we highlight those involving immunophenotypical patterns, proliferative capacity, and/or in vitro differentiation potential and the efficiency in therapeutic applications [34,36–44].

The need for standardization has been established and motivated the International Society for Cellular Therapy to set forth minimum criteria for the characterization of MSCs. These are:

1. adherence to plastic under conventional cultivation conditions;
2. the cell surface proteins expressed by MSCs include cluster of differentiation (CD): CD105, CD73, CD44, CD90, CD71, CD106, CD166, CD29 and Stro-1 ($\geq$95%) combined with no expression of CD45, CD14 or CD11. They also do not express co-stimulatory molecules CD80, CD86 or CD40 or adhesion molecules CD31, CD18 or CD56 or major histocompatibility class II HLA-DR ($\leq$2% positive).
3. demonstration of capacity to differentiate into osteoblasts, chondroblasts, and adipocytes in vitro [45,46].

Despite the many sources of MSCs already described, however, two tissues are used in the majority of MSC reports: bone marrow and adipose tissue. The volume of publications on bone marrow-derived MSCs (BMMSCs) is justified both because this tissue is the one from which MSCs were isolated for the first time and because of the improvement in hematological disease treatments [19–21,47]. Regarding adipose tissue, it was described as a source of MSCs [48,49]. Reports on adipose-derived MSCs (AMSCs) have become as common as the current BMMSC publications. Some authors have pointed them out as an ideal source for the isolation of MSCs. As an easy way to obtain a greater volume of tissue compared to other adult tissues, the procedure is less invasive and can be done under local anesthesia and, therefore, it results in less discomfort to the donor, as for example, liposuction in aesthetic procedures that can then be donated for the research [20,43,50–53].

14.2.2 Differences Between AMSCs and BMMSCs

Several studies comparing AMSCs and BMMSCs have been published, containing a great diversity of results, not infrequently conflicting. There are reports that AMSCs can be maintained for longer growing periods, with lower senescence rate, higher proliferation rate [39,41], increased differentiation potential, and greater stability than the cytogenetic BMMSCs [39].

Concerning the potential for differentiation, there are reports with differences for both of them. De Toni et al. [54], for example, reported higher rates of hematopoietic differentiation in vitro and in vivo from AMSCs than from BMMSCs. AMSCs also have been characterized as having the greatest potential for adipogenic differentiation and lower potential for osteogenic and chondrogenic differentiation than BMMSCs. However, the adipogenic, osteogenic, and chondrogenic potentials showed no difference in several reports. On the other hand, Zhu et al. [58] and Lindroos et al. [59] reported the occurrence of an influence of the donor's gender and age on the AMSC potential, but not on that of BMMSCs. Samples from male donors showed greater potential for osteogenic differentiation than those from female donors, which also showed a significant reduction in differentiation potential with increasing age of the donor, which was not observed in male donor cells [41,43,53–59].

Another distinction between AMSCs and BMMSCs is the immune phenotypic profile. In this context, there are also conflicting results and the markers CD34, CD49d, CD49f, CD54, CD106, and Stro-1 were the most frequently identified as differential between these MSCs [20].

The proteomic and transcriptomic profiles of AMSCs and BMMSCs present less controversial results, but need more study. There are some reports on the occurrence of different expression of approximately 10% of the genes. However, only 3.4% can be considered AMSC or BMMSC specific [39,43,60]. Such divergences are probably due to the differences between immunomodulatory properties [61].

MSC intrinsic properties have been described as molecular, immunocytochemical, and those having to do with isolation, expansion, differentiation, and cryopreservation. The fact that MSCs do not express the antigens of histocompatibility means that they can be used in allogeneic transplantation. Methods have been developed to isolate subpopulations of MSC fractions together by magnetic separation with the perspective that this subpopulation could obtain better results than the total population in replacement therapy for correction of a determined specific function. Magnetic sorting using magnetic beads has become a routine methodology for the separation of key cell populations from biological suspensions. Owing to the inherent ability of magnets to provide forces at a distance, magnetic cell manipulation is now a standardized process step in numerous processes in tissue engineering [62].

14.2.3 Mesenchymal Stem Cells in Cellular Therapy

The therapeutic effect and safe use of MSCs in the treatment of various diseases have also been extensively investigated, also showing a great diversity of organisms, models, and correlations between the target disease and its morbidity and/or mortality [20,63–65].

Similarly, the variety of diseases is as broad as that of BMMSC studies, including clinical conditions with relatively low morbidity, such as allergic rhinitis [66] and tendon injuries [67], to conditions with high morbidity and/or mortality, as in stroke [42] and spinal cord injury [68].

As of this writing, BMMSC and AMSC studies are in the clinical phase. The possibility of delivering, in certain diseases, cells that are committed to a specific type of cell in precursor form and maintaining their proliferation rate could provide transplanted cells with better integration into the host tissue, for example, neuron precursors.

14.2.4 Mesenchymal Stem Cell Expansion

Cell culture is a prerequisite for almost all of the potential MSC therapeutic applications. This is because even in tissue with high yield, for example, in the case of adipose tissue, the amount of AMSCs that can be isolated is usually insufficient to promote the desired therapeutic effect. In addition, there are factors that can provoke even smaller yield, such as the particular physiological condition of the donor organism, the presence of other disorders, or age [52,64,69–75].

The aim of MSC cultivation to increase their number is justified by the variability of cells used to start cultivation. For this purpose, the cells to be cultivated are arranged in a medium that attempts to mimic physiological conditions of the tissue and/or organism of origin. In this context, the incomplete knowledge of both the physiological conditions of origin, such as the difficulty in mimicking known specific conditions of, for example, 3D tissue architecture, and the cell–substrate interactions is a potentially interfering factor in cultivation [76].

The MSC cultivation step is required in studies aimed at the characterization of their potential for self-renewal and/or differentiation in vitro. Theoretically, because these potentials are appropriately known, it is possible to obtain both a high amount of MSCs and their differentiation into any tissue. In this context, where different sources of MSC are described and different methods of isolation and culture, the results are without being standardized [73–84].

Given this diversity, it will be necessary to apply principles of GMP in all stages of the study, from obtaining the sample to the evaluation of transplants performed. This makes it mandatory to keep detailed documentation of the parameters used in cell cultures, especially in cases in which changes are identified [46,85].

There are some reports on genetic modifications of MSCs in culture. These range from genetic alterations, such as amplification or deletion loci and changes in methylation pattern, structure, and/or number of chromosomes, to phenotypic changes, such as the expression of enzymes and/or CDs in the proliferative profile, differentiation potential, and/or even the morphology. Identifying these changes in MSCs, especially those intended for therapeutic applications, reveals the existence of a risk not yet properly quantified as well as a risk of transplanting cells with tumor potential. This risk is established by several changes reported in MSCs that were originally characterized as typical tumor cell markers [73–83].

14.2.5 induced Pluripotent Stem Cells

The iPSC was described from mouse adult fibroblasts that could be reprogrammed with all embryonic stem cell (ESC) characteristics by the introduction of four transcription

factors known to be expressed in ESCs (*Oct4, Sox2, Klf4,* and *c-Myc*) and selecting cells that expressed endogenous *Nanog* or *Oct4* [86].

Pluripotency is generated naturally during mammalian development through the formation of the epiblast, the founder tissue of the embryo. In the embryo, *Nanog* specifically demarcates the nascent epiblast, coincident with the domain of X-chromosome reprogramming. Without *Nanog*, pluripotency does not develop, and the inner cell mass is trapped in a prepluripotent, indeterminate state that is ultimately nonviable. The acquisition of *Nanog* is mandatory to gain full reprogramming of pluripotent cells. Otherwise, the somatic cells could be transfected with embryonic genes and then regain their pluripotency [87].

14.3 Substrate

The successful growth and maintenance of cells in vitro requires culture conditions that mimic in vivo conditions with respect to temperature, oxygen, carbon dioxide concentration, pH, osmolality, and nutrition. The tissue culture medium, the basal medium, provides these conditions as well as the growth factors and induced factors to differentiate the cells. Some cells require supplementation and serum to be added to the basal medium.

Serum is an effective growth-promoting supplement for practically all types of cells. These can be divided into specific polypeptides that stimulate cell growth (growth factors), carrier proteins, antioxidant agents, cell attachment factors, and nutrients as trace elements. Regarding the relation of cell to substrate, attachment factors guarantee anchorage to the substrate. With the development of cellular therapy, which needs to attempt GMP conditions, the use of a medium without serum is mandatory and justified as a way to avoid contamination by viruses and prions and the need to purify the final product of contaminating proteins as well as the great variability in serum components causing nonreproducible results [88].

In experimental cell biology, it is important to be aware of the inherent variability of serum, which renders it very difficult to study the specific effects of molecules such as growth factors, hormones, cytokines, adhesion molecules, or matrix components, all of which are present in serum at undefined and variable levels.

In this way, to mimic the natural conditions in ECM tissue, the substrate has an important role in culture for replacement of serum-containing medium. For these reasons, the matrix should be investigated for adhesion and growth properties of its integrated molecules as well as the functionality that promotes 2D or 3D architectures as in natural tissue. There is increased interest in developing scaffolds in 2D or 3D, while the monolayer in flask culture is losing its use.

Diverse substrate sources have now been developed in intention on promoting cell adhesion, proliferation, and induction to a differentiated state in undifferentiated MSCs for cardiomyospheres, myospheres, and neurospheres to obtain the precursos of cardiomyocytes, muscle cells, and neurons, respectively (Fig. 14.1). Each substrate could

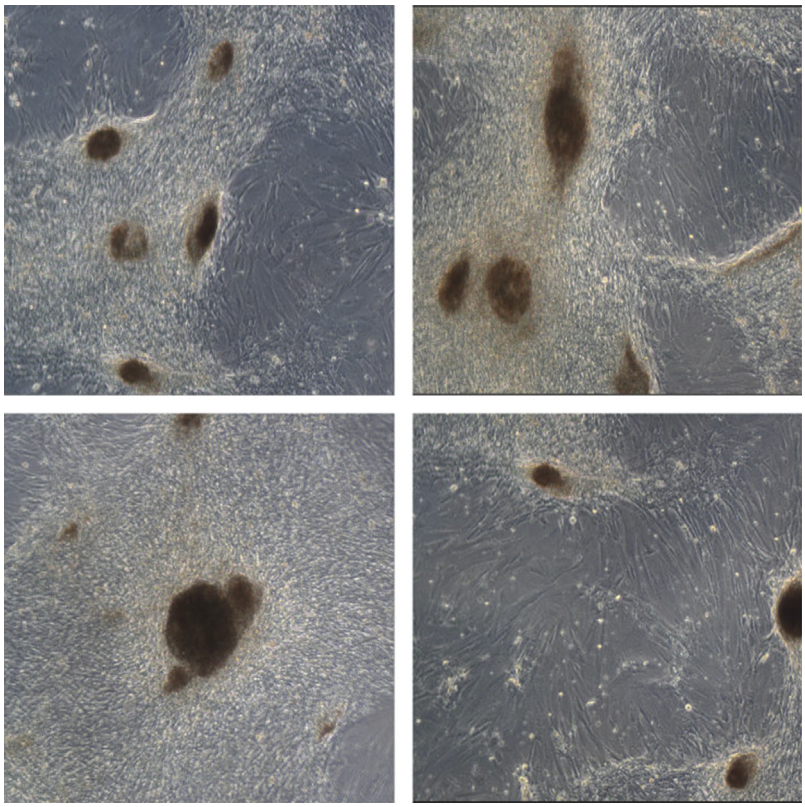

FIGURE 14.1 Myospheres from primary cultivation (rat). Inverted light microscopy, original magnification 100×.

induce different cell types. For example, collagen could be directed to epithelial cells as used to develop vessels and heart devices [12,13,89–98].

The substrate's functional properties depend on its nature and on its capacity to interact with diverse molecules or macromolecules of varying polarity. The cell–substrate interactions present different adhesive behaviors and could be selective regarding the induction medium toward certain types of cells. Cells are polyanionic surfaces that interact with their opposite, polycationic surfaces. The common polystyrene used in flasks that is treated with some molecules, for example, lysine, a polycationic layer of adsorbed polylysine, or one with altered electrostatic charge, has been shown to be able to modify the adhesion proprieties [99,100].

Cells of many kinds adhere firmly to glass or plastic surfaces that have been pre-treated with polylysine. The attachment takes place as soon as the cells make contact with the surface, and the flattening of the cells against the surface is quite fast. Cells that do not normally adhere to solid surfaces, such as some suspension mammalian cells in culture, could be attached to such surfaces. The attachment of cells to polylysine-treated surfaces can be exploited for a variety of experimental manipulations. In the preparation

of samples for scanning or transmission electron microscopy, the living material may first be attached to a polylysine-coated plate and subjected to some experimental treatment (fertilization of an egg, for example), then transferred rapidly to a fixative, and further passed through processing for observation. Each step involves only the transfer of the plate from one container to the next. The cells are not detached [101].

Particle surface modification through poloxamer adsorption can significantly alter the charge, adhesion, and consequently handling properties of a material. The charge reduction on polystyrene spheres achieved by this modification technique is dependent on the concentration, molecular weight, and conformation of poloxamer at the particle surface. Adsorption isotherms of poloxamers on polystyrene particles follow a Langmuir profile and there is an apparent correlation between the extent of adsorption and the poloxamer ability to reduce electrostatic charge [101].

In this way, the property of the attached fibroblasts could be used in replanting methods to obtain purity in a primary culture derived from skeletal muscles. The primary culture derived from skeletal muscles consists mainly of myoblasts and fibroblasts and the purification of myoblast cells from such a mixed population can be achieved. During the initial week or two of culture, selective growth and passaging conditions allow the myoblasts to become the dominant cell type, and after 3 weeks in culture, nearly 100% of the cells stain positive for the myoblast-specific proteins desmin or fast myosin [88,91].

14.4 Biomaterials

Biomaterials from plants and microorganisms such as bacteria and fungi could be a good choice for developings scaffolds, and the biopolymers were tried at first time and demonstrated successful results; these are explained by their versatility. Investigators have been looking for biomaterials apart from animal origin. Otherwise, they might incur in the same risks of medium supplementation with serum, with the virus and prion contaminations. These biomaterials needs to be xeno−free (without animal−derived components).

14.4.1 Biopolymers

The structures and functionalities of polysaccharides, proteins, and lipids allow their utilization with this aim in biomimetic and nanotechnology systems. Cellulose is one of the most studied biopolymers, and several sources of this polymer have been evaluated.

14.4.1.1 Cellulose

Cellulose is a linear polysaccharide consisting of monomer units of β(1 → 4)-D-glucopyranose; it presents long-chain variable molecular weight, with the empirical formula $(C_6H_{10}O_5)_n$, with n having a minimum value of 200. Total hydrolysis of cellulose produces glucose molecules, whereas partial hydrolysis produces cellobiose molecules. Some algae, fungi, and bacteria also produce this biopolymer, which comprises the

structural basis of the plant cell wall. The cellulose present in plants has the same chemical structure as the cellulose produced by bacteria, but there are morphological distinctions and they exhibit different physical and mechanical properties [102,103].

The synthesis of cellulose produced by *Gluconacetobacter xylinus* is a complex process and involves a glucose polymerization unit of a linear chain of β(1 → 4) glucans, the extracellular secretion of the chain, and the assembly and crystallization of these chains as knitted, resulting in a 3D gelatinous structure formed on the surface of a liquid medium. The physical and mechanical characteristics of the bacterial cellulose membrane arise from a single structure that differs from the plant pulp. The bacterial cellulose nanofibrils create a large surface area that allows the absorption of a large amount of water. The hydrogen bonds between the fibrillar units stabilize the entire structure, acquiring high mechanical strength, which results in a hard network structure. This linearity yields multiple elementary fibrils that aggregate into larger bundles, which can contain crystalline and amorphous regions. The degrees of crystallinity and crystal dimensions are dependent on the origin of the cellulose or on the modification to which it was submitted [104–106].

Specifically, cellulose from bacterial sources exhibits higher crystallinity and has distinct advantages over cellulose from other sources. The advantages include high purity, without the need to extract lignin or hemicelluloses, as well as high surface area [104].

The cellulose membrane has several characteristics that are desired in an ideal dressing for wounds, among which we can mention:

1. the presence of nanopores, which do not allow the entrance of microorganisms to the wound bed, but allow high throughput of water vapors;
2. ease of the sterilization process;
3. low production cost;
4. ability to shape the surfaces of the wound and elasticity [107].

Several studies are under development to improve bacterial cellulose membranes. Despite these membranes creating a barrier over the wound against microorganisms, this polymer shows no antimicrobial activity. The insertion of benzalkonium chloride, silver and copper nanoparticles, zinc oxide, and other bactericidal substances reduces the risk of infection [108–110]. The incorporation into these membranes of drugs such as propranolol, ibuprofen, and lidocaine allows the composition of a transdermal drug delivery, controlled by micropores formed by the mesh and nanostructured bacterial cellulose [111].

The fabrication of nanostructured membranes allows control of the thickness of the fibers that form the mesh and the properties at the molecular level of the original material. The large surface of this material due to volume increases its potential for interaction, creating functional surfaces. Sublimation (chemical vapor deposition), the sol–gel spin, self-assembly (layer by layer), the Langmuir–Blodgett technique, and electrospinning are some techniques for manufacturing nanostructured film [112,113].

The aforementioned characteristics have increased interest in producing polymer nanofibers, which began in the 1990s, bringing the possibility of producing 3D structures formed by nanonetworks and pores of varying sizes, serving as supports for tissue engineering. This approach is based on cell–substrate interactions. The identification of an optimal scaffold for cell implantation is a further requirement [114].

14.5 Scaffold Interaction With Mesenchymal Stem Cells

Curiously, the biofunctionalized scaffolds that have been seeded with MSCs interact with the cells in ways related not only to their charge but also to some unknown mechanisms. This may be due to the complexity of those cells that have pluripotency properties allied with their particular surface proteins, in particular adhesion molecules, for example, the endoglin known as CD105. On the other hand, scaffolds based on biopolymers have molecules that mimic the integrins (adhesion molecules). The MSC–substrate interaction plays a role in gene expression and gene suppression of their transcription factors. These facts could be inferred by the MSC differentiation state reported between different biopolymers. AMSCs seeded on cellulose have been maintained in the undifferentiated state, whereas AMSCs seeded on other biopolymers extracted from a Brazilian plant (protected for patent development) have been induced to neuron precursors, which express nestin, a biomarker for neurons, as well as CD271 (not published). The consequences of the differences in MSC–substrate interactions determine the purpose and potential.

14.5.1 Scaffold to Cellular Therapy

With this aim, Souza et al. [115] developed studies on both tissue engineering and delivery of cells for cellular therapy based on biofunctionalized scaffolds. The nanocomposites used were obtained after defibrillation using never-dried bacterial cellulose to form a pulp and to biofunctionalize the substrate, whose films were regenerated in the presence of hydrocolloid (10% w/w) with gellan gum, tamarind xyloglucan, and lysozyme. Afterward, this membrane was seeded with AMSCs and used as a curative for burned skin. The surface properties of this developed scaffold provided conditions for the adhesion and proliferation of AMSCs. It was demonstrated that the presence of stem cells may have accelerated the healing process and reduced the inflammatory infiltrate in the tissue. The nanostructured membranes seeded with MSCs were demonstrated to be efficient living bioactive dressings, effective for tissue regeneration (Figs. 14.2 and 14.3) [115,106].

14.5.2 Scaffold to Reprogram Mesenchymal Stem Cells to Neurons

In addition, a substrate as a functionalized scaffold biopolymer could reprogram MSCs based on its cell–surface interaction; neurospheres are an example. Neurospheres are agglomerates of precursors of neurons arranged as shaped spheres marked with the

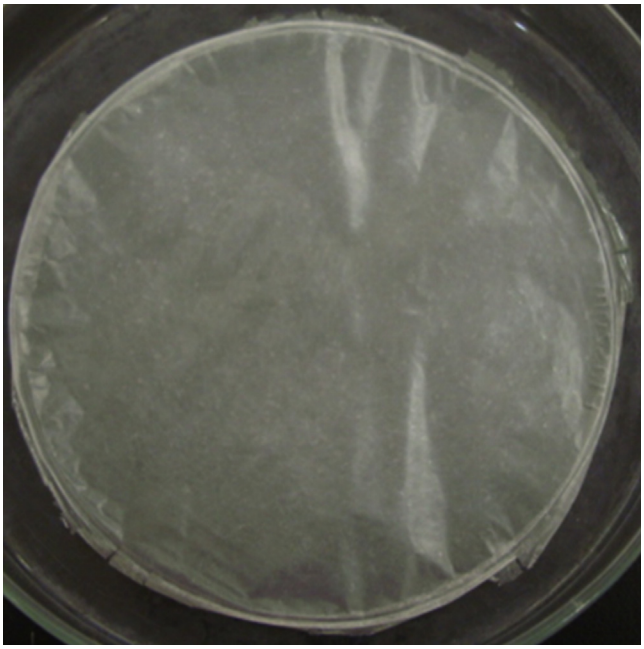

FIGURE 14.2 Cellulose in the presence of hydrocolloid (10% w/w) with gellan gum, tamarind xyloglucan, and lysozyme.

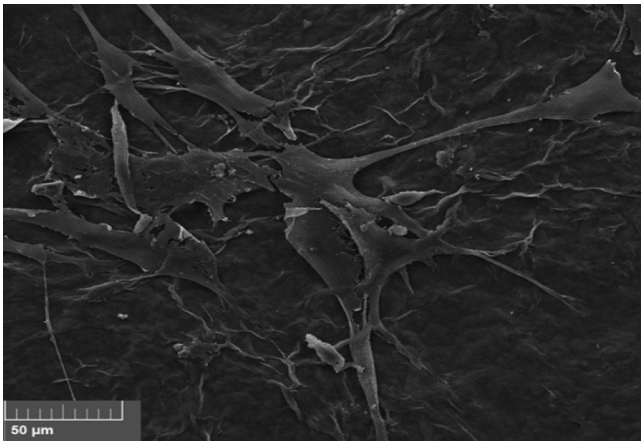

FIGURE 14.3 Nanostructured membrane reconstituted with cellulose, gellan gum, and lysozyme, seeded with mesenchymal stem cells. Electron microscopy, original magnification 1000×.

precursor neuron protein nestin and developed from undifferentiated MSCs seeded on the scaffold. The precursors of neurons could continue to differentiate in vitro or in vivo. In vivo, the neuron precursors may replace the neurons and repair the tissue to restore function. More preclinical studies are still needed to evaluate their effectiveness in cellular therapy. The mechanisms of this reprogramming are unknown, including its gene expression and metabolomics. These mechanisms should be understood. The

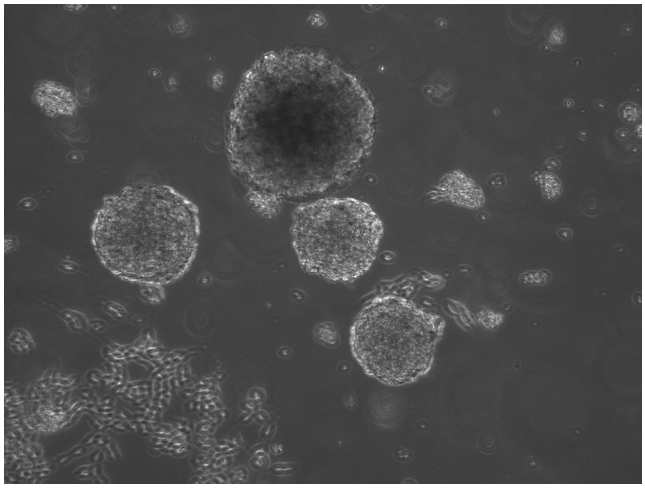

FIGURE 14.4 Neurospheres from human adipose mesenchymal stem cells seeded on biopolymer. Inverted light microscopy, original magnification 100×.

manufacture of cellular products obtained through the interaction of MSCs and a biofunctionalized scaffold requires further studies aimed at standardization of GMP conditions to be safe [95,96] (Fig. 14.4).

14.6 Conclusions

The versatility and variety of substrates as biofunctionalized scaffolds should be explored for the development of new approaches in tissue engineering based on their relations with stem cells and particularly MSCs.

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15

Biosurfactants as Antimicrobial and Antibiofilm Agents

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15.1 Introduction

Microorganisms are ubiquitous in the biosphere, existing on a tour of terrestrial and aquatic habitats that currently exist on earth. Diverse habitats present many selective pressure issues in addition to carbon and energy sources that have confronted microorganisms. Thus, survival under these conditions should require adaptation and the development of unique cellular biochemistry and metabolism by these microbes. To colonize these challenging environments, microorganisms developed strategies that enable them to survive as free-living organisms, associated with surfaces as biofilms or associated with other organisms, establishing various forms of relationships such as symbiotic and pathogenic. The resulting adaptive biochemical mechanisms involve production of varied metabolites with diverse activities, such as hydrolytic enzymes, surface-active compounds, and bioactive molecules, including ribosomally and nonribosomally synthesized peptides.

Biosurfactants are amphiphilic molecules, having hydrophilic and hydrophobic domains that allow them to exist at polar and nonpolar medium edges. Hence, they tend to accumulate at interfaces (air−water and oil−water) as well as air−solid and liquid−solid surfaces, resulting in the reduction of repulsive forces between dissimilar phases, allowing the phases to mix and interact easily. Specifically, biosurfactants can reduce surface (liquid−air) and interfacial (liquid−liquid) tension [1]. When surfactants are prepared in solution, a decrease in surface or interfacial tension can be observed until the molecule reaches the critical micellar concentration (CMC). At this concentration, biosurfactant monomers are likely to self-assemble in complex arrangements like micelles, vesicles, or bilayers mediated by hydrogen bonding or hydrophobic or van der Waals interactions [2].

The interaction of biosurfactants with a substratum surface can alter the surface hydrophobicity, interfering in the processes of microbial adhesion and desorption to biotic and abiotic surfaces [3]. Biosurfactants have also been reported to have biological activity,

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including antibacterial, antifungal, antiviral, and anticancer. As a result, several of these biomolecules have merited renewed interest in both clinical and hygienic sectors due to their potential to kill microorganisms and to disperse microbial biofilms [4–7].

Although biofilms play an important positive role in a variety of ecosystems, they also have many negative effects, including infections in clinical settings. Resistance to antimicrobial and host defense mechanisms is a property of the biofilm lifestyle, leading to treatment failure and infection recurrence. The possibility of developing pathogen resistance to or unwanted side effects of bioactive microbial metabolites is less likely than with chemical antimicrobials for several reasons, including new inhibitory mechanisms and the fact that some of them, as antimicrobial peptides, are a natural part of the human antimicrobial defense system. Also, these novel antimicrobials can be used in combinatory approaches with synergic effects to combat infectious pathogens and their antimicrobial-resistance derivatives in their sessile and planktonic forms [8–10].

In past years, nanotechnology has become increasingly important for the clinical field; once promising applications are already in development in the areas of nanoparticles, drug-delivery nanocarriers, or phage therapy. Owing to their self-assembling and biochemical properties, biosurfactants have been attracting increased attention in the field of nanobiotechnology and can be used in various ways: as components of (micro and nano) colloidal systems, as functionalizing nanoparticles, as components of vesicles due to self-assembly properties (as MEL-A), entrapped in phospholipid-based liposomes, and in gene transfection [11–13].

Therefore, this chapter brings to light the state of the art of biofilms as well as technologies based in microbial surface-active agents already in use or in development to prevent or decrease the risk of biofilm-associated infections. Also their use immobilized in liposomes or associated with nanoparticles will be discussed.

15.2 Biofilms

Biofilms are complex microbial communities adhered on the surfaces of solid–liquid, liquid–air, liquid–liquid, or solid–air interfaces, embedded in their own microbial-originated matrix of protective and adhesive extracellular polymeric substances (EPSs), which are mainly polysaccharides, lipids, and proteins [14]. This matrix along with carbohydrate-binding proteins, pili, flagella, other adhesive fibers, and extracellular DNA acts as a stabilizing scaffold for the three-dimensional biofilm structure. This way of life in which microorganisms adopt a multicellular behavior facilitates their survival in diverse environmental niches, including those natural, such as living tissue of plants and animals, or artificial, such as clinical devices, food-contact surfaces in the food industry, or pipeline distribution systems and water storage or natural water systems [14,15]. In these environments, biofilm cells show greater resistance to environmental challenges, including desiccation, pH fluctuations, biocides, and antimicrobial therapy, than their planktonic counterparts.

The switch from planktonic to biofilm bacterial growth happens in response to environmental changes and comprises varied regulatory mechanisms and signaling pathways

that can result in alterations in gene expression and spatiotemporal reorganization of bacterial cells [16]. This alters the expression of surface adhesion molecules and virulence factors and nutrient utilization and equips bacteria with varied properties that enable their existence in unfavorable conditions. In hospital settings, for instance, biofilm formation on vents and medical equipment allows pathogens to persist as reservoirs that can promptly spread to patients. Inside the host, pathogens can subvert immune defenses when associated with biofilms, being, therefore, associated with long-term disease.

Biofilm formation occurs as a result of sequential events: microbial surface attachment, cell proliferation, matrix production, and detachment. The conditioning film, formed by a layer of electrolytes and macromolecules such as DNA, proteins, biosurfactants, and humic acids adhered to the surface, is considered a precursor for the initial attachment of planktonic cells [17]. Once the microorganisms reach critical proximity to the surface, the determination of adhesion depends on the net sum of attractive or repulsive forces generated between the two surfaces and influenced by surrounding hydrodynamic forces [15,18]. Bacteria generally reach an irreversible attachment that may overcome shear forces and keep a persistent grip on the surface. This contact activates responses that result in changes in gene expression, up-regulating factors favoring sessility, such as those implicated in extracellular matrix formation [18,19]. This stage is controlled by *quorum sensing* (QS), an intracellular communication system designated for cell-density and population-based gene regulation [20].

A biofilm is considered mature when adherent bacteria produce EPSs, which contribute to the biofilm's vitality in several environmental conditions, protecting the biomass from desiccation, predation, oxidizing molecules, radiation, and damaging agents [14,21]. The matrix also acts as a virulence factor and confers to bacteria enhanced antimicrobial resistance and protection from host immune responses [22]. The EPSs act as an adsorbent or reactant, reducing the amount of antimicrobials available to interact with the biofilm; as well, their structure physically reduces the diffusion of antimicrobial agents by walling off access to regions of the biofilm. In this stage, it is possible to observe cavities that work as water transporter channels and planktonic cells throughout the biofilm community and also provide a unique environment for optimum nutrient absorption and waste disposal [16].

The final stage is the release of microorganisms back into their surroundings, where they return to their free-living state. Biofilm dispersal can be due to several reasons, including modifications in nutrient availability, oxygen oscillations and increase in toxic compounds, or varied stress-inducing conditions [23–25].

15.3 Current Approaches to Preventing or Removing Biofilms

As discussed previously, planktonic cells and young biofilms are often more susceptible to antimicrobial agents than mature biofilms. The large amounts of EPSs in the mature

biofilms can act as a diffusion barrier to antimicrobial agents. The high cell density in the mature biofilms can induce cell-to-cell communication (QS) systems, which up-regulate the expression of genes contributing to antimicrobial resistance [26,27] and release of protecting DNA in addition to increased transmission of resistance markers within the biofilm community [28]. Also, competition for nutrients can lead to differentiation of subpopulations and spatial physiological heterogeneity in mature biofilms, which may cause further antimicrobial resistance [21]. This pattern has been observed in several biofilm-forming bacteria on equipment or on indwelling medical devices and implants such catheters, mechanical heart valves, pacemakers, prosthetic joints, and contact lenses. This poses a critical problem because it enables pathogens as *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas* to persist as reservoirs that can readily spread to patients. Thus, occurrence of antimicrobial-resistant isolates of these species is global and is a serious problem, because of the limited efficacy of antimicrobial drugs and hospital hygiene and the resistance of biofilm-associated clinical strains. Notable biofilm-associated infections include chronic cystitis, endocarditis, otitis media, periodontitis, prostatitis, wound infections, and catheter- and stent-associated infections [29].

For *S. aureus,* the polymerizable mucopolysaccharide secreted by methicillin-resistant *S. aureus* (MRSA) at the biofilm surface limits penetration of antibiotics, increases the amount of time required for vancomycin to penetrate the biofilm, and decreases the permeability ratio [30]. The long-term exposure of biofilms to drugs may promote adaptation to stress, thus further increasing bacterial resistance to antimicrobials [31,32]. Cells of this bacterium on a stationary phase, like those existing in biofilms, exhibited thickened cell walls and lower antimicrobial activity of vancomycin in vitro, thought to be attributable to the absence of actively replicating cells [33]. In another study, 40 MRSA isolates recovered from bloodstream infections displayed a four- and an eightfold increase in the minimal inhibitory concentration, MIC_{50} and MIC_{90}, respectively, when in biofilm. In time-kill studies, 15 mg/L vancomycin showed bactericidal activity against low-biofilm-producing strains with only a 1.8 log(10) CFU/mL difference in bacterial kill, compared to high-biofilm-producing strains ($p < .001$) (phenotype of 60% of the isolates). In studies based on biofilm formation during peritoneal dialysis, all the antimicrobials tested were effective in laboratory MIC tests, but lost their efficacy against *S. aureus* biofilms, except for gentamicin [34].

As exposed, the development of novel effective biofilm control methods is essential. The control of biofilm formation might be achieved by killing bacteria or targeting developmental stages of the biofilms like microbial attachment to surfaces, altering biofilm structure development and differentiation, killing of biofilm cells, and induction of biofilm dispersion. A large number of new strategies and approaches have been developed in the past few years, including antimicrobial locks, in the case of biofilm control in catheters [35]; the use of QS inhibitors [36]; bioactive microbial metabolites, including biosurfactants, exopolysaccharides, and bacteriocins [37,38]; enzymes that

dissolve biofilms by depolymerizing either polysaccharides or extracellular DNA [9,39]; and electrical fields [40], ultrasound [41], or photodynamic therapy [42] for enhancing antimicrobial penetration of biocides. Also, coadministration of enzymes or biosurfactants and antimicrobial [8,43] and lytic phages [44] has been evaluated. Drug-delivery nanocarrier systems such as liposomes and nanoparticles also have come up as attractive methods with a great potential in the treatment of biofilm infections, because of varied factors such as good biocompatibility and the wide range and extent of drugs they can be loaded with [11,45]. Another important point is the protection provided by the entrapment of the drug in the biological milieu, reducing toxicity and allowing the drug to reach the specific target.

15.4 Surface Active-compounds as Potential Antibiofilm Agents

Biosurfactants are amphipathic molecules produced by a variety of bacteria, yeasts, and filamentous fungi. Because they present both hydrophilic and hydrophobic moieties, these molecules are able to interact with surfaces, lowering surface and interfacial tensions, and to form micelles, emulsifying immiscible substances. These biomolecules can be excreted to the extracellular medium or remain attached to the cell surface, being denominated particulate biosurfactants [1].

The surface-active properties of biosurfactants provide the same activities of detergency, emulsification, deemulsification, foaming, and dispersion phases, making them potentially applicable in varied industrial sectors, including agriculture, food, cosmetics, pharmaceuticals, and petrochemicals. Other advantages of these biomolecules that have attracted the attention of researchers and industry are specificity, minimum toxicity, elevated biodegradability, extensive applicability, and efficiency at pH and temperature extremes [1,46–49]. In the biomedical field, biosurfactants are getting more attention for their antibiofilm and antimicrobial activity because of lower toxicity for plants and animals, high biodegradability, low irritancy, and compatibility with human skin [50]. The only factor that works against the widespread use of biosurfactants is the economics of their production; however, much effort has been given to facilitating the optimization process at both the engineering and the biological levels. Our group evaluated the production of these molecules from biodiesel refinery coproducts such as glycerol, diatomaceous earth impregnated with esters from filters used in biodiesel purification, and macaúba (*Acrocomia aculeata*) [47].

Biosurfactants are classified according to their chemical composition and microbial origin. The major classes include varied structures such as glycolipids, lipopeptides, polysaccharides, or complexes of proteins, phospholipids, fatty acids, and neutral lipids [1]. Thus, it is logical to expect varied properties and biological activities for different biosurfactants. Conversely, these molecules can be modified to suit different applications

by genetic engineering, biologically or biochemically altering the growth substrate or growth conditions. The physiological roles of these biomolecules are often unclear, but many are essential for the survival of microorganisms in the environment, including increased surface area and bioavailability of hydrophobic substrates, antagonistic activity, binding to heavy metals, bacterial pathogenesis, QS, biofilm formation, and desorption surfaces [51].

These tensoactive molecules can spontaneously adsorb to surfaces, altering properties such as wettability and charge [3,52] or even making changes in bacterial cell surface structures. Loss of lipopolysaccharides (LPSs) was observed in *Pseudomonas aeruginosa* strains treated with rhamnolipids (RLs) and this resulted in increased changes in cell surface hydrophobicity and decreases in the outer-membrane proteins of bacteria [53,54]. Successive studies confirm that RL biosurfactants play an important role during the first steps of biofilm formation during cell attachment and when microcolonies emerge [5,55]. These biomolecules are responsible for maintaining the transport channels and directly influence biofilm structure, namely the creation of mushroom-like filaments [4,5]. RLs may be used to exclude other invasive species from the biofilm structure. Such a strategy allows the microbial community to keep its uniform structure and exclusiveness over a given niche [4]. A mannose-rich polysaccharide produced by *E. coli* impairs *S. aureus* adherence and colonization of the mature *E. coli* biofilm [56]. A uropathogenic strain of *E. coli* was shown to induce surface physicochemical alterations by releasing into the environment a group II capsular soluble polysaccharide, preventing biofilm formation by a wide range of gram-positive and gram-negative bacteria [57]. Polymers assembling on surfaces can increases their hydrophobicity and modulate microbial interaction with interfaces by limiting initial bacterial adhesion and by interfering with subsequent cell−cell contact within the biofilm. Also, a 546-kDa exopolysaccharide (A101) isolated from marine *Vibrio* sp. not only inhibited biofilm formation of many gram-negative and gram-positive bacteria but also disrupted established biofilms of *P. aeruginosa*, but not *S. aureus*. The polymer also affected cell−cell interactions of both *P. aeruginosa* and *S. aureus*, whereas the cell−surface interaction inhibition occurred only in *S. aureus* [58].

Other roles for biosurfactants include antibacterial, antifungal, and antiviral activities [38,50]. Because of their tensoactive properties, many surfactants can cause membrane disruption and cellular lysis by increasing membrane permeability, causing metabolite leakage by altering the physical membrane structure or by disrupting proteins; thus, interfering with important membrane functions such as energy generation and transport [59]. This has also been reported to lead to toxicity, lysis, pyrogenicity, mitogenicity, and immunogenicity, among other effects. Also, human-derived biosurfactants have received increased attention because of their role in immunity and defense [60]. In this context, the use of biosurfactants as alternatives to antibiofilm antimicrobial agents has extensively been explored [52,61].

15.4.1 Lipopeptides

Lipopeptides (LPs) are low-molecular-mass biosurfactants produced by a wide spectrum of microorganisms including species of *Bacillus, Streptomyces*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, and *Arthrobacter* [7,62,63]. Structurally, LPs are composed of a hydrophilic peptide attached to a hydrophobic lipid or fatty acid. Among the bacterial LP producers, *Pseudomonas* and *Bacillus* have received the most attention. The advances in genome sequencing have highlighted the genus *Bacillus* as a potential source of antibiotic-like compounds, including aminoglycosides, polyketides, and several small proteinaceous and peptidal structures such as bacteriocins, oligopeptides, and LPs and other unusual antibiotics. The nonribosomal peptide and polyketide groups include toxins, siderophores, pigments, antibiotics, cytostatics, and immunosuppressants [7].

LPs from *Bacillus* are classified into three families (surfactin, iturin, and fengycin) depending on their amino acid sequence, the type of peptide cyclization, and the nature of, length of, and substitutive groups that compose the fatty acid chain. Thus, each family has variants within the same peptide length, but changing the type and position of amino acid residue. Also, each variant can have homologs of varied length and isomeric state of the fatty acid chain [64]. The surfactin family, for instance, includes the heptapeptide variants of the esperin, lichenysin, pumilacidin, and surfactin groups. The peptide chain is linked to a β-hydroxyl fatty acid (C12−C16) with linear, iso, or anteiso, branches. The iturin family has the variants iturin A and C; bacillomycin D, F, and L; and mycosubtilin, in which the heptapeptide is linked to a β-amino fatty acid chain of variable length (C14−C17). The third family includes fengycins A and B, also known as plipastatins if Tyr9 is D-configured. These decapeptides are linked to a β-hydroxyl fatty acid chain (C14−C18) that can also be in linear, iso, or anteiso forms, saturated or not. Furthermore, the described LP families of *Bacillus* have specific mechanisms of cyclization. In addition, various other LPs have been identified for *Bacillus* species, including kurstakin heptapeptide from *Bacillus thuringiensis* [65], the 12-amino-acid-containing maltacines from *Bacillus subtilis* [66], polymyxins from *Bacillus polymyxa* with high content in diaminobutyric acid [67], and the surfactin-like bamylocin A from *Bacillus amyloliquefaciens* [62].

LPs belonging to the surfactin, iturin, and fengycin families, are formed by nonribosomal peptide synthetases and/or polyketide synthases [37]. The nonribosomal peptide synthetases lead to a remarkable heterogeneity among the LPs as described below.

Surfactin: Surfactins are one of the most effective biosurfactants first isolated from *B. subtilis*; they can decrease water surface tension from 72 to 27 mN/m, at low concentrations like 0.005%. These biomolecules have several pharmaceutical applications such as inhibition of clot formation, formation of ion channels in membranes, and antibacterial, antifungal, antimycoplasma, antiviral, and antitumor activities [64,68,69]. Unfortunately, surfactins can also be indiscriminately cytotoxic with hemolytic activity [70].

Mireles et al. [71] demonstrated that surfactin from *B. subtilis* is able to inhibit *Salmonella enterica* biofilm adhesion on precoated urinary catheters and polyvinyl chloride microtiter wells. They also reported a similar effect for *E. coli*, whereas this pretreatment was completely ineffective against *P. aeruginosa*. Fengycin-like and surfactin-like molecules produced by *B. subtilis* and *Bacillus licheniformis* have also been described to cause dispersion of up to 90% of gram-positive *S. aureus* ATCC 29213 biofilms and up to 97% of *E. coli* CFT073 biofilm [72]. It was shown that surfactin induces nanoripples in lipid bilayers, which may explain the biosurfactant action on biofilm permeability or integrity, probably through the formation of some kind of channels within the biofilm increasing penetrability [73]. Surfactin has an anionic nature; the antiadhesive effect can be due to the electrostatic repulsion between bacteria and the molecules of surfactin adsorbed onto the polystyrene surface [74]. Three surfactin-producing *B. subtilis* strains, C4, M1, and G2III, which were obtained from honey samples and intestines of the bee *Apis mellifera* L., have been described to exhibit antagonistic potential against six different bacteriocin-resistant *Listeria monocytogenes* strains. Surfactin produced by the *B. subtilis* subsp. *subtilis* C4, G2III, and M1 strains inhibited these pathogens at concentrations of 0.125, 0.250, and 1 mg/mL, respectively [75].

Furthermore, the N3 LP, which is produced by *B. amyloliquefaciens* M1 strain, shows broad-spectrum antibacterial potential, including activity against multidrug-resistant *Vibrio* species, particularly *Vibrio anguillarum*, and *Shewanella aquimarina*, which was isolated from sick marine animals. This LP contains surfactin isoforms with a regular amino acid sequence (GLLVDLL) and hydroxy fatty acids that are 12−15 carbons in extent. The treatment with this molecule also resulted in injury to the cell membrane of *V. anguillarum* and rupture of the bacterial cell [76].

Fassi et al. [77] described the use of surfactin to increase the effect of enrofloxacin against planktonic *Mycoplasma pulmonis*. Rivardo et al. [72] reported a synergistic increase in the efficacy of various antimicrobials (ampicillin, cefazolin, ceftriaxone, ciprofloxacin, piperacillin, tobramycin, and trimethoprim/sulfamethoxazole) in the eradication of uropathogenic *E. coli* CFT073 biofilm when associated with V9T14 LP biosurfactant from *B. licheniformis*. The authors hypothesize that the action of V9T14 biosurfactant lies in its interaction with the bacterial membrane, which results in pore formation and altered membrane integrity, leading to an increase in entrance and efficacy of the antimicrobial drug.

Iturin: This cyclic LP produced by *B. subtilis* presents antimicrobial activity. The antifungal activity involves pore forming that forms small vesicles in the plasma membrane of yeast resulting in metabolite leakage. Iturin A is able to penetrate the cell wall without causing damage, but the membranes of cytoplasmic organelles are affected. Furthermore, it also significantly enhances the electrical conductance of biomolecular lipid membranes. Iturin A has been investigated as an effective antifungal molecule for deep mycosis. Other variants of the iturin group, including bacillomycin D and bacillomycin Lc, also present antimicrobial activity against *Aspergillus flavus*, but the varied lipid chain length apparently interfered with the activity of the LP against other fungi [78].

An LP from *B. subtilis*, showed antifungal activity, modifying the morphology and structure of the cell membrane of a yeast [59].

Fengycin: Antimicrobial and antibiofilm activities have been ascribed to fengycin LPs. Like most the aminopeptides, fengycin seems to act by raising the plasma membrane permeability of the target cell. It was observed that fengycin purified from *B. thuringiensis* strain SM1 was subject to self-assembly, presenting a globular micellar structure. The interfacial modification enhanced the spectrum of the microbial action confined to fungi to include some activity against both gram-positive *S. epidermidis* and gram-negative *E. coli* (MIC 500 μg/mL) [79]. The fungitoxic activity can be attributed to inhibition of the enzymes phospholipase A2 and aromatase [80]. Culture supernatant of *B. amyloliquefaciens* ANT1 containing several LP antibiotics, including surfactin, iturin A, and fengycin B, showed antimicrobial and antibiofilm activities against *Bacillus cereus* ATCC 17778, *Pseudomonas* ATCC 15442, *Aeromonas hydrophila* ATCC 7966, and *Aspergillus niger* ATCC 9642 [81]. More recently, it was shown that a mixture of iturin and fengycin produced by *B. amyloliquefaciens* strain AR2 inhibited *Candida* biofilm formation as well as dislodging preformed biofilm; the dosage of 6 mg/mL of biosurfactant had similar fungicidal activity for both planktonic cell and biofilm [63].

Polymixins: These compounds constitute a class of nonribosomally synthesized cyclic LPs, generally produced as secondary metabolites of *Bacillus* sp. There are two polymyxins available for clinical use, colistin (i.e., polymyxin E) and polymyxin B. They were discovered in the 1940s and were never subjected to contemporary drug-development procedures. They have a narrow antibacterial spectrum, mainly against gram-negatives. Clinical use of colistin and polymyxin B waned in the 1970s because of the early experience of nephrotoxicity and neurotoxicity after intravenous administration, related to apoptosis of kidney tubular cells, due to oxidative stress. However, the rapid increase in resistance to all other antimicrobials has demanded their resurgence in clinical contexts [82]. Parenteral colistin is much more commonly used internationally, although injectable polymyxin B is available in a number of countries, such as Brazil, Singapore, and the United States; in these three countries, both antimicrobials are available [83].

The molecular structure of polymyxin comprises a cyclic peptide chain and a hydrophobic tail. Each member of the polymyxins differs in the structure of fatty acids and variations in the amino acid residues. The LPs of the polymyxin B and E groups share a similar primary sequence with the only difference being at position 6, which is occupied by D-Phe in B and D-Leu in E. Another important difference between polymyxin B and colistin is that the former is administered parenterally as the sulfate salt, whereas the latter is administered as the sodium salt of colistin methanesulfonate, an inactive prodrug that undergoes hydrolysis in vivo and in vitro to form the active entity colistin [83].

The bactericidal action of the polymyxin antibiotics is thought to be initiated by their interaction with LPS of gram-negative bacteria, which causes LPS aggregation raising the surface charge of the LPS. The antibiotic may be internalized and then attached to the bacterial phosphatidylglycerol-rich membrane leaflets, which in sequence causes

leakage of cellular contents at higher peptide concentrations [84]. The mechanism of action of polymyxins involves an initial stage of interaction with lipid A of the LPS, leading to self-promoted uptake of polymyxins across the membrane, followed by cell death.

Polymyxins have been shown to inhibit alternative nicotinamide adenine dinucleotide dehydrogenase and malate:quinone oxidoreductase in *Mycobacterium smegmatis*; no such enzymatic study has been reported in gram-negatives [85]. It was also suggested that rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway [86]. In addition, the mechanisms of resistance to polymyxins involve alterations in the chemical composition or loss of LPS, thereby decreasing the interaction with polymyxins.

A complex of antibiofilm compounds produced by *Paenibacillus polymyxa* containing fusaricidin B and polymyxin D1 and surfactins reduced the biofilm biomass of *B. subtilis*, *Micrococcus luteus*, *P. aeruginosa*, *S. aureus*, and *Streptococcus bovis*. This complex of biosurfactants also inhibited the formation of multispecies biofilms such as self-assembling marine biofilm in coincubation assays by 99.3% and disrupted previously established biofilms by 72.4% [87]. Polymyxin E (colistin) is indicated as an early destructive therapy to set back the onset of chronic *P. aeruginosa* infections, which normally form biofilms, or irregular colonization in cystic fibrosis patients, in a combination of oral ciprofloxacin with colistin inhalation [88].

Tarquinio et al. (2014) investigated the activity of tobramycin and polymyxin E against biofilms of *P. aeruginosa* in polyvinyl chloride endotracheal tubes, simulating a critical condition, which is biofilm formation in medical devices [89]. These drugs were not effective, neither isolated nor combined, against biofilms formed overnight. However, bactericidal and bacteriostatic effects were observed against planktonic cells, and when coating strategies were employed, tobramycin used in a concentration of more than 2× the MIC, alone or combined with polymyxin E, was more bactericidal than polymyxin E alone after overnight biofilm exposure.

The cyclic LPs produced by *Pseudomonas* were initially classified into four major groups, viscosin, amphisin, tolaasin, and syringomycin, considering their structural characteristics [90]. But, some structurally new LPs were identified, including arthrofactin of *Pseudomonas* (formerly *Arthrobacter*) sp. strain MIS38 [91], putisolvins I and II of *Pseudomonas putida* [92,93], pseudofactin of *Pseudomonas fluorescens* BD5 [94], and pseudodesmins A and B of a *Pseudomonas* strain obtained from salamander skin [95]. Furthermore, several linear LPs were also described, including syringofactins of *Pseudomonas syringae* pv. tomato strain DC3000 [96] and peptin31, a linear derivative of syringopeptin produced by *P. syringae* strain 31R1 displaying activities against *Rhodotorula pilimanae*, *Rhizoctonia solani*, and *Trichoderma harzianum* and also hemolytic and antibacterial activities [97].

Putisolvin: *P. putida* PCL 1445 produces two surfactant-active compounds referred to as putisolvins I and II, which influence the development of bacterial biofilms on polyvinyl chloride, but can be effective dispersal agents when added before or after the

development of biofilms of other *Pseudomonas* sp. strains [92]. The only difference between the compounds is that Val11 in putisolvin I is replaced by Ile or Leu in putisolvin II, which accounts for the mass difference of 14 Da. A possible mode of action of putisolvins I and II during the inhibition of biofilm formation could be by binding to the cell surface or to components of the cell surface, thereby influencing the outer membrane hydrophobicity and consequently the adhesion.

Pseudofactin: This is a cyclic lipodepsipeptide produced by *P. fluorescens* with a structure based on that of a palmitic acid bound to the terminal amino group of an eight-amino-acid peptide chain. The C-terminal carboxylic moiety of the last amino acid generates a lactone with the hydroxyl of the third amino acid, which is a threonine. Pseudofactin II produced by *P. fluorescens* BD5 lowered the adhesion to three types of surfaces (glass, polystyrene, and silicone) of bacterial strains of five species, *E. coli, E. faecalis, Enterococcus hirae*, *S. epidermidis*, and *P. mirabilis,* and two *Candida albicans* strains [94]. Pretreatment of a polystyrene surface with 0.5 mg/mL pseudofactin II inhibited bacterial adhesion by 36−90% and that of *C. albicans* by 92−99%. The same concentration of pseudofactin II dislodged 26−70% of preexisting biofilms grown on previously untreated surfaces. Pseudofactin II also inhibited the initial adhesion of *E. faecalis*, *E. coli*, *E. hirae*, and *C. albicans* strains to silicone urethral catheters. Total growth inhibition of *S. epidermidis* was observed at the highest concentration tested (0.5 mg/mL), which caused a partial (18−37%) inhibition of other bacteria and 8−9% inhibition of *C. albicans* growth.

15.4.2 Glycolipids

Glycolipids are formed by various types of mono-, di-, tri-, and tetrasaccharide carbohydrates combined to hydrophobic fractions of one or more long-chain aliphatic fatty acids. The most investigated glycolipids are rhamnolipids produced by *Pseudomonas* sp. [98]; mannosylerythritol lipids produced by *Pseudozyma antarctica* (previously *Candida*) [99]; trehalose lipids produced by *Rhodococcus* sp., *Nocardia* sp., and *Mycobacterium* sp. [100]; and sophorolipids synthesized by yeasts [101].

Rhamnolipids: These comprise one or two molecules of rhamnose bound to one or two molecules of β-hydroxydecanoic acid. Although originally isolated from *P. aeruginosa*, analogues of this biomolecule have been isolated from strains of *Burkholderia* [102], *Renibacterium salmoninarum*, *Cellulomonas cellulans*, *Nocardioides*, and *Tetragenococcus koreensis* [103]. RLs are involved in *Pseudomonas* sp. biofilm formation by way of promoting motility, the inhibition of attachment, and the destruction of the matrix, preserving channels throughout the biofilm for transport of water and oxygen [104]. RLs have shown antimicrobial activity against a wide variety of fungi and pathogens in concentrations from 0.4 to 35 μg/mL. A mixture of RLs produced by *P. aeruginosa* 47T2 showed excellent antimicrobial properties, and low MIC values were found for *Serratia marcescens* (4 μg/mL), *Enterobacter aerogenes* (8 μg/mL), *K. pneumoniae* (0.5 μg/mL), *S. aureus* and *S. epidermidis* (32 μg/mL), *B. subtilis* (16 μg/

mL), and phytopathogenic fungal species: *Chaetonium globosum* (64 μg/mL), *Penicillium funiculosum* (16 μg/mL), *Gliocadium virens* (32 μg/mL), and *Fusarium solani* (75 μg/mL) [105]. The biosurfactants produced by *Rhodococcus erythropolis* exhibited high inhibitory activity against *E. coli*, *P. aeruginosa*, and the fungi *A. niger* and *A. flavus*, among the tested organisms [106]. The molecules affected the morphology of cells, showing enlarged, elongated, empty hosts or fragmented ones, consistent with extremely low viability. Direct action on the surface structure of the bacterial cell has also been related. In *P. aeruginosa* at low concentrations of RL, a decrease in the proportion of LPSs was observed, increasing the hydrophobicity of the cellular surface and causing changes in the membrane proteins and therefore on the surface morphology of the cell [54].

RLs produced by *P. aeruginosa* generate 41−71% inhibition of bacterial adherence on polystyrene surfaces, as tested on *L. monocytogenes* [107]. They are also capable of eliminating and interrupting the formation of biofilms by *Bordetella bronchiseptica*, a pathogen of the respiratory tract in many mammals [108]. Moreover, the potent antibiofilm activity of RLs was evaluated against several microbial species associated with biofilm formation on voice prostheses and silicone rubber in the presence and absence of adsorbed RLs [109]. The antiadhesive activity of RLs at various concentrations was significant against all the strains and depended on the microorganism tested, with a maximal initial reduction of adhesion rate (66%) reported for strains of *Streptococcus salivarius* and *Candida tropicalis*. Furthermore, RL conditioning of silicone rubber caused a reduction of 48% in cell adherence of tested *S. epidermidis*, *S. salivarius*, *S. aureus*, and *C. tropicalis* strains. In another study, RLs reduced the initial attachment of the gram-negative *E. coli*, *P. putida*, and *P. aeruginosa* and the gram-positive *B. subtilis* on both hydrophilic glass and hydrophobic octadecyltrichlorosilane (OTS)-modified surfaces [110], whereas for *S. epidermidis*, these molecules reduced the attachment only on OTS-modified glass. RLs could inhibit the growth of *B. subtilis*, *S. epidermidis*, and *P. aeruginosa* PAO1 but not the growth of *E. coli*, *P. putida*, and *P. aeruginosa* E0340. Also, RLs were effective in changing the cell surface hydrophobicity of the tested strains, although no clear effect was observed on *B. subtilis*. Despite the observed trends on cell detachment, the responsible mechanism remains to be elucidated.

Studies have also demonstrated the activity of RLs against fungal biofilms. Dusane et al. [111] tested the potential of RLs to prevent biofilm formation and disrupt preestablished biofilms of the yeast *Yarrowia lipolytica*. In their study, precoating of microtiter plate wells with this glycolipid effectively reduced *Y. lipolytica* biofilm formation by 50% compared to cetyltrimethyl ammonium bromide (CTAB), which inhibited by 29%, and sodium dodecyl sulfate (SDS), which decreased biofilms by <10% at their respective MIC values. Moreover, RLs displayed 55% dispersion of *Y. lipolytica* biofilms (formed for 3 days in microtiter plate wells and treated for 1 h), whereas 35% and 40% disruption was observed with CTAB and SDS, respectively, at their respective MIC values. This biomolecule was able also to disrupt fungal biofilms of *C. albicans* formed on polystyrene surfaces [32]. The authors showed that antiadhesive activity of RLs on

Candida was concentration dependent. RLs from *Pseudomonas* spp. enhance the disinfection effect of NaOCl and peracetic acid/hydrogen peroxide on stainless steel surfaces contaminated with *L. monocytogenes* [112].

Lunasan and rufisan: *Candida sphaerica* UCP 0995 produces a biosurfactant known as lunasan, when using residual refined soy oil as a carbon source, and it has shown growth inhibition (5 mg/mL) as well as antiadhesive properties (10 mg/mL) on pathogens such as *S. aureus* (90% and 100%, respectively), *C. albicans* (64% and 100%), and *Streptococcus agalactiae* (92% and 100%) [113]. Antiadhesive and antimicrobial activities of rufisan, a biosurfactant produced by the yeast *Candida lipolytica* UCP0988, was described [114]. Crude biosurfactant presented antiadhesive potential at concentrations higher than 0.75 mg/L against most of the bacteria tested: *S. aureus*, *S. agalactiae*, and *Streptococcus mutans*. The antiadhesive properties were proportional to the concentration of the biosurfactant, whereas the antimicrobial activities were observed at higher biosurfactant concentrations.

Mannosylerythritol lipid: This biosurfactant contains 4-*O*-β-D-mannopyranosylmesoerythritol as the hydrophilic group and a fatty acid and/or an acetyl group as the hydrophobic moiety. The fatty acid chains can be short (2−8 carbon atoms) or long (10−18 carbon atoms). Mannosylerythritol lipid (MEL) is reported to be secreted by *Ustilago* sp. as a minor component along with cellobiose [115]. Regarding *Schizonella melanogramma* (shizonellin) [116], *Geotrichum candidum* [117], and *P.* (previously *Candida*) *antarctica,* it is secreted as a major component [118]. The type of MEL varies with the strain and the species of *Pseudozyma*. Hence its production can be used as an important taxonomic index to identify *Pseudozyma* yeasts. The MELs are classified as MEL-A, -B, -C, and -D considering the degree of acetylation at the C4 and C6 positions and their order of appearance on thin-layer chromatography. MEL-A is the diacetylated compound, MEL-B and MEL-C are monoacetylated at C6 and C4, respectively, and the completely deacetylated structure is known as MEL-D [119].

Mixtures of these four MELs, but predominantly MEL-A and MEL-B, isolated from *C. antarctica* T34 showed surface-active and antimicrobial activities, including activity against *C. albicans* [118]. Two MELs, schizonellin A and B, from the fungus *S. melanogramma* were effective against fungi, gram-positive bacteria, and some gram-negative bacteria [116].

Cellobiose lipids: Flocculosin, a cellobiose lipid from *Pseudozyma flocculosa*, possesses a C8-hydroxy acid and two acetyl groups in the cellobiose residue. It presents in vitro antifungal potential against various pathogenic yeasts that cause human mycoses, like *Candida lusitaniae*, *Cryptococcus neoformans*, *Trichosporon asahii*, and *C. albicans* [120]. It was also particularly effective against *Staphylococcus* species, including MRSA, and its antimicrobial potential was not modified by the presence of resistance mechanisms against methicillin and vancomycin [121]. This molecule was able to kill *C. albicans* cells quickly, in a dose-dependent manner. The K^+ efflux detected in flocculosin-treated cells suggested that the glycolipid acts by disrupting the cell membrane.

The cellobiose lipid secreted by *Cryptococcus humicola* presents 16-(tetra-*O*-acetyl-β-cellobiosyloxy)-2-hydroxyhexadecanoic acid as the major product and 16-(tetra-*O*-acetyl-β-cellobiosyloxy)-2,15-dihydrohexadecanoic acid as a minor product, whereas *Pseudozyma fusiformata* secreted mainly 16-[6-*O*-acetyl-2′-*O*-(3-hydroxyhexanoyl)-β-cellobiosyloxy]-2,15-dihydroxyhexadecanoic acid. These compounds exhibit similar fungicidal activities against different yeasts including pathogenic *Cryptococcus* and *Candida* species. *Filobasidiella neoformans* cells died after 30 min incubation with 0.02 mg/mL cellobiose lipids. The same effect on ascomycetous yeast, including pathogenic *Candida* species, is achieved at 0.1−0.3 mg/mL cellobiose depending on the test culture used [122].

Sophorolipids: This group is generally recognized as disaccharide sophoroses (2-*O*-β-D-glucopyranosyl-D-glucopyranose) β-glycosidically linked to the hydroxyl group at the last but one carbon of fatty acids. Sophorolipids (SLs) are produced by nonpathogenic yeasts, like *Candida bombicola*, *Candida apicola*, and *Candida bogoriensis*. Similar to other biosurfactants, SLs present antimicrobial potential. The mechanism of action is not merely restricted toward bacteria; they also present antifungal, antialgal, antimycoplasmal, and antiviral activities [123]. The biological activity of this group involves destabilization and alteration of cellular membrane permeability [124]. SLs inhibited the growth of several gram-positive bacteria in concentrations from 50 to 29,000 μg/mL, such as *B. subtilis*, *S. epidermidis*, *S. aureus*, *S. faecium*, *Propionibacterium acnes*, and *Corynebacterium xerosis* [125,126].

Biosurfactants could be useful to increase the efficacy of known antimicrobials and biocides. Coadministration of SL produced by C. *bombicola* ATCC 22214 enhanced the action of antimicrobials in representative gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*). Taking into consideration the self-assembly capacity of SL, it has been postulated that self-assembled SLs can span the bacterial cell membrane and thus make possible the entrance of antimicrobial molecules [127].

Trehalose lipids and succinyl trehalose lipids: Trehalose lipids (TL-1 and TL-2) do not show inhibition properties against gram-negative bacteria and yeast, but TL-1 presents antifungal activity by inhibiting the germination of conidia from *Glomerella cingulata* at 300 mg/L [128]. Succinyl trehalose lipids (STL-1 and STL-2) show antifungal and antiviral properties, as they are capable of inhibiting herpes simplex and influenza viruses at concentrations from 11 to 33 mg/L [1,129].

Trehalose lipids (TLs) synthesized by *Tsukamurella* sp. strain DSM 44370 jointly with trisaccharide and tetrasaccharide lipids presented partial activity against gram-positive bacteria, with the exception of *S. aureus*, whereas gram-negatives were poorly or not inhibited at all [130]. Studies performed to clarify the molecular interactions between this molecule and the lipid component of the cell membrane revealed that TLs improved the fluidity of phosphatidylethanolamine and phosphatidylserine membranes and formed domains in the fluid state, but did not change the macroscopic bilayer organization [131].

Xylolipids: Xylolipids produced by probiotic bacteria such as *Lactococcus lactis* using paraffin as a carbon source showed antibacterial activity against the pathogens *E. coli* and *S. aureus* resistant to multiple drugs, such as methicillin. These glycolipids have a great potential as an alternative to the use of pharmaceuticals for oral and dermal administration [132].

Oligosaccharide lipids: Oligosaccharide lipids (GL) produced by *Tsukamurella* sp. show certain inhibition properties against some gram-positive and gram-negative bacteria. GL-1, GL-2, and GL-3 inhibit the growth of *Bacillus megaterium* at concentrations of 50, 100, and 150 μg/mL, respectively [131]. *Tsukamurella* sp. DSM 44370 produces a mix of GLs when cultured in sunflower oil, but when using marigold oil as the carbon source, the production is increased 60% and the proportion is modified on GL-3 mainly. When these glycolipids are modified through microbial lipases, molecules that are biologically active against the activation of the Epstein−Barr virus are formed [133].

Other glycolipids: Complexes of glycolipids from *Brevibacterium casei* MSA19 have been reported to disrupt and significantly inhibit single and mixed biofilms of pathogenic and nonpathogenic bacteria of human and fish at concentrations of 30 μg/mL [134]. A glycolipid biosurfactant from *Lysinibacillus fusiformis* S9 restricted the biofilm formation of *E. coli* and *S. mutans* completely at a concentration of 40 μg/mL but did not show any bactericidal activity. The biosurfactant inhibited bacterial attachment and biofilm formation equally on hydrophilic and hydrophobic surfaces like glass and catheter tubing [135]. *Serratia marcescens* is capable of producing a glycolipid composed of glucose and palmitic acid that prevented the adhesion of *C. albicans*, *P. aeruginosa*, and *Bacillus pumilus*, as well as interrupting the formation of biofilms of these cultures in microtitration plates [136].

15.4.3 Biosurfactants From *Lactobacillus*

Biosurfactants isolated from lactobacilli have been shown to reduce the adhesion of pathogenic microorganisms to glass [137], silicone rubber [138], surgical implants [139], and voice prostheses [140]. Biosurfactant produced by *Lactobacillus paracasei* subsp. *paracasei* A20 was effective against gram-positive and gram-negative bacteria, yeasts, and filamentous fungi [141]. The biomolecule also showed antiadhesive activity against all the bacteria tested. But the antiadhesive activities were highest for *S. aureus*, *S. epidermidis*, and *S. agalactiae* compared to the values observed for *E. coli*, *C. albicans*, and *P. aeruginosa*, in contrast with the antimicrobial activity exhibited against these microorganisms at the same biosurfactant concentrations. Biosurfactants produced by *Streptococcus thermophilus* A and *L. lactis* 53 presented significant antimicrobial activity against different bacteria and yeasts obtained from explanted voice prostheses [142,143]. The total growth inhibition pattern of these biomolecules against *C. albicans*, *S. aureus*, and *S. epidermidis* was similar to those observed for the biosurfactants isolated from *L. paracasei* subsp. *paracasei* A20, with concentrations between 25 and 100 mg/mL [143]. Another probiotic strain, *Lactobacillus acidophilus*, was able to reduce the biofilm

formation of *S. mutans*, a primary dental cariogen, by inhibiting its attachment [144]. Velraeds et al. [137] also described the inhibition of pathogenic enteric bacteria adhesion by a biosurfactant produced by a *Lactobacillus* strain and then reported that the biosurfactant triggered a dose-related inhibition of the initial deposition speed of *E. coli* and other bacteria adherent on both hydrophobic and hydrophilic substrates. More recently, antimicrobial activities against clinical multidrug-resistant strains of *A. baumannii*, *E. coli*, and *S. aureus* (MRSA) by biosurfactant produced by *Lactobacillus jensenii* and *Lactobacillus rhamnosus* at 25−50 mg/mL was demonstrated. Antiadhesive and antibiofilm potentials were also proposed for the aforementioned pathogens ranging from 25 to 50 mg/mL. Biosurfactant caused membrane damage to *A. baumannii* and marked cell wall injury in *S. aureus* [145]. Our group showed that the combination of subinhibitory concentrations of biosurfactant produced by *L. jensenii* P6A and benzazoles presented a concentration-dependent synergism against *E. coli* and *C. albicans*.

15.4.4 High-Molecular-Weight Biosurfactants

Polymeric biosurfactants are synthesized by several bacteria and are made of lipoproteins, proteins, polysaccharides, LPSs, or complexes containing these molecules [146]. These high-molecular-weight biosurfactants generally possess effective emulsifying activity rather than an interfacial tension reducer, and are called bioemulsifiers. The best-studied biopolymer is emulsan, an LPS isolated from *Acinetobacter calcoaceticus* RAG-1 ATCC 31012, and its molecular weight is on average 1000 kDa [147]. Its surface activity is attributable to the presence of fatty acids in its hydrophobic portion, encompassing 15% of its dry weight, which are bound to the polysaccharide backbone through ester and amide bonds [148]. An additional high-molecular-weight biosurfactant is alasan, a complex of protein and an anionic polysaccharide, with a molecular weight of approximately 1000 kDa, isolated from *Acinetobacter radioresistens* [149].

Yeasts also can produce emulsifiers, which are particularly interesting because of the food-grade status of several species, which allows their use in pharmaceutical and food-related industries. Rufino et al. [114] reported that a polymeric biosurfactant produced by *C. lipolytica* UCP 0988 showed antiadhesive properties of 88% for *S. aureus* and 91% for *Lactobacillus casei*, at a concentration of 0.75 mg/mL, as well as antimicrobial activities (64.6%) at a concentration of 6 mg/mL for *S. mutans* HG985, and *E. coli* inhibition was 5% at 6 mg/mL.

Biosurfactants with antiadhesive properties produced by yeasts have been described by our group. A glycolipid-type biosurfactant produced by the yeast *Trichosporon montevideense* CLOA72 diminished biofilm formation by *C. albicans* CC obtained from the apical tooth canal by up to 85% in polystyrene microplates [52]. It was suggested that this inhibition was the result of interactions of the biosurfactant with the cell or the polystyrene plate surface and resulting modifications of net surface charge. We described also that the incubation of *C. albicans* CC cells in the presence of the glycolipid inhibited germ tube or hypha formation during biofilm formation on the polystyrene

plate surface, and the cells showed lower values of cell-surface hydrophobibity (evaluated by microbial adhesion to hydrocarbons test, MATH) compared to cells incubated without the biosurfactant. The inhibition of adhesion can result from both free surfactant molecules and micelles, which may adsorb on the cell surface of microorganisms and solid surfaces, blocking or facilitating microbial adherence to hydrophobic and hydrophilic interfaces [3]. Our group showed also that adhesion of a clinical isolate of *C. albicans* cells to epithelial buccal cells was reduced up to 87.4% with the use of biosurfactant at 16 mg/mL concentration. Measurements of the ζ potential of the cell surface showing an alteration of net surface charge ranged from −20.1 to −8.3 mV during the titration with biosurfactant.

15.5 Potential Use of Biosurfactants in Nanotechnology

15.5.1 Functionalized Nanoparticles

Nanoparticles are made of various inorganic or organic materials and range from 1 to 100 nm in size. Compared to bulk materials, nanoparticles present improved characteristics that might be of interest for several practical fields, such as optical, magnetic, and electric behavior under specific conditions, beyond their use as vectors for delivering substances to a specific target, such as drugs to their receptors [150]. Functionalization is the attachment of chemical (functional) groups to a polymer, material, or any other possible target, using routes of chemical synthesis. The mechanism by which nanoparticles reach functionalization is dependent on the functional group attached to them, and on the aimed at structure−target interaction. Despite several controversies on the biological context of nanoparticles, functionalization can improve the recognition (by any biological or chemical entity), vectorization, and distribution of nanoparticles, and potential toxic effects for humans, animals, and the environment might be reduced [151,152].

One might ask, why use biosurfactants to functionalize nanoparticles? The answer relies first on the efficiency of stabilization and dispersion compared to other chemical synthesis strategies and on applied biocompatibility and biodegradability concepts: the use of biosurfactants can effectively improve the desired characteristics of nanoparticles, and this complex can exist in contact with biological tissues or systems, and the environment, without causing significant injury, harm, or damage to them. Biosurfactants also reduce the formation of other aggregates than vesicles owing to electrostatic forces of attraction and provide standardized morphology of nanoparticles. Thus, it is of interest to replace chemicals that often offer compatibility risks and environmental impacts with these compounds [51,153].

The activity of $AgNO_3$ combined with the LP biosurfactant V9T14 from *B. licheniformis* was evaluated against a preformed *E. coli* biofilm on the Calgary Biofilm Device [8]. The combined use resulted in a reduction in the quantity of silver used to achieve greater antimicrobial impact. The concentrations of silver in the silver−biosurfactant solutions were 129- to 258-fold less than the concentrations needed when silver was used alone.

Silver nanoparticles (AgNPs) functionalized with Kocuran, an exopolysaccharide produced by *Kocuria rosea* strain BS-1 with repeating monosaccharide residues of D-glucose, D-mannose, D-galactose, and D-glucuronic acid (51.2 kDa), showed antimicrobial activity against *S. aureus* and *E. coli* [154]. The cell death was mainly due to hydroxyl radical induction and depletion of NADH. The improved antimicrobial activity of Kocuran-capped AgNPs may be credited to the surface functionalization with the EPS sugar residues, which makes possible the distribution and diffusion of glyconanoparticles into the cell wall, and the structure−activity relationship. The particles also inhibited the biofilm development by these bacteria in Kocuran-capped AgNP-coated silicone urethral catheters.

Reddy et al. [155] functionalized gold nanoparticles with surfactin and investigated the effects of proton concentrations and temperature on the morphology of these nanoparticles. Nanoparticles synthesized at pH 7 and 9 remained stable for 2 months, whereas aggregates were stable for only 24 h, at pH 5. At pH 7, nanoparticles presented a homogeneous distribution of shape and size. At pH 5, they were polydispersed, and at pH 9 they were anisotropic. The nanoparticles produced at room temperature were monodispersed and were more uniform compared to those formed at 4°C [155].

Spherical nickel oxide nanoparticles were synthesized by Palanisamy and Raichur [156], using RLs in a microemulsion prepared with *n*-heptane and water. The synthesized nickel particles were crystalline and spherical and presented piled lamellar sheets, as suggested by scanning and transmission electron microscopy. The particle size decreased as pH increased: at pH 11.6, particles were in the range of 86 ± 8 nm, and at pH 12.5 the particles were in the range of 47 ± 5 nm [156].

Purified RLs from *P. aeruginosa* were used to synthesize AgNPs by Kumar et al. [157]. These nanoparticles exhibited antimicrobial activity against both gram-positive and gram-negative pathogens and *C. albicans*, suggesting a broad-spectrum antimicrobial activity. The nanoparticles were spherical in shape and monodispersed, with average particle size of 15.1 nm, and had an adsorption peak at 410 nm [157].

Narayanan et al. [158] used RLs to functionalize ZnS nanoparticles of about 4.5 nm. The particles were stabilized in an aqueous environment and ranged up to 10 nm. The functional groups −OH, −CH2, −CH3, −C═O (carbonyl), −COO− (carboxyl), and −C−O−C− were successfully added to the nanoparticles, which moved from a narrow absorption (at 340 nm) to a broad emission spectrum (450 nm). Although the reason for the broadening of the emission spectra was not clear, this was used to confirm the capping efficiency [158].

Iron oxide nanoparticles were functionalized by Sangeetha et al. [159] with surfactin, RL, polyethylene glycol (PEG), and dextran. Cell viability assays using the L929 mouse fibroblast cell line indicated that pure surfactin, RL, and dextran showed cytotoxicity as the concentration increased; however, pure PEG exhibited biocompatibility at different concentrations. Fourier transform infrared (FTIR) and thermogravimetric analyses confirmed the presence of adsorbed moieties on nanoparticles [159].

15.5.2 Liposome Overview

Liposomes are closed lipid systems that are widely studied in various scientific fields, the pharmaceutical, cosmetics, and food sciences being the most advanced in their exploration. These systems were originally designed for studying the behavior of biological membranes; their biotechnological advantages due to controlled release and entrapment protection were first explored only in the 1970s, by G. Gregoriadis [160]. Controlled release provides a sustainable delivery of the substance of interest, and liposome entrapment has several advantages: bioactive compounds can support the presence of reactive species, exposure to extreme pH and temperature, high ion concentrations, and, often, light exposure. Hydrophilic and hydrophobic molecules can be put in the same vesicle: the first are localized in the aqueous core and the second in the membranes. Different delivery mechanisms, like rupture, permeability, or fusion to other membranes, are dependent on the surface that the liposome will have contact with; nevertheless, this increases even more the versatility of liposomes, considering that the rate of delivery might be different in each delivery mechanism [160,161].

The main components of liposomes comprise phospholipids, sterols, and antioxidants, and they may contain a single lipid membrane (unilamellar) or multiple membranes (multilamellar), depending on how the formulation is designed. Liposome size may range from nanometers to micrometers, and when the size is up to 100 nm, they are called nanoliposomes. The sizing can be achieved by techniques such as sonication and extrusion in polymeric membranes (made of materials such as polycarbonate). Several methods of liposome preparation are well described, such as lipid film hydration, reverse-phase evaporation, and injection into organic solvent systems [160].

Liposome nanotechnology relies largely on the biophysical properties of phospholipids and their interactions with the other components of liposome formulations: when dissolved in water or other polar solvents, phospholipids undergo spontaneous micellization, and then assemble into vesicles, mimicking cellular phospholipid bilayers. Proper combinations of liposome components determine characteristics such as membrane fluidity, allowing conformational flexibility. Loaded molecules become protected from degradation by external factors like free radical species and degrading enzymes [162,163]. This is especially important when liposomes are designed for crossing biological barriers such as tissues and bacterial biofilms.

The encapsulation of natural or synthetic antimicrobial molecules is of great potential to overcome microbial resistance. Synthetic drugs have been successfully encapsulated by several research groups, and strong increases in drug potency are expected and experimentally observed in most of the studies. Nevertheless, as the mechanisms of action of drugs are very specific, not only do antimicrobial drugs show a narrow spectrum of action, but the risk of developing resistance is only delayed and not avoided by the entrapment [6]. Hence, the investigation of antimicrobial alternatives that do not induce resistance is of paramount importance.

As discussed in previous sections, biosurfactants have been explored as new natural antimicrobial molecules with a broad spectrum of action. The entrapment of these molecules in liposomes can enhance their biological activity and also provide even more value to them, such that they might be used in formulations for clinical and nonclinical use against microorganisms. The subject, however, as of this writing, remains poorly investigated.

15.5.2.1 Interactions With Liposome Lipids

Hydrophobic moieties of biosurfactants and other natural antimicrobial compounds produced by bacteria usually comprise unsaturated or saturated hydrocarbon chains or fatty acids. In contrast, the main hydrophilic moieties are positively or negatively charged peptides (cations or anions), generating LPs, or mono-, di-, or polysaccharides, generating glycolipids. Thus, a range of structurally and chemically diverse molecules can be produced by different microorganisms, with different physicochemical properties [6,11].

Some of these molecules tend to aggregate at the interfaces where they are applied, using hydrogen bonds and hydrophobic and van der Waals interactions, forming an interfacial film that affects both the surface energy and the wettability of a surface. They also reduce the interfacial tension on the boundary existing between immiscible liquids, generating emulsions. In general, changes in the rheological behavior of any liquid or emulsified (semisolid) system are expected when biosurfactants are used, given that both micelles and liposomes can be formed. Glycolipids may form spherical micelles or disk-like or rod-like structures. MELs, in particular MEL-A, because of their molecular orientation and balance between hydrophilic and hydrophobic groups, can form vesicles larger than 10 μm, regarded as of giant size. On the other hand, RLs show a pH-sensitive alteration of structures: micelles can be formed at pH 6.8−7, lipid particles at pH 6.6−6.2, lamellar structures at pH 6.5−6.0, and 50- to 100-nm vesicles can be formed at pH 5.8−4.3. This conversion can be observed because of the presence of a carboxyl group on the side chain of the molecule [164,165].

Various issues can be raised regarding liposome entrapment of antimicrobial molecules: because the exposed biological features can interfere with the stability of liposome formulations, it is important to understand what events might be involved within phospholipid interactions and how these can interfere in entrapment efficiency. In this context, Deleu et al. [166] used biophysical techniques like cryogenic transmission electron microscopy and differential scanning calorimetry to study the mechanism of membrane perturbation by fengycin, using monolayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). The mechanism seems to be based on physicochemical properties that provide a concentration-dependent state transition: in low concentrations, fengycin is monomeric and nonperturbing. Its insertion does not affect the phospholipid interfacial assembly, being dispersed as a monomer on the hydrophobic core. At medium concentrations, self-association of fengycin with phospholipids can be observed, affecting the lipid bilayer. At high concentrations, the lipid bilayer is completely disrupted into micelles, and membrane leakage and bioactivity are seen [166].

Using isothermal titration calorimetry, Andrushchenko et al. [167] investigated the thermodynamics of the interaction of the cathelicidin peptides tritrpticin and indolicidin, and five tritrpticin analogues, with large unilamellar vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). A natural *E. coli* polar lipid extract was also used. All peptides exhibited poor binding to POPC, but presented stronger binding to anionic POPE/POPG and *E. coli* membrane systems. The electrostatic interactions between the highly positively charged peptides and the negatively charged membrane surface of these lipids help to explain this effect [167].

Exploring a similar approach to this question, Deleu et al. [168] investigated the effects of surfactin on membrane structure using fluid-disordered and gel-phase models of membranes, using 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and DPPC lipids, respectively. It was demonstrated that the surfactin concentration is critical for its behavior on the lipid membrane. Surfactin interacts with both the membrane polar heads and the acyl chain region, and the presence of rigid domains plays an essential role in surfactin entrapment. At concentrations below the CMC, surfactin inserted at the boundary between gel and fluid lipid domains, inhibited phase separation, and stiffened the bilayer without inducing any morphological changes in liposomes. At concentrations close to the CMC, phospholipids were solubilized. At higher concentrations, both fluid and rigid bilayers were dissolved into micelles and other structures of a wide size distribution.

Abbasi et al. [169] investigated the interactions of a monorhamnolipid produced by a *Pseudomonas* strain with dielaidoylphosphatidylethanolamine (DEPE) membranes. The authors observed that the amalgamation of monorhamnolipids into DEPE reduced the onset temperature of phase transition. The coexistence of lamellar and hexagonal phases in this complex was observed at 60°C with better miscibility of monorhamnolipids into DEPE in the fluid phase than in the gel phase. At this phase, however, pure DEPE is exclusively lamellar. The loss of stability was also demonstrated through shifts in the frequency of DEPE bands in FTIR analysis to higher wave numbers.

15.5.3 Gene Transfection With Liposomes

Ladokhin et al. [170] assessed the interactions of indolicidin with large unilamellar vesicles formed from POPC and POPG. It was observed that indolicidin binds strongly to both neutral POPC and POPG vesicles, but the process was reversible. Fluorescence assays showed that indolicidin was inserted in the bilayer interface and induced membrane permeabilization. Leakage induction was more effective with POPG (anionic rather than neutral) vesicles than with POPC vesicles [170].

Inoh et al. [163] developed different liposome formulations for gene transfection with MEL-A, MEL-B, and MEL-C. Only MEL-A enhanced the efficiency of gene transfection. Maitani et al. [12] prepared liposomes using cholesterol, L-dioleoylphosphatidylethanolamine (DOPE), and two biosurfactants, β-sitosterol β-D-glucoside (Sit-G) and

MEL-A, for transfecting a thymidine kinase gene. Vesicles of about 300 nm were generated, and using a luciferase marker gene and the measurement of the activity of the enzyme, it was demonstrated that Sit-G and MEL liposomes presented higher transfection efficiency compared to conventional liposomes.

MEL-A liposomes were also investigated by Igarashi et al. [171] in combination with DC-cholesterol and DOPE, aiming to increase the transfection efficiency of a plasmid in human cervix carcinoma HeLa cells. Vesicles of 169 nm were generated, and compared to conventional liposomes, MEL-A induced a significantly higher level of gene expression. Confocal microscopy indicated that the MEL-A liposome was widely distributed in the cytoplasm, and flow cytometry assays showed that DNA was strongly detected in the cytoplasm and around the nucleus. Also, Nakanishi et al. [13] showed that MEL-A liposomes remarkably promoted the efficiency of gene transfection into mammalian cultured cells with 1 h or shorter incubation time.

Inoh et al. [172] determined the efficiency of cationic liposomes containing MEL-A for small interfering RNA (siRNA) delivery, to interrupt gene transcription. Protein expression was strongly suppressed at 24 h after target cells were incubated for 30 min with MEL-A liposome-entrapped siRNA. Rapid siRNA delivery into the cytosol caused the suppression of protein expression, as demonstrated by confocal microscopic analysis. This transfection system was superior to commercially available transfection kits. Later, the group of Nakanishi et al. [173] investigated the effect of MEL-A in liposomes for transfection of plasmid DNA. Despite the entrapment efficiency being equal to that of conventional liposomes, the transfection efficiency of MEL-A liposomes was more than 10 times higher than that of conventional vesicles.

15.6 Conclusion and Perspectives

Biosurfactants comprise a set of molecules that are of great interest for various areas and represent a potential alternative to synthetic antimicrobial products. There is evidence that events of acquisition of resistance to antimicrobial biosurfactants due to natural selection are unlikely. When entrapped in liposomes, biofilms can be reached and disrupted more efficiently. The controlled release provided by liposomes preserves the chemical stability and antimicrobial activity for prolonged periods compared to free molecules. However, remaining challenges are to overcome the (often) unstable behavior of phospholipid–biosurfactant interactions, and to scale up the manufacturing process. Studies on liposome-entrapped biosurfactants will provide promising alternatives for safe and efficient control of microbial biofilm, such that drug resistance can be better managed than it is nowadays.

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16

Bacteriocins as Antimicrobial and Antibiofilm Agents

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16.1 Introduction

Biofilms are the predominant mode of growth for bacteria in most natural, industrial, and clinical environments, consisting of densely packed multispecies populations of cells encased in a self-synthesized polymeric matrix and attached to a tissue or surface [1]. Biofilms have been implicated in chronic disease (tooth decay, periodontitis, otitis media, necrotizing fasciitis, biliary tract infection, bacterial prostatitis, osteomyelitis, native valve endocarditis, cystic fibrosis pneumonia, and melioidosis), health care-associated infections (pneumonia, sutures, exit sites, urinary catheter cystitis), dissemination of community-acquired diseases, and effective hygienic processing and increased failure rate of antiinfective therapy, in addition to food-borne diseases. This surface-adhered complex bacterial community is highly resistant to antimicrobials compared to their planktonic analogues. Thus, the treatment of biofilms with antibiotics or other biocides is usually ineffective at eradicating them. As a result, novel therapeutic solutions other than the conventional antimicrobial therapies are an urgent need. Bacteriocins have attracted attention as potential substitutes for, or as additions to, currently used antimicrobial compounds, as such molecules are stable and potent against multidrug-resistant strains [2].

Bacteriocins are proteins or peptides ribosomally synthesized that are produced by species of the Bacteria and Archaea domains, and are usually active against strains of bacteria that are closely related to the producer strains or are unrelated (narrow and broad spectrum, respectively) [3–5]. Almost all bacteria can produce at least one bacteriocin and most of them remain unidentified [6]. Although the exact role of bacteriocins in nature is not well understood, these biomolecules may have an important role in colonizing or facilitating the introduction and/or dominance of a producer onto an already occupied niche [7]. Alternatively, bacteriocins may act as antimicrobial peptides, directly inhibiting competing strains or pathogens, or may function as signaling peptides, either signaling

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other bacteria through quorum sensing and bacterial cross-talk within microbial communities or signaling cells of the host immune system [8–10].

The exploitation of bacteriocins for microbial biofilm control is a relatively new research field. Nevertheless, most studies provide evidence of the promising potential of bacteriocins, given their potency, efficacy, resistance to heat and pH variation, possibility of chemical modification, low toxicity, availability of both broad- and narrow–spectrum activities, and the chance of in situ production by probiotic microorganisms [4,5]. Another appreciated characteristic of bacteriocins is their narrow inhibitory spectrum that provides an efficient means to direct activity toward certain pathogens without disturbing the commensal bacterial microbiota, which can contribute health-giving benefits to the mammalian host [11].

Bacteriocin molecules are also reported to inhibit relevant pathogens like Shiga toxin-producing *Escherichia coli* (STEC), enterotoxigenic *E. coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), *Agrobacterium*, and *Brenneria* spp. [5,12]. The potency of bacteriocins against clinically important pathogens varies both across and within the various peptide subclasses.

In past years, the application of encapsulation technology in liposomes for bacteriocins has been evaluated [13–16]. Liposomes are colloidal carriers that form spontaneously when certain phospholipids are hydrated in aqueous media [17]. They are composed of relatively biocompatible and biodegradable material and consist of an aqueous space surrounded by one or more layers of natural and/or synthetic lipids. As liposomes contain both lipid and aqueous phases, they can be utilized for the entrapment, delivery, and release of hydrophilic, hydrophobic, and amphiphilic compounds [18]. This technology has arisen with great potential in the treatment of biofilm infections, owing to good biocompatibility, stability, ample range and extent of drugs that they can carry, as well as the protection provided by the encapsulation of the drug in the biological milieu, decreasing toxicity and allowing the drug to reach the specific site.

Therefore, in this chapter, we describe the various classes of bacteriocins and the contribution of these metabolites as well as the drug-delivery nanocarrier technologies for planktonic cells and biofilm control.

16.2 Classification

Historically, bacteriocins are classified according to the producer microorganism, chemical structure, heat stability, molecular mass, enzymatic sensitivity, presence of modified amino acids, amino acid sequence homologies, gene cluster organization, and mechanism of action. Because of the large diversity of bacteriocins, several approaches have been taken to classify these biomolecules [3–5,19,20].

One way used to classify the bacteriocins of gram-negative organisms divides these biomolecules into small peptides, such as microcins (Mccs), and larger proteins, such as colicins (Cols). Mccs have previously been divided on the basis of the presence (class I) or absence (class II) of posttranslational modifications [21] (Table 16.1). Class I Mccs includes MccB17, MccC7-C51, MccD93, and MccJ25 of low molecular mass (<5 kDa)

Table 16.1 Classification Scheme for Gram-Negative Microcins

Class	Characteristics	Microcin	Receptor	Translocator	Molecular Mass (Da)	Producing Strain	References
I	Low-molecular-mass peptides (<5 kDa), posttranslationally modified	MccB17	OmpF	OmpF, SbmA	3094	*Escherichia coli*	[23]
		MccC7-C51	OmpF	OmpF, YejABEF	1177	*E. coli*	[22]
		MccD93	—	—	<1000	*E. coli*	[24]
		MccJ25	FhuA	Fhua, TonB, ExbBD, SbmA	2107	*E. coli*	[25]
II: Larger (5—10 kDa) peptides, with or without posttranslational modifications	Class IIa: Require more than one gene for synthesis and assemblage of functional peptides	MccL	Cir	Cir, TonB, ExbBD, SdaC	8884	*E. coli*	[26,27]
		MccV	Cir	Cir, TonB, ExbBD	8741	*E. coli*	[28]
		MccN	OmpF, OmpC, Phoe	TolAQR	7274	*E. coli*	[29]
	Class IIb: Linear peptides carrying a C-terminal siderophore as posttranslational modification or not at C-terminus	MccE492	FepA, Cir, Fiu	FepA, Cir, Fiu, TonB, ExbBD	7886	*Klebsiella pneumoniae*	[30,31]
		MccM	FepA, Cir, Fiu	FepA, Cir, Fiu, TonB, ExbBD	7284	*E. coli*	[32]
		MccH47	FepA, Cir, Fiu	FepA, Cir, Fiu, TonB, ExbBD	4865	*E. coli*	[32]
		I47[a]	FepA, Cir, Fiu	FepA, Cir, Fiu, TonB, ExbBD	—	*E. coli*	[33]
		G492[a]	FepA, Cir, Fiu	FepA, Cir, Fiu, TonB, ExbBD	—	*K. pneumoniae* RYC49	[32]

[a]Predicted by genome sequence in silico analysis.

and the class II Mccs are of higher molecular mass (5−10 kDa). Class II Mccs can be divided into subclasses IIa (such as MccL, MccV, and MccN24), which require three different genes to synthesize and assemble functional peptides, and IIb (MccE492, MccM, and MccH47), linear peptides with or without posttranslational modifications at the C-terminal region [22].

Cols are categorized into two groups (A and B) according to translocation across the outer membrane of sensitive bacteria (Table 16.2). Group A Cols use the Tol protein system, for instance: ColE1−E9, ColA, ColK, ColN, ColS4, ColU, Col28b, ColDF13, and group B uses the Ton system, such as Col5, Col10, ColB, ColD, ColM, MccV, ColIa, and ColIb [34].

As Cols reach and enter the target cell, they can be divided into three major classes based on the mechanisms of action:

1. pore formation, including ColA, ColB, ColE1, ColIa, ColIb, ColK, and ColN;
2. nuclease activity, with DNase, 16S rRNase, and tRNase activities, also with ColE2 to E9; and
3. peptidoglycanase activity: these proteins can digest the peptidoglycan precursor, leading to a failure to produce peptidoglycan and, thus, bacterial death, e.g., ColM [24].

The classification of bacteriocins from gram-positive bacteria used today has been modified in several steps based on the initial classification scheme proposed by Klaenhammer [3] (Table 16.3). The bacteriocins were grouped in four major classes (I−IV) based on their probable structure and mode of action as predicted at that time. In this classification, class I included lantibiotics (peptides <5 kDa posttranslationally modified resulting in lanthionine, β-methyllanthionine, dehydroalanine and dehydrobutyrine), class II comprised small (<10 kDa) unmodified heat-stable peptides, class III comprised large (>30 kDa) heat-labile molecules, and class IV contained complex molecules bound to lipids or carbohydrates. Both class I and class II bacteriocins are secreted by ATP-binding cassette (ABC) transporter proteins. Three subgroups of class II bacteriocins were also included: pediocin-like or *Listeria*-active with a YGNGVXC motif near the N-terminus and a GG leader peptide (IIa), two-component peptides with a GG leader peptide (IIb), and thiol-activated peptides that require reduced cysteine for activity (IIc). Nes et al. [76] regrouped the class II bacteriocins, retaining the pediocin-like bacteriocin in class IIa and the two-peptide bacteriocins in class IIb, but changing class IIc to include bacteriocins that contain a typical signal peptide and that are secreted by the translocase general secretion (Sec) pathway. These authors defined the circular bacteriocins as class IId and excluded class IV bacteriocins. In contrast, van Belkum and Stiles [77] subdivided the class II bacteriocins considering the number of cysteine residues and resultant disulfide bridges as a criterion for classification. In their classification, the bacteriocins that are secreted by the signal peptide pathway are not classified as a separate group because different types of bacteriocins are produced by the Sec pathway with a signal peptide as part of the prepeptide. In 2003, Kemperman et al. [78]

Table 16.2 Classification Scheme for Gram-Negative Colicins

	Colicin	Antibacterial Activity	Receptor	System Translocation	Molecular Mass (Da)	Producing Strain	References
Group A	A	Pore-forming	BtuB	OmpF, TolABQR	62,989	*Citrobacter freundii*, *Escherichia coli*	[35–37]
	E1	Pore-forming	BtuB	TolC, TolABQR	57,279	*E. coli*	[38]
	K	Pore-forming	Tsx, OmpFA	OmpA, OmpF, TolAQR	59,611	*E. coli*	[39,40]
	N	Pore-forming	OmpF	OmpF, TolABQR	41,696	*E. coli*	[41]
	S4	Pore-forming	OmpW	OmpF, TolABQR	54,085	*E. coli*	[42]
	U	Pore-forming	OmpA	OmpF, LPS, TolABQR	66,289	*Shigella boydii*	[43]
	Alveicin A	Pore-forming	—	TolABQR	43,027	*Hafnia alvei*	[44]
	Alveicin B	Pore-forming	—	TolABQR	38,183	*H. alvei*	[44]
	Marcescin 28b	Pore-forming	Omp4, OmpA, F, LPS	OmpF, TolABQR	47,462	*Serratia marcescens*	[45,46]
	E3	16S rRNase	BtuB	OmpF, TolABQR	57,960	*E. coli*	[47,48]
	E4	16S rRNase	BtuB	OmpF, TolABQR		*E. coli*	[37]
	E6	16S rRNase	BtuB	OmpF, TolABQR	58,011	*E. coli*	[37,49]
	Cloacin DF13	16S rRNase	LutA, OmpF	TolAQR	59,293	*Enterobacter cloacae*	[49–51]
	E2	DNase	BtuB	OmpF, TolABQR	61,561	*E. coli*, *Shigella sonnei*	[35,47,52]
	E7	DNase	BtuB	OmpF, TolABQR	61,349	*E. coli*	[53]
	E8	DNase	BtuB	OmpF, TolABQR	70,000	*E. coli*	[54,55]
	E9	DNase	BtuB	OmpF, TolABQR	61,587	*E. coli*	[55,56]
	E5	tRNase	BtuB	OmpF, TolABQR	58,254	*E. coli*, *S. sonnei*	[37]

Continued

Table 16.2 Classification Scheme for Gram-Negative Colicins—cont'd

	Colicin	Antibacterial Activity	Receptor	System Translocation	Molecular Mass (Da)	Producing Strain	References
Group B	Pyocin AP41	DNase	*orf1—tolQRA*, *tolB*, *oprL—orf2*	OmpF, TolAQR	84,000 and 10,000	*Pseudomonas aeruginosa*	[57,58]
	Pyocin S1	DNase	Ferripyoverdine	TonB, ExbBD	65,600 and 10,000	*P. aeruginosa*	[59,60]
	Pyocin S2	DNase	Type I ferripyoverdine (FpvAI)	TonB, ExbBD	74,000 and 10,000	P. *aeruginosa*	[59—62]
	Pyocin S3	DNase	Type II ferripyoverdine (FpvAII)	TonB, ExbBD	81,385 and 17,047	P. *aeruginosa*	[63,64]
	Pyocin S4	tRNase	Type I ferripyoverdine (FpvAI)	OmpW, OmpF, Tol		*P. aeruginosa*	[62,64]
	D	tRNase	FepA	TonB-ExbBD	74,683	*E. coli*	[35,65]
	G	Membrane lysis	Fiu	TonB, ExbBD	5500	*E. coli*	[35,66]
	H	Membrane lysis	Fiu	TonB, ExbBD	100	*E. coli*	[66]
	Pesticin I	Muramidase	FhuA	FhuA, TonB-ExbBD	40,043	*Yersinia pestis*	[67]
	M	Inhibition of synthesis of murein and lipopolysaccharide	FhuA	FhuA, TonB-ExbBD	29,453	*E. coli*	[68]
	B	Pore-forming	FepA	TonB-ExbBD	54,742	*E. coli*	[35,69]
	Ia	Pore-forming	Cir	TonB-ExbBD	69,429	*E. coli*	[35,70]
	Ib	Pore-forming	Cir	TonB-ExbBD	69,923	*S. sonnei*	[35,70]
	5	Pore-forming	Tsx	TolC, TonB-ExbBD	53,137	*E. coli*	[39,71]
	10	Pore-forming	Tsx	TolC, TonB-ExbBD	53,342	*E. coli*	[72]
	Pyocin S5	Pore-forming	FptA ferripyochelin	—	—	*P. aeruginosa*	[64,73]
	Fy	Pore-forming	YiuR	TonB	54,000	*Yersinia frederiksenii*	[74]
	Q	Pore-forming	Cir	TonB, ExbBD	ND	*E. coli*	[75]

Table 16.3 Comparison of Classification Systems for Bacteriocins

Class	Subclass	Klaenhammer [3]	Nes et al. [76]	van Belkum and Stiles [77]	Cotter et al. [4,5]	Heng et al. [79]		Examples
I/lantibiotic	A	Lantibiotic	Lantibiotics	Lantibiotics	Lantibiotics	Type A—linear	A1—nisin-like	Nisin A, U, and Z; subtilin, epidermin, gallidermin, Pep5, streptin
							A2—all SA-F22-like	SA-FF22, lactin 481, salivaricin A, nukacin ISK-1, mutacin II
	B					Type B—globular		Mersacidin, cinnamycin, ancovenin, duramycin B and C
	C					Type C—multicomponent		Lacticin 3247, lichenicidin, mutacin I
II/nonlantibiotic	IIa	Pediocine-like or *Listeria*-active with YGNGVXC motif near N-terminus and GG leader peptide	Pediocine-like or *Listeria*-active with YGNGVXCXXXXVXV motif near N-terminus and GG leader peptide	Cystibiotics_ with two disulfide bridges with YGNGVXC motif near N-terminus	Pediocine-like or *Listeria*-active with YGNGVXC motif near N-terminus and GG leader peptide	Type IIa—pediocin-like		Pediocin PA1, enterocin CRL35, carnobacteriocin BM1
	IIb	Two-peptide bacteriocin, requires both peptides for activity	Two-peptide bacteriocin, requires both peptides for activity	Cystibiotics with one disulfide bridge with YGNGVXC motif near N-terminus	Two-peptide bacteriocin, requires both peptides for activity	Type IIb—multicomponent		Lactococcin G, lactacin F, ABP118
	IIc	Thiol-activated peptides that require reduced cysteine for activity	Bacteriocins secreted by signal peptide pathway	Cystibiotics with one disulfide bridge but without YGNGVXC motif near N-terminus	Cyclic peptide, N- and C-termini are covalently linked	Type IIc—miscellaneous		Reuterin 6, anacyclamides
	IId	—	—	Thiolbiotics with one or no cysteine residues	Single non-pediocin-like peptides			Divergicin, MccV, MccS, epidermicin NI01, lactococcin A
	IIe	—	—	Two-component peptides with GG leader peptide	Contain a serine-rich carboxy-terminal region			MccE492, M
	IIf	—	—	Atypical bacteriocins	—			
III		Large, heat-labile proteins	Large, heat-labile proteins	Large, heat-labile proteins	Bacteriolysins: large, heat-labile proteins, often murein hydrolases	Large (>10 kDa) proteins; type IIa—lysins, type IIIb—nonlytic		Lysostaphin, enterolysin A, caseicin 80, helveticin J
IV		Protein complexes	—	—	—	Cyclic peptides		Enterocin AS-48

recommended recognition of a new group (class V) comprising ribosomally synthesized, unmodified head-to-tail ligated cyclic antibacterial peptides.

Later, Cotter et al. [4] reviewed this classification system for lactic acid bacteria bacteriocins and proposed a two-class division: I, lanthionine-containing bacteriocins/lantibiotics, and II, non-lanthionine-containing bacteriocins. Moreover, they also suggested that class III (large heat-labile murein hydrolases) should not be classified as bacteriocins but instead be named bacteriolysins, and class IV (the lipid- or carbohydrate-containing bacteriocins) be withdrawn. It was further foreshadowed that the Klaenhammer class II subgroups IIa and IIb be retained and that the class V (cyclic peptides) proposed by Kemperrmam et al. [78] be reassigned as class IIc. Subclass IId was proposed to include all of the remaining linear non-lanthionine-containing bacteriocins as certain Mccs, such as MccV and MccS, whereas the subclass IIe includes MccE492-like bacteriocins (formerly known as the class IIb Mccs) [5].

Heng and Tagg [79] disproved many statements made by Cotter et al. [4] and proposed a new classification designed to fit all bacteriocins produced by gram-positive bacteria. Class III (large bacteriocins) was retained and subdivided into IIIa (bacteriolysins) and IIIb (nonlytic proteins); the cyclic bacteriocins (class IIc) were reassigned as class IV; and as a consequence of upgrading the cyclic bacteriocins to their own class, subgroup IIc became the repository for all unmodified class II inhibitors other than the *Listeria*-active (IIa) and multicomponent bacteriocins (IIb).

Arnison et al. [20] recommended a nomenclature system for the biosynthesis of ribosomally synthesized and posttranslationally modified peptides (RiPPs), including only molecules $<$10 kDa and excluding posttranslationally modified proteins, for which more than 20 distinct compound classes have been described. Among the RiPPs, bacteriocins are detected in many classes, such as the lanthipeptides (lantibiotic nisin), linear azol(in)e-containing peptides (MccB17), lasso peptides (MccJ25), thiopeptides (thiostrepton, philipimycin, MccJ25), Mccs (MccB17, MccC7-51), linaridins (cypemycin), proteusins (polytheonamide A), sactipeptides (sactibiotics: subtilosin A, thuricin CD), bottromyces (bottromyces A2), cyanobactins (patellamide, anacyclamide A10), and glycocins (sublancin 168).

16.3 Mechanisms of Action

The bactericidal mechanisms of bacteriocins can be divided into those that work primarily at the cell envelope and those that work mainly within the cell, affecting gene expression and protein synthesis. Nisin, some lantibiotics, and class II bacteriocins inhibit cell wall biosynthesis by interacting with lipid II, which is the carrier of peptidoglycan monomers across the cytoplasmic membrane and is the target of vancomycin [80,81]. Because the binding sites of these bioactive molecules are in different locations, bacteriocins retain activity against vancomycin-resistant gram-positive pathogens [82].

Most bacteriocins kill target cells by inducing permeabilization of the cell membrane, and the activity is generally specific, given that they use specific receptors on the target cell surfaces. Nisin and various lantibiotics use lipid II as a docking molecule to ease pore formation in the cell membrane, leading to loss of membrane potential and cell death [83,84]. In addition, it has been shown that different bacteriocins produced by both gram-positive and gram-negative species can use the mannose phosphotransferase system (Man-PTS) membrane components on sensitive cells as receptor molecules, inducing pore formation. These bacteriocins comprise pediocin-like bacteriocins, lactococcins A and B (lactococcal bacteriocins), and MccE492 from *Klebsiella pneumoniae*, which can target the Man-PTS in *E. coli* membranes [85–87]. Other receptors related to the activity of bacteriocins are increasingly being reported including the maltose ABC transporter for class IIc bacteriocin, garvicin ML [88]; a Zn-dependent metallopeptidase for class IId bacteriocin LsbB [89]; and an undecaprenyl pyrophosphate phosphatase for the class IIb bacteriocin lactococcin G [90].

The class IIe peptide MccE492 uses the FepA, CirA, or Fiu receptors, all of which are iron siderophore receptors for its translocation to the periplasm. To exert its bactericidal activity via pores in the inner membrane, MccE492 needs TonB at the level of the interaction with the cytoplasmic membrane of the target cells [87,91]. However, the interaction of MccE492 with the TonB system is independent of the presence of the receptors. The RiPP proteusin polytheonamide B acts through a mechanism that involves pore formation, but precisely how this occurs and whether a receptor is involved still have yet to be elucidated [92]. The peptide depolarizes the bacterial cytoplasmic membrane, simultaneously decreasing the membrane potential and the intracellular K^+ contents, which is consistent with the formation of transmembrane ion channels [21,22,92,93]. Large-conductance mechanosensitive channels (MscL) are crucial for the activity of sublancin 168, a lantibiotic produced by *Bacillus subtilis* strain 168. MscL may serve either as a direct target for this lantibiotic or as a gate of entry to the cytoplasm [94].

The polycyclic peptides duramycin, duramycin B, duramycin C, and cinnamycin inhibit the phospholipase A enzyme indirectly by specifically sequestering the substrate phosphatidylethanolamine [95,96].

Other bacteriocins can kill their target cells by interfering with DNA, RNA, and protein metabolism. MccB17 and MccJ25 pass through the outer membrane via the OmpF and FhuA TonB-dependent porin pathways, respectively. The inner-membrane protein SbmA transports the Mccs through the inner membrane to the cytoplasmic face. Inside the cell, MccB17 targets DNA gyrase [97], whereas MccJ25 inhibits transcription by blocking the secondary channel of RNA polymerase [98]. The translocation of MccC7-C51 through the inner layer of the *E. coli* cell wall occurs via the YejABEF transporter. Then, the Mcc is processed by one of the many broad-specificity cytoplasmic aminopeptidases of the bacterium, producing a modified aspartyladenylate that inhibits aspartyl-tRNA synthetase, blocking mRNA synthesis [20].

Thiostrepton, micrococcin P1, thiazomycin, and several other thiopeptides interfere with bacterial protein synthesis by a direct interaction with the 50S ribosomal subunit. These molecules bind near the GTPase-associated center that engages a number of translation factors during initiation and elongation. The ribosome–thiopeptide complex prevents conformational alterations communicated from the translation factors to the ribosome, ultimately halting translocation along the mRNA template. Bottromycins block aminoacyl-tRNA binding to the 50S ribosome. Another subset of thiopeptides, including the thiomuracins and GE2270A, inhibits protein synthesis by binding to elongation factor Tu (EF-Tu) [20,99]. This ligation occludes a portion of the recognition site for the aminoacyl-tRNA substrate between EF-Tu and aminoacyl-tRNA.

An additional activity of lantibiotics including nisin, subtilin, and sublancin is the ability to prevent spore outgrowth by *Bacillus* and *Clostridium* species [100,101]. It was demonstrated that in *Bacillus anthracis* the initiation of germination is essential for the action of nisin. This lantibiotic rapidly and irreversibly inhibits spores growth by preventing the establishment of oxidative metabolism and the membrane potential in germinating spores and the shedding of the external spore structures [102].

Garvicin A and lactococcin 972 have been shown to inhibit cell wall biosynthesis, most probably by inhibiting septum formation [103,104]. Garvicin A is specifically active against other *Lactococcus garvieae* strains, whereas lactococcin 972 inhibits only closely related *Lactococcus* spp. The processes of cell elongation and septum formation are linked in gram-positive cocci, taking place at an annular band located in the equatorial zone of the cell. *Lactococcus lactis* cells treated with these bacteriocins presented only a primordial septum and suffered an elongation that preceded the arrest of macromolecular synthesis and the death of the cell [104]. This suggests that both bacteriocins inhibit septum invagination rather than septum initiation.

In past years, diverse studies have suggested that the continuous exposure of bacteria to bacteriocins may select cells resistant to them. Bacteriocin resistance may be either innate or acquired, and seems to be a complex phenomenon arising at different frequencies and by different mechanisms, even among strains of the same bacterial species [4]. This observation has encouraged studies focused on understanding the molecular mechanisms involved in resistance, on definition of the frequency at which susceptible organisms can develop resistance to a given bacteriocin, and on the definition of strategies that can be used to overcome such resistance. Resistance mechanisms generally involve changes in the bacterial cell envelope, which result in reduction or loss of bacteriocin binding or insertion, bacteriocin sequestering, bacteriocin efflux pumping, and bacteriocin degradation. Other general forms of resistance include the formation of spores or biofilms, which are a common mechanistic response to many classes of antimicrobials, and specific antilantibiotic mechanisms such as production of the nisin lytic protein nisinase and the phenomenon of immune mimicry [105].

16.4 Antimicrobial and Antibiofilm Activities of Bacteriocins

Table 16.4 shows a summarized overview of the antibiofilm activity, biomedical applications, and potential therapeutic uses of bacteriocins. The lantibiotics nisin, planosporicin, Pep5, epidermin, gallidermin, mersacidin, mutacin B-Ny266, lacticin 3147, and actagardine have strong antimicrobial activity against emergent pathogens such as *Streptococcus pneumoniae*, MRSA, VRE, various mycobacteria species, *Propionibacterium acnes,* and *Clostridium difficile* in vitro [127]. Even though lantibiotics are generally believed to be poorly active against gram-negative bacteria, purified lantibiotics such as nisin and epidermin have been found to kill various species such as *Neisseria* and *Helicobacter*, *Campylobacter*, *Haemophilus*, *E. coli,* and *Salmonella* species, in which the outer membrane is damaged [99,127,154].

Nisin A is mostly studied as a food additive, and it has been granted GRAS (generally recognized as safe) status. This bacteriocin is used as a food preservative particularly in dairy products, canned foods, plants, protein foods, and cured meat. Structurally, nisin A is a lantibiotic with 34 amino acid residues posttranslationally modified, with one lanthionine, four methyllanthionine rings, and unusual residues such as dehydroalanine and dehydrobutyrine [155]. The molecule is highly active against gram-positive bacteria such as *Listeria monocytogenes*, *S. aureus*, *Bacillus cereus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *Micrococcus flavus*, *Streptococcus mutans*, *Streptococcus sanguinis*, and *Enterococcus faecalis*, also promoting spore growth inhibition of *B. anthracis* and *Clostridium botulinum* and inhibiting food spoilage by *Clostridium butyricum*, *Clostridium tyrobutyricum*, and *Clostridium sporogenes* [156,157].

Nisin presents several variants, like F [158], Q [153], U [159], and Z [160], but the activity spectra are similar. Except for nisin U, produced by *Streptococcus uberis,* the variants are produced by *L. lactis* subsp. *lactis.* Nisins A and Z are structurally identical, except for a substitution of asparagine for histidine as amino acid residue 27 in nisin Z. The substitution has no influence on its antimicrobial activity, but provides nisin Z higher solubility and diffusion characteristics compared to nisin A [160]. Nisin U is active against mastitis-associated bacteria such as *Streptococcus pyogenes*, *S. uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Staphylococcus simulans*, *Staphylococcus cohnii*, *L. lactis*, *L. acidophilus*, and *Streptococcus mitis* [159]. Nisin F, described by De Kwaadsteniet et al. [158], inhibited the growth of *S. aureus* in the respiratory tract of rats when administered intranasally. The antimicrobial activity of low concentrations of this peptide (80−320 AU/mL) was slightly stimulated by lysozyme and lactoferrin. As of this writing, among the lantibiotics, only nisins A and Z have commercial applications.

Various studies have provided evidence that nisin can be active against *L. monocytogenes* and *Enterococcus hirae* growing in biofilms at surfaces such as stainless steel, rubber, and polyethyleneterephthalate on food-processing equipment [106,109].

Table 16.4 Bacteriocins Reported in the Literature With Antibiofilm and Antimicrobial Activities

Class I (Posttranslational Modifications)	Bacteriocin	Producer Microorganism	Antimicrobial Spectrum	Biomedical Application or Potential Therapeutic Use	References
(Lantibiotic/A)	Nisin A	*Lactococcus lactis* subsp. *lactis*	*Listeria monocytogenes*, *Staphylococcus aureus*, MRSA, *Bacillus cereus*, *Bacillus anthracis*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *Micrococcus flavus*, *Streptococcus mutans*, *Streptococcus sanguinis*, *Enterococcus faecalis*, *Actinomyces* sp., *Clostridium botulinum*, *Corynebacterium* sp., *Gardnerella* sp., *Lactococcus* sp., *Mycobacterium*, *Propionibacterium acnes*	Food preservative: inhibition of pathogenic and toxigenic bacteria, inactivation of endospore formers. Antibiofilm activity: *L. monocytogenes* and *Enterococcus hirae* in stainless steel chips; *S. aureus*, *Staphylococcus epidermidis*, and *E. faecalis* in catheters; oral biofilm-associated diseases. Treatments: peptic ulcer in humans, bovine mastitis, experimental gingivitis in dogs. Commercially available.	[106–110]
Lantibiotic/AI	Epidermin	*S. epidermidis*	*S. aureus*, *S. epidermidis*, *P. acnes*	Topical treatment of acne, eczema, folliculitis, impetigo. Use in personal care products	[111,112]
Lantibiotic/AI	Gallidermin	*Staphylococcus gallinarum*	*S. aureus*, *S. epidermidis*, *P. acnes*	Inhibition of biofilm formation of *S. aureus* and *S. epidermidis*. Topical treatment of acne, eczema, folliculitis, impetigo. Use in personal care products	[112–114]
Lantibiotic/AI	Pep5	*S. epidermidis*	*S. epidermidis*, *P. acnes*	Inhibition of *S. epidermidis* adhesion to silicone catheters. Topical treatment of acne	[115]
Lantibiotic/AI	Subtilin	*Bacillus subtilis*	*P. acnes*, *Staphylococcus* spp., *Streptococcus* spp., *Clostridium* spp., *Bacillus*	Food preservative: inhibition of pathogenic and toxigenic bacteria, inactivation of endospore formers	[116]
Lantibiotic/AI	Mutacin B-Ny266	*S. mutans*	MRSA, VRE	Treatment of multidrug-resistant bacteria	[117,118]
Lantibiotic/A	Nukacin ISK-1	*Staphylococcus warneri*	MRSA	MRSA biofilm	[2]
Lantibiotic/A	Amylolysin	*Bacillus amyloliquefaciens* GA1	*L. monocytogenes*, *S. aureus*, *S. epidermidis*, *B. cereus*	Antimicrobial activity	[81]
Lantibiotic/B	Mersacidin	*Bacillus* sp.	MRSA, VRE, *P. acnes*	Topical treatment of acne and staphylococcal and enterococcal infections. Active against MRSA in murine model	[119–121]

Lantibiotic/B	Actagardine	*Actinoplanes liguriensis*	MRSA, VRE, *Clostridium difficile*	Bovine mastitis, oral hygiene product, treatment of acne and CDAD	[122]
Lantibiotic/B	Duramycin	*Streptomyces cinnamoneus*	*Bacillus subtilis*	Treatment of cystic fibrosis increasing chloride secretion in lung epithelium and dry eye syndrome	[123]
Lantibiotic/C	Lacticin 3147	*L. lactis* subsp. *lactis*	*L. monocytogenes*, *B. cereus*, *C. botulinum Clostridium tyrobutyricum*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *S. mutans*, MRSA, VRE, PRSP	Prevent food spoilage and disease. Treatment of bovine mastitis, staphylococcal and enterococcal infection	[124–127]
Lantibiotic/C	Mutacin 1140	*S. mutans*	*Streptococcus sobrinus*, *S. sanguinis*, *Streptococcus oralis*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus parasanguinis*	Oral biofilm-associated diseases.	[128,129]
Sactibiotics	Subtilosin A	*B. subtilis*, *Bacillus atrophaeus,* and *B. amyloliquefaciens*	*E. faecalis*, *Streptococcus pyogenes*, *L. monocytogenes*, *Gardnerella vaginalis*	Treatment of bacterial vaginosis (vaginal biofilm)	[130,131]
Sactibiotics	Thuricin CD	*Bacillus thuringiensis*	*C. difficile*	Inhibition of *C. difficile* in an ex vivo model of the colon. Treatment of CDAD	[11,132]
Bottromycin	Bottromycin A2	*Streptomyces bottropensis*	MRSA, VRE, *Mycoplasma*	Nosocomial infections	[133]
Glycocin	Sublancin 168	*B. subtilis* 168	*S. aureus*, *B. cereus*, *S. pyogenes* inhibition of spore growth of *Bacillus* and *Clostridium*	Food preservative in milk and dairy products, and bioactive against *S. aureus* in infected mice	[94,100,134–136]
Thiopeptide	LFF571	*Catellatospora* sp.	*C. difficile*	Treatment of CDAD	[137]
Thiopeptide	Nocathiacin	*Nocardia* sp. and *Amycolatopsis* sp.	Clinically important multidrug-resistant pathogens (MRSA, MREF, VRE, and PRSP)	Control of *S. aureus* systemic infection (mouse model)	[69–71,138–140]
MccC7-C51 type bacteriocins	MccC7	*E. coli* H22	*Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Salmonella*, *Shigella*, and *Yersinia*	Inhibition of *Shigella flexneri* in intestinal tract of gnotobiotic mouse model	[141]
Lasso	MccJ25	*E. coli*	*E. coli*, *Salmonella enterica*, *Shigella*	Antimicrobial potential in mouse model of *Salmonella* infection	[142]
Linear azole- containing peptide	MccB17	*E. coli*	*Escherichia*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Pseudomonas* spp.	Antimicrobial activity	[143,144]
Class II (Unmodified or Cyclic)	**Bacteriocin Example**	**Producer Microorganism**	**Antimicrobial Spectrum**	**Biomedical Application**	**References**
IIa peptides	Pediocin PA-1	*Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Pediococcus parvulus*, *L. plantarum*, *Bacillus coagulans*, *Enterococcus faecium*	*L. monocytogenes*, *E. faecium*, *Leuconostoc mesenteroides*, *Aeromonas* sp., *Yersinia* sp., *S. aureus*, *Serratia liquefaciens*, *Pseudomonas fluorescens*	Food preservative against food spoilage and food-related health hazards. Commercially available.	[145]

Continued

Table 16.4 Bacteriocins Reported in the Literature With Antibiofilm and Antimicrobial Activities—cont'd

Class I (Posttranslational Modifications)	Bacteriocin	Producer Microorganism	Antimicrobial Spectrum	Biomedical Application or Potential Therapeutic Use	References
IIa peptides	Enterocin CRL35	*Enterococcus mundtii*	*L. monocytogenes*, herpes simplex virus I and II	Food preservative	[146]
IIa peptides	Carnobacteriocin A (CbnA)	*Carnobacterium piscicola*, *Carnobacterium maltaromaticum*	*Listeria* sp., *Enterococcus* sp., *Carnobacterium* sp.	Food preservative	[147]
IIb	Abp118	*Lactobacillus salivarius* subsp. *salivarius*	*Listeria*, *Salmonella*, *Campylobacter*	Antilisterial activity in mouse model. Probiotic microorganisms for pigs and poultry—control of pathogenic strains in the microbiota.	[148,149]
IIc	Enterocin AS-48	*E. faecium*	*L. monocytogenes*, *B. cereus*, *S. aureus*, *Staphylococcus carnosus*, *E. coli*, *S. enterica*, *Alicyclobacillus acidoterrestris*, *Bacillus* spp., *Paenibacillus* spp., *Geobacillus stearothermophilus*, *Brochothrix thermosphacta*, *Lactobacillus sakei*	Food preservation	[151]
IId	Epidermicin NI01	*S. epidermidis*	*Staphylococcus hominis*, *S. warneri*, *E. faecalis*, MRSA	Promising candidate for clinical use against multiresistant gram-positive pathogens	[152]
IId	Lactococcin A	*L. lactis*	*L. lactis*	Food preservative, narrow spectrum	[153]
IIe	MccE492	*Klebsiella pneumoniae*	*E. coli*, *K. pneumoniae*, *S. enterica*, *Enterobacter cloacae*	Remains to be elucidated	[32]
IIe	MccM	*E. coli*	*E. coli*, *S. enterica*, *E. cloacae*	Remains to be elucidated	[32]

CDAD, *clostridium difficile*-associated diarrhea; *MREF*, multidrug-resistant *Enterococcus faecium*; *MRSA*, methicillin-resistant *Staphylococcus aureus*; *PRSP*, penicillin-resistant *Streptococcus pneumoniae*; *VRE*, vancomycin-resistant enterococci.

Nisin A from *L. lactis* UQ2, a bacteriocinogenic strain isolated from Mexican-style cheese, reduced >5 log cycles of *L. monocytogenes* Scott A in planktonic and sessile cells on stainless steel chips, in biofilms per chip, in competitive-exclusion tests at 37°C [106].

Nisin A can be also used in the control of oral biofilm-associated diseases, as demonstrated in vivo on the treatment of gingivitis in beagle dogs [107], and in tooth decay [108]. This bacteriocin was effective in eradicating *E. faecalis* and *Streptococcus gordonii* cells in pure culture, and was similar to $Ca(OH)_2$ regarding the removal of these species from the root canal system [108]. It also inhibited the growth of oral bacteria in planktonic form at low concentrations, and slowed the development of polymicrobial biofilm by altering its development and reducing biofilm biomass and thickness in a dose-dependent manner. The molecule is a cationic bacteriocin with biological activity on membranes, and the possible mechanism of action is associated with disruption of the coaggregation process, which is essential for stable biofilms [110].

It was also shown that nisin A and lacticin Q have strong bactericidal activities against MRSA, both in planktonic and in biofilm cells (nisin showed the highest bactericidal activity), whereas vancomycin showed activity against planktonic cells only [2]. It was suggested that pore formation leading to ATP efflux is important for the bactericidal activity against biofilm cells and the pore size needs to be large enough for ATP flow, probably higher than 1.5 nm in diameter. The pore size and stability time vary among bacteriocins. The pore diameter of lacticin 3147 is around 0.6 mm, which induces ion leakage, but not ATP [161]. SA-FF22 produced by *S. pyogenes* FF22 disrupts the membrane potential by forming small short-lived pores (from 0.5 to 0.6 nm in diameter and with life spans of a few milliseconds) [162]. Nisin A forms large stable pores on membranes containing lipid II, with diameters around 2.5 mm and with life spans of a few seconds [163]. Lacticin Q forms large toroidal pores on membranes without lipid II, with diameters ranging from 4.6 to 6.6 nm that induce ATP leakage [164]. Another possibility is that lipid II, a docking molecule for nisin A, contributes to specific binding to targeted cells, although it is not required for lacticin Q activity.

It is important to mention that the effectiveness of individual bacteriocins might be enhanced when combined with other antimicrobials or membrane-active substances. Nisin, for instance, presented synergistic activity with polymyxin E and clarithromycin when tested against *Pseudomonas aeruginosa* [165], and with Ramoplanin and other drugs against MRSA and VRE strains [166]. This molecule can be used combined with other compounds such as chelating agents [167]. It was reported that ε-polylysine (ε-PL) and nisin present synergic behavior against *E. coli*, *B. subtilis*, and *S. aureus*, and it was proposed that nisin promoted the uptake of ε-PL into cells, forcing their interaction with intracellular DNA [168].

Staphylococcus spp. strains can produce lantibiotics such as gallidermin, epidermin, Pep5, and nukacin [2,115,169]. Gallidermin and epidermin are natural variants produced by *Staphylococcus gallinarum* and *Staphylococcus epidermidis,* respectively, which differ from epidermin by a leucine rather than an isoleucine at position 6 [169]. Gallidermin is

effective against *S. aureus* and *S. epidermidis*, which are involved in biofilm formation in several implanted medical devices such as stents, catheters, and others. Among the hypotheses to explain its mechanism of action is the repression of biofilm-related genes such as *atl* (autolysin) and the operon *ica* (intercellular adhesin), associated with *N*-acetylglucosaminyltransferase synthesis and biofilm formation [113,114].

As of this writing, there are several lantibiotics in clinical studies, including duramycin, produced by *Streptomyces cinnamoneus,* for the treatment of cystic fibrosis, by increasing chloride secretion in lung epithelium [123], a disease in which lungs are infected with bacterial biofilms, and a derivative of actagardine for the treatment of *C. difficile* infections [170,171]. Additionally, mutacin 1140 from the oral pathogen *S. mutans*, strain JH1140, has been developed to control supragingival biofilm formation (SMaRT Replacement Therapy, developed by Oragenics), excluding other *S. mutans* strains. It is worth mentioning that some people, after a period of 14 years, retained the SMaRT strain after being inoculated just once [128,129].

Bacillus strains are able to produce varied lantibiotics. Amylolysin from *Bacillus amyloliquefaciens* GA1 showed an antibacterial spectrum directed toward gram-positive bacteria including *L. monocytogenes* (0.5 μM), *S. aureus* (0.4 μM), and *S. epidermidis* (2.8 μM), or *B. cereus* (0.2 μM) [81]. The peptide did not inhibit the growth of either basidiomycetous or ascomycetous yeasts (i.e., *Cryptococcus neoformans* and *Saccharomyces cerevisiae*, respectively) or gram-negative bacteria (i.e., *E. coli* and *P. aeruginosa*). However, it is effective against *Enterococcus faecium,* with a MIC value of 0.1 μM, and against *E. faecalis* to a lesser extent.

Subtilin, a pentacyclic lantibiotic of 32 amino acid residues, is a natural analogue of nisin [172]. Mersacidin produced by *Bacillus* sp. strain HIL Y-85, 54728 [173], inhibits the growth of MRSA strains in vivo in mice [120]. The peptide inhibits cell wall synthesis of MRSA strains with efficiency equal to that reported for vancomycin [174]. Mersacidin is also active against *P. acnes* and may thus be used in the treatment of acne [119]. However, its mechanism of action differs from that of vancomycin, which provides the option of using the two substances in combination [175]. Another strain, *B. subtilis* subsp. *spizizenii* DSM15029, has been described as an unsuccinylated entianin AMP producer. This peptide differs from subtilin at three amino acid positions and is effective against *S. aureus*, *E. faecalis*, and other gram-positive bacteria [176].

More recently, Chopra et al. [177] identified sonorensin, a novel peptide produced by *Bacillus sonorensis* MT93. This peptide is part of the heterocycloanthracin subfamily of bacteriocins, a set of presumed peptides containing heterocyclic moieties of oxazole and/or thiazole. Sonorensin has a 6.27-kDa molecular mass and presents antimicrobial activity against food-borne pathogens like *L. monocytogenes* and *S. aureus*. The authors also demonstrated its effectiveness against biofilms of *S. aureus.*

Thiopeptides are mostly active against gram-positive bacteria with potent in vitro activity, but their poor solubility made their large-scale production undesirable. Nocathiacin I and derivatives, the main components of nocathiacins, thiazolyl peptides isolated from *Nocardia* sp. and *Amycolatopsis* sp., exhibit activity in vitro with a MIC in

the ng/mL range against many clinically important multidrug-resistant pathogens including MRSA, multidrug-resistant *E. faecium*, VRE, and fully penicillin-resistant *S. pneumoniae* [138,139]. In addition, nocathiacin I showed excellent efficacy in vivo in a systemic *S. aureus* infection mouse model [140]. Philipimycin from *Actinoplanes philippinensis* MA7347 showed strong antibacterial activities against gram-positive bacteria, including MRSA, and exhibited MIC values ranging from 0.015 to 1.0 μg/mL. It was effective in vivo in a mouse model of *S. aureus* infection exhibiting an ED_{50} value of 8.4 mg/kg [178].

Thiazomycin from *Amycolatopsis fastidiosa* was highly potent in vitro against gram-positive bacteria and was highly efficacious in vivo against *S. aureus* in mice. It showed activity against bacteria resistant to β-lactams, vancomycin, macrolides, quinolones, and other antimicrobial-resistant bacterial phenotypes, such as protein synthesis inhibitors (linezolid, macrolide, gentamicin, chloramphenicol, and tetracycline), indicating no cross-resistance [179].

Thiomuracins produced by a rare actinomycete bacterium typed as a *Nonomuraea* species inhibited the growth of *S. aureus* and *E. faecalis*, but had no measurable activity against *E. coli* and *P. aeruginosa*. Possibly, this poor antimicrobial activity can be explained by the low penetration of the molecules in gram-negative bacterial outer membranes [180]. GE2270 A and its derivative LFF571 (Novartis) exhibit activity against *C. difficile* and various gram-positive bacteria, excluding *Bifidobacteria* and lactobacilli [137].

Among the other posttranslationally modified bacteriocins, the sactibiotic thuricin CD produced by *Bacillus thuringiensis* exhibits antimicrobial activity comparable to the activities of vancomycin and metronidazole, both used to treat *C. difficile*-associated diarrhea (CDAD) in an experimental model of the human distal colon [181]. Notably, nevertheless, thuricin CD did not modify significantly the commensal microbiota composition, whereas vancomycin and metronidazole caused a remarkable increase in Proteobacteria abundance compared to organisms of other phyla. Thuricin CD consists of two different peptides, Trn-α and Trn-β, that work synergistically to kill *C. difficile* isolates, including CDAD-associated strains [132]. Bottromycin A2 exhibits potent activity against MRSA and VRE by targeting translation mechanisms [133].

Subtilosin A, a sactibiotic, is also an uncommon example of a modified bacteriocin produced by gram-positive bacteria that is active against some gram-negative bacterial strains [130]. Subtilosin A displays a narrow spectrum of activity against *E. faecalis*, *S. pyogenes*, and *L. monocytogenes* [130], as well as *Gardnerella vaginalis* [131]. The pathway of bacterial killing by this peptide remains to be described. Like nisin, the antimicrobial activity of subtilosin may be due to interactions with membrane-associated receptors [84].

The molecular mechanism of the action of subtilosin against *L. monocytogenes* remains unclear. However, the mechanism against the vaginal pathogen *G. vaginalis* involves depleting the transmembrane pH gradient portion of the protonmotive force, causing an efflux of intracellular ATP and eventually cell death [131,182]. Subtilosin A

inhibits *G. vaginalis* in vitro and has strong potential for inclusion in alternative bacterial vaginosis (BV) therapies because it has spermicidal activity, being atoxic to epithelial cells and lactobacilli at the human vaginal mucosa [183,184]. BV is a polymicrobial biofilm infection, dominated by dense clusters of *G. vaginalis* and, to a lesser extent, *Atopobium* species that probably have a role in potentiating failures of the antimicrobial treatment recommended by the Centers for Disease Control, metronidazole and clindamycin orally or intravaginally [185]. Noll et al. [186] showed that subtilosin A acts synergistically when combined with glycerol monolaurate, lauric arginate, and ε-PL against pathogens associated with BV, but not with human lactobacilli. The cell-free supernatants of overnight cultures were active against *L. monocytogenes*. The first report of antiviral activity for this peptide was for subtilosin from *B. amyloliquefaciens* KATMIRA 1933 [187].

Various unmodified class II bacteriocins have been described with antimicrobial activity against gram-positive bacteria, including lactic acid bacteria class IIa bacteriocins, pediocin PA1, leucocin A, and others, which are effective against *L. monocytogenes* [188] and other gram-positive pathogens [189]. A synergic effect was observed for the combination of the class IIa enterocin CRL35 with several antibiotics against *L. monocytogenes* [190]. Pediocin PA1 has been used commercially for food preservation. The Irish laboratory Quest explored a fermented product of *Pediococcus acidilactici*, a pediocin PA1-producing strain, and generated ALTA 2431. Patents from the United States and Europe cover its use for this purpose [191].

Class IIb bacteriocins are composed of a dimer of peptides like lactacin F [150,192] and ABP-118 [193]. These molecules are involved in the formation of an active poration complex, the mechanism of action of this class. ABP-118 presented antilisterial activity in a mouse model [148].

The class IV peptide enterocin AS-48, an antimicrobial enterococcal bacteriocin, has been studied for use as a food preservative, as it is active against food-borne pathogenic strains like *E. coli*, *Salmonella* spp., *L. monocytogenes*, *S. aureus*, and *B. cereus* and the food-spoilage bacteria *Bacillus* spp. and *Paenibacillus* spp. [194–197].

Licheniocin 50.2 from *Bacillus licheniformis* VPS50.2 is effective against *L. monocytogenes*, MRSA, and hemolytic streptococci [198]. Regarding class IId bacteriocins, epidermicin NI0 produced by *S. epidermidis* strain 224 has also exhibited potent antimicrobial activity in the nanomolar range against pathogenic gram-positive bacteria including MRSA, enterococci, and biofilm-forming *S. epidermidis* strains. The peptide is highly stable against protease and keeps its antimicrobial potential in a wide range of pH, from 2 to 10. Bacterial exposure to this peptide has no effect on the development of resistance mechanisms [152]. There are few cases of class IIa bacteriocins produced by gram-positive bacteria that are effective against gram-negative pathogens [199].

Bacteriocins of gram-negative bacteria usually have a narrow spectrum of action restricted to closely related species, different from those of gram-positive organisms that are active against many strains of gram-positive species, but generally inactive against gram-negative bacteria. Jordi et al. [200] found that 20 strains of *E. coli* could express Col,

which inhibited five STEC strains of serotypes O26, O111, O128, O145, and O157:H7. These *E. coli* can cause diarrhea and hemolytic uremic syndrome in humans. Under conditions simulating the cattle rumen environment (anaerobiosis and 30% rumen fluid), the Cols ColE1, ColE4, ColE8-J, ColK, and ColS4 can inhibit the growth of STEC. Purified ColE1 and ColN were active against enterotoxigenic *E. coli* F4 (K88) and F18 in vitro, which caused postweaning diarrhea and edema disease in piglets [201]. The same group showed also that the inclusion of purified ColE1 in the diet of young pigs reduced the incidence of postweaning diarrhea by F18-positive *E. coli* [202].

The antimicrobial spectrum of modified Mccs has been investigated. MccC7-C51 was described to be active against some strains of *Escherichia*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Proteus*, and *Yersinia* spp. [203]. Lasso-type bacteriocins were active against *Escherichia* and *Salmonella* spp. strains [204], and MccB17, a linear peptide with an azole moiety, was effective against several gram-negative species, including *Escherichia*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Shigella*, and *Pseudomonas* spp. [143,144]. Several Mccs (subclasses IId, MccV, MccL, IIe, MccE492, MccM3, and MccH47) were effective against some gram-negative strains [26,32,205,206]. Bacteriocin-producing *E. coli* strains could inhibit biofilm formation by non-bacteriocin-producing *E. coli* strains on catheter-related materials [207]. Other studies explored the influence of bacteriocins on biofilm formation by competing organisms [208,209]. Bacteriocin-producing strains have a selective advantage within multispecies biofilms, and it is possible that bacteriocins evolved to function largely in biofilms rather than as planktonic cells.

Purified ColA-43864 bacteriocin produced from *Citrobacter freundii* exhibited antimicrobial activity against a wide range of gram-negative bacteria and killed cells within biofilms in a dose-dependent manner [210]. The purified ColA-43864 eradicated a large number of cells within a biofilm, supporting the model that diffusion of ColA-43864 into the biofilm is not a limiting factor. But because of the biofilm complexity, it is possible that there are microenvironments within the biofilm where Col would not function because of suboptimal pH or other conditions [210].

16.5 Antimicrobial and Antibiofilm Investigations With Liposome-Entrapped Bacteriocins

Marciset et al. [211] encapsulated thermophilin 13A in liposomes. This is a broad-spectrum antimicrobial bacteriocin obtained from *Streptococcus thermophilus*. Thermophilin 13 is composed of the antibacterial peptide ThmA and the enhancing factor ThmB, which increases the activity of ThmA around 40 times. Both free and liposome-entrapped forms presented antilisterial activity; however, the bacteriocin exerted a pore-forming activity in both liposome lipids and targeted bacterial membranes.

Benech et al. [13] compared the antimicrobial activity of liposome-entrapped nisin Z, using proliposome H (Pro-lipo H), and free nisin Z, produced in situ by a strain of

L. lactis, on the inhibition of *Listeria innocua* in cheddar cheese. The nisin Z-producing strain and nisin Z-containing liposomes did not affect cheese production or chemical composition. After 6 months of monitoring, cheeses produced with the *L. lactis* strain presented 10^4 CFU/g of *L. innocua* and 12% of initial activity of the bacteriocin was preserved. Cheeses made with encapsulated nisin Z, on the other hand, contained less than 10 CFU/g of *L. innocua,* and 90% of the initial nisin activity was preserved.

Similarly, using different proliposome formulations, Laridi et al. [212] encapsulated nisin Z in various proliposomes (Pro-lipo H, Pro-lipo S, Pro-lipo C, and Pro-lipo DUO) and assessed its stability in milk under fermentation. Liposome encapsulation efficiency ranged from 9.5% to 47%, the last being for Pro-lipo H. Long-term stability of liposome-entrapped nisin Z was demonstrated for 27 days at 4°C in various media, including milk with different fat levels.

Park et al. [16] developed a pH-sensitive liposome-entrapped bacteriocin to control kimchi fermentation. The liposomes were prepared by reverse-phase evaporation using dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, dioleoylphosphatidylcholine, and cholesterol. The bacteriocin liposome was stable at pH 6 and 7, but ruptured at pH 4. The pH of samples treated with bacteriocin liposomes was preserved at 4 or higher, whereas untreated samples showed pH 3.58 or lower. The pH of kimchi samples treated with the bacteriocin−liposomes was 3.9 during 90 days storage, whereas that of the untreated samples was 3.32. The growth of lactic acid bacteria was controlled in treated samples, but kept increasing in untreated samples.

Sosunov et al. [14] assessed the antimicrobial potential of five bacteriocins obtained from bacterial culture supernatants in vitro against a *Mycobacterium tuberculosis* strain, using mouse macrophages, and in vivo in high-dose infection of inbred mice. In this model, four of the five tested bacteriocins were more potent antimycobacterials than rifampicin in equal concentrations. Whereas the molecules presented no toxicity to mouse macrophages, they also did not inhibit mycobacterial growth in these cells at 0.01−0.1 mg/L. However, when used entrapped in liposomes, bacteriocin 5 isolated from *E. faecalis* was able to inhibit mycobacterial growth inside macrophages without damaging the cellular membrane.

Also exploring bacterial growth control in food, Malheiros et al. [213] developed liposomes of soybean phosphatidylcholine and encapsulated an antimicrobial peptide, P34, produced by a strain of *Bacillus* sp. The liposomes' diameter was around 150 nm. The antimicrobial assays were performed by agar diffusion using *L. monocytogenes* ATCC 7644. Interestingly, no significant difference was seen in the biological activity of free and encapsulated P34. The mechanism of action of the liposomes was adherence to the bacterial cell wall, but without fusion. Free bacteriocin inhibited the pathogen more quickly than the encapsulated P34; however, liposome-entrapped P34 was more stable throughout the study.

Later, Malheiros et al. [214] encapsulated the same peptide P34 in nanovesicles prepared with soy lecithin, and tested them against *L. monocytogenes* strains by direct addition to milk samples. After 4 days of storage at low temperature, the antimicrobial

activity of both free and encapsulated BLS P34 decreased around 50%, and this decrease was associated with interactions with milk proteins. However, no significant loss of activity was detected until 21 days of storage. The viable counts of *L. monocytogenes* exposed to free or encapsulated P34 were permanently inferior to those observed in controls without the bacteriocin. Like the previous study of this group, the encapsulation of P34 in nanovesicles did not influence its antimicrobial activity; however, the encapsulation in liposomes preserved the same inhibitory effect against *L. monocytogenes* for a prolonged time compared to free P34.

16.6 Conclusion and Perspectives

Bacteriocins represent a potential alternative to synthetic antimicrobial products. In clinical contexts, the emergence of drug-resistant pathogens makes the search for novel bacteriocins even more relevant. This prospect can be based on the isolation of new bacteria and assays for antimicrobial activity followed by characterization of the activity spectrum and of the biomolecule. However, this approach presents limitations related to the susceptibility of the indicator bacterial species to the various groups of Cols and the growth conditions used in the screening. This is mostly because bacteriocin production can be under the control of a quorum-sensing system, and the expression of various bacteriocins can require defined physicochemical conditions. In this context, genomic, transcriptomic, and proteomic advances can also contribute to the identification of novel bacteriocins and understanding of regulatory mechanisms, which are important for determining how to switch on production when desired or how to enhance production or activity. The generation of bacteriocin-overproducer recombinant strains or manipulation of these biomolecules to improve their overall efficacy and stability is also important to make their technological use feasible. An important point is the concern that continuous exposure of bacteria to bacteriocins may select for resistant cells, as observed for synthetic antimicrobials. An approach to avoid the emergence of bacteriocin-resistant bacteria may be the combined use of bacteriocins with different mechanisms of action, which may additionally allow the use of lower bacteriocin doses. Another possibility consists in the combined use of synthetic antimicrobials and bacteriocins. Whereas most synthetic drugs act as enzyme inhibitors, most bacteriocins target the membranes of sensitive cells, leading to cell death. Therefore, the development of cross-resistance to antimicrobials with different mechanisms of action is expected to occur less frequently. Bacteriocins are capable of inhibition of the main pathways used by synthetic drugs as well as novel pathways. In this context, knowledge of the mechanisms involved in resistance to bacteriocins and the target pathogen resistance profiles is essential for the development of effective bacteriocin-based microbial control strategies.

The strategy of entrapment of bacteriocins in liposomes can contribute to the effective use of these biomolecules. When entrapped in liposomes not only can

intracellular delivery be achieved, but also biofilms can be reached and disrupted more efficiently. The controlled release provided by liposomes can preserve the chemical stability and antimicrobial activity for prolonged periods compared to free molecules. Studies on liposome-entrapped molecules are highly necessary to accelerate the use of these safe and efficient molecules mainly against highly resistant microorganisms in clinical and industrial environments. As the technologies become more accessible, research shall evolve at a quick pace, and the association of these compounds with nanotechnologies, in the near future, will allow their use as effective options for therapeutic use against biofilm-related infectious diseases and in other contexts, such as environmental and industrial.

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17 Recombinant Veterinary Vaccines

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17.1 Introduction

Animal vaccines are almost as old as vaccination itself and the involvement of animals and veterinary medicine in vaccine development dates from the same period. When Edward Jenner first vaccinated farmhands against smallpox in the early 19th century, the antigen he used was obtained directly from cowpox pustules of infected cattle. In fact, the word vaccine is derived from the Latin *vacca*, literally meaning "cow." It would be a few years until the first formal veterinary vaccines were introduced, but the cowpox procedure was known to work in animals from the start. Soon after the dawn of vaccinology, safety issues began to be taken into account, especially regarding the live attenuated vaccines developed after the studies of Louis Pasteur and Robert Koch. However, these safety issues were less of a concern for animal vaccines, a trend that still persists, which allows for faster development of animal vaccines compared to human vaccines.

Vaccination is an effective method of preventing a wide range of diseases in diverse animal species. The field of vaccinology has yielded several effective vaccines that have significantly reduced the impact of a number of important diseases in both companion animals and livestock. In fact, today, the vast majority of licensed vaccines are live attenuated or killed/inactivated microorganisms [26,34,55]. Live attenuated vaccines that contain targeted deletions of genes known to be responsible for virulence can be, nevertheless, very effective because they induce both cellular and humoral immune responses [9,42]. However, mechanisms of attenuation are often poorly defined and the risk associated with the potential for reversion to a virulent phenotype is a major concern [40,54]. Killed/inactivated vaccines are typically safer, although they may be less effective than attenuated vaccines, because, although they are able to induce strong antibody responses, cellular immune responses are usually poor. However, given that there are still several diseases that lack an efficient vaccine, a need remains for better and safer vaccines to prevent, control, or eradicate these diseases [13,26,41]. To this end, efforts to develop more effective vaccines against a large number of diseases using recombinant DNA technology are in progress around the world.

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The technique of using genomic information to study vaccine development in silico, without needing to cultivate the pathogen, is called reverse vaccinology [40,50]. This genome-based approach started with the complete genome sequence of the human pathogen *Haemophilus influenzae* in 1995 [17]. Since then, a large amount of whole genome sequencing of human and/or animal pathogens has been performed [39,53,55,56]. The advent of genetic engineering has given scientists the ability to rationally design both individual microbial components and whole organisms [6]. Furthermore, the application of functional genomics and structural biology has allowed the identification of many promising new antigens [49,50]. Molecular approaches, together with a clearer understanding of the genes responsible for virulence and identification of the determinants of protective immune responses, have provided new methods for the development of novel vaccines against infectious or metabolic diseases.

Reverse vaccinology starts from the pathogen genome sequence on which a computational analysis is carried out to predict in silico which genes are most likely to be potential vaccine candidates. The selected genes can be cloned and expressed in heterologous systems, followed by immunization in an animal model and evaluation of the immune response elicited by the antigens regarding their potential as vaccine candidates. Different antigens can be combined simultaneously, allowing for the development of vaccines against multiple strains of a pathogen. This aspect is especially attractive for animal vaccines, for which vaccine cocktails are a useful vaccination option. One drawback of reverse vaccinology is that it cannot be used to predict polysaccharides or lipids, which are often included in vaccines as active compounds.

17.2 Types of Recombinant Vaccines

Recombinant veterinary vaccines can be broadly grouped into three kinds: DNA (or RNA), subunit recombinant, and vectored vaccines. The recombinant protein (for subunit vaccines) is made in a heterologous system in the laboratory and injected into the vaccine recipient, whereas the immunogenic protein associated with a DNA vaccine is generated by the cells of the host. Recombinant DNA vaccines are based on the expression, by the host, of biological constructs encoding proteins from specific pathogens. Recombinant vector vaccines are experimental vaccines that use an attenuated virus or bacterium to either express and multiply the antigen in the host organism or introduce DNA into host cells. A fundamental step in recombinant vaccine development is the cloning of a DNA fragment insert into a plasmid vector. Many plasmids are commercially available for such uses. The gene to be replicated is inserted into copies of a plasmid containing genes that make cells resistant to particular antibiotics. The gene is also inserted into a multiple cloning site, which is a short region containing several commonly used restriction sites allowing the easy insertion of DNA fragments. Fig. 17.1 shows typical features of prokaryotic (used for subunit and vectored vaccines) and eukaryotic (used for DNA vaccines) plasmid vectors.

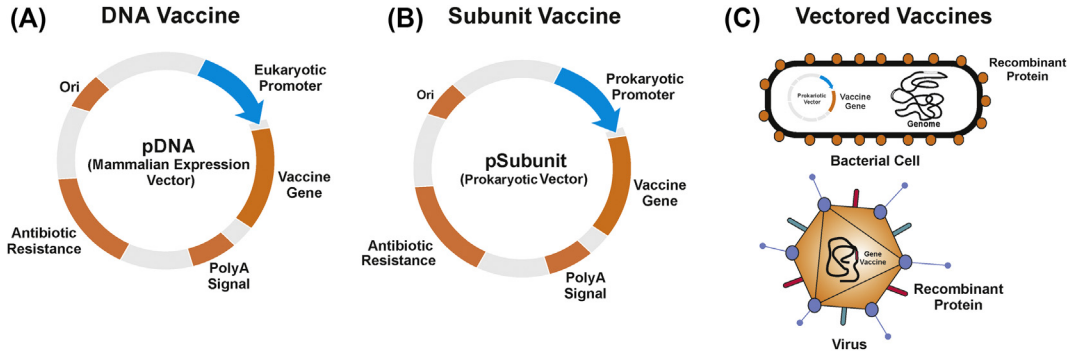

FIGURE 17.1 The transcriptional unit in plasmid vectors consists of the coding sequence of the gene of interest placed under the control of a strong promoter. A bacterial origin of replication (Ori) and an antibiotic resistance gene allow replication and selection of the plasmid in bacteria. (A) Diagram showing the typical features of an eukaryotic expression vector used to construct DNA vaccines. (B) Diagram showing a prokaryotic plasmid vector used for cloning and expression of recombinant proteins used as subunit vaccines. (C) Recombinant attenuated bacterium used as a vaccine vector, and virus vector vaccine expressing a heterologous antigen.

17.2.1 DNA Vaccines

DNA vaccines offer the potential for further advancements in the production of safe and effective veterinary vaccines. A DNA (or RNA) vaccine can be defined as a plasmid containing a viral, bacterial, or parasite gene that can be expressed in mammalian cells (in the case of infectious diseases) or a gene encoding a mammalian protein (in the case of noninfectious diseases, such as cancer, autoimmunity, and allergies) [6,11,34]. The gene of interest is inserted into a plasmid, along with appropriate genetic elements such as strong eukaryotic promoters for transcriptional control, a polyadenylation signal sequence for stable and effective translation, and a bacterial origin of replication. The plasmid is transfected into host cells and transcribed into mRNA, which is then translated, resulting in the production of an antigenic protein by the host cellular machinery. The expressed proteins should have authentic posttranslational modifications (e.g., glycosylation) and tertiary structure, and consequently, conformational epitopes of the antigen should be effectively represented. The expressed proteins are recognized as foreign by the host immune system and can induce cellular and humoral immune responses (Fig. 17.2). These vaccines can be administered via a number of routes and techniques, which can alter the immune response and distribution of the protein [12,41,45]. Currently, some DNA vaccines have already been approved for commercial use in animals (see Table 17.2).

17.2.2 Vectored Vaccines

Vector vaccine technology uses a vector to deliver a protective protein(s) to the immune system of the vaccinated host. The vector will usually be a virus or bacterium.

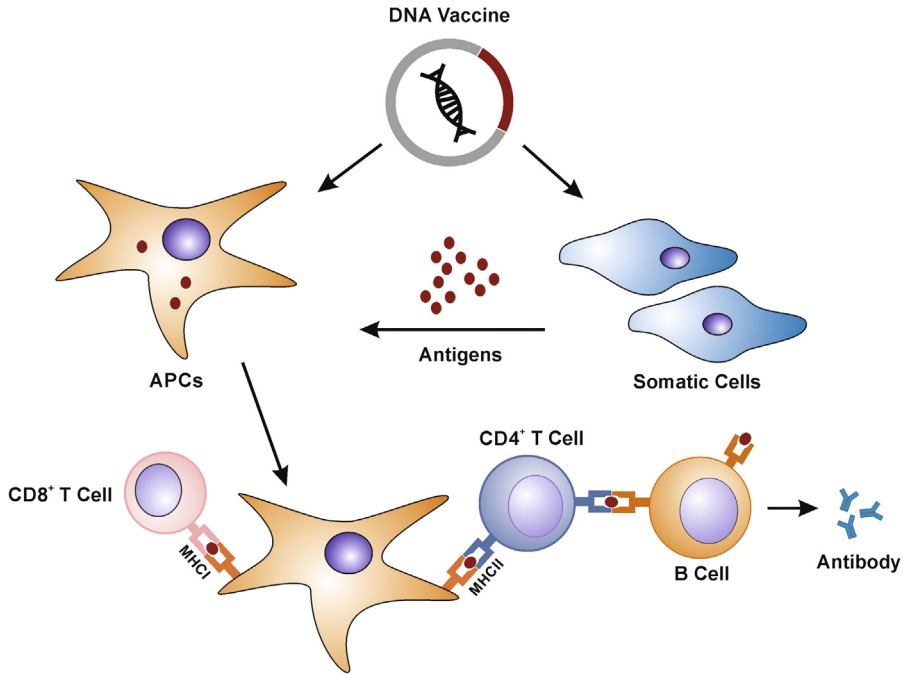

FIGURE 17.2 DNA vaccination induces a strong humoral and cellular immune response against the plasmid-encoded antigen. The delivery of antigens occurs by the intracellular route via induced MHCI-restricted $CD8^+$ responses, whereas antigens presented through the extracellular route activate MHCII-restricted $CD4^+$ responses. *APC*, antigen-presenting cell.

No extensive purification would be required and several live delivery systems have been shown to elicit strong long-lasting immunity without the need for adjuvants [12,44,54]. Both bacteria and viruses have been investigated for delivery of foreign antigens. The nucleotide sequence of these vectors has been modified using genetic engineering by inserting one or several exotic antigens and adequate upstream (promoter) and downstream (terminator) regulatory sequences. For example, the hemagglutinin of the influenza virus is responsible for attachment of the virus to cell receptors and for fusion of the viral and cellular membranes. In horses, the best-known vector vaccine against influenza comprises two canarypox recombinant viruses expressing hemagglutinin from the H3N8 lineage. This nonreplicative canarypox-based vaccine induces humoral immunity, primes cell-mediated immunity, and demonstrates a long duration of immunity. Another approach in vectored vaccines is that involving attenuated bacteria, which can also be engineered to express exotic antigens or overexpress antigens of interest. Cattle vaccinated with a BCG strain overexpressing the Ag85B antigen (protein found on the bacterial surface) showed improved protective immune responses against *Mycobacterium bovis* under experimental conditions [42].

17.2.3 Subunit Vaccines

This approach consists of transferring a gene encoding an antigen to a nonpathogenic host, thereby making the production of the antigen safer and generally more efficient. The recombinant antigen will then be isolated, and the subunit vaccine will be delivered as a purified recombinant protein [26]. The first of these vaccines was the highly pure surface antigen of the human hepatitis B virus expressed in yeast [11]. These novel approaches were based on studying the microorganism in terms of pathogenicity and immunogenicity to identify factors involved in virulence suitable as vaccine candidates, and have been the basis of development of some currently available vaccines. Additional benefits of this type of vaccine are that they incorporate proteins in their most native form, facilitating correct protein folding and reconstitution of conformational epitopes [8,15,18,19]. By incorporating more than one protein into a subunit vaccine, it is possible to derive immunity to more than one strain or serotype of a bacterial pathogen [12].

Cloning of the gene coding for the antigen is often necessary to better characterize and produce the identified antigen, and thus the new protein can enter vaccine development. *Escherichia coli* has been used extensively as a host for heterologous protein expression, but potential limitations include the yield, folding, and posttranslational modifications of the recombinant protein [24,49]. An alternative host to *E. coli* is the methylotrophic yeast, *Pichia pastoris*. This yeast strain has emerged as a powerful and inexpensive expression system for the heterologous production of recombinant proteins that allows techniques for genetic modifications, secretion of expressed proteins, posttranslational modifications, and a high yield [20,21]. The continued use of alternative expression systems has many potential advantages; foremost among these are other yeast species and plant-based systems, which may provide access to oral vaccines as well as enhanced stability of antigens. Expression of antigens in heterologous systems provides both manufacturer and user safety by eliminating the need to use a virulent or partially virulent microbe to provide immunity. Potential drawbacks of subunit vaccines are their moderate immunogenicity and requirement for adjuvants to generate robust immune responses. Types and some of the characteristics of recombinant vaccines are shown in Table 17.1.

17.3 Immune Responses Induced by Vaccination

The severity of disease depends on host susceptibility factors, the infecting species or strain, and the infective dose. Host susceptibility factors, such as immune competence, age, sex, and animal species and breed, have been identified as risk factors. Specific acquired immunity operates through clonal selection, whereby the B lymphocytes that produce antibodies display them on the cell surface, and when the T helper and cytotoxic lymphocytes that recognize an antigen are activated, these cells multiply and carry out immune defensive actions. In addition, immune memory is established because part of the lymphocytes that responded to the primary infection are preserved and may later

Table 17.1 Types and Characteristics of Recombinant Vaccines

Type of Recombinant Vaccine	Characteristics
Subunit (proteins)	Well-defined composition
	No risk of pathogenicity
	Possibility of cost-efficient production and purification
	Primarily humoral immune response
	Need for adjuvant
DNA-based	Humoral and cellular immune responses (antigen presentation by both MHC class I and class II molecules)
	Long-term persistence of immunogen
	Risk of integration into host genome not completely excluded
	Unstable and quite expensive production (for RNA vaccines)
Vector-based	Cellular and humoral immune responses
	In vivo amplification systems available
	Inefficient transfection

be reactivated in response to a second invasion by the same type of microorganism. Cellular memory depends on the persistence of antigen-sensitized T cells that are able to rapidly respond to a secondary infection. In this context, vaccination acts in a way similar to primary infection. A protective immune response to vaccination may be due to humoral immunity, the action of sensitized T lymphocytes (cellular immunity), or a combination of these factors. Recombinant subunit vaccines induce protective immunity without the risk of side effects or immune reactions caused by other parts of pathogenic bacteria, and are known to generally induce predominantly T-helper 2 (Th2) responses with weak or absent Th1 responses. Differences in the immunity induced by recombinant antigens have been observed, which can be influenced by differences in vaccine constructs, the route of immunization, the correct folding, and/or other post-translational modifications that may contribute to the antigen's capacity to generate antibodies [10,14,21].

Vectored vaccines can generate antigens for long periods after immunization, in contrast to a subunit vaccination. This increased presence and level of vaccine antigen contributes to and helps sustain a durable immune response, even augmenting the selection of higher affinity antibody-secreting cells. In subunit vaccines, the use of multiple antigens in the same immunization may represent a promising alternative [19]. Prime–boost strategies, using DNA or protein immunization in conjunction with a vectored vaccine, have also been explored as a means to avoided preexisting immunity [22]. The enhanced memory response is in part linked to the intrinsic augmentation of immunity induced by the vector.

Mucosal immunology is gaining increasing attention as an area of great potential for the development of animal vaccines. Indeed, mucosal surfaces are the major point of

entry for infectious agents into the body. The mucosa contain several defined lymphoid tissues that respond specifically to invading antigens, and this immune response can be either cellular or humoral [immunoglobulin A (IgA)] [10,30,31]. The characterization of a protective immune response requires the definition of which aspects of the immune reaction are responsible for the protection afforded (systemic, humoral, mucosal, or cellular). All this information is necessary to ensure that a given in vitro potency test is also relevant for assessing in vivo efficacy and for ensuring that the relevant immune response is being measured against the appropriate antigens.

The tools of molecular biology and understanding of the mechanisms of the immune response have helped to expand the development of vaccines. The understanding of the molecular mechanisms of innate immune responses has dramatically increased, leading to the discovery of new classes of receptors such as Toll-like receptors, Nod-like receptors, and Rig-like receptors. These molecules have evolved to sense microbial infection and trigger an immune response adapted to the invading pathogens. Importantly, the innate immune reactions triggered by these receptors are also required to enhance and modulate the antigen-specific immunity [25,52,57]. The reverse vaccinology approach and the immune response elicited are represented in Fig. 17.3.

17.4 Veterinary Vaccine Production

Recombinant vaccines are still controversial with the general public, especially with respect to the aspects of the technology that are poorly understood by nonscientists. Thus, a concern with mutation, for instance, hinders the acceptance of naked DNA vaccines for human use, although most are known to be safe and several DNA vaccines are in human clinical trial stages. The same can be said of vectored vaccines, using either viruses or bacteria. In veterinary medicine, however, public opinion is less of a concern, and products that are "taboo" for human use are readily accepted, with concerns being productivity (in the case of livestock) and economic viability. Following this trend, the first DNA vaccine for veterinary use was licensed in 2005, against the equine West Nile virus (WNV), whereas as of 2015 there are still no DNA vaccines for human use.

Vaccine development for the veterinary market has several advantages, especially concerning licensing, and some disadvantages compared to the development of human vaccines. Considering livestock vaccines, the guiding force behind acceptance will be economic viability and food safety. Therefore, while tissue reactions against foot and mouth disease (FMD) vaccines are a concern, the loss in productivity and the discarded meat around the local inflammation site is the greater problem, with animal welfare usually considered a secondary issue. This focus on productivity is especially strong in underdeveloped and developing countries, where animal welfare is only now being discussed. The development of livestock vaccines is further facilitated by the relatively short life expectancy of these animals, with beef-producing cattle rarely reaching 3 years of age, swine reaching no more than 200 days, and poultry being slaughtered at no older than 40 days; thus there is no need for long-term safety concerns. Likewise, the generation of relatively short-term immunity will not be a problem in several animal species.

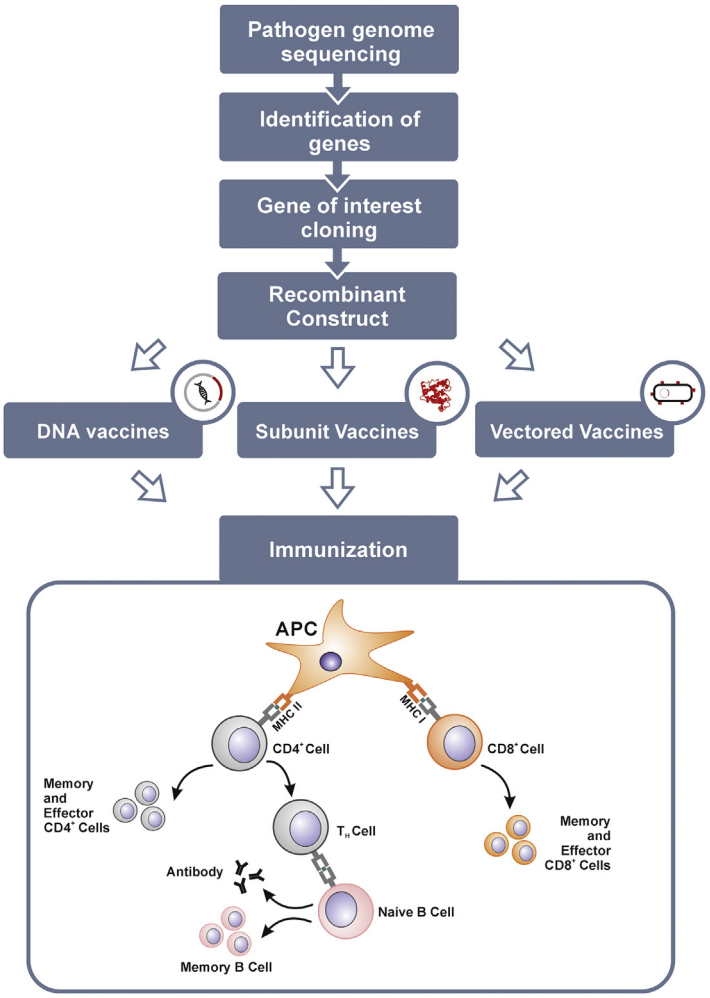

FIGURE 17.3 Schematic of different approaches to recombinant vaccine development and the cellular and humoral immune responses elicited by vaccination. *APC*, antigen-presenting cell; T_H, T-helper cell.

Of course, there are long-lived animals that must be vaccinated, such as dairy cattle, pets, and wildlife. The disadvantages brought on by the diversity of the veterinary market will be considered further on.

Another advantage in the development of veterinary vaccines is that researchers can work directly with the target species. Like vaccines for humans, most initial research occurs in laboratory animals such as rats and mice, as these are cheaper, and a vast array of biological resources are available. Nonetheless, the advancement from these lab animals to the target species is usually immediate, because the final preclinical assays can be conducted in the latter, and usually overlap with the clinical trials themselves. These advantages, compared to human vaccines, have resulted in the commercialization of several recombinant veterinary vaccines, a selection of which is listed in Table 17.2.

Table 17.2 Selection of Recombinant Veterinary Vaccines Commercially Available as of 2015

Vaccine	Animal Species	Pathogen	Vaccine Type	Manufacturer
EURIFEL FeLV	Cats	Feline leukemia virus	Vectored	Merial
PUREVAX Feline Rabies	Cats	Rabies virus	Vectored	Merial
Gavac	Cattle	*Ripcephalus* (*Boophilus*) *microplus*	Subunit	Heber Biotec
TickGUARD	Cattle	*R.* (*Boophilus*) *microplus*	Subunit	Fort Dodge
RECOMBITEK rDistemper	Dogs	Canine distemper virus	Vectored	Merial
PUREVAX Ferret Distemper	Ferrets	Canine distemper virus	Vectored	Merial
Apex-IHN	Fish	Infectious hematopoietic necrosis virus	DNA	Novartis
ProteqFlu TE	Horses	Influenza virus and tetanus toxin	Vectored	Merial
PROTEQ-FLU/RECOMBITEK	Horses	Influenza virus	Vectored	Merial
RECOMBITEK Equine WNV	Horses	West Nile virus	Vectored	Merial
West Nile-Innovator DNA	Horses	West Nile virus	DNA	Fort Dodge
Innovax ILT	Poultry	Infectious laryngotracheitis virus	Vectored	Merck
Trovac-AI-H5	Poultry	Avian influenza virus	Vectored	Merial
Vaxxitek HVT + IBD	Poultry	Marek disease virus	Vectored	Merial
Vectormune FP-ND	Poultry	Newcastle disease virus	Vectored	Biomune
Raboral	Raccoons and coyotes	Rabies virus	Vectored	Merial
HIDATIL EG95	Sheep and goats	*Echinococcus granulosus*	Subunit	Tecnovax
Bayovac CSF E2	Swine	Classical swine fever virus	Vectored	Bayer
P. Circumvent	Swine	Porcine circovirus	Subunit	InterVet
PleuroStar APP	Swine	*Actinobacillus pleuropneumoniae*	Subunit	Novartis
Porcilis APP	Swine	*A. pleuropneumoniae*	Subunit	Intervet
Porcilis Pesti	Swine	Classical swine fever virus	Vectored	Intervet
PRO-VAC Circomaster	Swine	Porcine circovirus	Subunit	Vencofarma
Suvaxyn PCV2	Swine	Porcine circovirus	Vectored	Fort Dodge

Arguably, the most significant advantage in recombinant veterinary vaccine development, compared to human vaccines, is the looser restrictions on adjuvants. Subunit vaccines may be the most straightforward and safest approach to developing recombinant vaccines; however, these are the least immunogenic. This is further hindered by the fact that the only adjuvants approved for human use are aluminum crystals, which have very limited immune boosting capabilities, compared to other available adjuvants. The combination of protective antigens and adjuvants or delivery systems may result in much-needed effective recombinant vaccines. By definition, an adjuvant is an agent that stimulates the immune system and increases the host response to an antigen without itself conferring a specific antigenic effect [3,26]. Some adjuvants act by sequestering antigens in physically restricted areas, termed depots, to provide an extended time

period of antigenic stimulation. This depot effect is essential for the efficacy of the majority of human and veterinary vaccine adjuvants, particularly with vaccines consisting of pathogen subunits or recombinant subunits [24]. The immunomodulatory effects are dependent upon the particular adjuvant used in conjunction with specific antigens. Thus, several veterinary vaccines are emulsions in oil. This relatively old-fashioned technology is, nonetheless, a powerful approach that allows for a strong inflammatory response and slow antigen liberation, exactly what recombinant subunit vaccines lack. Likewise, naked DNA vaccines may require (though several studies indicate otherwise) in vivo electroporation, or the addition of substances that enhance membrane permeability, for optimum function. These methods are readily available for animal use, though none are yet considered safe for human use. The same can be said of vectored vaccines, of which rabies or WNV vaccines, vectored in canarypox and vaccinia viruses, for example, are already available for the animal market, whereas no vectored vaccine is yet in use in humans (in the United States or Europe as of 2014).

Although the advantages and the relative ease in the development of recombinant veterinary vaccines mean that there are more of these currently commercially available, there are a few disadvantages that cut back research funding. The most important disadvantage concerning the veterinary vaccine development is market size, compared to human vaccines. These differences in market size are reflected in funding volume. For instance, the US National Institutes of Health allocated over $25 billion in support of human medical research in 2012. In the same period, the National Institute for Food and Agriculture's budget for veterinary medicine was almost 100 times less. Privately financed research follows the same trend; a successful veterinary vaccine will return a fraction of what a human vaccine returns, and that will depend on market acceptance, which brings us to another setback in vaccine development.

The second most important difficulty in veterinary vaccine development is the large number of species, both of hosts and of disease agents, which can suffer from or cause veterinary ailments, respectively. Thus, economically important species, such as livestock and mainstream pets, will be favored in vaccine development over wildlife, alternative meats, and exotic pets. Likewise, diseases that cause greater economic losses will, naturally, receive greater priority; those that cause more severe health problems may not necessarily be prioritized. This uneven distribution of research funds and effort is understood and expected; however, this means that several diseases and animals are currently unprotected.

17.5 Recombinant Vaccines Against Bacterial and Fungal Diseases

17.5.1 Bacterial Vaccines

Novel approaches in recombinant DNA technology, genomics, and structural biology have revolutionized the way vaccine candidates are developed and will make a

significant impact on the generation of safer and more effective animal vaccines [34,40,50]. Reverse vaccinology has primarily been applied to bacterial pathogens to identify proteins that can be formulated into subunit vaccines (one or more protein antigens) [12,19,26,31]. Currently, most of the bacterial vaccines licensed for animal use include live attenuated and inactivated or killed microorganisms, with varying degrees of efficacy and side effects. The development of effective vaccines against bacterial diseases with a wide range of protection and few side effects is a basic requirement for any new human vaccine. Indeed, such characteristics are just as important in animal vaccine development [12]. Research into recombinant vaccines has identified antigens that can protect distinct animal species against several infectious bacterial diseases.

An example under development as of this writing is a recombinant vaccine against leptospirosis. It is a zoonotic disease and a major public health concern in tropical and subtropical regions. There are numerous descriptions of problems associated with heat-killed, whole-cell (bacterin-type) vaccine preparations, including severe side effects (pain, nausea, fever), short-term immunity, and serovar-restricted protection [1,7,12,28,46]. Consequently, research has focused on leptospiral recombinant antigens capable of eliciting protective immunity, especially identifying virulence determinants such as the surface-exposed antigens and those present in multiple serovars/serogroups of bacteria. Some these antigens have been identified as being protective in experimental trials [8,12,21,47].

Several studies have evaluated recombinant proteins in various forms of administration and formulations, seeking to develop more effective bacterial vaccines. For example, some recombinant antigens were evaluated individually, and others were associated with attenuated bacterial or viral vectors and evaluated as cocktail or fused antigens. However, researchers have presented divergent results on vaccine efficacy for the same antigen, particularly when using a subunit formulation. Structural differences in the recombinant proteins are probably the simplest explanation for these observations. This is a recognized problem in recombinant protein expression; however, it should be possible to overcome this by using alternative expression systems and accurate mapping of the protective epitopes.

17.5.2 Recombinant Vaccines Against Fungal Infections

Although fungi, namely yeast, are major players in recombinant vaccine development as cell factories, vaccines to control fungal diseases are not a major concern. Few studies toward the development of recombinant vaccines against fungi of veterinary importance have been undertaken. Genera such as *Sporothrix*, *Aspergillus*, and *Cryptococcus*, as well as a myriad of fish microsporidia, are known to cause important diseases in pet and production animals; however, little effort has been undertaken toward developing any vaccines against these diseases, let alone recombinant vaccines [5]. *Candida* spp. are the most studied fungi regarding recombinant vaccines; however, most of these studies have been focused on human candidiasis [32]. Nevertheless, a human preparation will probably work in animals, though there will be limited commercial interest to push it forward.

17.6 Recombinant Vaccines Against Parasitic Diseases

Parasites affecting health in veterinary medicine can be divided into three major groups for the sake of control and vaccine development strategies, i.e., protozoan parasites, helminths, and ectoparasites. Generally, parasites pose a greater challenge for recombinant vaccine development, regardless of the major group. Their eukaryotic genomes are larger and more complex than those of viruses and bacteria, and even those of most fungi of veterinary relevance. A sequenced genome is the first step for a reverse vaccinology approach. However, although genomes of viruses and bacteria have been sequenced with ease, with several isolates being sequenced at once, this is not always possible with eukaryotic parasites. Although advances in technology are reducing costs, the production of immunogenic subunit parasitic antigens continues to pose challenges, such as posttranslational modifications, glycosylation, phosphorylation, and lipidation, among others.

Immune protection strategies similar to other, simpler disease agents can also be used against protozoan parasites, because they infect tissues or circulate in the bloodstream. Gut parasites and ectoparasites, on the other hand, will pose a greater challenge, because most of the parasite is "external" to the host and antigens are mostly hidden from the immune system. Strategies to control helminths may target those stages of the life cycle that occur in the host's tissues, or even involve IgA stimulation against antigens of the parasite's exterior. However, the multicellular, mostly inaccessible, ectoparasites pose the greatest challenge to researchers, who must work either with mouth antigens or with hidden antigens, because of the lack of any other exposure of the parasite to the host's immune system.

17.6.1 Protozoan Parasites

Protozoan parasites cause a myriad of diseases of veterinary importance. In most cases, the parasites require more than one host, usually an intermediate arthropod host, for the completion of the cycle. This means that protozoan agents are more prevalent in tropical and subtropical climates, where the arthropod hosts are more abundant. Cattle ticks do not occur beyond parallels 33, for example; therefore, babesiosis and anaplasmosis are also not a concern beyond these latitudes. It is a similar case for protozoan diseases that require bloodsucking flies and/or mosquitoes for their transmission. Consequently, these diseases are more common in underdeveloped and developing countries of the tropics, and vaccine production should be focused, not only on the control of these diseases, but also to allow the importation of naïve animals from outside these regions. Nevertheless, there are currently few commercial vaccines against protozoan agents.

Leishmaniasis is one of the most important protozoan zoonoses in the world, causing disease mostly in dogs and humans. Leish-Tec (Hertape Calier Saúde Animal S.A., Brazil) is a leishmaniasis vaccine licensed in Brazil. Although it was released without greater field studies, the recombinant A2 antigen used has been shown to be protective [16]. This antigen is a virulence factor of the amastigote stage of the parasite, and was foremost

among others studied as potential vaccine candidates. Leish-Tec is an example of how strong adjuvants can make simple subunit vaccines viable. It uses saponin, a proinflammatory adjuvant, in its preparation, which has been shown to be a potent adjuvant for Th1 cytokines [51].

Although the only commercially available recombinant vaccine against protozoan diseases up to 2013 was Leish-Tec [33], other antigens are expected to soon follow in its footsteps. Successful, or partially successful, experimental vaccines against the livestock and pet parasites *Toxoplasma*, *Babesia*, *Neospora*, and *Giardia*, as well as avian coccidiosis, indicate that this is the most promising market for future veterinary recombinant vaccines, at least as far as parasitic diseases are concerned.

17.6.2 Helminths

Medically relevant helminths can be divided into three greater families, namely, nematodes (roundworms), trematodes (flatworms), and cestodes (tapeworms). These large, multicellular parasites have been eluding vaccine development for years. Although a few traditional (nonrecombinant) antihelminth vaccines have been commercialized, namely against the cattle lungworm *Dictyocaulus viviparus,* there are, as of this writing, trials under way for a vaccine against ovine haemonchosis in Australia. Though theoretically more feasible than ectoparasite vaccines because of the "closer" relation with the host, success has been limited, possibly due to a lack of promising protein antigens, and experts have recommended more focus on glycans as vaccine targets for a long time now [23]. Furthermore, a possible approach to helminth control would be the stimulation of mucosal immunity through IgA; however, this approach is still hardly ever used.

Nematodes (roundworms) are the most important helminths in veterinary practice, causing the greatest productivity and economic losses in livestock (and other production species such as poultry and fish), and major health risks in the pet market. Efforts to control roundworms through vaccination have thus far been frustrated, but there has been a concentration of resources to develop anti-*Haemonchus* spp. vaccines, because of the economic importance of this parasite in sheep and goats, and its ability to rapidly develop resistance to drugs. These efforts have produced some promising antigens, especially gut glycoproteins [4]. However, recombinant approaches have not yet granted protective immunity, though minor amounts of purified native protein are protective and have been commercialized in Australia (Barbervax). To circumvent the lack of protection, production of the recombinant proteins in a helminthic expression system, *Caenorhabditis elegans,* has been attempted, but no better results have been obtained thus far [43].

Cestodes (tapeworms) are generally not a burden on production animals and cause minor health risks in pets. Nonetheless, these parasites are economically important because several of them cause food-borne or otherwise zoonotic diseases. There are major concerns regarding *Taenia* spp., infecting pigs and cattle, and the canine/ovine parasite *Echinococcus granulosus*. A recombinant vaccine targeting the larval stages of

E. granulosus in ruminants has been commercialized in Argentina, although success has been limited. The anti-rEG95 vaccine (HIDATIL EG95) claims to hinder the capability of oncospheres to cross the gut wall and cause infection [27]. Likewise, vaccine attempts against other tapeworms have been more successful against larval-stage antigens [29] than the adult worm. Nevertheless, from an epidemiological point of view, the control of the parasite in the definitive host may be more effective. Although the definitive hosts of *Taenia* spp. are humans, few studies have been conducted on vaccines at this level. Vaccines against canine echinococcosis, on the other hand, are a possibility, and several research efforts are being made in the development of recombinant vaccines for this host [38].

Trematodes (flatworms) of importance in veterinary medicine are the liver flukes of the *Fasciola* genus, specifically *Fasciola hepatica* and, to a lesser extent, *Fasciola gigantica*. Although they are zoonotic and do cause some economic losses, especially in sheep, these parasites are generally contained in endemic areas and environmental control is feasible. Consequently, the least effort, among cestodes, has been put in developing vaccines against flatworms. Nonetheless, the "internal" colonization of tissues (as opposed to "external" colonization of the gut) facilitates immune access, and promising recombinant antigens have been identified, though no commercial vaccine is available yet [35].

17.6.3 Ectoparasites

As stated, ectoparasites offer the greatest challenge for vaccine development, not only because of their size and complexity, but also because most of the parasite never enters the host, and usually only the mouthpiece is exposed during feeding. Unlike for human medicine, ectoparasites are a major concern in veterinary medicine, both as causative agents of disease and as vectors for other, mostly protozoal, agents. Ticks cause great losses in cattle populations, and fleas and mites are a prominent concern in pet markets worldwide. Though the need, and indeed the economic opportunity, for the development of vaccines against fleas, ticks, and mites has been pointed out [37], major efforts have been concentrated on the development of recombinant vaccines against the cattle tick *Ripcephalus* (*Boophilus*) *microplus*.

Considering the complexity and difficulty expected in the development of ectoparasite vaccines, it is surprising that one of the first recombinant vaccines available for veterinary use was against *R. microplus*. First introduced (and developed) in Australia in 1994, TickGUARD (Fort Dodge, Australia) is a recombinant subunit vaccine produced in and purified from *E. coli*. The same antigen, midgut protein BM86, was later produced in yeast by Cuban researchers, and marketed in Cuba (and some other countries, including Brazil) as Gavac (Heber Biotec SA, Cuba). The BM86 antigen is a midgut protein responsible for nutrient absorption by the tick. These commercial vaccines have seen limited success outside their countries of origin, with protection rates dropping when the vaccines were used in Brazil, for instance [2]. Explanations for this difference

include an alleged variation in the protein between locally circulating strains. However, studies have shown that BM86 is highly conserved, even among different tick species, and that efficacy does not seem to be associated with variations in the gene [58]. Furthermore, BM86 is a concealed or hidden antigen and is, therefore, not naturally exposed to the immune system. Antibody levels do not rise with each reinfestation, and the vaccine's effectiveness is only partial, intended to be used concomitant with conventional drugs.

There are no commercial vaccines available to control other ectoparasites of veterinary importance as of this writing. Nonetheless, research toward their development is ongoing. Because there is no natural immune response against flea antigens, all potential antigens studied have been "hidden," usually from the midgut of the parasite [48], following the, possibly misleading, experience of BM86. Promising results have been obtained [36]; however, progress on these vaccines is slow, and the associated flea bite allergy antigens have received more attention.

Likewise, the development of vaccines against mites and myiasis-causing flies (or fly bots) has yet to produce promising results. The analysis of expressed sequence tags seems to be an interesting approach to finding functional antigens in these complex parasites [36]. However, other than some homologs found between house dust mites and the sheep mite *Psoroptes ovis* that seem promising, other agents have still to produce convincing antigens worthy of greater research investments.

17.7 Recombinant Vaccines Against Viral Diseases

Licensed recombinant viral vaccines in veterinary medicine include those protecting against pseudorabies, rabies, canine distemper, Newcastle disease, and a strain of avian influenza. Vaccines are useful tools to control avian influenza especially when biosecurity and stamping out strategies alone are not successfully implemented. Inactivated vaccines are the most widely used avian influenza vaccines although viral-vectored vaccines based on fowlpox or Newcastle disease virus are also used in some countries [41,54]. Oral rabies vaccines were first proven to be successful in the late 1960s and have been used for prevention and control of wild species from rabies over the world. Oral immunization in free-roaming dogs is one of the most practical approaches to protect humans from rabies in developing countries. The development of recombinant DNA technology has initiated a new era in rabies control. Various recombinant constructs (e.g., animal poxviruses or human or canine adenovirus as the vector) expressing the rabies glycoprotein were tested in various target and nontarget wildlife species by the oral route. The only recombinant vaccine that is now produced and used in large quantities is a vaccinia-based recombinant product expressing the rabies glycoprotein (VRG). VRG shares many basic properties with the parental vaccinia virus (Copenhagen strain) but differs in other ways, which make the vector virus safer. The deletion of the thymidine kinase gene dramatically decreases the pathogenicity of the vaccine in mice when it is

injected intracerebrally and intraperitoneally. In addition, no viral spread from currently known sites of viral replication has been observed, and oral vaccination of dozens of animal species, including wild animals, has not revealed any residual pathogenicity [41].

17.8 Conclusions and Perspectives

Traditional vaccines are generally protective, and the same advances expected in human recombinant vaccines are expected in veterinary vaccines. Biologically engineered vaccines provide targeted immunity and eliminate the need to work with dangerous agents; they reduce the time required for the onset of immunity, with excellent safety characteristics. Nevertheless, some issues are particular to animal vaccines, and recombinant DNA technology may be the best alternative to tackle these. The most relevant issue to be tackled in the near future is the differentiation among vaccinated and exposed animals. Most countries use serological tests in their disease control strategies; thus, vaccination interferes in the control. For instance, BCG vaccines are mostly vectored for cattle and pigs, because the antibodies would jeopardize control through tuberculinization. Likewise, FMD vaccines in countries that are disease-free are used only in outbreak situations, because herd-wide vaccination may result in international sanctions, mainly regarding the exportation of live animals, but also concerning meat and meat products. Recombinant vaccines, capable of protecting animals with a strong immune response against one or few antigens, are a safe bet to circumvent these restrictions, because the diagnosis of a positive animal could be made while ruling out vaccine immunity.

Recombinant vaccines are undoubtedly the future of veterinary disease prevention. In this light, the drivers for recombinant vaccine development must be more potent, safer, better characterized vaccines, in addition to developing vaccines that provide broader protection against multiple antigens.

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18

Development of Process to Produce Recombinant Component for Acellular Pertussis Vaccine

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18.1 Introduction

18.1.1 Pertussis Disease

Pertussis, also known as whooping cough, is a disease of the respiratory tract that affects humans of all ages and has worldwide distribution. The disease cause great morbidity and mortality in young children, especially in babies less than age 1 year. This disease occurs as the result of infection caused by *Bordetella pertussis*, which releases a number of virulence factors, including pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin, and fimbriae [1,2].

Pertussis is primarily a toxin-mediated disease. The bacteria attaches to the cilia of the respiratory epithelial cells, producing toxins that paralyze the cilia and causing inflammation of the respiratory tract, which interferes with clearing of pulmonary secretions [3].

Main symptoms of this disease are violent coughing, which often provokes difficulties in breathing [2]. Severe and potentially fatal complications include convulsions, bronchopneumonia, and encephalopathy [4].

Typical pertussis, as defined by the World Health Organization [5], consists of three phases: catarrhal, paroxysmal and convalescent. The catarrhal phase, during which the disease is most contagious, manifests as a cold-like illness with coryza and a mild cough. The onset of these symptoms is usually 7–10 days after exposure. Proliferation of *Bo. pertussis* and its subsequent abundance in nasopharyngeal secretions provide a means of aerosol transmission via droplet spread or direct contact [6].

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Progression from a mild asymptomatic cough to convulsive or violent attacks (paroxysmal cough) indicates onset of the paroxysmal phase. Excessive mucous production, vomiting, and lymphocytosis are additional manifestations that are commonly observed during this period of infection. A feature of the paroxysmal cough is the characteristic "whoop" sound that occurs as a result of inhalation through a narrow glottis. Coughing attacks can occur frequently and may be so severe that exhaustion and apnea can occur. This stage can last for up to 4 weeks, after which most who experience it enter a convalescent phase characterized by a gradual recovery. However, the severe cough can reemerge in the presence of an opportunistic infection [4].

18.1.2 Historical Aspects

Whooping cough was recognized as a human acute respiratory disease in the 16th century. The etiologic agent, *Bo. pertussis*, was isolated and identified by Bordet and Gengou in 1906 [7].

In the 16th century, pertussis was one of the most common childhood diseases and a major cause of childhood mortality in the United States. Before the availability of pertussis vaccine in the 1940s, more than 200,000 cases of pertussis were reported annually. During the 6 years from 1940 through 1945, more than one million cases of pertussis were reported [8]. Whole-cell pertussis vaccines were first licensed in the United States in 1914 and became available combined with diphtheria and tetanus toxoids (as DTP) in 1948 [8].

After the introduction of whole-cell pertussis vaccine in the 1940s, the incidence of pertussis gradually declined, reaching 15,000 reported cases in 1960 (approximately 8 per 100,000 people). By 1970, the annual incidence was fewer than 5000 cases per year, and during 1980−1990 an average of 2900 cases per year was reported (approximately 1 per 100,000 people). Since widespread use of the vaccine began, the incidence has decreased more than 80% compared with the prevaccine era [8].

18.1.3 The Pathogen and the Toxin

Bo. pertussis is a small gram-negative aerobic coccobacillus that appears singly or in pairs [9]. It is fastidious and requires special media for isolation [8]. This bacterium produces an array of virulence factors, some of which are considered to have a key role in the establishment of *Bo. pertussis* infection. These include FHA, pertactin (P69), PT, and adenylate cyclase toxin. Other factors such as fimbriae, dermonecrotoxin, tracheal cytotoxin, tracheal colonization factor, serum resistance factor, and lipopolysaccharide have important roles leading to clinical manifestations of pertussis [10,11].

Regarded as the central virulence factor during infection, PT elicits a number of deleterious consequences including leukocytosis, splenomegaly, histamine sensitization, hypoglycemia, and hypoproteinemia [12].

PT is one of the most complex soluble bacterial proteins [8] (Fig. 18.1). It is a multimeric 105-kDa protein composed of five different subunits: S1, S2, S3, S4, and S5 [13]. The S1 subunit catalyzes the adenosine diphosphate (ADP) ribosylation of G proteins in target

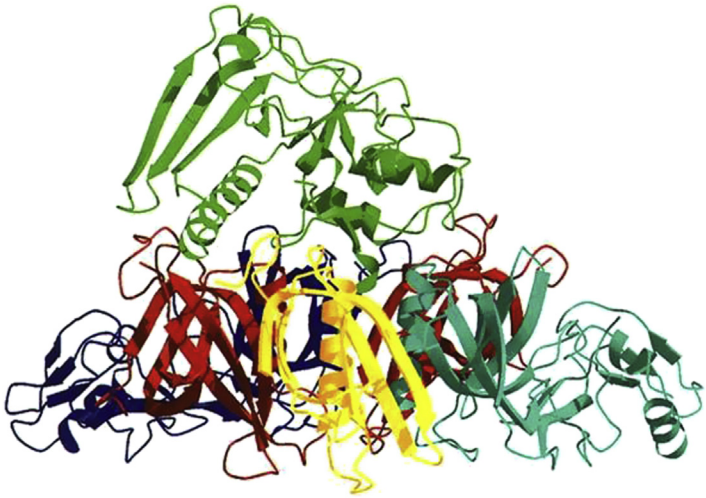

FIGURE 18.1 Schematic of pertussis toxin. The holotoxin is viewed perpendicular to the fivefold axis of the B-oligomer. *Reprinted from P.E. Stein, A.B. Boodhoo, G.D. Armstrong, S.A. Cockle, M.H. Klein, R.J. Read, The crystal structure of pertussis toxin, Structure, 2 45–57, copyright 1994, with permission from Elsevier.*

cells of mammals, whereas subunits S2 to S5 (oligomer B) are responsible for the binding of S1 to these cells. ADP ribosyl transferase enzymatic activity is associated with the domain located in the 180 N-terminal amino acids of S1 (termed C180), whereas the rest of this subunit is involved in G protein binding and interaction with the oligomer B [14].

Besides enzymatic activity, mainly owing to Arg9, Arg13, and Glu129 residues [15], the subunit S1 is also recognized as the immunogenic portion of PT once it promotes the maturation of dendritic cells. These cells are in charge of activation of antigen-specific T- and B-cells, which mediate adaptive immunity against *Bo. pertussis* [16].

This subunit expressed in *Mycobacterium bovis* Bacillus Calmette–Guérin was able to induce protective immunity against the toxin itself and against *Bo. pertussis* in a model of intracerebral challenge in mice [17]. In addition, a DNA vaccine comprising a plasmid encoding this subunit was able to induce immunity [18].

18.1.4 Epidemiology

The disease is endemic and has epidemic cycles of 3–5 years. It ranks fifth among vaccine-preventable diseases in children under age 5 years. Since 2010, a global trend of increasing pertussis cases has been observed, with reports in California and elsewhere on the American continent [19].

There were 16 million cases of pertussis worldwide in 2008; 195,000 children died of the disease, most of them in developing countries. However, pertussis is not confined in the developing world. There has been a resurgence of the disease in many developed countries, including the United States, Australia, England, Wales, and Ireland. In Australia, the incidence of pertussis has increased from 349 reported cases in 1991 to 38,722 in 2011 [20].

Even with high vaccine coverage (vaccination was responsible for avoiding 37 million cases of illness and 587,000 deaths in 2002), the fall of immunity 5–10 years after the last vaccine dose left adolescents and young adults susceptible to the disease, mainly owing to decreased protective antibody titer [21,22]. This reduction in immunity among adults may be related to the reduced circulation of *Bo. pertussis* in the vaccinated populations as well, which causes less frequent exposure to antigens, and thus less strengthening of natural immunity [23]. This can be considered a serious problem, because an increased risk of complications exists with increasing age, including hospitalizations for pneumonia and sinusitis [24]. Moreover, adolescents and adults may serve as reservoirs for the pathogen and could be an infection source for children, beginning the cycle of transmission to newborns and infants not yet immunized, and the disease remains endemic [25].

18.1.5 Pertussis Vaccines

Because of the emergence of new production technologies and regulatory requirements, vaccine production has had constant and deep transformations. These new products have high value and are causing technological dependence in developing countries [26] that has led to the requirement for short-term replacement of some vaccines, including DTP, used in public health systems.

The DTP vaccine is composed of tetanus toxoid, diphtheria toxoid, and a detoxified bacterial suspension of *Bo. pertussis* [27]. This vaccine has been effective in immunization against pertussis. However, several adverse effects have been reported, such as local effects (redness and swelling), systemic effects (fever, vomiting, and diarrhea), and even seizures [28]. Furthermore, these effects are exacerbated with age, and therefore the last dose of vaccine is given to people age 5 or 6 years.

Many groups have started to study the possibility of producing a acellular pertussis vaccine (DTaP) vaccine that contains only purified antigens of *Bo. pertussis*. A literature review [29] showed that these acellular vaccines have been well tolerated and highly immunogenic in adolescents and adults. These vaccines, which have an antigenic content more than 50% lower than formulations for children and reduced adverse effects, demonstrated an estimated efficacy of 92% in a study among American teenagers [25].

Initially, the approach to production of this vaccine was a bacterial culture and subsequent purification of some proteins recognized as being immunogenic. The major disadvantage of this procedure is that purification of components causes a low yield, because only that fraction of the cell is exploited for vaccine production. Only one dose of acellular vaccine can be produced from the same fermentation broth volume that produces 10–20 doses of whole-cell vaccine [30]. In addition, the vaccine generally has low immunogenicity owing to the chemical detoxification process by which it must pass [15].

Actually, several vaccines available on the market allow the vaccination of adults and young babies (Table 18.1). However these vaccines are produced by multinationals and are too expensive to be acquired by public health programs.

Table 18.1 Composition of Main Commercial Acellular Pertussis Vaccines

Component	KINRIX (GSK)	Pentacel (Sanofi Pasteur)	TETRAXIM (Sanofi Pasteur)	Adacel (Sanofi Pasteur)
Diphtheria toxoid	25 Lf*	15 Lf*	30 I. U.**	2 Lf*
Tetanus toxoid	10 Lf*	5 Lf*	20 I. U.**	5 Lf*
Pertussis toxoid	25 μg	10 μg	25 μg	2.5 μg
Filamentous Hemagglutinin	25 μg	5 μg	25 μg	5 μg
Pertactin (69 kDa)	8 μg	3 μg	–	3 μg
Fimbriae	–	5 μg	–	5 μg
Age indication	4–6 years	6 weeks–4 years	2–13 years	4–64 years

*Limit of flocculation;
**International Units.

18.1.6 Recombinant Vaccines

The production of DTaP by recombinant technology can be accomplished in two main ways. The first, already analyzed in the review of Loosmore and colleagues [31], consists of the mutation of *Bo. pertussis* to change certain amino acids in the S1 subunit of the toxin, which is responsible for its toxicity. The second most studied technology is currently the overexpression of components in microbial cells.

Burnette and colleagues [32] performed cloning of all subunits of PT in *Escherichia coli* to identify key functional sites and map epitopes to obtain candidates for an acellular vaccine. Furthermore, cloning attempts aimed at producing not only PT but also other possible components of the DTaP vaccine were performed in microorganisms such as *Bacillus subtilis* [33], *Pichia pastoris*, *Saccharomyces cerevisiae* [34], and *Bacillus brevis* [35], besides insect cells [36].

The advantages of using these organisms are the low cost of the process, mainly because of the high yield of protein [35], and the greater ease of purification. Furthermore, recombinant production has the advantage of eliminating the step of detoxifying the protein [37].

Table 18.2 shows that since 1987, many studies in developing pertussis vaccines that were performed were related to recombinant technology. These studies involved many different host cells, including *Bo. pertussis* itself, besides some bacteria such as *E. coli* and *Ba. subtilis* and some yeasts such as *P. pastoris* and *S. cerevisiae*. This research used subunit S1 from PT and other molecules as potential vaccines, with good results in producing antibody response.

18.2 Technological Development: Case Description

To develop recombinant vaccine, three steps are necessary. The first involves all of the procedures to get the recombinant plasmid. The second step has the objective of

Table 18.2 Research Development of New Generation of Pertussis Vaccines

Cell	Objective	Author	Year
Escherichia coli K12 ΔH1 Δtrp	Produce each of the five subunits of pertussis toxin as fusion proteins and verify their immunologic potential against intracerebral challenge with *Bo. pertussis* in mice	Nicosia et al. [38]	1987
Bacillus subtilis	Expression and secretion of pertussis toxin subunit S1 with an α-amylase secretion vector	Runeberg-Nyman et al. [39]	1987
Escherichia coli	Expression of each of the five subunits of pertussis toxin at nonfusion forms to use as vaccine components lacking toxic effects of traditional pertussis vaccines	Bumette et al. [40]	1988
E. coli	Expression of S1 subunit with site-specific mutagenesis in critical region. A mutant containing a single amino acid substitution (Arg9-*Lys) with reduced enzymatic activity (approximately 0.02% of control) while retaining protective epitope	Bumette et al. [32]	1988
E. coli	Expression of nonfusion form of S-1 subunit (rS-1) and catalytic peptide of S1 subunit that is composed of the first 180 amino-terminal residues (C180 peptide)	Barbieri et al. [41]	1989
Bordetella pertussis *Bordetella parapertussis* and *Bordetella bronchiseptica*	Construction of a number of genetically engineered alleles of pertussis toxin genes, replacing either one or two key amino acids within enzymatically active S1 subunit, and introduction into the chromosome of strains of *Bo. pertussis, Bo. parapertussis*, and *Bo. Bronchiseptica*, aiming to produce pertussis toxin molecules that are nontoxic and immunogenic	Pizza et al. [15]	1989
Ba. subtilis	Expression and secretion of S1 subunit and test of its immunogenic potential	Ölander et al. [33]	1991
Saccharomyces cerevisiae S150-*2B e Pichia pastoris GS115*	Expression of membrane protein pertactin (P69) from *Bo. pertussis* to high levels in multicopy transformants	Romanos et al. [34]	1991
Bacillus brevis	Expression and secretion of S2 subunit of pertussis toxin	Kozuka et al. [35]	1996
Streptococcus gordonii	Purification and immunogenicity of recombinant *Bo. pertussis* S1S3FHA fusion protein expressed by *S. gordonii* to produce mucosal vaccine	Lee et al. [1]	2002
	Production of DNA vaccine with plasmid DNA expressing pertussis toxin S1 subunit (pcDNA/S1) of *Bo. pertussis* and verification of its immunogenicity, for its ability to induce protection against PT challenge or *Bo. pertussis* infection in mice	Kamachi et al. [18]	2003
	Development of pertussis DNA vaccine expressing nontoxic C180 polypeptide of pertussis toxin S1 subunit	Kamachi et al. [42]	2007
Bo. pertussis	Construction of *Bo. pertussis* strains with enhanced production of genetically inactivated pertussis toxin and pertactin	Buasri et al. [43]	2012
Bo. pertussis BpCNIC0311	Synthesis of genetically detoxified toxoid (PTg). Filamentous hemagglutinin and type 2 pertactin aiming for suitable yields after purification and high immunogenic response	Quintana-Vázquez et al. [44]	2015

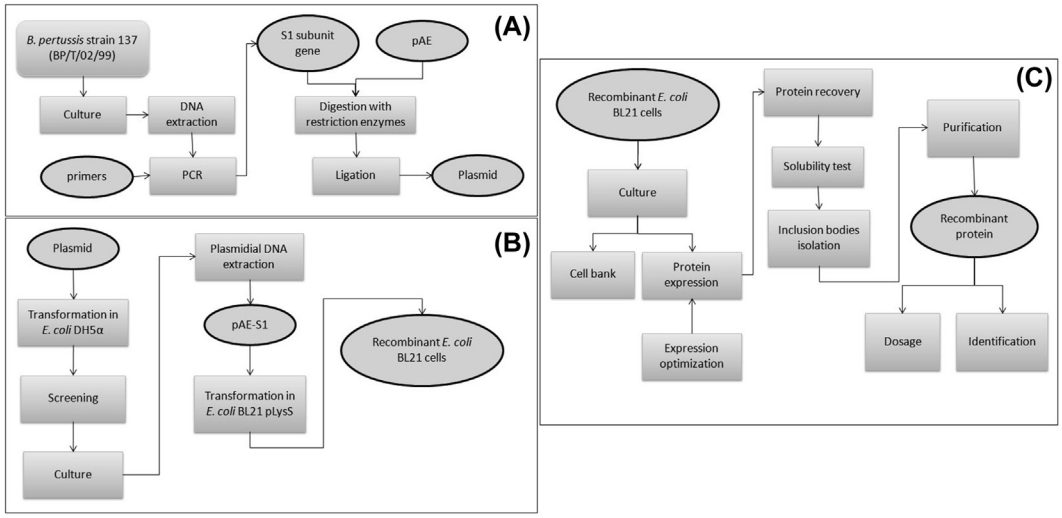

FIGURE 18.2 Example of flowchart of steps to obtain the recombinant plasmid (A), to obtain the recombinant cells (B), and to obtain recombinant S1 subunit of pertussis toxin (C).

cultivating recombinant cells, and the third is finally the production and purification of the recombinant protein (Fig. 18.2A–C).

An example is presented of a heterologous vaccine production using DTaP obtained from a recombinant antigen. It is produced by the cloning and expression of an immunogenic protein of *Bo. pertussis*. Fig. 18.2 presents the steps of the process for heterologous vaccine production.

18.2.1 Cultivation and Inactivation of *Bordetella pertussis*

Before the cloning step, the pathogenic microorganism has to be cultivated. For this, the freeze-dried content, of an ampoule of *Bo. pertussis* strain 137 (BP/T/01/99), is homogenized with sterile saline solution (NaCl 0.9%), inoculated in an Erlenmeyer flask containing 50 mL of Stainer–Scholte medium [45]. Then it is incubated in a shaker flask at 37°C at 150 rpm for 48 h. Gram staining is performed to confirm the identity and purity of the culture. Furthermore, the strain is transferred to plates containing solid medium charcoal agar base with niacin and incubated at 37°C for 5 days, and stored at 4°C.

Liquid cultures are distributed in 50-mL centrifuge tubes, to be inactivated in a water bath at 60°C for 30 min, and then centrifuged at 5000 × *g* for 20 min at 4°C for cell recovery; the pellets are stored at 4°C [46]. All of these procedures must be performed in a biosecurity area.

18.2.2 Extraction and Quantification of DNA of *Bordetella pertussis*

The pellet of one of the centrifuge tubes can be used for DNA extraction, which can be developed combining freeze–thaw and phenol–chloroform–isoamyl alcohol

techniques. To see whether the technique was successful and to quantify DNA, absorbance is read at 260 nm. Then an aliquot is applied to a 0.8% agarose gel and run at 80 V for 1 h. Next, it is colored with ethidium bromide to verify the quality of DNA.

In this example, the average concentration of the DNA samples determined by spectrophotometry at 260 nm is 3000 μg/mL. The concentration of DNA obtained is determined by the equation:

$$[\text{DNA}](\mu\text{g/mL}) = \text{Abs}_{260} \times 50 \times \text{FD}$$

where [DNA] is the DNA concentration (in μg/mL); Abs_{260} is the absorbance measured at 260 nm, and FD is the dilution factor of the sample.

18.2.3 Primers Design and Vector Choice

Some preliminary analyses are needed for proper amplification of the desired region of DNA. These analyses can be performed using the software Vector NTI (Invitrogen). This software is also useful for construction of the resulting plasmid in silico. The ptxA gene sequence encoding the S1 subunit of PT was obtained from GenBank (GeneID: 2,665,068, locus tag BP3783). Aiming the directional cloning of amplicons into the expression plasmid, restriction sites for the enzymes *Bam*HI and *Hind*III were introduced in forward and reverse primers, respectively. The region selected for amplification contains 684 bp and excludes the first 44 amino acids from the protein, eliminating the potential pathogenicity of the molecule. Including the nucleotides inserted by the primers, the fragment size expected after the polymerase chain reaction (PCR) is 698 base pairs (bp).

The vector used in that case report is the noncommercial vector pAE. The vector has several advantageous features, such as a replication origin in *E. coli*, multiple cloning sites, ampicillin resistance, being a strong promoter of phage T7, the possibility of isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, and the inclusion of a polyhistidine tail to the cloned protein.

18.2.4 Amplification of Gene of Interest by Polymerase Chain Reaction

PCR is carried out using commercial DNA polymerase following the manufacturer's protocol, using 1 μL of 10 ng/μL DNA template for each 50 μL of reaction mix. The conditions of PCR in this example are 95°C for 5 min (initial denaturation), 30 cycles of 95°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 1 min (extension), and 72°C for 5 min (final extension). The efficacy of amplification is determined by running samples on a 1.0% agarose gel at 80 V for 1 h.

The result of the gel is analyzed; if it shows only one band with the fragment of the desired size, it confirms the correct amplification of the gene. Furthermore, in negative control (a sample that passes through PCR procedure without primers) must be observed no bands (Fig. 18.3).

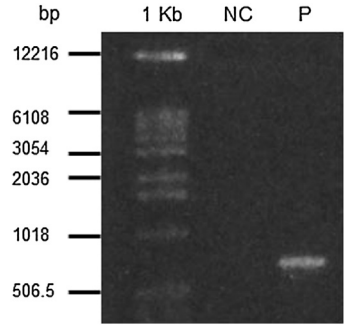

FIGURE 18.3 Agarose gel (0.8%) of polymerase chain reaction products. *1 Kb*, molecular weight marker; *NC*, negative control of PCR; *P*, amplified sample.

18.2.5 Preparation of Vector and Gene for Ligation

Both the gene obtained by PCR cloning (after purification) and the vector, in this case pAE, are subsequently digested with restriction enzymes suitable for this gene, according to the used primers. In this case, *Bam*HI and *Hind*III are used, and the digestion is carried out according to the manufacturer's protocol. Their concentrations are determined by spectrophotometry at 260 nm and the efficacy of digestion is determined in a 1.0% agarose gel.

The efficacy of digestion is proved if various bands can be observed at the circular vector sample and only one band with the expected size (2822 bp) can be observed on the digested vector.

Normally, on an uncut plasmid sample, four different bands can be seen, corresponding to variations in DNA folding, once not only the size but also the shape affects migration in electrophoresis. When plasmid is cut, it migrates as linear DNA, so the presence of only one band is expected [47].

On the other samples any difference is seen once digestion eliminates only a few nucleotides, and then there is no significant difference in molecule size.

After digestion, the ligation reaction with T4 DNA ligase is executed overnight at 4°C following the manufacturer's protocol.

18.2.6 Transformation of *Escherichia coli* DH5α for Plasmid Replication

The plasmids obtained are used to transform *E. coli* DH5α by heat shock according to the protocol for replication and verification of the success of ligation. In addition, a negative control is performed only with the vector.

The protocol for competent cells production is described. Ten isolated colonies of *E. coli* are grown in 20-mL Luria–Bertani (LB) medium at 37°C until $A_{600} = 0.5$ (about 3–4 h), and then centrifuged at 3000 × *g* at 4°C for 10 min in ice-cold sterile centrifugation tubes. The cell sediment is suspended in 20 mL ice-cold 0.1 M $MgCl_2$ solution and then centrifuged at 3000 × *g* at 4°C for 10 min. After that, the cell sediment is suspended

in 20 mL ice-cold 0.1 M $CaCl_2$ solution and incubated for 30 min at 4°C. After the incubation, cells are centrifuged at 3000 × *g* at 4°C for 10 min; the cell sediment is resuspended in 1 mL ice-cold 0.1 M $CaCl_2$ solution; 100 μL glycerol (sterilized by autoclaving) is added and 100 μL of the cell suspension is frozen at −80°C.

In the protocol of competent cell transformation, the suspension with the competent *E. coli* cells on ice (about 15 min) is thawed; 20 μL competent of *E. coli* cell solution is added to 5 ng (2 μL) plasmid DNA. The suspension is incubated for 30 min on ice and incubated for 90 s at 42°C and then for 2 min on ice. Then 200 μL LB medium is added and incubated at 37°C for 2 h while agitating.

The transformations are spread on Petri dishes containing LB medium supplemented with 100 mg/L ampicillin (once the vector has the gene of resistance to this antibiotic) and incubated overnight at 37°C.

In this case, the growth of six colonies are observed on the plate, which is seeded with a culture transformed with plasmid pAE-S1. These colonies are subjected to screening with phenol−chloroform to confirm the presence of the plasmid. After screening by electrophoresis (Fig. 18.4), four colonies (P1, P2, P3, and P4) have the same running pattern as the negative control samples. P5 has a plasmid without an insert compared with the pAE sample. P6 shows a different pattern, although the plasmid seems to be smaller than the pAE. Even so, this running difference can occur as a result of variations in plasmid folding, as described in Section 18.2.5. Thus P6 was previously chosen for plasmid extraction. Nevertheless plasmids from P4 and P5 were also extracted for comparison after double digestion, and thus confirm the presence of an insert.

The selected colonies are inoculated in 5 mL LB medium with ampicillin, incubated overnight on a rotary shaker at 180 rpm and 37°C, and used for the extraction of plasmids.

To confirm the presence of inserts in the extracted plasmids, a double digestion is performed with the same enzymes and verified on a 1.0% agarose gel.

Double digestion of the extracted confirms the expected results only if the digestion of the plasmid has a 698-bp band. In this case, it occurred only with the P6 colony. Thus

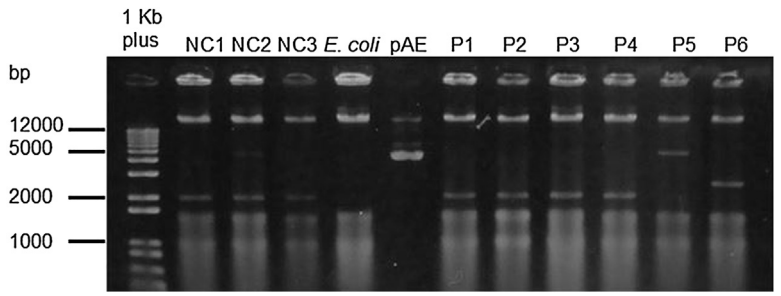

FIGURE 18.4 Screening of colonies after transformation. *1 Kb plus*, molecular weight marker; *E. coli*, DNA extracted from one *E. coli* DH5α colony; *NC 1, NC 2, NC 3*, DNA extracted from three colonies of negative control; *pAE*, uncut vector; *P1−6*, DNA extracted from the six colonies that had grown in plates with selective LB medium.

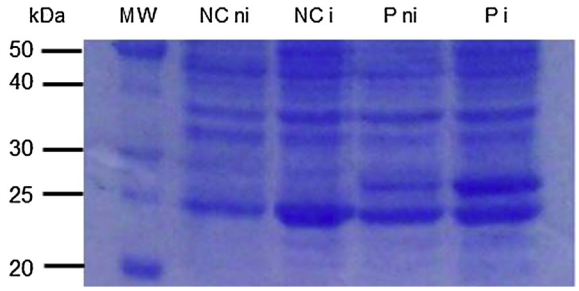

FIGURE 18.5 Verification of recombinant protein expression. *NC ni*, negative control before induction; *NC I*, negative control after induction; *P ni*, transformed cells before induction; *P i*, transformed cells after induction; *MW*, molecular weight marker.

this plasmid was chosen to transform *E coli* Bl21 pLysS cells and produce the recombinant protein.

18.2.7 Transformation of *Escherichia coli* BL21 pLysS and Induction of Recombinant Protein Expression

The selected plasmid is used to transforming *E. coli* Bl21 pLysS expression cells. Cells transformed by heat shock (as described previously) are inoculated in LB medium containing ampicillin and 25 mg/L chloramphenicol (to maintain the plasmid and pLysS) and incubated on rotary shaker at 180 rpm overnight at 37°C. Cells without transformation also incubated in nonselective LB broth are used as a negative control.

One milliliter of this preinoculum is inoculated in 10 mL LB medium with the same antibiotics and incubated on a rotary shaker at 37°C and 180 rpm until the culture reaches an absorbance [optical density (OD)] of 0.6 at 600 nm. Then, induction of expression is performed with 0.3 mM IPTG for 3 h. Confirmation of the recombinant protein expression is performed by electrophoresis sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel.

In SDS-PAGE a band can be observed with approximately 28 kDa overexpressed in the inducted sample (Fig. 18.5).

This confirms the expression of the recombinant protein and the efficiency of IPTG in the induction of protein expression, although a band can be also seen with less intensity corresponding to the recombinant protein in the noninducted ample. This shows that sometimes the system of expression control, regulated by the pLysS plasmid, is not efficiently completed.

18.2.8 Solubility Test to Verify the Presence of the Protein in the Supernatant or in Inclusion Bodies

One milliliter of the preinoculum is used to inoculate 9 mL of LB broth with antibiotics and is incubated overnight at 37°C and 150 rpm. Then the culture is used to inoculate

100 mL of the same medium, which is kept in a shaker under the same conditions until an OD of approximately 0.8 is reached. IPTG is added to a final concentration of 0.3 mM and induction occurs for 3 h. The final culture is centrifuged at 7000 × *g* and 4°C for 15 min; the pellet obtained is washed with PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4), centrifuged again under the same conditions, and resuspended in 10 mL resuspension buffer (20 mM NaH_2PO_4, pH 8.0). Four pulses of sonication of 20 s each at the same time interval are performed. Then centrifugation is performed at 15,000 × *g* and 4°C for 10 min to precipitate inclusion bodies. The supernatant is stored at 4°C and is called the soluble fraction. The pellet is resuspended in 10 mL solubilization buffer (20 mM NaH_2PO_4, 500 mM NaCl, 6M urea, pH 8.0) and a new cycle of sonication and centrifugation is carried out. The supernatant obtained is called the insoluble fraction.

The solubility test demonstrates whether the protein is mainly in the insoluble or soluble fraction. In this case, the recombinant protein is mainly in the insoluble fraction, meaning in the inclusion bodies, whereas a big part of other proteins is extracted in the soluble fraction.

This is expected for recombinant proteins expressed in *E. coli*; this result was also obtained by Burnette [32], whose results showed an expressed recombinant S1 subunit of PT at high phenotypical levels (7−22% of total cell protein content) in inclusion bodies. Choi and colleagues [48] demonstrated that overexpressed proteins are often produced in the form of inclusion bodies, and showed several advantages, such as much higher levels of protein accumulation (usually greater than 25% of total proteins) than soluble proteins. Also, inclusion bodies can be initially isolated in a highly purified and concentrated state by simple centrifugation, leading to a significant decrease in downstream processing requirements for removing contaminating host proteins; they also are resistant to proteolysis by *E. coli* proteases, allowing high-yield protein production [48].

Nevertheless this result can lead to some difficulties. The protein needs to be refolded to be soluble in a common saline buffer, which is not simple for some proteins.

18.2.9 Cell Growth and Protein Production Kinetics

Cell growth kinetics and its relation to the yield of the process must be observed. This is essential for process optimization and scale-up.

Thus during the fermentation process, 2-mL aliquots are collected every hour before verification of the influence of the induction time in cell growth and protein production. These fractions are centrifuged for 5 min at 11,000 × *g*. Pellets are resuspended in 200 μL solubilization buffer, pass through a cycle of four sonication pulses of 5 s with the same time interval, and are again centrifuged under the same conditions. The supernatant is then stored for further analysis in SDS-PAGE. To determine the growth rate (μ) at different phases of the culture, the following equation is used: $\mu = (\ln X_t - \ln X_0)/(t - t_0)$, where X_t and X_0 are the cell concentrations at times t and t_0, respectively. Cell

concentrations are determined by multiplying OD value by 0.35. Lima [49] described that for recombinant *E. coli* cultures, 1.0 U absorbance at 600 nm corresponds to a cell concentration of 0.35 g/L, on a dry basis.

In the kinetic analysis of cell growth of cells of *E. coli* BL21 pLysS, the lag phase was not observed for not-transformed cells. However, for cells transformed with the recombinant plasmid (P), a lag phase was observed until the third hour of incubation. After 5 h of cultivation, absorbance at 600 nm reached an average value of 0.7, and then induction with IPTG was performed. The exponential phase was observed from the third hour to half of the fifth hour of growth. After addition of the inducing agent, a decrease in the growth rate was observed, which was expected because overexpression and overproduction of heterologous protein impose metabolic burden stress and subsequently decreases in cell growth [50].

The graphic of the natural logarithm of the cell concentration as a function of time represents the variation in growth rate (μ). The calculated μ for each phase of the growth of transformed cells (P) is: 0.358 h^{-1} (lag phase), 0.626 h^{-1} (exponential phase), and 0.215 h^{-1} (after induction).

Cell concentration reached 0.42 g/L at the end of fermentation for both the negative control and transformed cells of the fermentation broth.

Analysis of protein expression in each fermentation time indicates that the maximum expression was achieved at the end of fermentation, 3 h after induction, and that there was little change in other proteins present in the sample during this time.

18.2.10 Optimization of Expression

The most important factors when dealing with the expression of recombinant proteins are the concentration of inducer (IPTG) and the time and temperature of induction. To study these factors, a fractional factorial $3^{(3-1)}$ experimental design can be used with three replicates at the center point. This type of design allows determination of the statistical significance of each factor studied. In this example, the levels studied of induction time (2, 3, and 4 h) were determined using the study previously described. The levels of other variables (temperature between 25°C and 37°C and IPTG between 0.1 and 0.9 mM) were set as are commonly used for recombinant protein expression in *E. coli* [51].

Using this kind of optimization methodology, in this example the best conditions achieved for the insoluble fraction of the culture were 37°C, 0.1 mM of IPTG, and 3 h of induction. In the soluble fraction the recombinant protein band was not observed for any condition. This study also showed that an increase of IPTG concentration has a negative effect on protein production, i.e., this variable must be used at a lower value to achieve maximal expression values. This is important when thinking about industrial processes, because IPTG is an expensive additive. If lower concentrations give a better response, costs are reduced.

Results obtained for the influence of IPTG concentration agree with those obtained by Cao and colleagues [52], who tested six different concentrations of this compound to

induce 1,3-propanediol oxidoreductase expression in *E. coli* and obtained the optimal value of 0.1 mM (the lower concentration tested).

18.2.11 Protein Purification

Purification is performed by affinity chromatography using a prepacked column HisTrap FF 5 mL from GE Healthcare. These columns are filled with nickel resin, which has affinity for polyhistidine tails present in the recombinant protein. Twenty-five milliliters of the clarified fraction is injected in the column after balance with binding buffer. Elution is performed using imidazole, a compound with greater affinity for nickel than polyhistidine, and thus which is able to disconnect histidine from the resin. All buffers contain urea to ensure that inclusion bodies are solubilized. The protocols were adapted from those recommended by the manufacturer (GE Healthcare).

After each chromatographic step, 150 μL of each fraction is aliquoted and stored at −80°C for subsequent dosage of total protein content [53] and protein profile electrophoresis analysis.

SDS-PAGE of the samples acquired at purification of the clarified fraction (obtained as described earlier) demonstrates that prior purification could be done but it still has some proteins that were eluted together with the recombinant protein (Fig. 18.6).

This may be because of the low concentration of imidazole (5.0 mM) in the binding and washing buffer. The column protocol recommends concentrations between 20 and 40 mM of this compound to decrease nonspecific binding. However previous tests (data not shown) indicated that higher concentrations led to the elution of the protein in binding and washing steps.

Other strategies can be tested to improve purification, including decreasing NaCl concentration in all buffers, facilitating the protein binding to the resin, and then increasing imidazole concentration to avoid nonspecific binding.

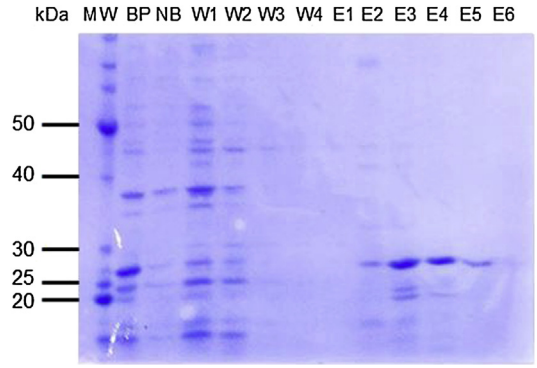

FIGURE 18.6 Samples of purification process in HisTrap FF column. *BP*, sample before injection in column; *E1, E2, E3, E4, E5, and E6*, eluted fractions; *NB*, not-bounded proteins; *W1, W2, W3, and W4*, fractions of column washing; *MW*, molecular weight marker.

Moreover, once proteins with similar molecular weight are excluded from the sample by affinity chromatography, two steps of ultrafiltration with membranes of 20 and 35 kDa, or gel filtration chromatography, can eliminate other proteins and concentrate the sample. It is also useful to change the buffer (to remove imidazole and urea and decrease the salt concentration) and promote protein refolding, so it can be used for biological tests. Finally, the protein is eluted in four fractions (E2, E3, E4, and E5); and their concentrations, determined by Bradford method are, respectively: 133.73; 328.902; 272.176, and 91.364 μg/mL. Therefore, the major fractions of the recombinant protein are E3 and E4. Analysis of the gel image by software gave the proportion of the band of recombinant protein in function of total bands of each sample as 54.28%, 71.53%, 75.21%, and 95.79%, respectively. Therefore, in these four fractions, it can be estimated that the total yield of protein obtained was 16.52 mg/L of the total culture; considering the purity, the yield is estimated to be 12 mg/L.

This result is more than three times higher than that obtained by Buasri and co-workers [43], who constructed *Bo. pertussis* mutant strains to produce genetically inactivated PT. They achieved protein expression of 3.77 ± 0.53 mg/L. Moreover, cultivation of *Bo. pertussis* requires a rich medium and thus is much more expensive than *E. coli* fermentation.

Another attempt to produce a fusion protein of subunits S1 and S3 of PT and FHA in *Streptococcus gordonii* achieved only 12 μg of protein per liter of culture [1]. Yet the expression of an S2 subunit of PT in *Ba. brevis* yielded 70 mg protein per liter of culture [35].

18.2.12 Protein Identification by DNA Sequencing

Both forward and reverse strains of the insert were sequenced in the ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Results obtained were compared with sequences of the BLAST (NCBI) database to confirm that the correct fragment had been amplified.

Comparison of the nucleotide sequence obtained from the cloned gene with the BLAST database resulted in 99 matches with 99% identity and an E-value of 0.0. The lower the E-value, or the closer it is to zero, the more "significant" the match is. These matches were mainly of *Bo. pertussis* PT S1 subunit (ptxA) gene, as expected. There were results from *Bordetella parapertussis* and *Bordetella bronchiseptica*, as well.

18.2.13 Identity Confirmation by Mass Spectrometry

A protein band obtained by SDS-PAGE is digested with trypsin (Promega) at 37°C for 24 h. The sample is hydrolyzed in a mixed-solution matrix (α-cyano-4-hydroxycinnamic acid II). The mass spectrum is obtained using the spectrophotometer matrix-assisted laser desorption and ionization—time-of-flight/time-of-flight Autoflex II (Bruker Daltonics, Germany). Some signals present in the mass spectrum MS were fragmented in mode mass spectrometry/mass spectrometry. Peaks are analyzed with the computer program FlexAnalysis 3.0 (Bruker Daltonics).

In this case, this analysis returned some hits according to PT S1 subunit (*Bo. pertussis*) with a score of higher than 37, which indicates identity or extensive homology (data not shown).

18.3 Conclusions

In the 21st century pertussis, also known as whooping cough, remains a disease that kills children and adults worldwide. Although its incidence has decreased since the first vaccination in the 1940s, it has not yet been eradicated.

For 30 years, many groups around the world have studied new technologies for pertussis vaccination that could be used not only for children but also for teenagers and adults. Even so, there is no cheap and widely distributed new vaccine for all countries, developed or not.

This chapter intended to show that there many new technologies can be studied with the aim of producing this important vaccine. A case report showed that it is possible to amplify a specific DNA fragment from *Bo. pertussis*, clone it into other kind of bacteria, and produce a heterologous protein able to immunize against whooping cough with high specificity and efficiency. This report also explained each steps required to develop such a subunit vaccine.

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19

Cloning and Expression of a Heterologous Protein With Imunological Potential Against *Corynebacterium diphtheriae*

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19.1 Introduction

Diphtheria mostly developed as an infection of the upper respiratory tract, causing fever, sore throat, and malaise. A thick, gray fibrin membrane, called a pseudomembrane, often forms over the site of infection as a result of bacterial growth, toxin production, necrosis of underlying tissue, and the host immune response [1]. It is a disease caused by *Corynebacterium diphtheriae* and can be a serious problem and even fatal, especially in children, owing to airway obstruction and tissue damage. The toxin that is produced by the pathogen can spread through the bloodstream and cause muscle paralysis, heart and kidney failure, and even death. About 5–10% of people who develop diphtheria can die; most experience permanent damage [2]. In 2000, 30,000 cases were observed and 3000 deaths were reported to be caused by diphtheria worldwide [3]. Even with vaccination campaigns, the disease is still a public health concern [1,3,4].

C. diphtheriae produces a toxin that can harm and destroy human tissues and organs. It often develops in the throat and sometimes the tonsils, but in the tropics it also causes ulcers on the skin. It can affect people of all ages, but it is most common in non-immunized children [1].

Because children are highly susceptible, artificial immunization at an early age is universally used. Toxoid is given in two or three doses (1 month apart) for primary immunization at age 3–4 months. A booster injection should be given about a year later,

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and it is advisable to administer several booster injections during childhood. Infants are usually immunized with a trivalent vaccine containing the diphtheria toxoid, pertussis vaccine, and tetanus toxoid (DPT or DTaP vaccine) worldwide [3].

As a preventable disease, it should be controlled as soon as the population is vaccinated. However, actual vaccines do not provide long-lasting immunity. The titer against diphtheria often disappears after some years and boosters are generally not provided to the population [3].

There have being some efforts in the development of a new class of vaccines, subunit vaccines capable of long-lasting immunity and with reduced side effects. The diphtheria vaccine has not yet achieved at this point, but it should be interesting for disease control, because the booster should be taken at any age, without restrictions [3].

19.2 Historical Aspects

Throughout history, diphtheria was a leading cause of death among children. Diphtheria and scarlet fever were sometimes difficult to distinguish. The illnesses were known by a variety of names, such as throat disease, throat distemper, throat ail, malignant croup, angina suffocative, gangrenous sore throat, canker ail, malignant quinsies, purid, malignant or pestilential sore throat; cynanche trachealis, malignant angina, "strangling angel of children," and "*Garotillos*," among others [5].

In the fourth century BC, Hippocrates described the symptoms of asphyxia and clinical evolution of what could be diphtheria. In Spain in 1613, an epidemic of diphtheria caused the year to be named "*El Año de los Garotillos*" ("Year of Strangulations") because of the many deaths caused by diphtheria suffocation [6].

In 1735 there was an epidemic in New England. In 1826, Pierre Philippe Bretoneau described the disease as a tough membrane that is formed in the throat, for the first time giving diphtheria disease its name. In England it was known as Boulogne sore throat, because it had spread in France in 1746 [6].

In 1883, Klebs demonstrated that *C. diphtheriae* was the agent of diphtheria. Loeffler, in 1884, had found that the organism could be cultured only from the nasopharyngeal cavity, and postulated that the damage to internal organs resulted from a soluble toxin [1].

Roux and Yersin discovered the diphtheria toxin in 1888, and showed that animals injected with sterile filtrates of *C. diphtheriae* developed organ pathology indistinguishable from that of human diphtheria; this demonstrated that a potent exotoxin was the major virulence factor [1,7].

In the 1890s the antitoxin against diphtheria was developed by Shibasaburo Kitasato and Emil von Behring. The first vaccine was developed in the 1920s. In 1923, Gaston Ramon, a veterinarian of the Pasteur Institute in France, developed the diphtheria toxoid [1].

With the development and administration of the diphtheria vaccine, the incidence of diphtheria decreased significantly, but it still is a public health problem [3].

19.3 The Pathogen and the Toxin

C. diphtheriae is gram-positive, nonsporulating, unencapsulated, nonmotile *Bacillus*. Corynebacteria are classified as Actinobacteria and are related phylogenetically to Mycobacteria and Actinomycetes. They belong to the kingdom Bacteria, phylum: Actinobacteria, order: Actinomycetales, suborder: Corynebacterineae, family: Corynebacteriaceae, genus: *Corynebacterium*, species: *C. diphtheriae*. They undergo snapping movements just after cell division, which brings them into characteristic forms resembling Chinese letters or palisades [8].

Not all the strains of *C. diphtheriae* are pathogenic. The pathogenicity comes from the toxin that is produced only if the *Bacillus* itself is infected (lysogenized) with a bacteriophage, which carries the genetic information. This is a high conserved polypeptide encoded by the bacterial chromosome, the tox-gene [9–11]. The structural gene of the diphtheria toxin has 535 residues, a 58-kDa exo toxin, and two subunits linked by a disulfide bond [1,10,12].

Outside the host cell, the toxin is inactive. The nontoxic fragment B (Mr 37,195) of the toxin attaches to the heparin-binding epidermal growth factor (HB-EGF), a member of the superfamily of growth factors. Just after the attachment in the cell surface, the highly toxic fragment A is detached from the N terminus of the polypeptide chain by cleavage with trypsin [13]. At this point, the fragment A (Mr 21,167), is able to catalyze the adenosine diphosphate (ADP)-ribosylation of elongation factor 2 in protein translation in practically all eukaryotic cells [9,14,15].

The toxin can produce local tissue necrosis, and when absorbed into the bloodstream, it causes toxemia and systemic complications including paralysis owing to demyelinating peripheral neuritis and cardiac failure due to myocarditis [10,16].

It has four biotypes according to colony morphology (mitis, intermedius, gravis and belfanti), as well as into lysotypes based on corynebacteriophage sensitivity [1]. Most infections have been caused by gravis or mitis biotypes, but the clinical and public health management is identical for all toxigenic strains [10].

The diphtheria toxin (DT) is a molecule synthesized as a single chain that can be cleaved by trypsin into two fragments, A and B. The A fragment is located at the N terminus of the polypeptide chain, and catalyzes the ADP-ribosylation of elongation factor 2 in practically all eukaryotic cells. This mechanism blocks the protein transcription, making it impossible for the cells to continue normal metabolism, and resulting in cell death [17].

19.4 The Disease

There are two types of clinical manifestation of diphtheria: nasopharyngeal and cutaneous. The symptoms of pharyngeal diphtheria may appear as mild pharyngitis, but can evolve to hypoxia because of airway obstruction by the pseudomembrane. The cutaneous type is characterized by skin lesions covered by a gray-brown pseudomembrane as well.

The lesions start as vesicles and quickly form small, clearly demarcated, and sometimes multiple ulcers [1,10,18]. Usually they appear on exposed parts, especially the legs.

Life-threatening complications, principally loss of motor function (e.g., difficulty in swallowing) and congestive heart failure, may develop as a result of the action of DT on peripheral motor neurons and the myocardium [1].

Classical nasopharyngeal diphtheria is characterized besides the membranous pharyngitis with fever, enlargement of the cervical lymph nodes, and edema, which results in a "bull neck" appearance. Although not always present, the pseudomembranes are typically gray, thick, fibrous, and firmly adherent. Dislocation of the membrane releases a great amount of toxin. There is gradually increasing hoarseness and stridor when it reaches the pharynges. It can cause nasal discharge of bloody secretions [1,10,16].

Diphtheria is no longer easily diagnosed on clinical grounds. Mild cases of the disease resemble streptococcal pharyngitis and the classical pseudomembrane may not develop, particularly in people who have been vaccinated. Because the disease is rare, principally in developing countries and regions with better hygienic and sanitary conditions, and many clinicians may never encounter a case, the clinical diagnosis is missed [1,19]. Not all laboratories routinely culture throat swabs for *C. diphtheriae*, which further increases the potential for missed or delayed diagnosis [10,19].

19.5 Epidemiology

Diphtheria is found worldwide, although it is not common in industrialized countries because of longstanding routine use of diphtheria–tetanus–pertussis (DTP) vaccine. However, large epidemics occurred in several Eastern European countries in the 1990s, and many cases have been reported in developed countries [20,21]. As an example, outbreaks emerged after the breakup of the former Soviet Union in the late 1980s. Therefore, in the constituent countries, vaccination rates fell so low that there was a surge in diphtheria cases. In 1991 there were 2000 cases of diphtheria in the USSR. By 1998, according to Red Cross estimates, there were as many as 200,000 cases in the Commonwealth of Independent States, with 5000 deaths [8].

The case-fatality rate for diphtheria has changed little over the past 50 years. The overall case-fatality rate for diphtheria is 5–10%, with higher death rates (up to 20%) among people younger than age 5 and older than age 40 years. Before that, the disease was fatal in half of cases [8].

Worldwide there are still cases of diphtheria, with 4887 cases reported by the World Health Organization (WHO) in 2011. In the same year, in south France, one case was registered, in a 40-year-old man [22]. Whereas diphtheria primarily affected younger children in the prevaccination era, an increasing proportion of cases today occur in unvaccinated or inadequately immunized adolescents and adults [20].

Much research involves diphtheria cases. They describe insufficient vaccination coverage; discontinuation of the vaccination schedule; insufficient boosters after

primary doses in childhood, and so on, trying to discover why diphtheria is still circulating. Because diphtheria is present only among humans, eradication of the disease is possible [17].

According to Myers, even with a booster, the protective rate for diphtheria was 94%, and people above age 19 years were more responsive to the vaccine [23].

Research developed 25–30 years ago [24] involved 403 vaccinated people screened for diphtheria antitoxin titers. The result was that 19% of the group was unprotected. The research reaffirms the continuous decline of diphtheria antitoxin titer after vaccination, even in people who were revaccinated at age 12. As a conclusion, the authors suggested that people who had their primary vaccination before age 2 years or revaccination more than 10 years ago should be revaccinated [24].

Adults were revaccinated and protective serum diphtheria antitoxin levels were attained by all patients receiving diphtheria toxoid [25]. Mild local reactions were reported by 22% of the patients, and the authors concluded that the revaccination is advisable.

Kadirova et al. [26] believed that resurgent cases are reported as a result of the lack of vaccination with the dissolving of the Soviet Union. In the same way, Galazka et al. [27] suggested high immunization coverage of target groups, prompt diagnosis, management of diphtheria cases, and rapid identification of close contacts with effective management to prevent secondary cases. These actions should be taken to prevent the incidence of new diphtheria cases [26,27].

Hardy [28] suggested that current vaccination recommendations also need to be reviewed to ensure that population immunity are adequate to prevent a resurgence of diphtheria in Europe and North America [28].

A vaccination campaign with DT vaccine targeting the adult population was analyzed by Christenson et al. [29] and a major improvement in immunity to diphtheria with only minor side effects was observed.

Nandi et al. [30] described the number of cases in India; 70% of affected patients were not previously immunized against diphtheria. The mortality rate was about 16% in early diagnoses and treatment with the antidiphtheritic serum and antibiotics were very important for the cure. It was also stated that inadequate immunization was responsible for continued outbreaks and deaths caused by diphtheria.

Research on the loss of immunity 10 years after vaccination revealed that antidiphtheria titers are usually below the protective amount [23,31,32]. Authors also showed that vaccination coverage is still at about 83%; according to WHO and UNICEF, there is a clear gap in the immunization campaign. Although the vaccine is effective in preventing diphtheria-related death, its overall effectiveness against symptomatic disease is only estimated to be 70–90%. Diphtheria outbreaks were reported among highly vaccinated populations in closed communities [32–37].

Karakus et al. [38] evaluated antibodies against the intact DT and DT subunits A and B in 1319 individuals. Those with a protective antibody level were reactive to subunit B; the authors suggested its use as a detection agent for the diphtheria titer.

Unfortunately, the situation is not different on the American continent: Many regions have outbreaks revealing that the vaccination coverage and immunity given are not efficient. There are endemic regions that often have outbreaks despite the vaccination program. Haiti, the Dominican Republic, and Brazil are countries that have noticed a great number of cases in the past 10 years. There are still sporadic cases in several countries, excluding those that have not reported to the authorities, and misdiagnoses [39].

19.6 Diphtheria Vaccines

Diphtheria vaccines are based on diphtheria toxoid, an inactivated bacterial toxin that induces protective antitoxin. Diphtheria toxoid combined with tetanus and pertussis vaccines (DTwP) has been part of the WHO Expanded Program on Immunization since its inception in 1974. In 1980–2000, the total number of reported diphtheria cases was reduced to less than 90% [16,18]. After the introduction of routine immunization with vaccines containing diphtheria toxoid in the 1940s and 1950s, the incidence of diphtheria declined dramatically [27].

Actual vaccines containing the diphtheria component are still the crude protein toxoid. The 58-kDa toxin is the antigen and is converted to a toxoid with formaldehyde and is crudely purified. The cells are separated by centrifugation or membrane filtration, and the supernatant is treated with formaldehyde at 0.75% and stored for 4–6 weeks at 37°C to allow complete detoxification [7]. Then, the components are extracted from the native's bacteria; the toxins from tetanus and diphtheria and the cell lysate for pertussis are the components of the DTwP vaccine [40].

There are four combination vaccines used to prevent diphtheria, tetanus, and pertussis. The first, DTP vaccine, has components that include diphtheria (D), tetanus (T), and pertussis whole inactivated cells (wP) [41].

The second combination, DTaP, was developed because of the high incidence of collateral effects. Efforts in the sense of producing a safer vaccine with fewer unpleasant reactions were done. In this way, antigens were selected to induce immunity with fewer antigens than the whole cell vaccine. This model of vaccine would be safer but more expensive. The rate of redness, fever, and pain is about 90% lower. This vaccine, which was developed in Japan in 1981, is indicated for children aged 6 years and younger to protect them from these three diseases. Older children, adolescents, and adults should not take this vaccine [41].

The third combination, the Tdap, is indicated for adolescents and adults. It was licensed in the United States in 2005. The difference from the previous vaccine is the reduced diphtheria and pertussis toxoids concentration to prevent side effects. The pertussis component is acellular like the DTaP, but with a lower concentration of antigen. Existing vaccines with this technology are Adacel (Sanofi) and Boostrix (GlaxoSmithKline) [41,42].

The fourth combination is the DT vaccine with diphtheria and tetanus components, just like the DTP vaccine, but without the pertussis component The Td vaccine has a lower antigen concentration and is indicated for adolescents and adults [41].

19.6.1 Subunit Vaccines

Since the 1920s, the diphtheria vaccine has been produced the same way: as a batch cultivation of the pathogen. The toxin is secreted from the cell, and only the supernatant is used to produce the vaccine. In this supernatant there is not only the necessary antigen for immunization but a large number of other components (from the media, cell residues, and cell metabolism products) are also present in this protein pool, which can contribute against the vaccine's side effects.

Producers conduct different downstream processes, with a low purity (around 70−80%) antigen end product [13]. A huge amount of research is being conducted in the sense of developing new technologies capable of improving the diphtheria vaccine with long-lasting immunity. One is the subunit vaccines [43].

The world already uses subunit vaccines, with safer results and rare side effects. The hepatitis B and the pertussis vaccine (aP) are already used and approved by the WHO, with success. The pertussis acellular vaccine contains inactivated pertussis toxin (protein) and may contain one or more other bacterial components. The pertussis toxin is detoxified either by treatment with a chemical or by using molecular genetic techniques, transforming the molecule of the toxin into a nontoxic epitope [44].

There are three forms of subunit vaccines: protein, polysaccharide, or conjugated protein-polysaccharide, using these molecules as antigen. The technology is based on the presence of an antigen to the immune system without viral or bacterial particles, using a specific, isolated protein of the pathogen. It can contain one or several antigens from the pathogen. A weakness of this technique is that isolated proteins, if denatured, may bind to antibodies different from the protein of the pathogen [45].

There is a subunit vaccine against diphtheria, the Td and the Tdpa, with the diphtheria toxin purified for chromatographic method and with lower antigen concentration. However, the cost of the downstream process is still an impediment to producing and delivering the vaccine throughout developing countries [5,41].

These kinds of vaccines also can provide a stronger immune response, with fewer side effects. This epitope for subunit vaccines can be obtained by purification processes, which can considerably increase the vaccine price. They can also be produced with the use of recombinant techniques. These techniques are leading the search for the ideal vaccine, because they are safer and cheaper, heat-stable, and easy to administer, preferably single-dose, and capable of inducing a broad immune response with lifelong memory in both adults and infants. The perspective is that the same may be developed for diphtheria as an improvement in immunization and the prevention of undesirable side effects [45].

As stated, subunit B is capable of inducing immunity against DT and much research in ongoing in this sense [38,46–49].

Table 19.1 lists some reports on the use of subunit B as a potential vaccine, with good results in producing antibody response and the ability to neutralize the whole DT when challenged.

Table 19.1 Research Developed Containing Diphtheria Toxin or Its Subunits as the Main Objective or a Collaborative Molecule

Cell	Objective	Author	Year
Escherichia coli	Synthesis of diphtheria tox-gene products	Murphy J. et al. [50]	1974
E. coli K12	Cloned diphtheria toxin fragment A	Leong D. et al. [51]	1983
	T cell–specific antibody identifies regions of B-chain active in cell entry	Colombatti M. et al. [52]	1986
E. coli	Construction and expression of diphtheria toxin-encoding gene derivatives in *E. coli*	Zdanovsky et al. [17]	1992
	Corynebacterium diphtheriae dtx R homologue from *Streptomyces lividans* and *Streptomyces pilosus*	Gtünter-Seeboth et al. [53]	1995
E. coli	Subunit B conjugated to meningococcal as vaccine to diphtheria toxin and meningococcal proteins	Johnson N. et al. [54]	1997
E. coli	Two recombinant fragments of diphtheria toxin	Lobeck K et al. [55]	1998
	Diphtheria toxin CRM	Randall K. H. [57]	2000
Mycobacterium bovis BCG	Diphtheria toxin CRM197 mutant	Miyaji E. N. et al. [58]	2001
Pichia pastoris	Targeted introduction of diphtheria toxin resistant mutation into chromosomal EF-2 locus of *P. pastoris* and expression of immunotoxin in EF-2 mutants	Liu Y. Y. et al. [59]	2003
E. coli	Recombinant diphtheria fusion toxin DT388IL3	Urieto J. O. et al. [60]	2004
Streptococcus gordonii	Recombinant diphtheria toxin fragment A	Lee C. W. et al. [56]	2004
E. coli BLR(DE3) pLysS	Murine C3d sequences to diphtheria toxin fragment B	Gor D. O. et al. [61]	2006
E. coli	Expression and purification of trivalent pertussis–diphtheria–tetanus toxin fusion protein	Aminian M. et al. [62]	2007
	Recombinant subunits A and B and new method of their estimation	Kaberniuk A. et al. [63]	2009
E. coli	Diphtheria toxin CRM197 mutant	Stefan A. et al. [64]	2010
	Biochemical and biological characteristics of cross-reacting material 197	Bröker M. et al. [46]	2011
Insect cells	Recombinant protein vaccines produced in insect cells	Cox M. M. J. et al. [47]	2012
	Subunit B using as drug delivery across blood–brain barrier	Chen Y. et al. [48]	2012
E. coli	A and B fragments of *C. diphtheriae* toxin toward recombinant diphtheria vaccine	Abulmagd S. et al. [49]	2013

19.7 Technological Development of a Diphtheria Vaccine: Case Description

Improvement in technology is necessary to reduce side effects and the vaccination of the world population with a better-quality product, as well as vaccination program uptake effort has been made to develop a new, safer product for immunization.

This case description presents a new diphtheria vaccine using recombinant technology. The steps of the development are shown in Fig. 19.1.

19.7.1 Diphtheria Culture and DNA Extraction

The strain of *C. diphtheriae* was received courtesy of the Technology Institute of Paraná-TECPAR (Park-Williams 8). A freeze-dried sample of the strain, which was reported as a producer strain of the diphtheria vaccine [65] was employed. The cultivation was carried out in a modified culture medium for *C. diphtheriae* [66] with 100 mg/mL casein

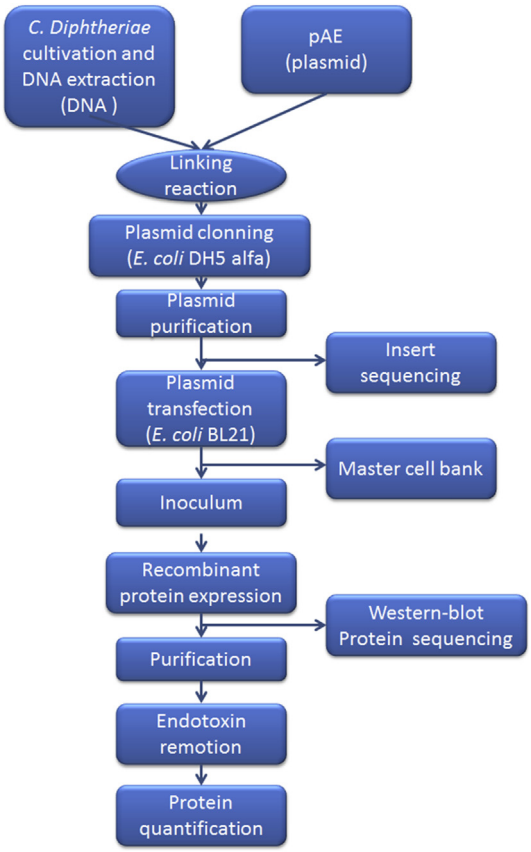

FIGURE 19.1 Flowchart of subunit vaccine development.

hydrolysate and incubated at 37°C (±1°C) for 72 hours [67]. The toxin was inactivated in a water bath at 100°C for 30 minutes.

The DNA extraction was performed by employing the defined protocol for gram-positive bacteria DNA extraction; 60% purity was obtained with a concentration of DNA obtained at 270 mg/mL.

For DNA amplification by polymerase chain reaction (PCR), two primers were designed, corresponding to DT subunit B [193—534 base pairs (bp), the immunologic portion of the molecule], with the assistance of the software Vector NTI (Invitrogen). The sense primer was designed with *Bam*HI, and the antisense with *Hin*dIII to enable the insertion in the right direction in the vector.

The sequence of the gene that contains 1026 bp totalized 1046 bp with the nucleotide containing the restriction sites for the enzymes mentioned. The initial 579 bp of the toxin sequence was excluded from the toxin activity portion [68].

Gene amplification was prepared with GoTaq Flexi DNA Polymerase (Promega), following the manufacturer's protocol. The PCR product was purified using the GFX PCR Kit DNA and Gel Band Purification (GE Healthcare), following the manufacturer's protocol. The PCR purification results were analyzed in 1.0% agarose gel electrophoresis, 80 V, for 1 hour to check their quality and concentration. Only one band appeared in the gel, between molecular weight markers 1636 and 1018, indicating the interest sequence (1046 bp).

19.7.2 Plasmid and Diphtheria Toxin Subunit B DNA Ligation

The pAE vector was chosen because it has several advantages: it is not commercial, it was replicated in *E. coli*, it has multiple cloning sites, it has an ampicillin-resistant gene, it is a strong promoter of T7 phage, and it involves isopropyl β-D-1-thiogalactopyranoside (IPTG) induction and the inclusion of a polyhistidine tail in the cloned protein. The binding reaction between the insert (DT subunit B) and plasmid pAE was performed using a T4 DNA ligase (Promega) overnight at 4°C following the manufacturer's protocol.

19.7.3 Plasmid Linking to Diphtheria Toxin Subunit B Insert Cloning

The plasmid containing the subunit B insert was transfected into *E. coli* DH5alfa with the thermic chock protocols (transformation of *E. coli*), to plasmid replication.

19.7.4 Insert Sequencing

Both DNA strains (forward and reverse) of the insert (DNA sequence of DT subunit B) were sequenced in capillary electrophoresis (ABI PRISM3100 Genetic Analyzer; Applied Biosystems). The results were compared with BLAST sequencing database (NCBI) and resulted in 99 hits with 99% identity and an E-value of 0.0 for "*C. diphtheriae* strain DIFT020 DT (tox) pseudogene, partial sequence" deposit. V01536.1. The result matched with sequence B of DT.

19.7.5 Protein Expression

The extracted plasmid was transfected into *E. coli* BL21 pLysS, using the same transfection protocol, to express the recombinant protein. To confirm the presence of plasmid with the insert, a screening was made and analyzed by electrophoresis (0.8% agarose gel). A cell blank was produced and stored at −80°C until use.

Cells cultivation was then scaled up to 100 mL (on a rotary shaker at 37°C ± 1°C at 180 rpm) and carried out until the culture reached an optical density of 0.6−0.8 at 600 nm. At this point, 0.3 mM IPTG was added to the culture and incubated under the same conditions for 3 hours. Protein expression was verified by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).

An overexpression band of about 40 kDa was observed after induction. The band indicated DT subunit B, because its weight was about 37 kDa. A recombinant protein band with lower intensity was detected in the noninduced fraction, which suggesting that the protein had been expressed before induction.

Protein solubility was verified by sonicating the pellet with phosphate buffer (20 mM Na_2HPO_4, pH 8.0) and dissolution in buffer (20 mM Na_2HPO_4, 500 mM NaCl, 6 M urea, pH 8.0) for further steps. Recombinant proteins can be secreted in supernatant or stored in inclusion corpuscles. Both fractions were verified by 10% SDS-PAGE. An advantage of protein expression in inclusion bodies is low toxicity to cells; these corpuscles are easily precipitated by centrifugation because they are substantially free of contaminant aggregates [69−71].

Choin et al. [72] developed a high-yield recombinant protein production system. The overexpressed proteins produced in inclusion bodies have several advantages. It is possible to accumulate high levels of recombinant protein (greater than 25% of total protein) compared with soluble proteins. The inclusion bodies may initially be isolated in a highly purified state and concentrate by simple centrifugation, decreasing downstream steps for removing contaminating proteins of the host cell. For these reasons, the insoluble fraction was chosen to carry out the following purification steps.

To verify the best cultivation conditions and the effects of inducer concentration (IPTG), temperature, and time on protein expression, an experimental design of fractional factorial 3 (3-1) with three repetitions at the central point was carried out. The best culture conditions were observed at 31°C, 0.5 mM IPTG, at 3 hours.

19.7.6 Protein Identity: Western Blot and Protein Sequencing

The protein band obtained by SDS-PAGE was analyzed by the method adapted by Shevchenko et al. [73]. The mass spectrum (MS) was obtained using the matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight-II Autoflex (Bruker Daltonics, Germany) spectrometer. Peaks were analyzed by Flex Analysis 3.0 software (Bruker Daltonics). The MS/MS spectra were combined and subjected to searches using Mascot

software. This analysis identified the insert sequence as "chain A, with refined structure of DT at 2.3 Å resolution," database NCBInr (NCBI Access No. gi576189). The protein expressed is positive to the sequence of DT subunit B.

The protein fractions (soluble and insoluble) were also analyzed by western blot whit anti-his6 antibodies that were positive, indicating the success of polyhistidine tail inclusion in the recombinant protein. Protein expression was verified in this step; it showed only a band without nonspecific reactions. For the presence of the polyhistidine tail, it is important to perform the purification step through nickel column affinity chromatography, the chosen purification mechanism.

19.7.7 Cell Growing and Protein Production Kinetics

During protein expression, 2-mL aliquots were collected every hour to evaluate the influence of induction time in cell growth. The cell concentration was determined by a spectrophotometer at 600 nm. Two kinetic parameters were used to evaluate bacterial cell growth: specific growth rate (μ) and growth velocity (dx/dt). Cell concentrations were determined by multiplying the absorbance values by 0.35. According to Lima [74], in *E. coli* recombinant cultures, 1.0 absorbance unit at 600 nm is equivalent to a cell concentration of 0.35 g/L dry weight.

After 5 h of cultivation, induction was performed. The addition of IPTG to the culture caused the metabolism to deviate to overexpression of the recombinant protein, generating cellular stress and reducing cell growth [72]. The culture was stopped and the cell concentration at the end of the cultivation time was approximately 0.360 g/L.

The highest expression of recombinant protein was achieved within 3 h after induction with IPTG, and there was no difference in the expression of other proteins present in the culture.

19.7.8 Purification

To perform chromatography, the cell pellet from 2 L cultivation was sonicated (four cycles of 20 s with the same time in an ice bath) with buffers. The obtained supernatant was analyzed with SDS-PAGE. The total protein content [75] and protein profile were determined by SDS-PAGE. The protein concentration of the samples obtained after the sonication cycles biomass was 1.300 g/mL and 1.100 mg/mL, respectively.

A prepacked HisTrap column FF 5 mL (GE Healthcare) with polyhistidine tail affinity was used. Purification was performed on an ÄKTA Purifier 100 automated system (GE Healthcare). Protocols were adapted according to the manufacturer's recommendations, with a flow rate of 5 mL/min. To avoid the presence of contaminants, elution gradient was performed with 5 column volumes of binding buffer (20 mM Na_2HPO_4, 500 mM NaCl, 6 M urea, 5 mM imidazole, dithiothreitol or DTT 2 mM, pH 7.4) and elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, 6 M uremia, 500 mM imidazol, DTT 2 mM, pH 8.0).

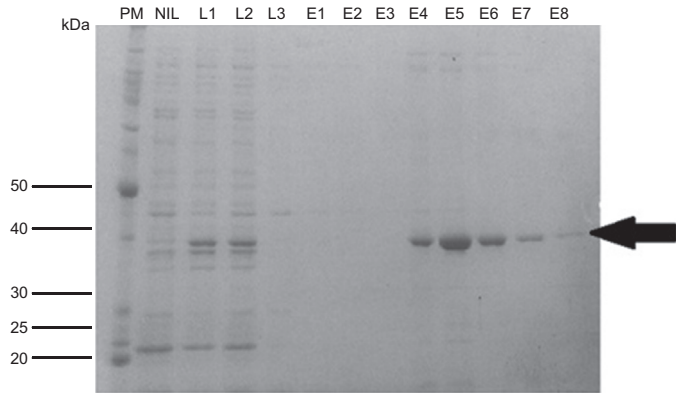

FIGURE 19.2 Purification process in HIS trap FF electrophoresis gel. *E1–8*, elution of recombinant protein; *L1–3*, wash; *NL*, unbound; *PM*, molecular weight.

Purification by liquid chromatography of the recombinant protein in a nickel affinity column is shown in Fig. 19.2. Electrophoresis on SDS-PAGE gel shows fractions obtained in the purification step.

The fractions collected from purification in elution showed that protein concentrations (g/mL) varied from 80.33 to 595.58 mg/mL. A pool of the fractions was made and called "purified protein" with a final concentration of 418 μg/mL. Endotoxins were removed using the resin X-114, 1% v/v. The final protein concentration was then 348 mg/L. Protein loss in the lipopolysaccharide removal process was about 9%. The sample, which was free of pyrogens, had a recovery of 33% and could be used for subsequent inoculation in animals for verification of immunogenicity. Protein production and recovery are in accordance with others' reports [76].

19.8 Conclusions and Perspectives

Much research has been conducted to develop new technologies capable of improving not only a vaccine against diphtheria but also other vaccines, with the aim of reducing adverse effects and ensuring long-lasting immunity. One new technology is the subunit vaccine [43].

Diphtheria has a subunit vaccine, the Td and Tdpa, using chromatographic methods for purification and lower concentrations of antigen. However the cost of the downstream process is still an obstacle for the production and distribution of this type of vaccine in developing countries [5,41]. These kinds of vaccines are also capable of granting a stronger immune response, with fewer incidents of side effects. Nevertheless epitopes obtained by purification processes can significantly increase the price of the vaccine [5].

A cheaper alternative is to develop recombinant vaccines. These techniques are leading the development of an ideal vaccine that is safer and cheaper, promotes higher thermal stability, in a single dose, and is capable of inducing an immune response with lifetime memory, which can be used in adults and children [38,46–49].

For diphtheria, research developed in this direction is a promising alternative for a safer and more effective vaccine [38,46–49].

Nascimento et al. demonstrated the DT subunit B in *E. coli* to obtain high levels of protein. However, at the end of the process, low quantities of the protein were recovered. In this case, the expressed protein was in an insoluble form, probably in inclusion corpuscles. The refolding process is also a concern when working with inclusion corpuscles, mainly when the right conformation is needed for its activity, such as in vaccines for conferring the right immunity [69,70,76].

The DT is able to bind to cell surface in HB-EGF receptor. The receptor is able to perform the internalization of molecules attached into it in human cells. This mechanism is being used as a vehicle for drugs, vaccines, antitumor medicine, and molecules of interest.

The diphtheria toxoid can be used as an immunogenic molecule, as an adjuvant, or attached to other drugs. Because of such properties there are possibilities of developing vaccines with recombinant DT or its subunits. These studies indicate promising immunization against the disease [46–52,54–57,61–63,77].

Cytotoxins blocking HB-EGF, diagnostic system tests, and serum therapy with recombinant DT DNA sequence are also being developed [59,77]. Research into therapeutic drugs, such as bacterial flagellin administered in combination with DT, or thymocytes that co-stimulate interleukin-17 producers, are successfully tested against microbes [78].

There are still further advances in the use of recombinant derivatives for the treatment and prophylaxis of oncology. One is the fusion of genetically modified toxins selected for acute myeloid leukemia blasts, using the first 388 amino acid residues of DT fused to human interleukin-3, which has achieved good results in the treatment of cancer [60].

The design of autoimmune drugs, controlling the immune system reaction to infectious diseases and other diseases, is also a promising target for a new approach to DT as an adjuvant agent for developing new drugs [43,79].

The use of recombinant technology with DT as a potential and improved vaccine is a promising solution for disease control in the near future. In the case description of this chapter, steps including immunogenic verification and the scale-up of protein production are still needed. However, research into new methods and technologies for vaccination improvement are certainly the best method to find an effective solution for disease control as well as a reduction of its treatment costs.

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20 Veterinary Rabies Vaccine

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20.1 Introduction

Rabies is acute encephalitis or meningoencephalitis resulting from to a lyssavirus infection [1]. It is known to be present on all continents except Antarctica. All warm-blooded animals are susceptible to rabies. It enters the body through wounds or by direct contact with mucosal surfaces. Rabies virus replicates in the bitten muscle and gains access to motor end plates and motor axons to reach the central nervous system [2,3]. Most deaths occur in the absence of postexposure prophylaxis.

In more than 99% of all human rabies cases, the virus is transmitted from dogs. Half of the global human population lives in canine rabies-endemic areas [4]. The most cost-effective strategy for preventing rabies in people is to eliminate it in dogs.

Canine rabies can be eliminated, as it has been demonstrated in North America, Western Europe, Japan, and many areas in South America. However, canine rabies is still widespread; it occurs in over 80 countries and territories, which are predominantly in the developing world. Mass parenteral vaccination programs remain the mainstay of canine rabies control. Coverage of 70% of dog populations has been sufficient to control canine rabies in several settings [4].

Pasteur developed the first rabies vaccine in 1885. It was composed of crude suspensions of desiccated, infected brain and spinal cord of rabbit, sheep, or goat brain. Occasionally Pasteur's vaccine led to vaccine-induced rabies [5]. Viral inactivation methods using phenol or betapropiolactone had been developed, increasing safety in vaccination; however, nerve-tissue vaccines enhance allergic neuroparalytic accidents caused by encephalitogen associated with myelin in adult mammalian brain. These reactions range from transient to severe paralysis, in some cases resulting in death [5].

A significant reduction in neuroallergic side effects was obtained with vaccine prepared in suckling mouse brain and inactivated with ultraviolet light or betapropiolactone

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[6]. It is imperative in the case of mouse suckling that they be under 9 days of age at the time of harvest [5].

After the 1950s, establishment of cell culture technology allowed significant progress in the propagation of virus in vitro and consequent development of viral vaccines [7]. Rabies vaccines developed and produced by cell culture technology have been demonstrated to be safer and more immunogenic than nerve-tissue vaccines [8].

Since 1984, World Health Organization has recommended discontinuation of the production and use of nerve-tissue vaccines and their replacement for cell culture vaccines [1]. In Brazil, nerve-tissue vaccine was replaced by cell culture vaccine in 2002 [1] for human public vaccination, and in 2010 for dog and cat mass vaccination.

Industrial production of viral vaccines preferably requires the use of continuous or immortalized cell lines as the basis for viral multiplication [7]. For human rabies vaccine production, the Vero cell line is the most widely used. For veterinary vaccine production, the cell lines used are baby hamster kidney cell line (BHK-21) and embryo hamster cell line [9–12].

The BHK-21 cell line was isolated from five 1-day-old hamsters [11] and consists of adherent fibroblasts that can also be adapted to suspension culture [13]. Suspension culture is widely used for veterinary vaccine production, owing to the lower costs of production and large cell quantities attained without the need of increasing superficial areas [13].

Effective in vitro maintenance and growth of animal cells requires culture conditions similar to those found in vivo with respect to temperature, oxygen concentration, pH, osmolality, and nutrients [14].

Culture medium composition is one of the most important factors in the culture of animal cells. Its functions are to provide appropriate pH and osmolality for cell survival and multiplication, as well as to supply all chemical substances required by the cells that they are unable to synthesize themselves [14].

Some of these substances can be provided by a culture medium consisting of low–molecular weight compounds, known as basal media. However, most basal media fail to promote successful cell growth by themselves and require supplementation with more complex and chemically undefined additives such as blood serum [14].

Fetal bovine serum (FBS) is the most frequently employed serum in animal cell culture. However calves, horses, and even human sera are also used [14]. FBS is the favorite because it has low immunoglobulin and high growth factor concentrations. However it is one of the most expensive components in culture medium [14]. Use of serum in cell culture is important because of nutritional factors, culture-stimulating factors (growth factors, hormones, and proteins), and protecting agents, both for biological protection (antitoxin, antioxidant, and antiprotease) and to prevent mechanical damage [14].

However using animal serum is potential risky for health, owing to the possible presence of adventitious agents such as virus and prions. In addition, serum can contain contaminants such as bacteria, fungi, and mycoplasmas, which can negatively affect cell culture. Another strong limitation to the use of sera is variability among different lots and

suppliers, which hinders standardization of the culture medium and the reproducibility of culture performance [14].

Furthermore, studies have demonstrated that bovine serum is the major reason for allergic reactions in dogs [15]. A research was designed to investigate the relationship between immunoglobulin E (IgE) reactivity to the vaccines and immediate-type allergic reactions after vaccination in dogs. Sera from 10 dogs that developed immediate-type allergic reactions such as circulatory collapse, cyanosis, dyspnea, facial edema, and vomiting within 1 h after vaccination with rabies vaccines and sera from 50 dogs that did not develop allergic reactions after vaccination were collected. Then, IgE reactivity to FBS and stabilizer proteins included in the vaccines was measured in sera that had high levels of IgE to the vaccines. Of the eight dogs that developed immediate-type allergic reactions and had high levels of serum-specific IgE to the vaccines, seven had specific IgE directed to FBS [15].

Later, researchers investigated IgE-reactive components of FBS using sera from 16 dogs that exhibited allergic reactions after vaccination. Immunoblot analysis revealed that several FBS proteins strongly reacted with IgE in the sera from dogs that showed postvaccination allergic reactions. The 66-kDa band was detected in the sera from 14 of the 16 dog serum samples analyzed in the immunoblot analysis for FBS, and it was speculated to be albumin based on its molecular weight; however, serum IgE reactivity to bovine serum albumin could be detected in only four of the 14 dog samples. These findings demonstrated that a variety of FBS components including albumin could function as allergens in dogs that developed allergic reactions after vaccination [16].

Many attempts have been made to develop culture media that do not need serum supplementation: that is, serum-free media (SFM) formulations. One of the greatest difficulties is to design a culture medium that ensures replicate cultures that show stable genetic, metabolic, and kinetic behavior [14].

Despite all of the developments seen, currently no standard culture medium can be used universally because each cell line has a specific metabolic profile and requires an appropriate medium composition [14].

For instance, Cellvento BHK-200 medium is an SFM formulated with no animal-derived component and optimized for the culture of suspension BHK-21 cells at high density, viability, and efficient propagation of viruses. The medium formulation was developed for the growth and maintenance of BHK-21 suspension cell lines used for viral vaccine production, and qualified for the production of foot and mouth disease virus (FMDV) [17].

Ex Cell 302 is an SFM specifically developed for the long-term growth of transformed Chinese hamster ovary (CHO) cells in suspension for the expression of antibodies or protein products. It was developed using only recombinant human proteins that have molecular weights less than 10 kDa [18].

VP-SFM is an SFM expressly designed for virus production, manufactured with no components of animal or human origin. VP-SFM is an ultra-low protein medium designed specifically for the culture of Vero cells. It is also appropriate for the growth of

COS-7 [African green monkey kidney cells (CV1 cells transformed with a defective mutant of SV40)], Madin–Darby canine kidney cell line, and BHK-21 in suspension. It is particularly suitable for growing viruses [19].

When using a new medium, the need for cell adaptation should be considered. The assessment of cellular performance in different culture media is usually challenging and time-consuming, because it requires the establishment of the concentration of total and viable cells over multiple subcultures. In addition, it may involve the quantification of residual nutrients, such as glucose and glutamine, of metabolic by-products such as lactate, ammonium, and alanine, and the concentration of the target product [14].

Cell adaptation is often carried out in static cultures employing tissue culture flasks. However problems seen at the time of adaptation can be minimized in suspension cultures, because this allows subculture during the log growth phase. Cultures in suspension also allow better medium oxygenation [14] and homogeneity.

Mammalian cells can be adapted to serum-free conditions by direct adaptation from serum-containing media or gradual weaning. Both procedures require healthy, viable cultures in the midlogarithmic growth phase. During the adaptation phase, growth rates will usually be somewhat slower than growth in media supplemented with serum.

Perrin et al. [20] adapted BHK-21 cells to grow in SFM developed by Merten et al. [21], for the production of an experimental rabies vaccine. Kallel and colleagues [22] also adapted the BHK-21 cell line to grow in different commercial SFM and evaluated the rabies vaccine produced. In both cases, adaptation was carried out with cells growing in monolayer.

This chapter presents a case study of a process of rabies virus production to a veterinary rabies vaccine, using the BHK-21 cell line in suspension adapted to commercial SFM. To achieve this technological development, the following strategies were used:

- Adapt the BHK-21 cell line to grow in different commercial SFM.
- Analyze the growth kinetics of adapted cells.
- Infect cells with rabies virus and analyze the behavior of infected cells.
- Verify the virus production capacity of SFM-adapted cells.
- Compare the results of adapted cells.

20.2 Case Study

20.2.1 Adaptation of Baby Hamster Kidney Cell Line-21 to Serum-Free Media

20.2.1.1 Cell Line

The BHK-21C13 LVI cell line, adapted to suspension, in passage 118 was used. This cell bank was coming from Panaftosa (the Pan American Centre for Foot-and-Mouth Disease) with 84 passages. Cells were adapted to grow at Dulbecco Modified Eagle's medium (DMEM)/Ham's F12 1:1 supplemented with 3% of FBS to generate the master and working cell bank. Cells were stored at −196°C.

20.2.1.2 Culture Media

Standard culture medium, composed of DMEM/Ham's F12 1:1 supplemented with 3% FBS, was used for control cell culture.

The following serum free media were used: VP-SFM (Gibco—Invitrogen cell culture), Ex Cell 302 (SAFC Biosciences), and Cellvento BHK-200 (Merck Millipore). Media were prepared following manufacturers' instructions. No antibiotic was added.

Cryopreservation culture media were prepared using 7% dimethyl sulfoxide, 46.5% SFM, and 46.5% media supernatant of centrifuged cells.

20.2.1.3 Strategies for Cell Adaptation to Serum-Free Media

Three different cell adaptation protocols were used.

20.2.1.3.1 DIRECT MEDIUM CHANGE

For direct medium change (D), the cell line growing healthily in standard culture media, with a cell density higher than 7.5×10^5 cells/mL, was centrifuged and resuspended in SFM at a concentration of 2.0×10^5 cells/mL. Subcultures were made until cell adaptation.

20.2.1.3.2 GRADUAL MEDIUM CHANGE

For gradual medium change (G), the cell line growing healthily in standard culture media, with a cell density higher than 7.5×10^5 cells/mL, had SFM added in culture gradually during subculture. An increased quantity of SFM was added only when cells were growing healthily in a previous adaptation phase. In the last adaptation step, cells were centrifuged to remove all serum from cultivation and resuspended in SFM.

20.2.1.3.3 GRADUAL SERUM REDUCTION (S)

For gradual serum reduction (S), the cell line growing healthily in standard culture media, with a cell density higher than 7.5×10^5 cells/mL, was centrifuged and resuspended in SFM, at a concentration of 2.0×10^5 cells/mL. SFM was supplemented with 3% FBS. Serum was decreased from culture gradually during subcultures. Serum was reduced only when cells had normal growth at a previous adaptation phase. In the last adaptation step, cells were centrifuged to remove all serum from cultivation and resuspended in SFM (Fig. 20.1).

During adaptation, a control culture in DMEM/Ham's F12 1:1 supplemented with 3% FBS (standard culture media) was held to compare growth behavior.

A direct media change protocol was used for adaptation of three serum-free culture media used in this development. The protocol of gradual media change was used to adapt cells to VP-SFM and Ex Cell 302 SFM. The gradual serum reduction protocol was used to adapt cells to Cellvento BHK-200 SFM. The choice of protocol followed the manufacturer's recommendations. Table 20.1 presents strategies used for cell adaptation.

20.2.1.4 Cell Cultivation

Cell cultivation was performed in spinner flasks (Techne, United Kingdom) of 500 mL working volume, with 400 mL culture medium, incubated at 37°C and agitated at 55 rpm.

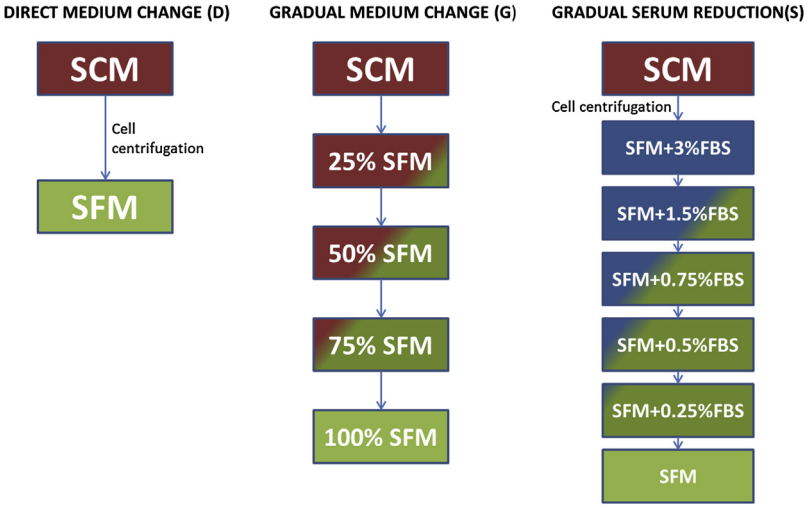

FIGURE 20.1 Protocols used for cell adaptation to serum free media. *SCM*, standard culture medium composed of DMEM/Ham's F12 1:1 supplemented with 3% of fetal bovine serum.

Table 20.1 Strategies Used for Baby Hamster Kidney Cell Line-21 Cell Line Adaptation to Serum-Free Media

	Serum-Free Media		
Adaptation Strategy	**VP-Serum-Free Media**	**Ex Cell 302**	**Cellvento Baby Hamster Kidney Cell Line-200**
Direct media change (D)	X	X	X
Gradual media change (G)	X	X	
Gradual serum reduction (S)			X

Inoculum was made with a cell density of 2.0×10^5 cells/mL. Subcultures (cells dilution) were made when growth reached 7.5×10^5 cell/mL density or higher.

Sometimes, during adaptation to SFM, even when cells did not reach 7.5×10^5 cells/mL, a subculture was done, usually to try to enhance growth conditions. In addition, if cells could not reach a cell density higher than 7.5×10^5 cells/mL, inoculum for subculture was increased to 5×10^5 cells/mL, to try to make cells reach a higher density.

Samples were collected daily for cell count, viability determination, morphology verification, metabolite analysis, and pH measurement.

20.2.1.5 Cell Count

Cell samples were diluted and stained with trypan blue (0.5%), then counted in a hemocytometer. Viable cells have an intact membrane and are not colored by trypan blue. Nonviable cells permit trypan blue to traverse the membrane and are colored by dye.

Total cells, viable cells, and cell viability were determined. Morphology, light refringence, and size were evaluated.

20.2.1.6 Metabolism Analysis

Glucose consumption and lactate production were determinate to verify growth limitation by inhibitory levels of lactate or by glucose scarcity.

A dual-channel biochemical analyzer YSI 2700 (Yellow Springs Instruments, EE.UU.) was used for glucose and lactate determination. Each channel contains immobilized enzyme on a polycarbonate membrane. One membrane has enzyme glucose oxidase immobilized and catalyzes the following reaction:

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2 \quad \text{[i]}$$

In other membrane, the enzyme lactate oxidase catalyzes reaction [ii]:

$$\text{Lactate} + O_2 \rightarrow \text{piruvate} + H_2O_2 \quad \text{[ii]}$$

Hydrogen peroxide generated independently in each membrane is oxidized in a platinum anode. Electron flow is linearly proportional to the concentration of hydrogen peroxide formed, and this, to the concentration of the substance in the sample. A 2% variation was considered acceptable between successive calibrations.

20.2.1.7 Sedimentation Tests

Cell lines adapted to SFM were submitted to sedimentation tests. They consisted of taking cell cultures, with a cell density over than 7.5×10^5 cells/mL, in a cold chamber (2−8°C) for 2 or 3 h until complete cell sedimentation. Culture media was carefully removed from the spinner (about 200 mL) and fresh media were added; then cells were incubated at 37°C and agitated at 55 rpm. This procedure was repeated for 3 consecutive days. Every day, samples were collected for cell count, viability, morphology, and metabolic analysis. To be approved in this test, cells had to keep their growth behavior and not grow in clumps.

A sedimentation test was performed to simulate industrial rabies virus production in which cells had to be settled for virus infection. Therefore, adapted cells had be robust enough for this operation.

20.2.1.8 Cryopreservation

Seed banks were made with cell lines approved in sedimentation tests. Cryotubes with $1 \times 10\text{^}7$ cells and 1 mL cryopreservation media were frozen in a −80°C freezer. A gradual temperature reduction rate of −1°C/min was achieved using Mr. Frosty (Nalgene) and isopropyl alcohol. After they reached −80°C, cells were stored in liquid nitrogen (−196°C).

20.2.1.9 Growth Kinetics

Growth kinetics was carried out with all serum-free adapted cell lines and control culture, growing in standard culture media.

Cell cultivation was performed with 400 mL culture medium at 37°C, in spinner flasks of 500 mL working volume (Techne, United Kingdom), agitated at 55 rpm. Inoculum was made at a cell density of 2.5×10^5. No subculture was done. Daily samples were collected for cell count, viability determination, morphology verification, metabolite analysis, and pH measurement. Cultivation was carried out until cell death.

Specific growth rate, μ (cells/h), was estimated by Eq. [20.1]:

$$\mu = (LnX_2 - LnX_1)/(t_2 - t_1) \quad [20.1]$$

where X represents the viable cell density (mL), t represents the time points of sampling (h); and subscripts 1 and 2 stand for two succeeding sampling points.

20.2.1.10 Adaptation to VP-Serum Free Media From Standard Culture Media

20.2.1.10.1 CONTROL CULTURE

Control cultures had standard behavior during subcultures. Cell morphology, size, and refringence were normal. Cell density reached 7.5×10^5/mL or higher after 2 days of cultivation; cell viability was higher than 80%, and usually cells doubled or tripled every day. Glucose consumption, lactate production, and pH values were similar during subcultures.

The growth kinetics of BHK-21 cells in standard culture media reached a maximal viable cell density of 8.1×10^5 cells/mL on the fourth day of cultivation. The glucose concentration was not limited to growth nor did lactate concentration reach inhibitory levels, according to what Cruz et al. [23] described. Cultivation lasted 6 days, in which there was no lag phase, 2 days of exponential phase, 3 days of stationary phase, and 1 day of death phase. Maximal specific growth rate obtained was $0.036\ h^{-1}$. Because glucose and lactate were not limited to growth, cells probably stopped growing because of ammonia production or the absence of another nutrient, such as glutamine.

20.2.1.10.2 DIRECT MEDIUM CHANGE

In adaptation using direct media change from standard culture media to VP-SFM (VPD), over 18 days and 10 subcultures, cells had abnormal morphology, size, and growth. In some subcultures, cells grew in clumps. There was much cell debris in the medium and viability was very low, reaching 18% on the fourth adaption day. In this period, between some subcultures, cells were centrifuged and half of the culture media was replaced to remove inhibitory metabolites, adjust pH, and supply nutrients. In the 11th subculture, cells started to have behavior similar to that of control cells, but in next subculture (12th), cells formed aggregates, so three more subcultures were carried out to confirm cell behavior. Because the cells maintained their behavior and clumps no longer formed, they were submitted to a sedimentation test. During the test, no clump formed and the culture reached a density of 1.8×10^6 cells/mL. Cells were considered adapted to VP-SFM culture media and were robust enough for virus production. A seed bank was made.

Adaptation to VP-SFM took 26 days and 15 passages.

The growth kinetics of cells by VPD had 1 day of lag phase, 1 day of log phase, 1 day of stationary phase, and 2 days of death phase. The maximal cell density, 8.2×10^5 viable cells/mL, was obtained on the third day of cultivation. Cultivation lasted 6 days, just like

Table 20.2 Growth of Baby Hamster Kidney Cell Line-21 Cells in Spinner in Different Serum-Free Media and Different Adaptation Strategies

	Maximal Cell Density (10^6 cell/mL)	Maximal Specific Growth Rate (h^{-1})	Time of Cultivation (day)
Control	0.81	0.036	6
VPD	0.82	0.052	6
EXD	2.09	0.113	13
EXG	0.95	0.030	17
CVTDvp	1.03	0.058	8
CVTGvp	1.09	0.041	8

CVTDvp, Adaptation to Cellvento BHK 200 media by direct media change using cells previously adapted to VP-serum-free media (SFM); *CVTGvp*, adaptation to Cellvento BHK 200 media by gradual media change using cells previously adapted to VP-SFM; *EXD*, adaptation to Ex Cell 302 media by direct media change; *EXG*, adaptation to Ex Cell 302 media by gradual media change; *VPD*, adaptation to VP-SFM media by direct media change.

the control culture. Growth was not limited by the glucose or lactate concentration [23]. Because the log phase was more acute, maximal specific growth rate obtained at 0.052 h^{-1} was higher than the control culture. Cells lasted less time in the stationary phase and cell death was more gradual than in the control culture. The maximal cell density was lowest among serum-free adapted cells (Table 20.2).

20.2.1.10.3 GRADUAL MEDIUM CHANGE

In adaptation using gradual media change from standard culture media to VP-SFM, the first step of media change was 75% standard culture medium and 25% VP-SFM. No alteration in cell behavior or metabolites was observed. After three subcultures, a new step of media substitution was done, with 50% of standard culture medium and 50% of VP-SFM. No alteration in cell behavior or metabolites was observed. The next step was 25% of standard culture medium and 75% of VP-SFM. In this step, the cell viability and cell growth decreased during the third and fourth subcultures, but in the next passage, cell growth behavior became normal and cell viability improved in the sixth subculture. Therefore the next media change step was done: 0% of standard culture medium and 100% of VP-SFM. In this adaptation stage, cell morphology was abnormal, clumps formed, and much cell debris was observed in the media. In some subcultures, cells were centrifuged and half of the culture media was replaced to remove inhibitory metabolites, adjust the pH, and supply nutrients. However, no improvement in cell morphology was observed, and glucose consumption and lactate production were higher than during previous adaptation steps and control cells. Because adaptation by direct media change was reached, this adaptation procedure was stopped and cells were not considered to be adapted.

Adaptation procedure took 49 days and was unsuccessful.

20.2.1.11 Adaptation to Ex Cell 302 From Standard Culture Media

20.2.1.11.1 CONTROL CULTURE

Control culture had standard behavior during subcultures. Cell morphology, size, and refringence were normal. Cell density reached 7.5×10^5 or higher after 2 days of

cultivation, cell viability was higher than 80%, and cells doubled or tripled every day. Glucose consumption, lactate production, and pH values were similar during subcultures.

20.2.1.11.2 DIRECT MEDIUM CHANGE

In adaptation using direct media change from standard culture medium to Ex Cell 302 EXD, over 26 days and 10 subcultures, cells had an abnormal morphology, size, and growth. There was much cell debris in the medium but cell viability did not decrease as much as in adaptation to VP-SFM by direct media change. The lowest viability value was 72%. In this period, between some subcultures, cells were centrifuged and half of the culture media was replaced. In the 11th subculture, cells started to have growth and metabolite behavior similar to the control culture. Three more subcultures were done to confirm cell behavior. Because cells maintained their behavior, they were submitted to a sedimentation test. During the test, no clump formed and the culture reached a density of 1.2×10^6 cells/mL. Cells were considered to be adapted to the Ex Cell 302 culture media and were robust enough for virus production. A seed bank was made.

Adaptation to Ex Cell 302 by direct media change took 36 days and 14 passages.

The growth kinetics of cells adapted to Ex Cell 302 SFM by direct media change EXD had atypical behavior. On the first day of cultivation, the cell density was 1.95×10^6 viable cells/mL, about 10 times higher than the inoculum. The maximal specific growth rate was $0.113\ h^{-1}$, twice as high as that obtained for other kinetics (Table 20.2). The maximal cell density, 2.09×10^6, was obtained on the third cultivation day. This cultivation was the one with the best energetic efficiency, because with a similar glucose concentration available, a higher cell density and lower lactate production were obtained compared with other growth kinetics. Glucose scarcity was obtained on the fifth day, but cultivation lasted for 13 days, which suggests that another energy source, probably glutamine, was used during the stationary phase. The death phase lasted only 1 day. Kallel et al. [22] also adapted BHK-21 cells to Ex Cell 302 SFM, but the maximal cell density obtained was 7×10^5 cells/mL and the maximal specific growth rate was $0.016\ h^{-1}$.

20.2.1.11.3 GRADUAL MEDIUM CHANGE

In an adaptation using gradual media change from a standard culture medium to Ex Cell 302 EXG, the first step of media change was 75% of the standard culture medium and 25% of Ex Cell 302. No alteration in cell growth behavior or metabolism was observed, but the cell morphology and size were abnormal and the media had much cell debris. However, after four subcultures, a new step of media substitution was performed: 50% of standard culture medium and 50% of Ex Cell 302. No alteration in cell behavior or metabolites was observed. The cell morphology and size became normal and cell debris disappeared. The next step was 25% of standard culture medium and 75% of Ex Cell 302. In this step, four subcultures were done. Cell growth behavior was normal and glucose consumption was higher, which could have resulted from the higher cell density. Therefore the next media change step was done: 0% of standard culture medium and

100% of Ex Cell 302. In this adaptation stage, no problems were observed. Morphology, growth, and metabolites had the same behavior as for control cells. Thus the cells were submitted to a sedimentation test. No clumps formed and the culture reached a density of 1.3×10^6 cells/mL. The cells were considered to be adapted to the Ex Cell 302 culture media and robust enough for virus production. A seed bank was made.

The growth kinetics of cells adapted to Ex Cell 302 SFM by gradual media change EXg reached maximal cell density, 9.5×10^5 viable cells/mL, on the 13th day. Cell growth was the slowest, with maximal specific growth of $0.030\ h^{-1}$. However it had the longest cultivation time, lasting 17 days. This cultivation had 1 day of lag phase, 2 days of exponential phase, 12 days of maintenance phase, and 2 days of senescence phase. Cultivation reached the lowest pH value at the death phase, 6.01.

Adaptation to Ex Cell 302 by gradual media change took 36 days and 18 passages. Adaptation to Ex Cell 302 by gradual media change took the same time as adaptation to Ex Cell 302 by direct media change, but more subcultures were done. This was because in adaptation to Ex Cell 302 by direct media change, cells took longer to reach a cell density higher than 7.5×10^5.

20.2.1.12 Adaptation to BHK From Standard Culture Media

20.2.1.12.1 CONTROL CULTURE

The control culture had standard behavior during subcultures. The cell morphology, size, and refringence were normal. The cell density reached 7.5×10^5 or higher after 2 days of cultivation. The cell viability was higher than 80% and cells doubled or tripled every day. In some subcultures, cells quadrupled in 24 h. Glucose consumption, lactate production, and pH values were similar during subcultures.

20.2.1.12.2 DIRECT MEDIUM CHANGE

In adaptation using direct media change from standard culture medium to Cellvento BHK-200, over 50 days and 15 subcultures, cells had an abnormal morphology, size, and growth. In some subcultures, cells grew in clumps. There was much cell debris in the medium. In this period, between some subcultures, cells were centrifuged and half of the culture media was replaced to remove inhibitory metabolites, adjust the pH, and supply nutrients. However in no subculture did cells reached a density higher than 5.8×10^5 cells/mL. No enhanced cell morphology or growth was observed with time, so this adaptation work was stopped without cell adaptation.

20.2.1.12.3 GRADUAL SERUM REDUCTION

In an adaptation using a gradual serum reduction from standard culture medium to Cellvento BHK-200, the first step was to centrifuge cells from standard culture medium and resuspend cells in Cellvento BHK-200 with 3% FBS. In this step of adaptation, cells had normal morphology, growth, and metabolism. Four subcultures were performed. The first step of serum reduction was done (reduction to 1.5%). Cells also had a normal morphology, growth, and metabolism. Therefore after three subcultures, a new adaptation step was done: Cellvento BHK-200 supplemented with 0.75% FBS. This step

presented no problem with cell morphology, cell growth, or metabolism. The next step of serum reduction was carried out (Cellvento BHK-200 with 0.5% FBS) and presented no cultivation problems. Cell growth, morphology, and metabolites were similar to those of the control.

In next step, Cellvento BHK-200 supplemented with 0.25% FBS, cells started to show abnormal growth. In some subcultures, even with media replacement, they could not reach a density of 7.5×10^5 cell/mL. The cell inoculum was increased to 5.0×10^5 cells/mL but cells could not double. The cell morphology became irregular; the media had much cell debris and different-sized cells in culture.

After 41 passages and 116 days trying to adapt cells, work was stopped without cell adaptation.

Because Cellvento BHK-200 is a culture medium developed for use in veterinary vaccine production, its price is about 10 times cheaper than other SFM used in this technological development. Adaptation of BHK-21 with this medium has an important economic perspective. Therefore, failure in adaptation using previous strategies led to attempts to try a new strategy. In the new strategy, cells already adapted to SFM, VP-SFM, were used to adapt to Cellvento BHK-200. Two adaptation protocols were used: direct media change and gradual media change.

20.2.1.13 Adaptation to BHK From VP-Serum-Free Media

20.2.1.13.1 CONTROL CULTURE

The control culture demonstrated standard behavior during the subcultures. The cell morphology, size, and refringence were normal. The cell density reached 7.5×10^5 or higher after 2 days of cultivation, the cell viability was higher than 80%, and cells doubled or tripled every day. In some subcultures, cells quadrupled. Glucose consumption, lactate production, and pH values were similar during subcultures.

20.2.1.13.2 DIRECT MEDIUM CHANGE

In the adaptation using direct media change from VP-SFM to Cellvento BHK-200 (CVTDvp), the first two passages had a lot of cell debris, but the cell morphology, size, and refringence remained normal. During subcultures, cells could not reach a density higher than 7.5×10^5 cells/mL, so in some subcultures inoculums with 5.0×10^5 cells/mL were used to make cells double and reach 1.0×10^6 cells/mL. In the 13th subculture, the cell density was 1.0×10^6 cells/mL, from an inoculum of 2.0×10^5 cells/mL. For the next two subcultures, the cells maintained their behavior and so were submitted to a sedimentation test. During the test, no clumps formed and the culture reached a density of 2.1×10^6 cells/mL.

After 42 days and 15 passages, cells were adapted to Cellvento BHK-200 culture medium and were robust enough for the virus production process.

The growth kinetics of cells adapted to Cellvento BHK-200 media by direct media change (CVTDvp) took 1 day of lag phase, 3 days of log phase, no days in stationary

phase, and 4 days of death phase. The maximal cell density, 1.03×10^6 viable cells/mL, was obtained on the fourth day of cultivation. Cultivation lasted 8 days, and growth was limited neither by glucose nor by lactate concentration [23]. The maximal specific growth rate obtained was 0.058 h^{-1}.

20.2.1.13.3 GRADUAL MEDIUM CHANGE

In the adaptation using gradual media change from VP-SFM to Cellvento BHK-200 (CVTGvp), the first step of media change was 75% VP-SFM and 25% Cellvento BHK-200. No alteration in cell growth behavior or metabolites was observed, but in the third passage cells grew in clumps. However a new step of media substitution was done: 50% VP-SFM and 50% Cellvento BHK-200. No alterations in cell behavior or metabolites were observed. The next step was 25% VP-SFM and 75% Cellvento BHK-200. In this step, cells showed standard behavior. Therefore the next media change step was done, but because adaptation to Cellvento BHK-200 failed in prior adaptation work, a more gradual media change was made in this adaptation process: 15% VP-SFM and 85% Cellvento BHK-200. In this adaptation stage, no problem was observed. A new adaptation step was performed in which 5% VP-SFM and 95% Cellvento BHK-200 were used. In this step, the subculture had a density of 1.1×10^6 cells/mL and a viability of 99%, so the last step was done with 0% VP-SFM and 100% Cellvento BHK-200. Three subcultures were carried out and no abnormal situation was detected. Cells were submitted to the sedimentation test. During the test, no clumps formed and the culture reached a density of 1.9×10^6 cells/mL. Cells were considered to be adapted to Cellvento BHK-200 culture media and robust enough for virus production. A seed bank was made.

The growth kinetics of cells by CVTGvp presented behavior similar to that of cells adapted to Cellvento BHK-200 by direct media change: 1 day of lag phase, 2 days of log phase, no day in stationary phase, and 5 days of death phase. Maximal cell density, 1.09×10^6 viable cells/mL, was obtained on the third day of cultivation. Cultivation lasted 8 days and growth was limited neither by glucose nor by lactate concentration. The maximal specific growth rate obtained was 0.041 h^{-1}.

Adaptation to Cellvento BHK-200 by gradual media change from cells previously adapted to VP-SFM took 42 days and 20 passages. Adaptation to Cellvento BHK-200 by gradual media change took the same time as adaptation to Cellvento BHK-200 by direct media change, but more subcultures were done. This was because in adaptation to Cellvento BHK-200 by direct media change, cells took longer to reach cell density higher than 7.5×10^5 cells/mL.

It was expected that in direct media change cells would adapt to new media without the need for subcultures. This result was not found in any culture media used. At least 14 passages for Ex Cell 302 and 15 passages for VP-SFM were needed for cells to adapt.

H. Kallel et al. [22] adapted the BHK-21 cell line to three commercial SFM, HyQ PF CHO, HyQ PF CHO MPS, and Ex Cell 302, in a single step by direct switch from serum-containing media, and adapted BHK-21 to Rencyte by gradual media change. In gradual

adaptation, 26 days were necessary for adaptation. In this study, cells grew in a monolayer. After adaptation, cells no longer adhered but grew in suspension-forming clumps.

Merten et al. [21] adapted BHK-21 cells to SFM MD SS2 using same protocol as Kallel and coworkers [22]; cells also grew as clump cultures. In both studies, clump cultures were able to produce rabies virus. However in an aggregated culture, it is not possible to have homogeneity. Cells in the middle of aggregates have difficulty accessing nutrients and it is more difficult to have a virus infection. Besides, cells in clumps do not grow as healthy as do cells free in suspension. Thus cells in suspension adapted to SFM without forming clumps are a great possibility for industrial use.

A summary of results of cell adaptation to SFM is shown in Table 20.3. Considering that 26 days and 15 passages were needed to adapt cells to VP-SFM, the actual value of passages to adapt BHK-21 cells to Cellvento BHK-200 culture media was 30 in direct media change and 35 in gradual media change, and it took 68 days.

Fig. 20.2 shows a comparison among kinetics studies. The cultivation of cells adapted to Ex Cell 302 by direct media change EXd obtained a twice-higher cell density and cultivation lasted twice as long as the control cultivation. The cultivation of cells adapted to Ex Cell 302 by gradual media change EXG lasted three times longer than the control culture with a similar maximal cell density. Although cells adapted to Cellvento BHK-200 (CVTDvp and CVTGvp) did not have a stationary phase, the cell density was higher than that of the controls on the third through sixth cultivation days. Cells adapted to VP-SFM (VPD) had maximal cell density similar to control culture, but cell death started earlier than for controls.

In this technological development, it is possible to affirm that BHK-21 cells are better adapted to Ex Cell 302 serum-free culture media, and direct media change adaptation led to better cell adaptation.

Table 20.3 Summary of Cell Adaptation to Serum-Free Media (SFM)

	Media Exchange			
Adaptation strategy	SCM → VP-SFM	SCM → Ex Cell 302	SCM → Cellvento BHK-200	VP-SFM → Cellvento BHK-200
Direct media change (D)	26 days 15 passages	36 days 14 passages	Not adapted	42 days 15 passages
Gradual media change (G)	Not adapted	36 days 18 passages	—	42 days 20 passages
Gradual serum reduction (S)	—	—	Not adapted	—

SCM, Standard culture medium composed of Dulbecco Modified Eagle's medium/Ham's F12 1:1 supplemented with 3% fetal bovine serum.

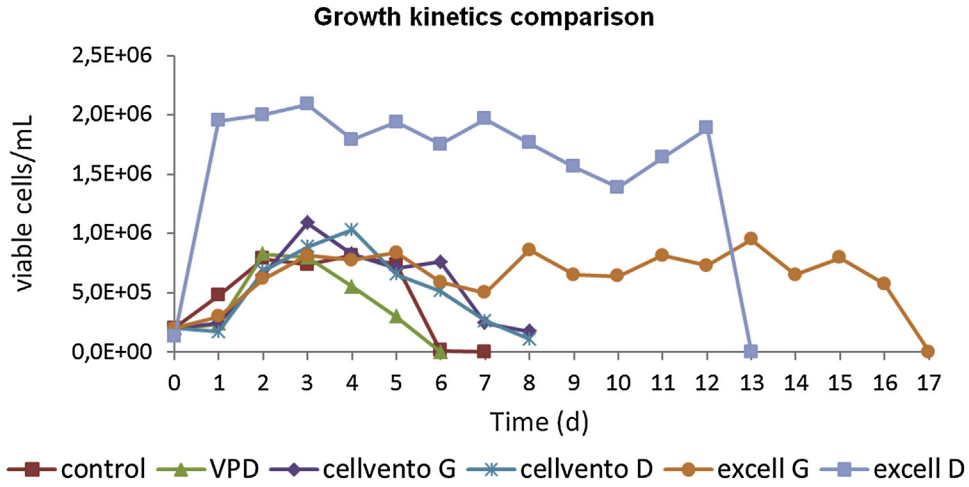

FIGURE 20.2 Growth kinetics comparison of all adapted cell lines.

20.2.2 Rabies Virus Production Using Baby Hamster Kidney Cell Line-21 Adapted to Serum-Free Media

All adapted cell lines were submitted to infection kinetics to verify the ability of adapted cells to produce rabies virus. A control culture was carried out using standard culture media for infection.

20.2.2.1 Culture Media

Standard culture medium for infection, composed of DMEM/Ham's F12 1:1 supplemented with 1% FBS, was used during virus infection of the control cell culture.

VP-SFM (Gibco—Invitrogen cell culture), Ex Cell 302 (SAFC Biosciences), and Cellvento BHK-200 (Merck Millipore) were prepared following the manufacturers' instructions. No antibiotic was added.

20.2.2.2 Virus Strain

Rabies Pasteur virus, supplied from the Federal University of Minas Gerais, was adapted to grow in BHK-21C13 in suspension in standard culture media for infection. The adapted virus generated TECPAR (Technology Institute of Paraná) master and working virus bank was stored at −80°C. The virus strain provided by the TECPAR working virus bank was used for this study.

20.2.2.3 Cell Infection and Virus Production

Cell infection was carried out on cultures with a cell density of about 1.0×10^6 cells/mL. The pH was adjusted to 7.4 with sodium bicarbonate 7.5%. Cells were settled in a cold chamber and 300 mL media were removed. Viruses in a multiplicity of infection of 0.015, and diethylaminoethanol (1% final volume) were added to the spinner flask and cells

were incubated at 33°C, over 35 rpm intermittent agitation (5 min of agitation and 20 min stopped) for 90 min. Media were added and spinners were again incubated at 33°C over 55 rpm.

Samples were collected daily for cell count, metabolite analysis, pH measurement, virus titer determination, and direct immunofluorescence assay. The pH was adjusted to 7.4, cells were settled in a cold chamber, and half the volume of media was harvested. Fresh media were added and spinners were again incubated. This procedure was continued for 6 days.

20.2.2.4 Direct Immunofluorescence Assays

Infected cell samples (50 μL) were settled in microscope slides, fixed with cold 80% acetone, and stained with Evans Blue dye (1:40,000) and fluorescein-labeled antirabies nucleocapsid immunoglobulin (Bio-Rad code 357-2114). Slides were analyzed under a fluorescence microscope to determine the level of cell infection. It was evaluated in terms of fluorescent focus inside the cells (Table 20.4). This procedure was adapted from Batista and collaborators [24].

Immunofluorescence assays were conducted to verify the evolution of cell infection.

20.2.2.5 Rabies Virus Titration

To determine the rabies virus titer, monolayers of BHK-21 cells on 96-well microtiter plates were infected with sample dilutions and incubated at 37°C with 5% CO_2 for 22 h. Cells were then fixed with cold 80% acetone, washed with phosphate-buffered saline, and incubated with fluorescein-labeled antirabies nucleocapsid immunoglobulin (Bio-Rad code 357-2114) for 1 h at 37°C [25–27]. The microtiter plates were then observed under a fluorescence microscope and the titer was expressed as the 50% fluorescent focus-forming dose (FFD_{50}), as calculated by the Spearman-Kärber method [28].

20.2.2.6 Infection Kinetics of Control Culture

During all infection kinetics studies, the maximal cell densities of cultivations were obtained after infection, owing to the media harvest that provided nutrients and removed metabolites. In the control cultivation, cell viability decreased right after cell infection. Glucose and lactate metabolism after infection was similar to the metabolism of

Table 20.4 Relation Between Infection Class and Fluorescence Focuses

Infection Class	Fluorescence Focus
0	Total absence of fluorescent focuses
I	1–25% of cells had fluorescence focuses
II	26–50% of cells had fluorescence focuses
III	51–75% of cells had fluorescence focuses
IV	76–100% of cells had fluorescence focuses

Table 20.5 Virus Titer During Infection Kinetics of Adapted Cells to Serum-Free Media

	Rabies Virus Titer $\log_{10}$ FFD_{50}/mL						
Serum-Free Adapted Cells	**1dpi**	**2dpi**	**3dpi**	**4dpi**	**5dpi**	**6dpi**	**Average**
Control	3.23	4.93	**5.47**	5.04	5.04	4.63	4.72
VPD	4.90	5.25	5.54	5.54	**5.62**	5.11	5.33
EXD	2.96	4.92	**5.54**	5.04	5.4	5.25	4.85
EXG	2.83	4.91	4.9	**5.32**	4.9	5.18	4.67
CVTDvp	3.53	4.63	4.9	**5.32**	**5.32**	4.64	4.72
CVTGvp	3.23	4.63	4.9	**5.05**	4.77	4.9	4.58

CVTDvp, Adaptation to Cellvento BHK 200 media by direct media change using cells previously adapted to VP-serum-free media (SFM); *CVTGvp*, adaptation to Cellvento BHK 200 media by gradual media change using cells previously adapted to VP-SFM; *dpi*, days postinfection; *EXD*, adaptation to Ex Cell 302 media by direct media change; *EXG*, adaptation to Ex Cell 302 media by gradual media change; *VPD*, adaptation to VP-SFM media by direct media change.

noninfected cells. Since the second day postinfection (dpi), more than 75% of cells were infected. Titer peak (bold in Table 20.5) coincided with maximal cell density on dpi 3.

Taking into account that in the industrial production of rabies vaccine in TECPAR only a harvest with $\log_{10}$ FFD_{50} higher than 3.8 is used for veterinary vaccine production, the first harvest was the only one that did not meet this requirement (Table 20.5).

20.2.2.7 Infection Kinetics of Cells Adapted to VP-Serum-Free Medium by Direct Media Change

In the infection kinetics of cells adapted to VP-SFM, the maximal cell density was 2.3×10^6 viable cells/mL. Cell viability decreased only after dpi 3. Infection class IV cells were obtained on dpi 3. After infection, glucose consumption increased when cells reached class IV of infection, all available glucose had been consumed during harvest, media were replaced, and glucose was supplied, even though all glucose was consumed. This may be because rabies virus is a glycoprotein, and glucose was used to produce it. Since dpi 1, the virus titer met the vaccine production requirement. In this study, the infection of cells adapted to VP-SFM reached the highest virus titer and the highest average of virus titer (Table 20.5). This cultivation was the only one in which the maximal cell density did not coincide with the peak virus titer.

20.2.2.8 Infection Kinetics of Cells Adapted to Ex Cell 302 by Direct Media Change

The infection of cells adapted to Ex Cell 302 by direct media change had the maximal cell density at dpi 3. A slight decrease in cell viability was observed right after cell infection. The virus titer peak (bold in Table 20.5) coincided with the maximal cell density and when more than 75% of cells were infected. Glucose consumption and lactate production increased after infection, but glucose scarcity was not observed. Only the first harvest did not reached the minimal virus titer for vaccine production.

20.2.2.9 Infection Kinetics of Cells Adapted to Ex Cell 302 by Gradual Media Change

The infection of cells adapted to Ex Cell 302 by gradual media change reached the maximal cell density on dpi 4. A slight decrease in cell viability was observed right after cell infection. Infection class IV was obtained on the second day after infection. The virus titer peak (Table 20.5) coincided with the maximal cell density. Glucose consumption and lactate production increased after infection, but glucose scarcity was observed only on dpi 6. Only the first harvest did not reach the minimal virus titer for vaccine production.

20.2.2.10 Infection Kinetics of Cells Adapted to BHK by Direct Media Change

The infection of cells adapted to Cellvento BHK-200 by direct media change reached maximal cell density at dpi 5. A high decrease in cell viability was observed right after cell infection. The virus titer peak (bold in Table 20.5) coincided with the maximal total cell density on dpi 4, and with the maximal viable cell density on dpi 5. One day after infection, less than 25% of cells were infected. On dpi 3 more than 75% of cells were infected. Glucose consumption and lactate production increased after infection. Glucose scarcity was observed only at the sixth harvest. The first harvest was the only that did not reach the minimal virus titer for vaccine production.

20.2.2.11 Growth Kinetics of Cells Adapted to BHK by Gradual Media Change

Infection of cells adapted to Cellvento BHK-200 by gradual media change reached the maximal cell density at dpi 4, coinciding with the virus titer peak. A high decrease in cell viability was observed right after cell infection. One day after infection, less than 25% of cells were infected. More than 75% of cell infection was observed on dpi 3. Glucose consumption and lactate production increased after infection, but glucose scarcity was observed only at the sixth harvest. Only the first harvest did not reach the minimal virus titer for vaccine production.

20.2.2.12 Comparison of Virus Titer in Serum-Free Adapted Cell

Fig. 20.3 shows the virus titer during infection kinetics studies. With the exception of cells adapted to VP-SFM that had all harvests with a virus titer meeting requirements for vaccine production, others had enough virus titer since dpi 2.

No relation was observed between the infection class and virus titer because even when less than 50% of cells were infected, the virus titer was higher than $10^{3.8}$. All adapted cells had the capacity to produce virus for vaccine production.

Cell adapted to VP-SFM culture media had the worst growth kinetics but the best virus production, demonstrating the importance of the virus infection study. VP-SFM is a culture medium developed for virus production, and probably has a formulation more

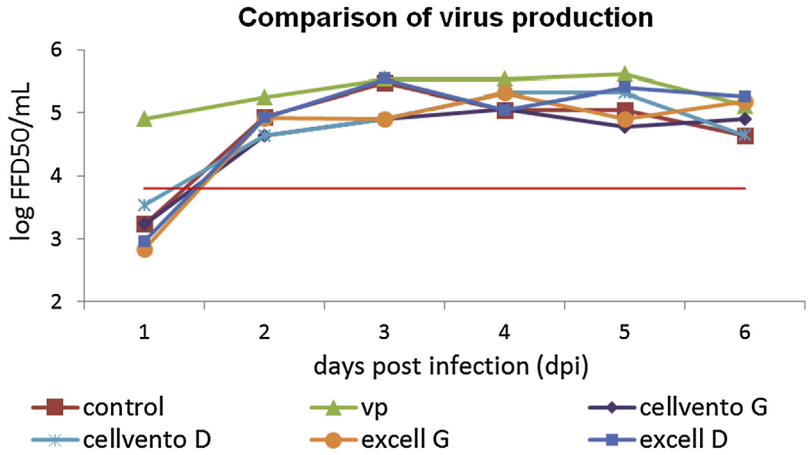

FIGURE 20.3 Comparison of virus production of all adapted cell lines. The *red line* (gray in print versions) is the cutoff line. Only the harvest of virus that results in the virus titer above the red line is used for vaccine production.

appropriate for this indication. Studies should be carried out to investigate whether increasing the glucose supply during infection enhances virus production.

The results presented here indicate that cells adapted to SFM can produce rabies virus for vaccine production. However, virus harvests have to be formulated and challenged in animals to confirm vaccine potency.

Perrin et al. [20] carried out cell infection in a bioreactor using a perfusion system. The best virus titer was achieved on the third to fifth days after infection. The results of this technological development were shown in a high virus titer from the second to the sixth day postinfection.

Kallel et al. [22] infected BHK-21 adapted to SFM with rabies virus. The study was carried out in spinners. The virus titer started to increase after dpi 3 and 5, depending on the media used. Virus peaks were obtained only on dpi 9.

Although cells adapted to VP-SFM culture media showed the best virus titer results, attention should be paid to Cellvento BHK-200 culture media. Because this culture medium was developed for a veterinary vaccine, mainly mouth-and-foot disease, which requires large-scale production and low prices compared with human vaccines, the Cellvento BHK-200 price is about 10 times cheaper than other SFM developed for human viral vaccine production (VP-SFM) or for recombinant proteins for human use (Ex Cell 302).

Table 20.6 compares culture media costs using SFM produce 10 million of doses of rabies vaccine. Therefore Cellvento BHK-200 culture medium currently seems to be the most adequate for veterinary rabies vaccine production.

Table 20.6 Cost Estimation of Serum-Free Culture Media for 10 Million of Doses of Vaccine Production

Culture Medium	Volume for 10 Million of Doses (L)	Medium Costs (EUR)
VP-serum-free media	10,000	400,000.00
Ex Cell 302	11,000	440,000.00
Cellvento Baby hamster kidney cell line-200	11,000	40,000.00

20.3 Conclusion and Perspectives

Dog rabies mass vaccination is an extremely important strategy to control human rabies infection. In Brazil, about 30 million of doses are used annually for dog and cat mass vaccination. Veterinary rabies vaccines available on the market are produced using serum for cell cultivation. Knowledge of side effects caused in dogs by serum in rabies vaccine leads to development of a vaccine produced with SFM. This case study showed that it is possible to substitute serum in the production process using commercial SFM. BHK-21C13 cell in suspension is able to adapt in the three serum-free culture media used in this technological development (VP-SFM, Ex Cell 302, and Cellvento BHK-200). Kinetics studies of cell growth and rabies virus production demonstrated that the best results in growth kinetics do not mean the best results in virus production. All adapted cells are able to produce rabies virus for vaccine production. Different adaptation protocols of cell adaptation to SFM do not interfere with the results of virus production. The best virus productivity was reached using VP-SFM culture media; however, taking into account economic aspects, the best result was achieved using Cellvento BHK-200.

Vaccine potency must be determined to prove virus quality. Because vaccine potency is good, scale-up studies using SFM should be carried out to permit serum substitution in industrial veterinary rabies vaccine production.

In addition, BHK-21 adapted to SFM can be used to produce other veterinary viral vaccines, such as foot-and-mouth disease. In this case, replacement of serum in cell cultivation leads to an important improvement in vaccine, because bovine serum may have adventitious agents, such as virus and prions, which can infect vaccinated calves.

List of Abbreviations

BHK	Baby hamster kidney cell line
CHO	Chinese hamster ovary cell line
COS	African green monkey kidney cells (CV1 cells transformed with a defective mutant of SV40)
CVTD	Adaptation to Cellvento BHK 200 media by direct media change
CVTDvp	Adaptation to Cellvento BHK 200 media by direct media change using cells previously adapted to VP-SFM
CVTGvp	Adaptation to Cellvento BHK 200 media by gradual media change using cells previously adapted to VP-SFM
CVTS	Adaptation to Cellvento BHK 200 media by gradual serum reduction
DEAE	Diethylaminoethanol

DMEM	Dulbecco Modified Eagle's medium
DMSO	Dimethyl sulfoxide
dpi	Day postinfection
EXD	Adaptation to Ex cell 302 media by direct media change
EXG	Adaptation to Ex cell 302 media by gradual media change
FBS	Fetal bovine serum
FBS	Fetal bovine serum
FFD_{50}	Fluorescent focus doses 50
FMDV	Foot and mouth disease virus
HCV	Hepatitis C vírus
HIV	Human immunodeficiency virus
IgE	Immunoglobulin E
MDCK	Madin-Darby canine kidney cell line
MDSS2	Serum free medium developed by Merten et al. [21].
MOI	Multiplicity of infection
NIL2	Embryo hamster cell line
Panaftosa	Pan American Centre for Foot-and-Mouth disease
PBS	Phosphate buffered saline
PV	Rabies Pasteur Virus
SCM	Standard culture medium, comprising DMEM/Ham's F12 1:1 supplemented with 3% of FBS
SCMI	Standard culture medium for infection, comprising DMEM/Ham's F12 1:1 supplemented with 1% of FBS.
SFM	Serum free medium
TECPAR	Technology Institute of Paraná
UFMG	Federal University of Minas Gerais
UFPR	Federal University of Paraná
VPD	Adaptation to VP-SFM media by direct media change
VPG	Adaptation to VP-SFM media by gradual media change
VP-SFM	Commercial serum free medium developed for human viral vaccine production
WHO	World Health Organization

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21

Development of a Rabies Vaccine in Cell Culture for Veterinary Use in the Lyophilized Form

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21.1 Rabies Virus and Rabies Vaccines

21.1.1 Rabies: Disease, Epidemiology, and Burden

Rabies is a viral zoonosis that occurs in more than 100 countries and territories, as shown in Fig. 21.1 [1]. There were 61,000 estimated cases of death from rabies in the world in 2010, according to the informal group Partners for Rabies Prevention. The estimated annual cost of rabies is US $6 billion, covering pre- and postexposure treatments and loss of productivity owing to the premature deaths of workers [2].

The infection usually occurs when an infected animal causes a transdermal bite or scratch in the victim. Transmission may also occur when infectious material, usually saliva, comes into direct contact with the victim's mucosa or with fresh skin wounds. The incubation period is typically 1–3 months, but it may vary from less than a week to more than a year [3].

Inoculated virus is transported to the central nervous system (CNS). Upon arrival in the brain, it replicates and disseminates rapidly in many different tissues including the salivary glands [3]. The acute disease includes pain or paresthesia close to the bite site and is often associated with fever, fatigue, and weakness in associated limbs. Nonspecific neurologic symptoms including headache and anxiety are often experienced before the development of encephalitis, and it is usually only at this point that patients seek medical help and admission to the hospital [4].

As the virus spreads through the CNS, progressive fatal encephalomyelitis develops, characterized by hyperactivity and fluctuating consciousness and, in cases of furious

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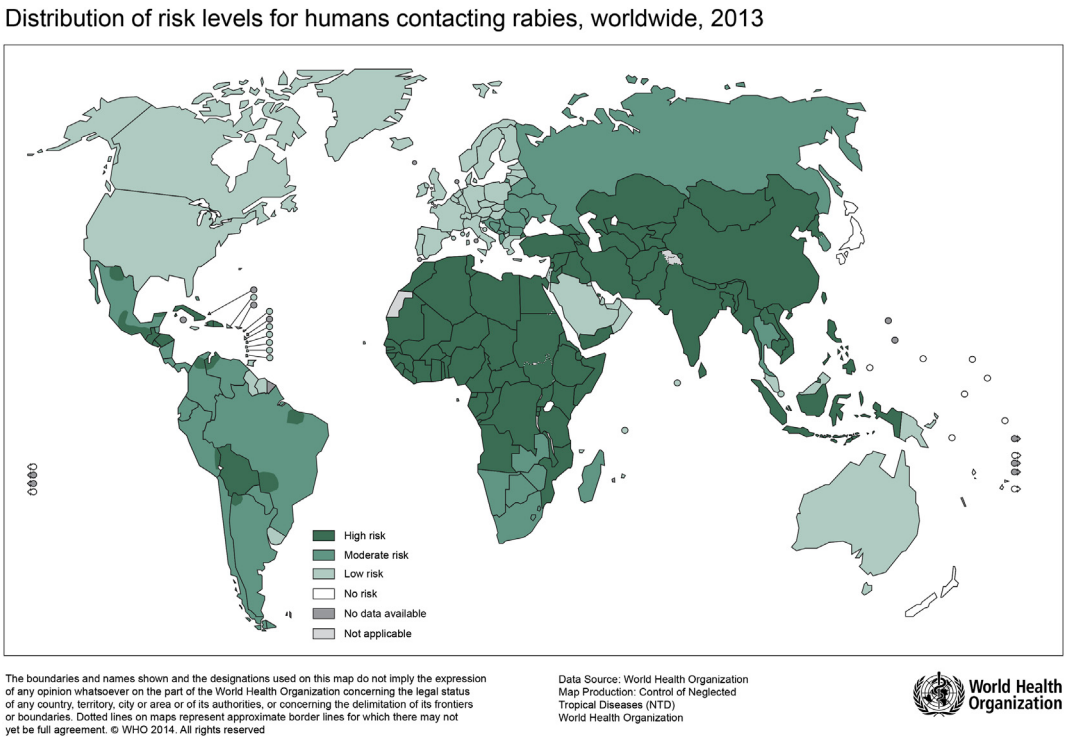

FIGURE 21.1 Rabies worldwide distribution and risk exposition [1].

rabies, hydrophobia, aerophobia, or both. Death occurs by cardiorespiratory arrest within a few days [3].

21.1.2 Rabies: Classification, Proteins, and Cell Infection

The rabies virus (RABV) belongs to the order Mononegavirales and family Rhabdoviridae. This family has three genera: *Vesiculovirus* (whose prototype is the vesicular stomatitis virus), *Ephemerovirus* (whose prototype is the bovine ephemeral fever virus), and *Lyssavirus* (whose prototype is the RABV) [5]. RABV and variants known as "rabies-related" virus belong to one of the 14 *Lyssavirus* genotypes, all of which are capable of producing a rabies-like encephalomyelitis [1].

The *Lyssavirus* stable infective particles (virions) have a bullet-shaped form with an average length of 180 nm and average diameter of 75 nm [6]. Lyssaviruses, like other Rhabdoviruses, consist mainly of RNA (2–3%), protein (67–74%), lipid (20–26%), and carbohydrate (3%) as integral components (percent total mass) of their structure [7].

The RABV genome is a nonsegmented, single-stranded RNA that has a negative-sense polarity. This implies that the minus-strand genome RNA, when free of protein, is not infectious for the victim's cells [8]. The genome analysis reveals the presence of five proteins: a

nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), an RNA polymerase RNA-dependent (L), and a glycoprotein (G), which carries the main antigenic sites [3,9].

The cycle of infection and replication of RABV can be summarized in the stages: (1) adsorption of virions to host cell, (2) penetration of the virion into the cell, (3) removal of protein−RNA complex proteins, (4) transcription of viral RNA, (5) translation of mRNAs, (6) processing of proteins, (7) viral RNA replication, (8) assembly of virions, and (9) budding [10].

In the virion morphogenesis, the proteins N, P, and L newly synthesized in the cytoplasm adhere to nascent viral RNA to form the nucleocapsid. The nucleocapsid is then stabilized by the M protein, which is responsible for giving the nucleocapsid its bullet-shaped morphology. The protein G, which requires glycosylation, is processed in rough endoplasmic reticulum and then goes to the host cell membrane. Finally, the nucleocapsid−protein M interacts with cytoplasmic membrane fragments that contain protein G to finish virion assembling and proceeds to budding of these new virion to spread the virus further [8,11].

21.1.3 Rabies Vaccines: Definition, Generations, and Use for Humans and Animals

A vaccine is any preparation intended to produce immunity to a disease by stimulating the production of antibodies. The most common method of administering vaccines is by injection. However some vaccines are given by mouth or nasal spray [12]. The first rabies vaccine was developed by Louis Pasteur and Emile Roux in 1885 [13].

Speaking specifically about rabies, there were three generations of vaccine production: the first generation used brains of rabbits, sheep, cows, horses, and other animals as substrate for virus cultivation. In 1955, Fuenzalida and Palacios described a vaccine using suckling mouse brain as substrate [14], and Peck and collaborators described a vaccine using duck embryonated eggs as substrate [15]. The second generation used cell cultures to produce rabies vaccine. In 1960, Fenje announced the first vaccine to be produced on hamster kidney tissue [16]. In the following years there were many reunions and congresses that debated research all over the world to standard the work, which enabled the development and then rapid commercialization of vaccines for veterinary use. This development benefitted from four favorable factors: (1) cell lines such as baby hamster kidney (BHK) were accepted for the production of vaccines for use in animals; (2) viral multiplication is excellent in these cells; (3) these systems permitted industrial scaling-up of the cultures; and (4) the addition of adjuvant was authorized for these veterinary vaccines [17]. The third generation of rabies vaccines is available. The vaccinia−rabies glycoprotein recombinant vaccine is currently applied on a large scale in some European countries for the immunization of wildlife. The canarypox recombinant vaccine has been considered and successfully tested for human immunization [18]. However it is first necessary to understand the differences between vaccines for human and veterinary use.

21.1.3.1 Rabies Vaccines for Human Use

Vaccines for human use have existed for more than 40 years and can be obtained as (1) nerve tissue vaccines and (2) concentrated and purified cell culture or embryonated egg-based vaccines (CCEEVs) [2].

Nerve tissue vaccines have a series of disadvantages compared with CCEEVs, such as the induction of more severe adverse reactions and less immunogenicity. Because of these, the World Health Organization (WHO) recommended discontinuation of nerve tissue vaccines for human use in 1984 and their replacement by CCEEVs. Many developing countries followed this recommendation, including Brazil, Bangladesh, China, Chile, India, Indonesia, Mexico, Paraguay, and Thailand. Other countries such as Ecuador, Peru, Myanmar, and Pakistan are investigating affordable ways to substitute nerve tissue. Nevertheless in a few countries, mainly in Asia and Latin America, populations at high risk for rabies still depend on nerve tissue for postexposure prophylaxis [2].

CCEEVs have proved to be safe and effective against RABV and are used for pre- and postexposure prophylaxis; they have been administered to millions of people across the globe [19]. CCEEVs contain RABV that has been propagated in cell substrates such as: (1) human diploid cells; (2) Vero cells; (3) primary chick embryo cells; and (4) embryonated duck eggs. Chick embryo and Vero cells are comparatively safe and effective; however the others are less expensive. In the process, after growth in cell culture (or embryonic egg), the viral harvest is concentrated, purified, inactivated, and lyophilized. To stabilize the vaccine, human albumin or processed gelatin can be used. These types of vaccines are made for individual dose by intramuscular injection and do not contain preservatives such as thimerosal. The result is a vaccine with more than 3 years of shelf life that must be stored at 2–8°C and protected from sunlight [2,19]. The WHO recommendations for characterization, production, and control apply only for inactivated rabies vaccines produced in cell culture or embryonated eggs [2].

The minimal acceptable potency of CCEEVs is 2.5 international units (IU) per intramuscular dose, as determined in the mouse protection potency test [20]. That way, quick CCEEVs administration after exposure allied with proper wound management and simultaneous administration of rabies immunoglobulins is almost invariably effective in preventing rabies, even after high-risk exposure [19].

In general, CCEEVs are safe and well-tolerated. However some adverse effects may occur, depending on a series of factors. The most common adverse effects observed in vaccinated people are (1) minor, transient erythema, pain, or swelling occurring at the site of injection in 35–45% of patients; and (2) transient fever, headache, dizziness, and gastrointestinal symptoms in 5–15% of vaccinated people. Serious adverse events are rare and include Guillain–Barré syndrome [21].

CCEEVs provide immunologic memory that presumably persists for the life of the individual even after titers of neutralizing antibodies decline [2]. Some studies have indicated that periodic booster doses of vaccine are not required after primary rabies vaccination, except as an additional precaution for people whose occupation puts them

at continual or frequent risk of exposure [22,23]. However, the WHO recommends that even vaccinated individuals who are subsequently exposed to rabies should receive a short course of postexposure prophylaxis [2].

For special cases of exposure to RABV, the rabies vaccine must be complemented with rabies immunoglobulin [24]: (1) people with incomplete vaccination; (2) people with class III exposure (single or multiple transdermal bites or scratches, licks on broken skin, or contamination of mucous membrane with saliva from licks and exposure to bats); (3) immunocompromised individuals, such as patients with AIDS, with class II exposure (nibbling of uncovered skin, minor scratches, or abrasions without bleeding). For class I exposure (touching or feeding animals, licks on intact skin, or contact of intact skin with secretions or excretions of a rabid animal or human) there is no requirement for postexposure prophylaxis [2,22].

Three classes of immunoglobulins are available for passive immunization: human rabies immunoglobulin, equine rabies immunoglobulin, and highly purified F(ab′)2 fragments produced from equine immunoglobulin [25]. Rabies immunoglobulin should be given along with the first dose of vaccine in and around the wound site. Human immunoglobulin should be given at 20 IU/kg body weight, whereas equine immunoglobulin requires 40 IU/kg body weight because it has a shorter half-life in humans. Equine immunoglobulin is considerably less expensive than the human product, and most new equine preparations are potent, highly purified, and safe, with few adverse events [2].

21.1.3.2 Rabies Vaccines for Veterinary Use

Veterinary vaccines have been developed for use against rabies in domestic mammals and wildlife. Vaccines should be administered by or under the supervision of a competent person such as a veterinarian, according to the producer's recommendations. Three types of vaccines are used for domestic animals: (1) live replication-competent vaccines for oral use; (2) injectable modified-live virus vaccines; and (3) injectable inactivated vaccines [2].

Live vaccines for oral use may be appropriate under specific conditions, although the parenteral route is preferred for dog vaccination. The oral route should be used as a complement to parenteral mass vaccination campaigns, to improve vaccination coverage of the dog population by targeting individuals inaccessible for injectable vaccines, such as stray dogs and isolated dogs. Because oral rabies vaccines are costly and safe distribution tends to be time-consuming, the cost–benefit ratio of administering these vaccines to dogs should be carefully assessed. The most common use of oral vaccines is to immunize wild animals. In this case, the liquid vaccine usually contained in a sachet or blister pack should be incorporated in bait, preferably adapted to the target species with regard to taste, size, and texture [2].

Modified-live vaccines are produced from a modified egg-adapted strain of virus serially passaged in embryonated chicken eggs and from strains adapted to cell culture. Examples of this vaccine are those made with virus adapted to cell culture, such as the

SAD and ERA strains. These vaccines can cause rabies in vaccinated animals; therefore their production and vaccination should be discontinued owing a lack of safety [2].

Injectable inactivated (killed) vaccines are the ones most used in domestic animal species because they are safe and less expensive than other kinds. Inactivated vaccines are produced in cell culture with either primary cells or continuous cell lines infected with an adapted strain of RABV. Inactivated rabies vaccines can be used in combination with bacterins such as *Leptospira* and other viral agents such as canine distemper virus and canine parvovirus. Combined vaccines currently available for cats include various antigens such as feline panleukopenia virus, feline calicivirus, and feline herpesvirus [2].

Speaking specifically about veterinary vaccine production in cell culture, after cell growth and virus production, it is necessary to inactivate the virus. β-Propiolactone (BPL), UV light, and acetylethyleneimine are used as inactivating agents, and BPL is the one most used [26]. Once inactivated, adjuvants are added to increase the immune response to the antigen. The most used are aluminum hydroxide, aluminum phosphate, and saponin; less commonly used are oil adjuvants [27,28]. Inactivated rabies vaccines are available in liquid or lyophilized form [2].

A case of study presented in Section 21.3 of this chapter shows an example of the freeze-drying process using different stabilizers.

21.1.4 Perfusion and Multiplicity of Infection

Animal cells grow substantially slower than most microorganisms and lack a protective cell wall; thus they are also more fragile. Therefore growth is limited to gentle culture conditions and relatively low cell concentrations. One way to increase cell concentration yet maintain gentle culture conditions is the perfusion method [29].

A perfusion culture is one in which waste medium is continuously removed from the culture and the displaced medium is replenished with fresh medium. The constant addition of fresh medium and elimination of waste products provides cells with the environment they require to achieve high cell concentrations and with higher productivity [29].

In the context of virus multiplication in cells, the concept of multiplicity of infection (MOI) must be explained. MOI is a term frequently used in virology that refers to the number of virions that are added per cell during infection. If one million virions are added to one million cells, the MOI is 1. If 10 million virions are added, the MOI is 10. Add 100,000 virions, and the MOI is 0.1 [30].

A high MOI is used when it is expected that every cell in the culture is infected. By contrast, low MOI is used when multiple cycles of infection are required. However, it is necessary to determine the virus titer to calculate the MOI [30].

MOI calculations establish some conditions. The most frequent is that all cells in a culture are identical concerning the ability to be infected. Deviations in size and surface properties can be disregarded for some cell cultures. However when it comes to multicellular animals there are substantial differences in cell types that will affect

susceptibility to infection. Under these conditions, it is experimentally difficult to determine how many virions infect different cells [30]. Perrin and collaborators tried to develop a rabies vaccine using BHK-21 cell suspension culture in a bioreactor with perfusion, with satisfactory results [31]. They used different MOIs for each stage of adaptation of the cells from monolayer to suspension: 0.003, 0.01, 0.1, and 0.3 cells^{-1}. Kallel and coworkers tested various serum and protein-free media to produce rabies vaccines. In spinners, the MOI selected was 0.1 cells^{-1}. In bioreactors the MOI was 0.1 or 0.3 [32]. Work with the same MOIs was conducted in Vero cells by Trabelsi and collaborators [33,34]. Guidolin and collaborators also tested two different MOIs (0.07 and 0.046 cells^{-1}) to produce rabies vaccines in BHK cells, and obtained satisfactory potency results [35].

21.2 Freeze-Drying: Process and Use in Vaccines

21.2.1 Lyophilization: Basic Process

Lyophilization, or freeze-drying, is defined as a stabilizing process in which the substance is first frozen and the quantity of the solvent is reduced by sublimation (primary drying). After, a secondary drying (desorption), the reduction achieves values that no longer support biological growth or chemical reactions, resulting in powder or a cake. The lyophilization process is composed of five phases: (1) formulation, (2) freezing, (3) primary drying submitted to vacuum, (4) secondary drying, and (5) closure of recipients [36].

The main principle involved in freeze-drying is sublimation, in which water passes directly from a solid state, in this case ice, to a vapor state without passing through the liquid state. The material to be dried is first frozen; after that it is subjected to heat under a high vacuum, using conduction and/or radiation, so the frozen liquid sublimes, leaving only dried components of the original liquid. The driving force for the removal of water during freeze-drying is the concentration gradient of water vapor between the drying front and the condenser [37,38].

Lyophilization is a multistage operation in which each step is critical. The major important characters of this scenario are all well-known and should be under strict control to achieve a successful operation: (1) the product, the active principle, which must keep its prime properties; (2) the surrounding medium with its bulking agents, stabilizers, antioxidants, cryoprotectors, lyoprotectors, and other possible constituents, which provide stabilization of the product; (3) the equipment, which must be flexible, reliable, and adapted to the desired use of freeze-drying (mass production of sterile/nonsterile drugs or ingredients, experimental research, and technical development); (4) the process, which has to be adapted to individual cases according to specific requirements and low-temperature behavior of the different products under treatment; (5) the final conditioning and storage parameters of the finished product, which will vary not only from one substance to another but also in relationship to its "expected

therapeutic life" and marketing conditions. In other words, a freeze-dryer is not a conventional balance; it does not perform in the same way with different products. There is no universal recipe for a successful freeze-drying operation, and "this material cannot be freeze-dried" can be said only after challenging the system with all and each successive step of the process [39].

An important part of freeze-drying is the energy involved in the process. Energy in the form of heat must be applied to the product to encourage the removal of water in the form of vapor from the frozen product. The heat must be carefully controlled, because applying more heat than the evaporative cooling in the system can warm the product above its eutectic or collapse temperature. Heat can be applied in the system in two ways: directly applied through the thermal conductor shelf, such as is used in drying; or using ambient heat, as in manifold drying [40].

Heat enters the products by one of several mechanisms: (1) direct contact between the container base and the shelf; (2) conduction across the container base and then through the frozen mass to the drying front (sublimation interface); (3) gaseous convection between the product and residual gas molecules in the chamber; and (4) radiation, which is low owing to the low temperature encountered in freeze-drying. Of all four mechanisms, convection is certainly the most important [41].

Before the freeze-drying process, there is a stage of sample preparation. Because the product that will undergo lyophilization may be a solid, liquid, paste, or emulsion, great care must be taken not to impede its fundamental properties [39]. The sample preparation may include product concentration, formulation revision by the addition of components to increase stability and/or improve processing, or a decrease in high–vapor pressure solvent or increase in the surface area. To achieve those goals, several methods can be used: freeze concentration, solution phase concentration, formulation to preserve product appearance, formulation to stabilize reactive products, formulation to increase the surface area, and a decrease in high–vapor pressure solvents [37].

The basic freeze-drying process is divided into three main stages: freezing, primary drying, and secondary drying. There is also an optional stage, the annealing stage.

21.2.1.1 Freezing Stage

The first step in lyophilization is to freeze samples that are aliquotted into containers. The containers are placed on a freeze-dryer shelf and the samples are either cooled from ambient temperature or cooled rapidly on a prechilled shelf [41].

Freezing is a critical period because the microstructure established by the freezing process usually represents the microstructure of the dried product and the product must be frozen to a low enough temperature to solidify completely. Two types of constituents in a solution will be cooled: the major part of products that are subjected to freeze-drying consist primarily of the solvent, usually water, and the solute, the materials dissolved or suspended in the solvent [37].

During this critical stage, all fluids in the sample become solid bodies, crystalline, amorphous, or glass. Usually water results in a complex ice network, but it might also be

imbedded in glassy structures or remain more or less firmly bound within the interstitial structures. This process is critical because two processes can occur at the same time: the solutes concentrate and might crystallize; the sample undergo volumetric expansion that might induce mechanical stresses that combine with the osmotic shock owing to the increasing concentration of interstitial fluids [39].

The freezing point of a sample can be determined using four methods: (1) the theoretical thermodynamic value, (2) cryomicroscopy, (3) differential scanning calorimetry (DSC), and (4) measurement of temperature and resistance. The most common method is DSC [42].

The freezing method and the final temperature of the frozen product can affect the posterior freeze-drying of the sample. Rapid cooling results in small ice crystals, which is useful in preserving structures to be examined microscopically; however the product becomes more difficult to freeze. Slower cooling results in large ice crystals a lower restrictive channel in the matrix during the drying process [37,39].

Products freeze in two ways, depending on their constitution. First there are eutectic mixtures, which are mixtures of substances that freeze at a temperature lower than the surrounding water. The lowest possible melting temperature overall of the mixing ratios for the involved component species is called the eutectic temperature; in other words, a product will be frozen only when all of the solute suspension is properly frozen, which is achieved only when the eutectic temperature is reached. It is important to freeze the product to below the eutectic temperature before beginning the freeze-drying process [37,42].

The second type of frozen product is a suspension that undergoes glass formation during the freezing stage, which happens to polymers rather than eutectic mixtures. In this case there is a concept called the glass transition temperature (Tg). For each polymer or polymer mixture, there is a different Tg, and when the sample is cooled below this temperature, it becomes hard and brittle, like glass. Above the TG the product retains its amorphous form. When freeze-drying occurs below the Tg the entire suspension becomes increasingly viscous as the temperature is lowered. Finally the products freeze at Tg, forming a vitreous solid, which makes the freeze-drying process extremely difficult [43,44].

21.2.1.2 Annealing Stage

Once the product has been frozen, the drying process can be facilitated by an additional annealing process. Annealing is a holding step at the Tg. Annealing can be carried out as a way point during the initial cooling, but more commonly it is a postfreezing warming and holding step, followed by recooling. The freezing step and any postfreezing temperature deviations above Tg will determine the texture or morphology of the product [45].

Annealing is an optional step occasionally used to crystallize the formulation component. If the solute separates out in crystalline form, it is known as the eutectic temperature. In contrast, if an amorphous form is formed, the temperature is referred to

as the Tg. Determination of this critical temperature is important for the development of an optimized lyophilization cycle [37,41].

The annealing process is most beneficial in formulations with amorphous components with relatively low Tg values and that require long drying times, or when crystalline components are not completely crystallized. The annealing process in not good for the freeze-drying process in all cases and its use must be evaluated for effectiveness because when excipients crystallize during increases in temperature, they can eliminate their stabilizing effects. That is the reason for the optional choice of this stage [41].

21.2.1.3 Primary Drying Stage

After the product is frozen, the pressure within the freeze-dryer is reduced using a vacuum pump to provide conditions under which ice can be removed from the frozen samples via sublimation, resulting in a dry, structurally intact product. To achieve good primary drying, it is necessary to optimize the balance between the temperature and the pressure inside the chamber [42].

In pharmaceuticals, for example, the chamber pressures range from 30 to 300 mTorr, depending on the desired product temperature and characteristics of the freeze-dryer. The chamber pressure needs to be lower than the vapor pressure of ice to ease sublimation of ice and the transport of water vapor to the condenser where it is deposited as ice, a phenomenon called cold trap. If the chamber pressure exceeds the vapor pressure at the sublimation interface, mass transfer stops. However, if the chamber pressure is too low, the heat transfer to the product is limited, resulting in slower sublimation rates [37,42].

During this stage, frozen samples are maintained below the product collapse temperature and the eutectic melting temperature. The product collapse temperature is highly influenced by the Tg and is unique for each formulation. Below the Tg, the amorphous phase exists as glasslike material, inhibiting large-scale molecular movement. Therefore, sublimation efficiency during the primary drying stage is maximized by operating as close to Tg as possible yet below that temperature. If the Tg is exceeded during the primary drying phase, it can result in cake collapses, which in turn can lower the rate of water sublimation/desorption, cause physicochemical changes in the product and resulting in an inferior product. Drying just below the Tg (at least 2−5°C) not only increases the efficiency of the primary drying phase, but also prevents cake collapses and results in more uniform distribution of bound water at the completion of the lyophilization process [41,46].

The end of the primary drying stage is indicated by a visible temperature increase that occurs upon completion of sublimation of water from ice. Although a large percentage of the water is sublimed during the primary drying phase, approximately 20−50% of more tightly associated solvent water is still present in the dried cake [41,47].

Because primary drying cycles can require more than a week to complete, the choice of temperature and sublimation rate needed to avoid cake collapses must be balanced with practical and economic considerations [41].

21.2.1.4 Secondary Drying Stage

After primary freeze-drying is complete and all ice has sublimed, in the area where ice has been removed, desorption of water from the cake occurs. This process is called secondary drying and has started in the primary drying phase. The product appears to be dry but the residual moisture content may be as high as 7−8%. Continued drying is necessary at a warmer temperature to reduce the residual moisture content to optimal values. This process is called isothermal desorption because the bound water is desorbed from the product. It is not an easy task because overdrying might be as bad as underdrying. For each product appropriate residual moisture has to be reached under given temperatures and pressures [37,39].

Secondary drying is normally continued at a product temperature higher than ambient but compatible with the sensitivity of the product, typically maintaining the product at a temperature between 20°C and 40°C for several hours. In contrast to processing conditions for primary drying, which use low shelf temperature and a moderate vacuum, desorption drying is facilitated by raising the shelf temperature and reducing the chamber pressure to a minimum if it is not already low enough. At this stage, it is possible to increase the shelf temperature because the product has an increased thermal stability and a higher Tg owing to previous dehydration. However, the temperature must not be increased rapidly, so the ramp rate to the secondary drying temperature needs to be moderate (0.1−0.3°C/min) for amorphous substances to avoid surpassing the Tg of the lyophilized cake and avoid cake shrinkage [37,41,42].

Also, water remaining during secondary drying is more strongly bound, and thus requires more energy for its removal. Decreasing the chamber pressure to the maximum attainable vacuum has traditionally been thought to favor desorption of water. The rate of desorption and the obtainable moisture level are controlled by diffusion within the solute phase and desorption from the surface, and therefore mostly depend on product temperature [37].

The duration of the secondary drying phase is dictated by the desired residual moisture level in the freeze-dried product. This residual moisture is a small portion of strongly bound water molecules that stabilize the product by hydrogen bonding interactions. Usually higher residual moisture levels translate to an increase in the product degradation rate because sorbed water can promote molecular mobility and increase product deterioration, both chemically and physically. Therefore, staying within a specified range of residual moisture levels can be vital to maintaining a desirable level of product integrity [41,48].

21.2.2 Components of a Freeze-Dryer and Methods of Freeze-Drying

The essential components of a freeze-dryer are the chamber, shelves and shelf fluid system, condenser, refrigeration system, vacuum system, and control system.

The chamber is a vacuum-tight box that contains a shelf or shelves for processing the product and that can also be fit with a stoppering system. It is typically made of stainless

steel and is usually highly polished on the inside and is insulated and clad on the outside. The door-locking arrangement can be made with a hydraulic or electric motor [49].

The shelves have an elaborate design because of the several functions it performs. Shelves act as heat exchangers, removing energy from the product during freezing and supplying energy to the product during the primary and secondary drying segments of the freeze-drying cycle. Depending on the type of freeze-dryer, it can have one or more shelves and can be manufactured in sizes up to 4 m^2 in area [50].

Energy exchange demanded by the freeze-drying process during the drying stages of the cycle is traditionally done by circulating a fluid through the shelves at a desired temperature. Temperature is set in an external heat exchange system consisting of cooling heat exchangers and an electrical heater. Fluid circulated is normally silicone oil and the shelves are connected to the silicone oil system through fixed or flexible hoses. This is circulated at a low pressure in a sealed circuit using a pump [51].

The condenser serves as a cold trap. It is designed to trap the solvent, usually water, during the drying process. The process condenser consists of coils or sometimes plates that are refrigerated to the desired temperature. These refrigerated parts may be in a vessel separate from the chamber (external condenser) or they may be located within the same chamber as the shelves (internal condenser). For an internal condenser the refrigerated coils or plates are placed beneath the shelves on smaller machines and behind the shelves on larger machines, although the position of the condenser does not affect the trapping performance [52].

Concerning the refrigerating system, the product to be freeze-dried is either frozen before it is put into the dryer (prefreezing) or frozen while on the shelves; either way it requires a considerable amount of energy. Compressors (or sometimes liquid nitrogen) supply cooling energy. Because the compressor may perform two tasks, cooling both the shelves and the condenser, usually multiple compressors are needed [37].

A vacuum must be applied to remove the solvent in a reasonable time during the drying process. To achieve a low vacuum, a two-stage rotary vacuum pump is used. Typical mechanical vacuum pumps used in freeze-dryers are oil lubricated; however oil-free mechanical pumping systems are available. For large chambers, multiple pumps may be used. The vacuum pumping system, in conjunction with the condenser system, provides the necessary pressure to conduct the primary and secondary drying processes [36,37].

Control may be entirely or usually fully automatic for production machines. The control elements required are shelf temperature, pressure, and time. A control program sets these values as required by the designed process. The time may vary from a few hours to several days. Other data such as product temperatures and process condenser temperatures can also be recorded and logged [37].

Three methods of freeze drying are most commonly used: manifold, batch, and bulk [53].

In the manifold method, flasks ampoules or vials are individually attached to the ports of a drying chamber. The prefrozen product is quickly attached to the drying

chamber or manifold to prevent warming. The vacuum must be quickly created in the product container and the operator relies on evaporative cooling to maintain the low temperature of the product. This procedure can be used only for relatively small volumes and product with high eutectic and collapse temperatures. Heat input can be affected simply by exposing the vessels to room temperature or via a circulating bath [42].

In batch drying, large numbers of similar-sized vessels containing the same product are placed together in a tray dryer. The product is usually prefrozen on the shelf of the tray dryer. Precise control of the product temperature and the amount of heat applied to the product during drying can be maintained. Vials located in the front portion of the shelf may vary in terms of heat exchange from the vials in the center of the shelf. These slight variations can result in small difference in residual moisture. Batch drying allows closure of all vials at the same time under the same atmospheric condition, ensuring a uniform environment in each vial and uniform product stability during storage. The vials can be stoppered in a vacuum or after backfilling with inert gas. Stoppering the vials under a slightly reduced pressure of dry nitrogen gas seems to be the favorite option [39,42].

Bulk drying is generally carried out in a tray dryer as in batch drying. However the product is poured into a bulk pan and dried as a single unit. Although the product is spread throughout the entire surface area of the shelf and may be the same thickness as the product in the vials, the lack of empty spaces within the product mass changes the rate of heat input [42].

Manifold drying has some advantages over batch tray drying. Because the vessels are attached to the manifold individually, each vial or flask has a direct path to the collector. This removes some of the competition for molecular space created in a batch system and is most ideally realized in a cylindrical drying chamber, where the distance from the collector to each product vessel is the same. However, for some products in which precise temperature control is required, manifold drying may not be suitable. In addition, batch drying is used to prepare large numbers of ampoules or vials of one product and is commonly used in the pharmaceutical industry [42].

21.2.3 Freeze-Drying Applications

The most extensive use of the lyophilization process is involved in the health-care industry. This includes the lyophilization of pharmaceuticals such as chemical compounds, parenteral formulations, vaccines, and in vivo and in vitro diagnostic products. Included in this category are biotechnology products that are mainly protein based [36].

Another common use of lyophilization involves veterinary products. These products range from vaccines for individual household pets to large-scale applications such as the inoculation of herds of cattle and sheep or large flocks of poultry. These lyophilized products serve not only to protect animals and the investment of the producer but also to improve the quality of the product. These products also protect the consumer from diseases that may be transmitted by the consumption of animal products [37].

Freeze-drying is commonly applied to foods as well. The most widely known freeze-dried food product is coffee. However, freeze-dried coffee is not a lyophilized product in the true sense. Other freeze-dried food products may be used as additives, such as berries in breakfast cereals, or are complete products that eliminate the need for refrigeration, such as ice cream that can be eaten without reconstitution. The process has been popularized in the form of freeze-dried ice cream, an example of astronaut food, and is also applicable for products in which weight can be a factor, such as in backpacking. These freeze-dried meals, such as freeze-dried beef, require reconstitution by the addition of water and enhancement of flavor by heating the product. Except in special cases such as freeze-dried coffee, freeze-dried foods cannot compete economically with frozen or canned food products [36].

In chemical synthesis, products are often freeze-dried to make them more stable or easier to dissolve in water for subsequent use. In bioseparation, freeze-drying can also be used as a late-stage purification procedure, to remove solvents. Furthermore, it is capable of concentrating substances with low molecular weight that are too small to be removed by a filtration membrane [37].

Other applications of lyophilization include the freeze-drying of floral products, taxidermy, and the recuperation of wet books and manuscripts. There has been only limited success in the freeze-drying of floral products: only the flower portion of the plant appears to respond to the drying process, and there is often a slight change in color. Freeze-drying has been applied to the taxidermy of small animals. Many small mammals such as squirrels and raccoons have been successfully freeze-dried to provide specimens with a lifelike appearance. Despite success with this technique, it is currently used on a limited basis. Freeze-drying has been instrumental in the rescue of precious manuscripts, books, and documents that incurred water damage as a result of a fire or a flood. By quickly freezing all of the documents and then freeze-drying the documents in batches, many documents and valuable manuscripts have been restored to nearly their original condition [36].

21.2.4 Freeze-Drying Use for Vaccines

Freeze-drying is an established process to improve the stability of labile drugs, especially proteins and complex vehicles such as viral vaccines, viral vectors, liposomes, and lipid–DNA complexes. However, during freezing and subsequent drying, the drug is exposed to diverse stress factors that can cause a significant loss of activity. During freezing, drug stability can be influenced by exposure to ice–water interfaces, salt and drug concentration effects, pH shifts owing to selective crystallization of buffer species, and mechanical damage by growing ice crystals. During drying, removal of stabilizing hydration shells can influence the stability of the drug, to mention only the most common known degradation causes [54,55].

Currently, most vaccines must be stored at refrigerated temperatures or stored as frozen formulations because of progressive loss of antigen thermal stability. To sustain

vaccine activity, a cold chain ranging from −22°C to 8°C is usually required to maintain vaccine temperature below that of ambient from the location of manufacture to the site of vaccine administration, using refrigerated trucks to distribute the vaccines. Unfortunately, in many remote areas of the world it is not technologically possible to maintain the cold chain temperature because it is error-prone, logistically difficult, and expensive. In those areas the lack of refrigerators, power failures, and other problems can expose vaccines to elevated temperatures for several weeks, which can ultimately make the vaccine lose its therapeutic properties [41].

Lyophilization has permitted the development of vaccines that would otherwise be too unstable for effective use, and approximately one-third of vaccines in use today are prepared using lyophilization. Although it has been effective in certain cases, this technique has several drawbacks:

1. the process does not guarantee increased stability;
2. many lyophilized vaccines still require storage at 4−8°C; the rabies vaccine is one of them;
3. many adjuvants included to enhance immunogenicity cannot be frozen, such as aluminum salts;
4. lyophilized formulations must be reconstituted and are therefore more complicated to administer;
5. clinicians prefer a liquid product for safety and ease of administration;
6. the freeze-drying process is expensive and laborious; and
7. lyophilization requires complex processing, which can lead to antigen instability [41].

Problems concerning vaccine stability are far from solved. The main reason for these problems is a lack of understanding regarding the mechanisms involved in the thermal degradation of vaccines, both in solution and in the dried state. Dried or not, the major barrier to the development of more thermally stable vaccines is a lack of detailed information regarding the mechanisms by which vaccine potency is lost. In most cases there is no information about the molecular basis of the observed instabilities. In contrast, considerably more information is available regarding the mechanisms of inactivation for pharmaceutical peptides, proteins, and DNA than for common viral- and bacterial based vaccines. A better understanding of actual mechanisms of inactivation of specific vaccines should permit a much more rational approach to developing thermostable vaccines by systematic formulation or through alterations in the intrinsic nature of the vaccine entity itself [41].

Ideally, successfully lyophilized vaccines have a uniform appearance with no obvious signs of cake collapse and less than 2% residual moisture, that can be safely stored at ambient temperatures for 1−2 years and are quickly and easily reconstituted to an active solution. A few vaccines consist of relatively “simple” homogeneous components. Others, however, more typically contain combinations of proteins, polysaccharides, nucleic acids, and/or lipids, arranged in a viral or bacterial macromolecular complex.

In large part because of their natural ability to generate a robust immune response that is optimally protective, viral particles or bacteria make up approximately half of available vaccines. The current vaccine thermal instability problem, however, most often lies within these macromolecular complex-based vaccines. For this reason, vaccine component stability will first be addressed in terms of these individual macromolecular components [41].

Therefore to ensure drug stability in the freeze-drying step and subsequent storage, stabilizing excipients have to be employed. Cryoprotectants such as salts, polyols, and sugars are used to stabilize the solution therapeutic in the freeze-thawing process. Lyoprotectants, especially the disaccharides sucrose and trehalose, can be used to stabilize the drug during drying. In addition, bulking agents such as mannitol can be added to enable faster drying times and maintain lyophilisates with an attractive appearance [54,55]. Hubálek described a list of different cryoprotectors used in microorganism conservation [56]: sulfoxides; alcohols and derivatives; saccharides and polysaccharides; amides and imides; heterocyclic compounds; amino acids, proteins, peptides, and polypeptides; complex compounds; and surfactants.

Disaccharides such as sucrose and trehalose are inert and have been used to stabilize liposome, protein, and viral formulations. Excipients such as mannitol, sucrose, glycine, glycerol, and sodium chloride are good tonicity adjusters. Amino acids such as glycine can lower the Tg if it is maintained in the amorphous phase [37].

21.2.5 Advances and Alternatives in Freeze-Drying

21.2.5.1 Advances

Freeze-drying is a process that has been used for decades. Yet there still plenty of room for improvement in many areas. The main objectives are related to equipment improvement, process improvement, and standards adoption.

The equipment has an important role in the lyophilization process; therefore equipment manufacturers should be held accountable for the quality and performance of their equipment [57]. Instead of worrying about the software, enterprises must think about improvements in freeze-dryer design and instrumentation. Regarding the drying chamber, the freeze-dryer can be enhanced in chamber design/configuration, construction materials, finishing materials, insulation, leak prevention, design and construction of doors and handles (to prevent the gorilla effect), shelves construction and spacing, heat transfer and the fluid chosen for this purpose, gas conductance, and shelf temperature. With regard to cleaning, choices are manual, cleaning-in-place, and flooding. Related to the condenser, there is room for improvement in plate and coil configuration, capacity, temperatures, isolation valves, and use of external and internal condensers. Regarding the vacuum system, the foreline (connection between the vacuum pump and the chamber) design and prevention of backstreaming of oil vapors derived from the vacuum pump are important [57].

When process improvement is mentioned, the main objective is to exit the trial-and-error process design to enter rational vaccine design. More effort must be made to include the lyophilization aspects of a formulation as a part of the acceptance criteria [57]. The stability of several vaccines has greatly benefitted from the development of lyophilized formulations; however a significant number of vaccines have yet been successfully stabilized by this approach [41]. Ideally, vaccines should follow some stages so as to be well developed: preformulation (characterization) of the active ingredient, formulation design and accelerated or normal stability testing, and formulation process development. A variety of techniques exist to characterize vaccine formulations physically, but some are more informative and easily accessible than others. On the other hand, a variety of hydrodynamic, spectroscopic, and calorimetric techniques are readily available for routine characterization of vaccine moieties that can provide a multidimensional picture of a vaccine that is potentially sensitive to stability changes when combined [41]. The use of each technique depends on the desired final product and the inherent need of each active ingredient.

In addition, it is time for all parties involved in the lyophilization process to adopt statistical standards and standard methods that will be applicable to the lyophilization process. Such standards, if effective, will lead to improved quality of lyophilized products, a reduction in costs, and a general increase in productivity. They will also increase the sale of freeze-drying equipment because of greater confidence in the process and its associated equipment. The ultimate goal is to develop standards of confidence in assessing the performance and quality of a product that will, in time, eliminate the need for a regulatory agency. The public is better served by an industry that can regulate itself rather than one that has to be closely regulated by a government agency [57].

Inside the lyophilization world there is even room for unconventional improvements and changes. All lyophilization operations have been carried out with aqueous solutions although other solvents can sublimate from the frozen state, including mineral solvents such as ammonia and carbon dioxide, and organic solvents such as diethyl amine, chloroform, cyclohexane, dimethylsulfoxide, and benzene [39]. However the use of other solvents must be studied before their application as vaccines because of the toxicity some of them can bring to the products.

Other efforts are used to change the batch system to a continuous system. For more than 65 years, freeze-drying has been almost exclusively done within the pharmaceutical and biological industries using batch operation. In contrast, the food industry, which had to face large-scale production, progressively shifted from batch to semicontinuous and even purely continuous operations. The results of adopting a continuous method are a substantial increase in productivity and a more homogeneous product [39]. In addition, the dimensions and weight of a continuous freeze-dryer are smaller compared with freeze-dryers used for batch drying, and the losses in case of malfunctioning are smaller in continuous freeze-dryers [57].

Finally, Worral and collaborators developed a method called Xerovac, in which the liquid vaccine formulation is placed on preheated shelves (at 40°C) of a conventional

freeze-dryer, reducing the pressure to 600 Torr [58]. When evaporation begins, the product temperature decreases and the pressure inside the chamber is carefully controlled to maintain the product temperature above the freezing point during the entire evaporation process. The method is used for live attenuated Rinderpest and Peste des Petits ruminant vaccines. Under these conditions viruses are capable of resisting 45°C for 14 days with minimal loss of potency. The degree of thermotolerance was similar to current thermostable freeze-dried vaccines but provided a shorter, cheaper, and simpler process in the absence of the potentially harmful freezing step [41,58].

21.2.5.2 Alternatives

There are alternatives to producing dried products rather than freeze-drying; mainly spray-drying and spray–freeze drying. Both methods require the liquid formulation to be sprayed through a nozzle to produce droplets within a desired size range. For spray-dried formulations, these liquid droplets are sprayed into a high-temperature environment to facilitate evaporation. One major advantage of spray-drying with respect to vaccines is the potential to create dried formulations of aluminum adjuvant-containing vaccines. It is also possible that the production times and product uniformity for dry powder delivery formulations could be significantly more desirable than those for standard lyophilized formulations [41].

An improvement in this method is nano spray-drying, which has been used to manufacture a dry powder for inhalation containing ketoprofen lysinate, a nonsteroidal antiinflammatory drug able to control inflammation in patients with cystic fibrosis. This method was able to obtain a free-flowing powder with satisfying aerosol performance. Different from a standard spray-drying apparatus characterized by a pneumatic nozzle, in this case liquid feed droplets are generated by a piezoelectric system, vibrating a thin stainless-steel membrane [59].

Spray–freeze dried products require the droplets to be sprayed into a low-temperature bath (using liquid nitrogen) to freeze the droplets. The frozen particles are then dried in a standard freeze-dryer [41]. Uses of spray–freeze drying include pulmonary, colony, nasal, and ophthalmic drug deliveries, needle-free intradermal injection, and apparent solubility enhancement [60].

In addition to the challenges mentioned previously for standard lyophilization and dehydration techniques, these spraying technologies include the introduction of new air–liquid interfaces that could lead to macromolecular structural perturbations. Other challenges include the extreme high and low temperatures necessary to dry or freeze the liquid droplets effectively, which have the potential to be damaging; and degradation of macromolecules owing to increased surface areas. The high surface areas of the spray-dried products may not consistently lend themselves to the production of stable dried vaccines when antigens are particularly labile, and there is a possible need to dry-fill vials as a result of manufacturing such formulations, which could be a costly and wasteful process [41].

Also, there are other drying techniques with a potential not yet realized, such as air-drying, foam vacuum-drying, microwave vacuum-drying, supercritical fluid co-nebulization, and supercritical fluid co-precipitation, which could find applications in the preparation of more stable vaccines [41].

Air-drying is the most common method but it has the disadvantage of need longer drying periods and higher drying temperatures [61].

Supercritical-assisted nebulization has been used successfully to develop a stable dried powder formulation from aqueous protein solutions [63].

In microwave vacuum-drying, the heat is not transferred to a material. Instead, the material is induced to heat itself by directly transforming the electromagnetic energy into kinetic molecular energy [61]. Advantages of this method are a reduction in drying time, lower energy demand, and a high-quality product. Major uses of microwave vacuum-drying are pharmaceutical and food applications such as the drying of carrots [63] and potatoes [64]. This technique also can be used for fine chemicals in the chemical industry and semiconductor manufacturing [61].

21.3 Case Study

In the first two sections of this chapter, a review was presented concerning the RABV, the mechanisms behind cell infection with such a virus, and how this can be used to produce rabies vaccines. In addition, the reviewed explained how the lyophilization process occurs and how it can be used to produce vaccines in the freeze-dried form, thus enhancing their stability and shelf life.

To exemplify the matters explained in the first part of the chapter, an experiment was conducted to test different inocula of RABV in cell culture, to learn a bit more about MOI and the importance of an equilibrated amount of virus in the moment of infection to ensure good virus production, therefore producing a good vaccine, in this case for veterinary use.

After evaluating the results of the different MOIs tested, the harvests collected from the vessel with the best result were inactivated and used to formulate 12 different combinations of excipients that were freeze-dried using the same protocol, to study the effects of different excipient classes in the final product. The freeze-dried vessels were also evaluated.

21.3.1 Technological Aspects

To perform the experiment, it was necessary to follow a series of stages to achieve the objectives. Fig. 21.2 shows a scheme of all of the stages during the experiment.

21.3.1.1 Virus Infection Study

The medium used to grow BHK-21 was composed of a combination of Dulbecco's modified Eagle medium and Ham's F12 nutrient mixture medium, pH 7.8. The medium

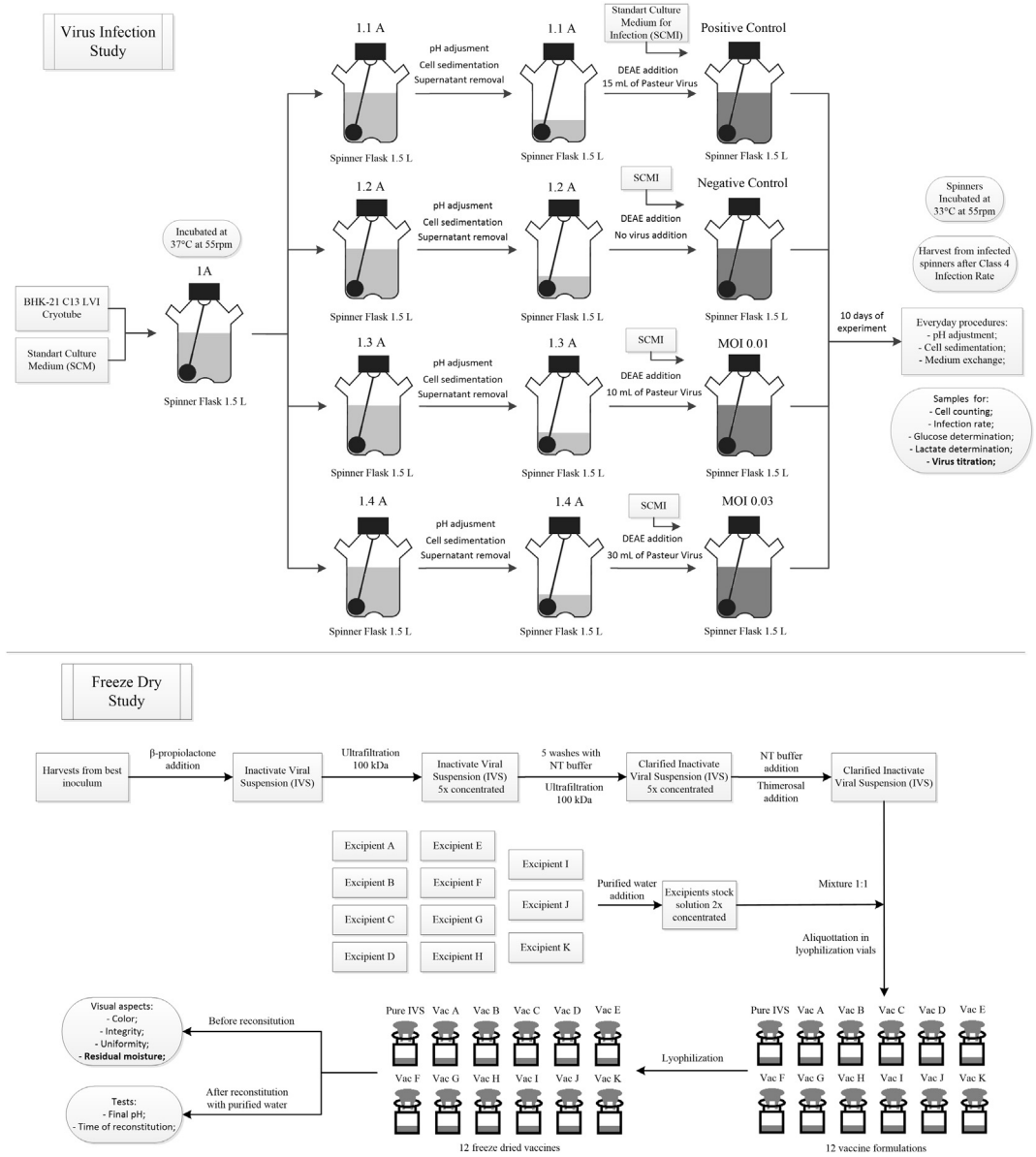

FIGURE 21.2 Scheme for case of study. The top part concerns the virus infection study and the bottom part concerns the freeze-dry study.

used before cell infection was called the standard culture medium (SCM) and was supplemented with 3% fetal bovine serum (FBS). After infection with virus particles, the medium used was called the SCM for infection (SCMI) and was supplemented with 1% FBS.

The cell line used was BHK-21C13 LVI [Pan American Centre for Foot-and-Mouth Disease (Panaftosa)] with 84 passages. During the experiments, the cell line had 118 passages.

The RABV strain was Pasteur virus adapted for the infection of BHK-21C13 cells; it had one passage. The strain came from Minas Gerais' Federal University. During the experiments, the virus from the work bank was used directly to infect the spinners.

The cells were counted in a hemocytometer. Readings were performed using an Olympus optical microscope with ×100 magnification. The cells were stained with 0.5% (w/v) Trypan blue at a dilution in which at least 20 cells could be counted per quadrant [65].

A Cryotube with BHK-21 cells from the work bank was thawed at 37°C and used as the cell inoculum in a 500-mL spinner (named 1A) with 80 mL SCM. The spinner was incubated at 37°C at 55 rpm agitation and cell counting was made every day. When it achieved at least 800,000 cells/mL, more medium was added to dilute the cells to 200,000 cells/mL and the spinner was incubated again. This proceeded until the spinner had 500 mL SCM.

After that, 250 mL of the first spinner was transferred to a 1.5-L spinner. SCM was added to this second spinner to complete 1.25 L, which left it with 200,000 cells/mL again; this second spinner was incubated at 37°C at 55 rpm. When the concentration reached 1 million cells/mL, the sample was divided into four spinners (1.1A, 1.2A, 1.3A, and 1.4A), each completed to 1.5 L with SCM. The four spinners were incubated under the same conditions. When the concentration of the four spinners reached at least 1 million cells/mL each, three of the spinners were infected with the virus strain.

The virus titer for the experiment was $10^{6.18}$ fluorescent focus-forming dose (FFD) per milliliter, titrated in a microplate. The MOI calculations were made using the formula shown in Fig. 21.3.

To study the effects of different initial virus concentrations in the spinners, three different MOI were selected for infection: MOI 0.01 and 0.03 FFD/cell and 1% of the total flask volume. The inoculum used corresponding to 1% of the total flask volume (for example, 15 mL in a 1.5-L spinner) was the positive control because of its recurrent use for rabies vaccine production on a bioreactor. Using the formula, it was calculated that the positive control had MOI 0.015 FFD/cell.

When 1 million cells/mL was reached in the spinners, the infection protocol was started. On day 0 of infection, the pH was adjusted to 7.4 with sodium bicarbonate 7.5% (w/v) and the spinner was placed in a cold room (2–8°C) for 2 h to sediment the cells. The supernatant was carefully removed from the spinner, leaving a final volume of

$$Vol_{inoculum} = \frac{MOI \times Cel_{Tot}}{Anti\log_{10} Tit}$$

FIGURE 21.3 Formula for calculating the correct volume for the infection of spinners. *Antilog Tit*, antilogarithm of virus titer (FFD/mL); Cel_{tot}, total number of cells, multiplying cell concentration for volume of the flask; *MOI*, multiplicity of infection selected (FFD/cell); $Vol_{inoculum}$, volume of inoculum required for the infection (in mL).

300 mL. Then diethylaminoethanol-dextran 0.5% (w/v) was added at 1% of the remaining volume to optimize adsorption of the viral particles in the cells. The spinner was incubated at 33°C in intermittent agitation: 20 min stopped and 5 min with 30–35 rpm agitation for 90 min.

The four spinners were categorized: spinner 1.1A became the positive control and received 15 mL virus; spinner 1.2A became the negative control and was not inoculated with virus; spinner 1.3A became MOI 0.01 and received 10 mL virus; and spinner 1.4A became MOI 0.03 and received 30 mL virus.

After inoculation, the volume was completed to 1.5 L with SCMI and incubated at 33°C at 55 rpm. The assay lasted 10 days after infection. The next days were called days postinfection (dpi). On each day, samples were taken for cell counting, infection rate, pH evaluation, and glucose and lactate determination, and two samples were frozen for subsequent virus titration. The pH was adjusted to 7.4 with 7.5% sodium bicarbonate and the spinner was placed in a cold room for cell sedimentation for 2 h. The maximum volume of medium was discarded without losing cells, and new SCMI was added to achieve 1.5 L again; then the spinner was incubated in 33°C at 55 rpm. From 3 to 8 dpi, the medium was harvest instead of discarded, placed in 2-L roller bottles, labeled, and stored at −80°C.

After 10 days of experiment, the growth curves for the four spinners were measured in terms of viable cells (Fig. 21.4).

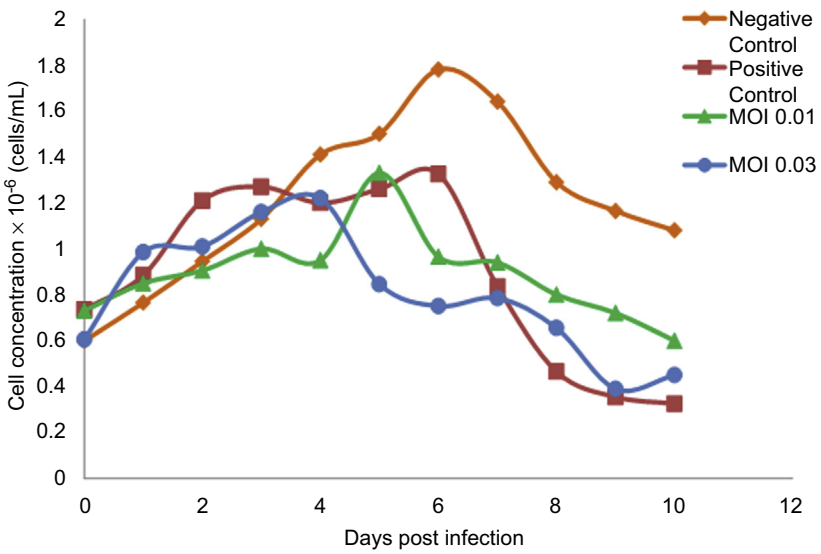

FIGURE 21.4 Cell growth curves obtained for cultivation of four spinners in development experiments. After 10 days of infection, the negative control of assay achieved maximal cell concentration of 1.78 million cells/mL (viable cells) at dpi 6. The positive control achieved maximal cell concentration of 1.33 million cells/mL (viable cells) at dpi 6. The spinner MOI 0.01 reached a maximum of 1.33 million cells/mL (viable cells) at dpi 5. The spinner MOI 0.03 achieved a maximum with 1.22 million cells/mL (viable cells) at dpi 4.

The growth peaks for the different infected cultures were different: 6 dpi for positive control, 5 dpi for MOI 0.01, and 4 dpi for MOI 0.03. The peak for MOI 0.03 was achieved more quickly, probably because the virus concentration was the highest and started to have an effect on the culture earlier than the others. Nevertheless, the infected cultures achieved similar peaks of concentration and the growth oscillations were practically constant.

Results for the four spinners were similar to those obtained by Kallel and collaborators, although the four spinners used a higher amount of medium volume. In addition, cultures with the same volume in the bioreactor had better cell production than the spinner did [32]. This happened because the bioreactors possess better control for temperature, dissolved oxygen, pH, and agitation. Thus using bioreactors with the same volume as that of the experiment (1.5 L) would increase cell production. In addition, using a perfusion system coupled with a bioreactor would have even better results.

After counting viable and total cells, it was possible to calculate the viability as a percentage. Viability was obtained as a percentage, dividing viable cells by total cells and multiplying by 100. From the results, the highest, lowest, and mean values for viability were obtained (Table 21.1). The viability of the negative control was highest because of the absence of the virus. Therefore cell concentration was limited by other factors such as the absence of gas, pH, temperature control, agitation, and accumulation of some metabolites and debris that could interfere in cell growth. The virus does not kill the cell because new virions are released outside by budding. Uninfected cells replicate normally, although one that is infected cell no longer replicates, just produces more virus until its death. Therefore, with a lower quantity of virus as inoculum, the tendency is to achieve better viability because the cells take a longer time to become infected and die. The spinner with MOI 0.01 had the best viability among infected spinners because of the lower quantity of virus inoculated.

Chapman and collaborators studied the viability of BHK-21 cells infected with RABV and kept at two temperatures, 33°C and 36°C [66]. MOI 0.05 was used in this experiment, corresponding to 1% of the final volume of the vessel, just as for the positive control. The viability of cells after infection at 36°C decreased from 3 dpi to almost 0% [66]. However, the viability of infected cells at 33°C remained above 90%, just as was found in this experiment.

Table 21.1 Rate of Cell Culture Viabilities for Four Spinners

Spinner	Highest Viability	Lowest Viability	Mean Viability
Negative control	98.3% (6)	88.2% (0)	95.4%
Positive control	96.2% (3)	66% (8)	87.7%
Multiplicity of infection 0.01	95.4% (7)	85.6% (8)	91.1%
Multiplicity of infection 0.03	97.5% (3)	75.7% (8)	90.8%

Numbers in parentheses are days postinfection when viability was achieved.

$$\mu = \frac{ln\,(X_n) - ln\,(X_{n-1})}{(t_n - t_{n-1})}$$

FIGURE 21.5 Formula for calculating specific growth rate of cell cultures. n and n^{-1}, two succeeded sampling points; μm, specific growth rate (h^{-1}); t, time (h); X, cell concentration (cells/mL).

The specific growth rate (μ) can be calculated with a formula adapted for cell concentration values obtained in the assay (Fig. 21.5). The specific growth rate measures the rate of cell multiplication and is calculated every day after infection.

A summary of the specific growth rate for the spinners is shown in Table 21.2. A characteristic of animal cells such as BHK-21 is that with optimized growth conditions there is a low specific growth rate, between 0.01 and 0.05 h^{-1}. μ is significantly lower than specific growth rates shown by microorganisms, which makes the industrial process costlier [67]. The four spinners achieved a μ_{max} compatible with the optimized cultures, although the spinners had poor process control, which means that the cultures grew satisfactorily.

Every day a sample of each spinner was taken in a microtube and centrifuged at 800 rpm for 5 min. Then the supernatant was injected in a YSI 2700 Select Dual-Channel Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH) to determine the residual glucose and lactate production concentration. The results for metabolite consumption are shown in Table 21.3.

Table 21.2 Maximum Specific Growth Rate for Four Spinners

Spinner	μ_{max} (h^{-1})	Days Postinfection of μ_{max}
Negative control	0.010	1
Positive control	0.013	2
Multiplicity of infection 0.01	0.014	5
Multiplicity of infection 0.03	0.020	1

Table 21.3 Minimum Glucose Concentration and Maximum Lactate Production for Culture Cells in Four Spinners During 10 Days of Infection

Spinner	Minimum Glucose Concentration	Maximum Lactate Concentration
Negative control	1.20 g/L (8)	2.33 g/L (8)
Positive control	2.63 g/L (6)	1.42 g/L (7)
Multiplicity of infection 0.01	2.04 g/L (5)	1.81 g/L (5)
Multiplicity of infection 0.03	2.78 g/L (8)	1.14 g/L (5)

Numbers in parentheses are days postinfection when viability was achieved.

Animal cells need glucose and glutamine as carbon sources. In glucose metabolism practically all glucose is converted into lactose. Thus, the relation between glucose consumption and lactate production represents an important parameter for process monitoring and provides information about the metabolic flow inside cells [67,68]. Cruz and collaborators studied the effects of lactate inhibition on BHK cells [69]. When lactate concentration achieved 28 mM (which corresponds to 2.52 g/L), cell growth was reduced [61]. Therefore, in none of the spinners did the lactate reach a limiting concentration. In addition, in none of the spinners was the glucose totally consumed.

The direct immunofluorescence test was adapted from Batista and collaborators [70]. The lamina was observed in an Olympus immunofluorescence microscope with ×100 magnification. Samples were evaluated in terms of fluorescent focus inside the cells. The results are listed in Table 21.4.

Table 21.5 summarizes results for the infection rate found in the three infected spinners. Fig. 21.6 shows examples of the classification of the infection rate. However the infection rate did not reflect the production of viruses inside the cells and the quality of viruses produced. Direct immunofluorescence is a tool for process control and classifies

Table 21.4 Classes and Requirements for Evaluate Direct Immunofluorescence

Class	Requirements
0	Total absence of fluorescent focuses
1	Form rare fluorescent focuses to 25% fluorescent focuses
2	Form 25–50% fluorescent focuses
3	Form 50–75% fluorescent focuses
4	>75% fluorescent focuses

Table 21.5 Infection Rates of Virus Inside Cells

Days Postinfection	Positive Control	Multiplicity of Infection 0.01	Multiplicity of Infection 0.03
1	Class 1	Class 1	Class 1
2	Class 3	Class 2	Class 4
3	Class 4	Class 4	Class 4
4	Class 4	Class 4	Class 4
5	Class 4	Class 4	Class 4
6	Class 4	Class 4	Class 4
7	Class 4	Class 4	Class 3
8	Class 3	Class 4	Class 3
9	Class 3	Class 4	Class 3
10	Class 3	Class 4	Class 4

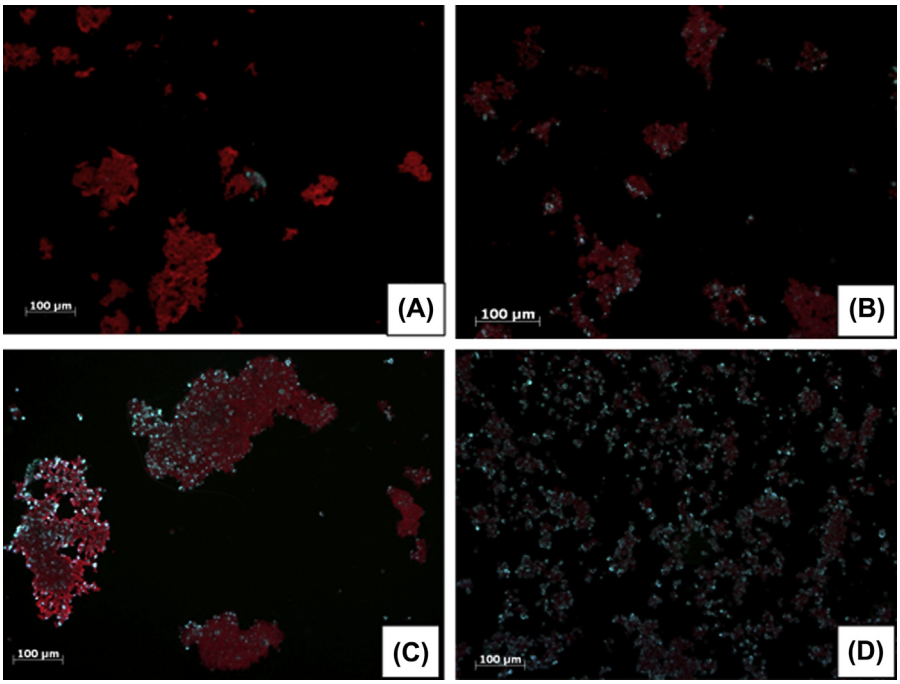

FIGURE 21.6 Examples of classes in direct immunofluorescence assay. Cells are stained in red; virus is green. Magnification of images is 50 times; evaluation is performed 100 times. (A) Class 1: from rare focus to 25% of fluorescent focuses (Pos. Control at dpi 1); (B) Class 2: 25–50% of fluorescent focuses (MOI 0.01 at dpi 2); (C) Class 3: 50–75% of fluorescent focuses (Pos. Control at dpi 2); (D) Class 4: more than 75% fluorescent focuses (MOI 0.03 at dpi 4).

only the infection rate inside the cells. This technique can be used to predict on which dpi the harvests can be initiated. Normally when the culture reaches Class 4 it means that harvests can be made. This happened for all spinners at dpi 3, and on that day the harvests were initiated.

The RABV titration of samples was made following the Fuches protocol, which was previously validated by Moura and colleagues [71,72]. The virus titration is an important tool for evaluating viral production and quality at several stages of vaccine production [63]. It is a relatively fast in vitro test (compared with other in vivo tests) to evaluate the infection of virus produced in other cells and to compare the results with a standard virus. Samples for different dpis were centrifuged at 800 rpm for 5 min before distribution on a microplate and only the supernatants were used in the titration. Three microplates with 96 wells were made, each with 10 samples (one for each dpi) of one infection, in duplicate.

The virus samples from the three infected spinners collected during the 10 days of infection were titrated. The titration for the standard virus was $10^{5.37}$ FFD/mL. The maximal titration value reached by positive control was $10^{5.98}$ FFD/mL at dpi 4. The

maximum for MOI 0.01 was $10^{5.37}$ FFD/mL at dpi 3, and for MOI 0.03 was $10^{5.77}$ FFD/mL at dpi 4. Harvests of virus from infected spinners were made from dpi 3–8 and kept in an ultrafreezer at −80°C.

The harvests are used for vaccine production, usually from a bioreactor. After several attempts to produce a vaccine with great potency using different titers, it was decided to use a cutoff at $10^{3.8}$ FFD/mL and that harvests with a virus titer lower than the cutoff should not be used to produce a rabies vaccine. Fig. 21.7 shows the titration curve for the three infected spinners along with the cutoff.

On the days when harvests occurred, all of the infected spinners had six harvests above the cutoff. However, the positive control and MOI 0.03 obtained two more harvests above the cutoff that were not collected. Between the harvests above cutoff, it can be seen that the positive control had the largest graphic area above the cutoff.

This experiment was able to tell the influence in the different MOIs in the spinners and compare the results with an uninfected spinner. However it was important to obtain growth curves of the four spinners to analyze and compare the infected cultures with the negative control in terms of kinetics, specific growth rate, and metabolite consumption and production. Based on all of the information shown and calculated, it can be concluded that the positive control is the best inoculum for spinner infection. This viral inoculum is made by adding a viral volume corresponding to 1% of the volume of the vessel used in the experiment, which is the best way to infected a BHK-21 culture with RABV.

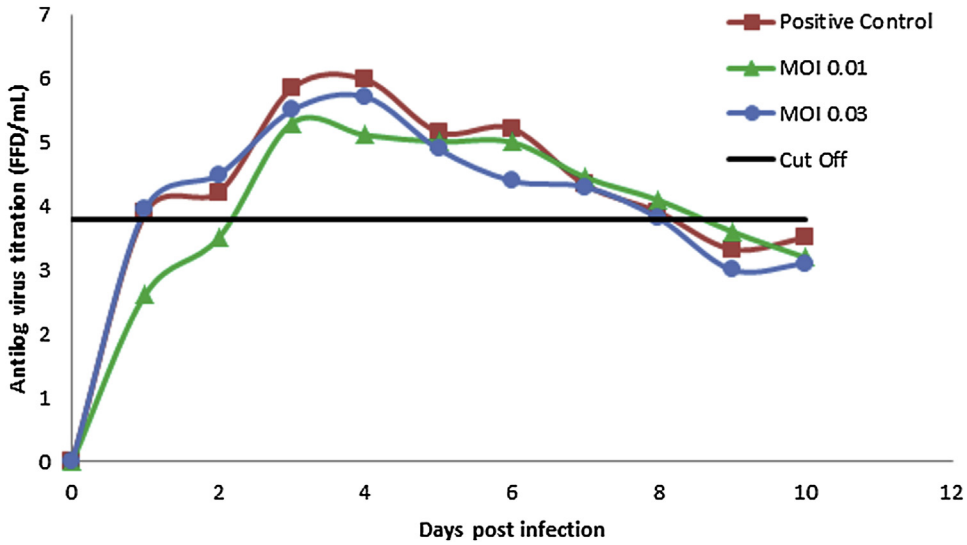

FIGURE 21.7 Virus titration curves for the three infected spinners. Only harvests above cutoff are used for rabies vaccine production. The maximum titration value reached by positive control was 105.98 FFD/mL at dpi 4. The maximum for MOI 0.01 was 105.37 FFD/mL at dpi 3, and for MOI 0.03 was 105.77 FFD/mL at dpi 4. The best graphic area above cutoff means the best inoculum. In this case, the best inoculum corresponds to the positive control.

21.3.1.2 Freeze-Dry Study

Harvests from the positive control were used for lyophilization experiments. However it is necessary to inactivate the harvest and concentrate it before its formulation and use in lyophilization.

The harvests that had titration above the cutoff were merged to form a pool of RABV. The mixture was inactivated using BPL and became an inactivated viral suspension (IVS).

Harvests from the spinner usually have some cellular debris and other undesirable metabolites. Thus it was necessary to clarify this IVS, washing it with NT (NaCl and Tris) buffer solution. The NT buffer used in the exchange is composed by NaCl 130 mmol/L and Tris-HCl 50 mmol/L, with pH adjusted to 7.6. Before the buffer exchange, it was necessary to concentrate the IVS to improve the wash results, and to use a minor amount of buffer solution.

The buffer exchange step was performed in a Pellicon tangential filtration system with 100 kDa of porosity (Merck-Millipore, Billerica, MA). The membrane was coupled with a peristaltic pump set up for 55 rpm. First, the IVS were concentrated from 2 L to 400 mL, discarding permeate. Next, buffer was continued added to the IVS to wash, totalizing five times volume, also discarding permeate. Finally, the volume was reestablished with the same buffer and thimerosal was added to preserve the IVS. The IVS were stored at cold room.

For lyophilization experiments, 11 excipients were selected. A summary of the selected excipients, their respective classes, and their estimated prices is given in Table 21.6.

Excipients were diluted in purified water (Milli-Q) for lyophilization experiments and prepared concentrated two times to prepare stock solutions of each excipient, which were used during all experiments. For sugars and polyalcohols, the stock solution was

Table 21.6 Type and Cost of Selected Excipients

Excipients	Type	Estimated Cost (US$) (per kg)
Sucrose	Sugar	$86
Trehalose	Sugar	$4211
Lactose	Sugar	$193
Sorbitol	Polyalcohol	$55
Mannitol	Polyalcohol	$232
Proline	Amino acid	$1263
Serine	Amino acid	$1280
Glutamic acid	Amino acid	$162
Dextran 40	Polymer	$4522
Polyethylene glycol 3350	Polymer	$254
Gelatin	Polymer	$142

10% (w/v) concentrated, and for amino acids and polymers it was 2% (w/v) concentrated.

Each vaccine is a combination of IVS with a different excipient solution. The vaccine formulation was done by mixing together the IVS and the excipients at 1:1 proportion, homogenizing it and making 1-mL aliquots in 7.5-mL glass vials. The vials were partially closed with a rubber stopper to permit water vapor from sublimation to escape. Five vials were prepared for each excipient solution. An additional of five 1-mL vials were prepared with only the IVS diluted in purified water. This unprotected formulation was used to compare results.

The lyophilization experiments were developed in a freeze-dryer (Edwards RC-300; Edwards Vacuum, Crawley, West Sussex, UK). After preparation of the partially closed glass vials with vaccine formulations, the vials were disposed in the trays of the equipment. The trays were placed inside the freeze-dryer and the protocol was started.

The correct way to conduct this experiment would be to test each formulation separately, with more control parameters to optimize the protocol for each formulation. However, this experiment was made to determine the effects of different excipients in the freeze-dried cakes from rabies vaccines, so it was necessary to freeze-dry all formulations together using the same IVS and protocol.

The protocol (Table 21.7) was established based on other experiments related to lyophilization. It is useful to see the different times for freezing, primary drying, and secondary drying. The vaccines were not frozen before lyophilization, and parameters such as product temperature and DSC were not made. This protocol was used to freeze-dry the different vaccine formulations.

After freeze-drying occurred, the lyophilized cakes for the 12 formulations were obtained (Figs. 21.8 and 21.9).

The lyophilized vials were visually evaluated using three parameters: color, integrity, and uniformity. The color of the IVS with no excipient was white, so the excipient must not make modifications in the cake color to pass the color test. The cakes must have uniform mass distribution to pass the uniformity test. The cakes must not present cracks to pass the integrity test. Table 21.8 summarizes the visual aspects results for color, uniformity, and integrity.

Table 21.7 Description of Lyophilization Protocol

Step	Temperature (°C)	Duration (h)
Freezing	−42	3
Primary drying	−40 to 5	24–96
Secondary drying	+25	3

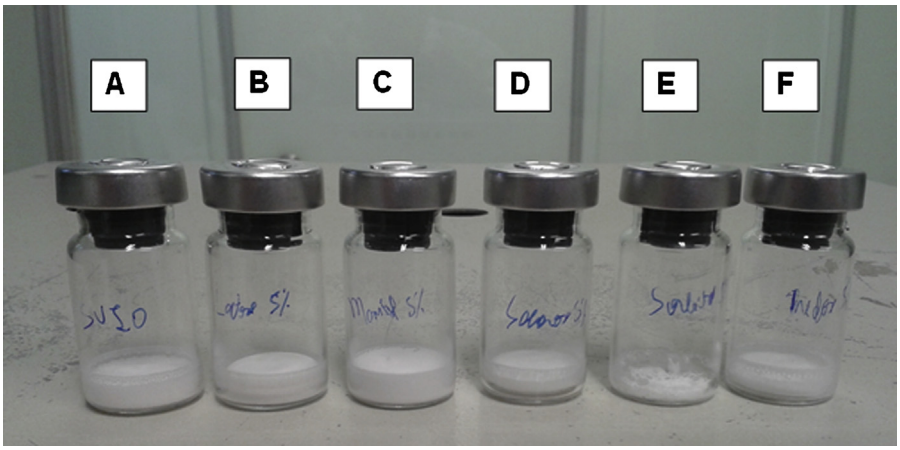

FIGURE 21.8 Lyophilized cakes, part 1. (A) IVS without excipient; (B) lactose 5%; (C) mannitol 5%; (D) sucrose 5%; (E) sorbitol 5%; (F) trehalose 5%. All sugars resulted in good cakes as well as mannitol, the IVS without excipient formed a heterogeneous cake and the sorbitol cake collapsed.

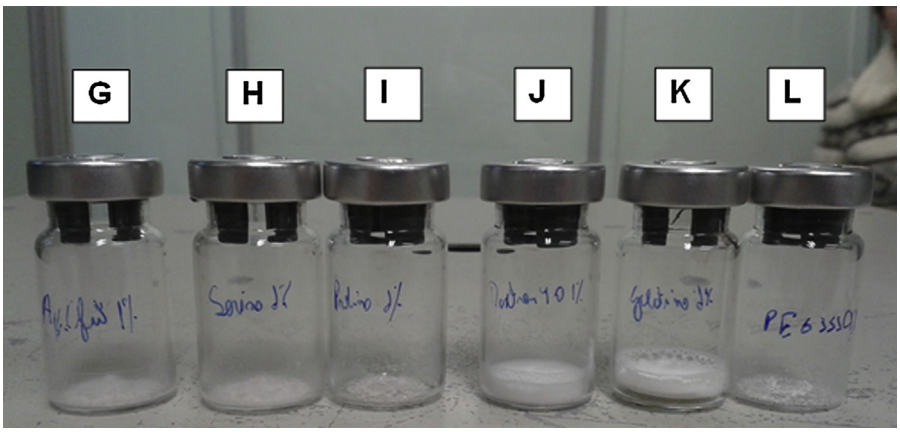

FIGURE 21.9 Lyophilized cakes, part 2. *G*, glutamic acid 1%; *H*, serine 1%; *I*, proline 1%; *J*, dextran 40 1%; *K*, gelatin 1%; *L*, PEG 3350 1%. All amino acids formed collapsed cakes, as well as PEG 3350. Dextran 40 formed an excellent cake and gelatin presented a heterogeneous cake.

Lyophilization of amino acids as excipients did not produce good cakes. The samples presented collapsed during lyophilization (a process also known as melt back). The same phenomenon happened with sorbitol. PEG 3350 cakes also melted back but they appeared to be drier than the sorbitol and amino acids cakes. IVS and gelatin did not pass the uniformity test because bubbles appeared. All excipients did not aggregate color in the cake and passed the color test. The best excipients in this step were sucrose, lactose, trehalose, and dextran.

Table 21.8 Visual Aspects Evaluation of Lyophilized Cakes

Excipient	Color	Uniformity	Integrity
Pure inactivated viral suspension	X		X
Sucrose	X	X	X
Trehalose	X	X	X
Lactose	X	X	X
Sorbitol	X		
Mannitol	X	X	X
Proline	X		
Serine	X		
Glutamic acid	X		
Dextran 40	X	X	X
Polyethylene glycol 3350	X		
Gelatin	X		X

X, cakes that passed the tests.

Table 21.9 Time and pH Evaluation of Reconstituted Cakes

Excipient	Time of Reconstitution	Final pH
Pure inactivated viral suspension	X	7.58
Sucrose	X	7.52
Trehalose	X	7.56
Lactose	X	7.54
Sorbitol	X	7.55
Mannitol	X	7.58
Proline	X	7.56
Serine	X	7.32
Glutamic acid	X	3.47
Dextran 40	X	7.56
Polyethylene glycol 3350	X	7.57
Gelatin		7.4

X, formulations that achieved the specified time of reconstitution.

Then the cakes were reconstituted with purified water. Two other parameters were evaluated under this condition: time of reconstitution and pH. The time of total reconstitution of cakes must not exceed 1 min. Results are shown in Table 21.9.

The only excipient that did not pass the time of the reconstitution test was gelatin, which did not solubilize in purified water in less than 1 min. Although the other excipients passed, the time of reconstitution was better in pure IVS, sucrose, mannitol, and dextran. Lactose and trehalose took awhile longer to become homogenized. The excipients with worse cakes, like all amino acids and PEG, took more time for reconstitution but achieved total homogenization in less than 1 min.

Table 21.10 Residual Moisture Results for Lyophilized Cakes

Excipient	RM1	RM2	RM3	Mean Residual Moisture
Pure inactivated viral suspension	4.16	3.99	2.73	3.63% ± 0.78%
Sucrose	2.30	2.98	2.32	2.53% ± 0.39%
Trehalose	2.51	3.33	3.18	3.01% ± 0.44%
Lactose	2.34	2.86	3.28	2.83% ± 0.47%
Sorbitol	NC	NC	NC	NC
Mannitol	1.93	2.78	2.72	2.48% ± 0.47%
Proline	NC	NC	NC	NC
Serine	NC	NC	NC	NC
Glutamic acid	NC	NC	NC	NC
Dextran 40	3.15	2.95	2.74	2.95% ± 0.21%
Polyethylene glycol 3350	NC	NC	NC	NC
Gelatin	3.42	3.10	2.85	3.12% ± 0.29%

NC, sample was not calculated.

The Brazilian regulatory agency (MAPA) demands that the reconstituted vaccine have a pH between 6.8 and 8.5. The only excipient that did not pass the pH test was glutamic acid, which turned the vaccine more acid than was recommended [73].

The residual moisture test was done by volumetric Karl Fischer titration using Titroline KF equipment (Schott SI Analytics, Mainz, Rhineland-Palatinate, Germany). The three best lyophilized vials of each formulation were selected for the test. The samples of each vial were weighted and titrated using Karl Fischer equipment. Table 21.10 shows the results for residual moisture.

Cakes obtained for the amino acids and PEG 3350 could not be removed from lyophilized vials for injection in the titration equipment because the cakes were melted back and difficult to collect. Therefore, the residual moisture of these excipients could not be calculated. MAPA recommends that residual moisture not pass 3% [73]. The IVS without excipient addition and gelatin did not pass the test. The trehalose was slightly above that recommended but because the test is influenced by many factors (temperature, operating time, and air humidity), it can be said that trehalose passed the test; furthermore, the lyophilized trehalose cake will be reevaluated. The other excipients analyzed had residual moisture under regulatory claims.

The IVS with no excipient formed a cake that passed in the visual aspects tests and almost passed the residual moisture test. This happened, because there could still be traces of sugar, fetal bovine serum, amino acids and other metabolites that probably stabilized the IVS during lyophilization. Lang and Winter demonstrated that sodium chloride interferes in lyophilization in concentrations over 60 mmol/L by aggregating molecules [55]. The sodium chloride concentration in NT buffer is 50 mmol/L, so the presence of this salt only improves the tonicity of the vaccine.

The amino acids cakes melted back. It probably occurred because of its ineffectiveness in protecting the IVS when used as the only excipient, even though the

excipients were present in the pure IVS. It may have better results when associated with excipients of a different kind. Probably with the addition of amino acids, the Tg would be lower.

The book *Laboratory Techniques in Rabies* describes two protocols using gelatin as an excipient [26]. In this experiment, gelatin provided good cakes but after reconstitution it did not homogenize within the desired time.

Trehalose was described as a good cryoprotector and lyoprotector by Gupta and collaborators and Sarkar and collaborators for a respiratory syncytial virus and peste des petits ruminants vaccine, respectively [74,75]. Caricati also tested different excipients for rabies vaccine lyophilization and obtained interesting results for trehalose, sucrose, and PEG 3350 [76]. This experiment also had good results for trehalose and sucrose in terms of visual aspects and residual moisture. However PEG 3350, which is used as a stabilizing agent in lyophilization, had poor results. This could be because PEG is usually coupled with other excipients to serve as a stabilizing agent; in this experiment, it was used alone.

Lang and Winter also tested mannitol as an excipient. When used alone, mannitol had good results for appearance and residual moisture. When coupled with trehalose, the resultant cake had a robust crystalline matrix, leading to a more elegant final product and enabling shorter drying times [55].

Sucrose at concentrations of 1−68% has frequently been used for the cryopreservation of microorganisms [56]. Law and Hull tested the stabilizing effect of sucrose for respiratory syncytial virus and its infectivity, and obtained good results [77]. Sehgal and Das studied the effect of freezing on the bean mosaic virus using sucrose as a stabilizer, and also obtained good results [78].

Dextran, sorbitol, and mannitol are inert excipients. They provide elegance to lyophilized cakes because they easily crystallize under low temperatures and are employed in biopharmaceutical vaccines that use lower doses per vial and consequently have lower mass. In these cases, the inert excipients provide more volume and can be coupled with amorphous excipients generating a two-phase solid: one amorphous with the active principle and one crystalline with the inert excipient.

21.3.2 Conclusions

The growth curves were determined for four BHK-21 cells cultivated in spinners under different viral inocula. Parameters were calculated and discussed to comprehend cell growth before and after the infection and how the initial quantity of virus influenced cell growth. The virus titration showed that the positive control (1% of spinner volume) was the best viral inoculum.

Furthermore, the vaccine was formulated and freeze-dried with different excipients. The IVS with no excipient alongside trehalose, sucrose, lactose, mannitol, and dextran 40 presented lyophilized cakes with the best appearance. This experiment shows the trial-and-error method, a common practice when freeze-drying a vaccine. However, the correct approach is the rational design along with better parameter controls to truly

understand the necessities of the antigen to remain active. A rational design would help choose the best excipient before freeze-drying as well as design an adapted lyophilization protocol, which would result in a better product to be tested in vivo.

21.4 Perspectives

RABV is a well-known virus that has been studied for more than a century. The infection mechanisms are well-described and a large number of vaccines are available for human and veterinary use for pre- and postexposure to the virus. If the potency of a vaccine could be measured by some mathematical model that matches results with biological tests, it would enhance production and eliminate in vivo tests. Novel methods have been proposed to substitute potency tests as well as mathematical models to predict the effectiveness of vaccines; however in most cases in vitro and in vivo results do not match. Therefore these tests and models need to be improved to substitute biological tests in the future.

Freeze-drying is currently the most commonly used drying method to improve the stability of biopharmaceuticals. Since its beginning, most efforts to improve the process focus on increase drying rates to reduce residual moisture, decrease the cycle duration to optimize time, and enhance process efficiency to improve production. One important part that can no longer be overlooked is the characterization and formulation of vaccines to improve the rational design of products, which in turn improves the stability of vaccines, the main objective when freeze-drying. Also, effort must be spent on improving equipment and in some cases finding alternatives to freeze-drying to obtain pharmaceutical products that are safer, more effective, and cheaper.

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22 Antileishmanial Biocompound Screening

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22.1 Introduction

Leishmaniosis is a disease that can manifest as a visceral or cutaneous form. The prevalence is estimated to be 12 million people in 98 countries, with about 1.5 million new cases annually. The infection is caused by protozoa of the genus *Leishmania* Ross, 1903 and is transmitted by the bite of female sandflies. Infections in humans are caused by more than 20 species of *Leishmania* [1]. The visceral form is characterized by affected internal organs and the cutaneous form can demonstrate sores on skin and mucosa (cutaneous or mucocutaneous forms). The severity of some manifestations impairs psychological and physical health and has social and economic impacts. Leishmaniosis is considered by the World Health Organization [1] to be the second most important protozoan disease with regard to public health. Available treatments for leishmaniosis have serious toxic side effects. Moreover, the drugs are expensive and there is the possibility of developing resistance.

Derivatives of pentavalent antimony (Pentostam) or meglumine antimoniate (Glucantime) are the reference standard treatment. They require a hospital stay of several weeks and have significant side effects. In addition, many cases of resistance are reported in India. Thus there is increasing use of amphotericin B or miltefosine as a lipid (despite the significant cost), since shorter hospital stay and increased tolerance are often observable.

Many molecules are tested each year to find new treatments for less severe cases. Few of them show in vivo activity and go beyond the preclinical stage of development. However the disease continues to evolve and cases of resistance are worrying, so it is essential to continue research.

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22.2 Primary Chemotherapy With Antimonials

Organic antimonials were first used a century ago as leishmanicidal compounds by Gaspar Vianna in 1913 for the treatment of cutaneous leishmaniasis (CL). The first commercial drug developed was urea stibamine, in India in 1922, but it had severe toxic effects. Other chemotherapeutics, also antimonials {based on pentavalent antimonials [Sb (+5)]} have been introduced as drugs of choice for treatment and have been used successfully for 60 years [2].

Pentavalent antimony compounds remain the first-line pharmaceuticals of choice for the treatment of leishmaniosis [3]. The compounds (Fig. 22.1A and B) that have this active principle have the trademarks names Glucantime (*N*-methyl meglumine antimoniate; manufactured by Aventis Pasteur) and Pentostam (sodium stibogluconate; Wellcome, manufactured by GlaxoSmithKline). Glucantime is widely prescribed as the initial treatment for CL and visceral leishmaniosis (VL). It is usually administered by intramuscular injections.

To be active against *Leishmania* spp., Sb (+5) has to enter the infected cell and cross the phagolysosomal membrane to attack intracellular amastigotes. To combat the infection, Sb (+5) must be reduced to the active form of the drug against the parasite, the trivalent antimonial [Sb (+3)] [4,5]. The whole molecular mechanism of antimonials, however, is not fully understood. Antimony compromises the thiol redox potential of the cell by inducing the efflux of intracellular thiols and by inhibiting trypanothione reductase [6].

However, pentavalent antimonials have toxic side effects and limited efficacy to control parasite proliferation, and drug resistance is frequently encountered [9,47]. Although its effectiveness still saves lives [10], it is an aggressive substance as a chemotherapeutic. This drug causes serious side effects such as cardiac, hematologic, liver, pancreatic, and renal toxicities that can result in death [11]. Because of the aggressiveness of the side effects, treatment often stops before the cure occurs, thereby contributing indirectly to the worldwide increasing frequency of resistance to antimonials [12,13]. Chemotherapeutic regimens for treating parasitic diseases have been derived empirically and are not particularly selective for the metabolic machinery of the parasite. Unsurprisingly, therefore, antiparasitic drugs are noted for causing moderate to severe toxicity in the human host [14].

Since the 1940s, a serious public health problem has been described: resistance to antimonials by some strains of *Leishmania* [15]. Although pentavalent antimonials still have their basis in treatment, resistance to this class of drug is becoming so high in some parts of the world that it is quickly becoming obsolete [13,16]. For example, the treatment of kala-azar by conventional dosages has failed particularly in northeast India since the 1970s, with 60% unresponsiveness reported [17].

Adenosine triphosphate–binding cassette (ABC) transporters mediate Glucantime sequestration in cellular organelles [18]. In leishmaniosis, ABC transporters are responsible for much of the decreased sensitivity to antimonials. Active movement of

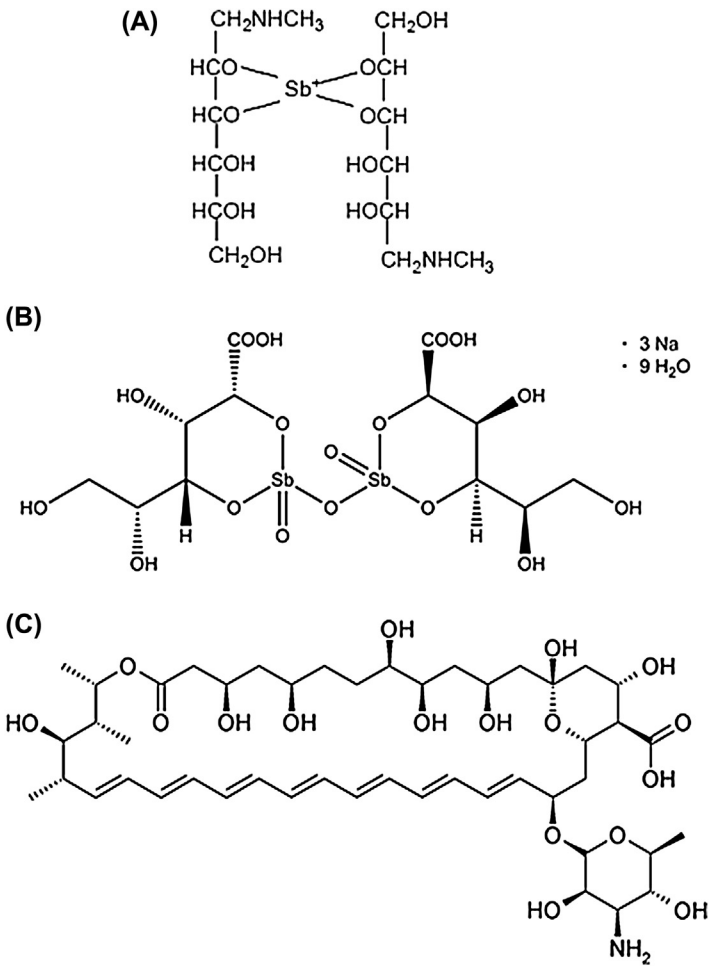

FIGURE 22.1 (A) Meglumine antimoniate (Glucantime). Systematic name: hydroxy-dioxostiborane; (2R,3R,4R,5S)-6-methylamino -hexane-1,2,3,4,5-pentol (B) Sodium stibogluconate (Pentostam). Systematic name: 2,4:2′,4′-*O*-(oxydistibylidyne) bis [D-gluconic acid] Sb,Sb′dioxide trisodium salt nonahydrate (C) Amphotericin B. Systematic name: (1R,3S,5R,6R,9R,11R,15S,16R,17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S,36R,37S)-33-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo14,39-dioxabicy-clo [33.3.1] nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylic acid. *Adapted from: IUPAC (International Union of Pure and Applied Chemistry), Organic and Biochemical Nomenclature Recommendations (2010). Available at: www.iupac.org, and Disanejercito, Direccion de Sanidad del Ejercito Salud Operacional, Leishmaniosis (2008). Available on: http://www.disanejercito.mil.co/Salud_Operacional_Leishmaniosis.htm.).*

drugs across membranes through these transporters is a cause of therapy failure. Previous reports evidenced that the augmented expression of ABC transporters enhances drug extrusion and disposition [4] and constitutes one of the main impediments to successful chemotherapy against parasites of the *Leishmania* genus [19].

Administration of therapeutic drugs combined with ABC transporter blockers is one way to actively avoid the increasing frequency of drug resistance. Glibenclamide (specific blocker) enhances the Glucantime effect to decrease the infection rate of macrophages [18]. Furthermore, the selective inhibition increases the cytoplasmic concentration of Glucantime and efficacy. However further investigations into some arsenite-resistant strains revealed that increased activity and expression of topoisomerase II (necessary for kinetoplast DNA) induces a multidrug-resistant phenotype [20,21] (Fig. 22.1A and B)

22.3 Second Generation of Antileishmanial Drugs

Pentavalent antimony compounds have been the mainstay of leishmanicidal therapy for half a century [3], but over the past decade, alternative drugs or new formulations of other standard drugs have become available and registered for use in some countries, whereas other drugs are under clinical trial for both forms of the disease [22–24]. Some representative second-line drugs are presented in the following subsections.

Second-line drugs have not experienced widespread use, mainly owing to the easy development of clinical resistance, toxicity, cost, and possible contribution to the increased incidence of leishmaniosis–HIV co-infections [22]. Similar to antimonial derivatives (Glucantime), drugs such as *bis*-amidines (pentamidine) and polyene macrolides (amphotericin B) exhibit high liver, kidney, and heart toxicities. Still others, including paromomycin, allopurinol, and sitamaquine have been reported to have variable cure rates. Although miltefosine is effective in some cases, parasites can develop resistance against it [25]. Fluconazole, taken orally, was shown to be effective against CL [13].

Therapies combining second-line antiparasitic substances are currently being considered, especially to avoid the development of parasite resistance. Some drugs that were already tried include atovaquone, roxithromycin, and edelfosine [9]. A tested combination of paromomycin and other leishmanicidal agents with sodium stibogluconate in a standard dose gave no optimistic results. Immunotherapy combined with chemotherapeutic agents using stimulating cytokines or vaccines is still is a future promise [17].

22.3.1 Amphotericin B

Amphotericin B (Fig. 22.1C) is a polyene amphoteric antibiotic that binds to cell membranes and alters their permeability, creating pores and leakage of ions [30]. It has been proven to be highly effective for the treatment of resistant strains of *Leishmania* to antimonials, in the form of amphotericin B deoxycholate (Fungizone) [2,26].

Amphotericin B makes complexes with substituted sterols, thus causing the pores that alter ion balance and result in death of the parasite [16].

It was first developed as an antifungal compound, valuable for use in the treatment of systemic fungal infections [27] by interacting with fungi cell membrane sterols and preferentially with ergosterol. Like fungi, *Leishmania* also has ergostane-based sterols as its major membrane sterol [28].

However, amphotericin B is an unpleasant drug because of its toxicity and the need for slow-infusion parenteral administration over 4 h [24]. It was also found to be highly nephrotoxic. Often a serious acute reaction is noted after the infusion, consisting of high fever, shaking chills, hypotension, anorexia, nausea, vomiting, headache, drowsiness, generalized weakness, dyspnea, and tachypnea.

The use of lipid formulations containing amphotericin B has been one of the greatest advances in the treatment of leishmaniosis, minimizing side effects of its use. Various safer colloidal and lipid formulations have been prepared [12]. Lipid formulations of amphotericin B, although expensive and administered parenterally, represent a major advance for treating VL, e.g., liposomal amphotericin B (AmBisome) [29].

The standard dosage of amphotericin B for treating New-World CL is 0.5−1.0 mg/kg per day through 20 days. An urgent technological need for this kind of treatment is an understanding of the immune response to leishmaniosis and field-applicable and affordable methods for diagnosis and treatment. Although introduced more recently, amphotericin B is not widely used because of cost of the treatment with this drug [12].

22.3.2 Pentamidine

Pentamidine, a cationic diamidine (Fig. 22.2A) is a drug used against resistant parasites, but close clinical control of the patients is required because of side effects [30]. Pentamidine accumulates in the mitochondria; indeed, it was shown that this could greatly enhance the efficacy of mitochondrial respiratory chain complex inhibitors [31].

This drug does not accumulate in the mitochondria of in vitro pentamidine-resistant mutants, and the cytosolic fraction of the drug is extruded outside the cell [32]. Similarly to Glucantime, a possible candidate for the required efflux pump is the ABC transporter that was isolated by Coelho et al. [33], while selecting for pentamidine resistance. Acquired resistance has been reported against pentamidine (about 25%), although it was initially used with prolonged dosage as a second-line drug [17].

22.3.3 Miltefosine

Miltefosine (Fig. 22.2B), which belongs to the alkylphosphocholine group, was approved for the treatment of human leishmaniosis [13]. It has been developed for treatment of both VL and CL by Zentaris, Impavido, and the World Health Organization [34]. Miltefosine is the first oral leishmanicidal drug; it was put in use in 2001, although it was originally developed as an anticancer agent [35]. An advantage is that it does not require refrigeration. There is a governmental program in India to face pentavalent antimonial

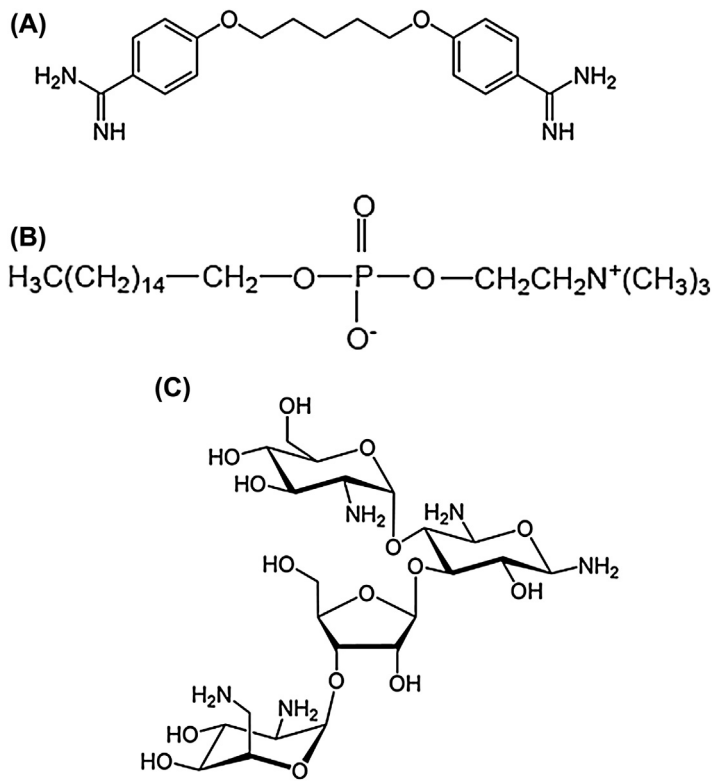

FIGURE 22.2 (A) Pentamidine. Systematic name: 4,4′-[pentane-1,5-diylbis(oxy)]dibenzenecarboximidamide (B) Miltefosine. Systematic name: 2-(hexadecoxy-oxido-phosphoryl)oxyethyl-trimethyl-azanium (C) Paromomycin. Systematic name: (2R,3S,4R,5R,6S)-5-amino-6-[(1R,2S,3S,4R,6S)-4,6-diamino-2-[(2S,3,4,5R)-4-[(2,3,4,5R,6S)-3-amino-6-(aminomethyl)-4,5-dihydroxy-oxan-2-yl]oxy-3-hydroxy-5-hydroxymethyl)oxolan-2-yl]oxy-3-hydroxy-cyclohexyl]oxy-2-(hydroxymethyl)oxane −3,4-diol. *Adapted from: IUPAC (International Union of Pure and Applied Chemistry), Organic and Biochemical Nomenclature Recommendations (2010). Available at: www.iupac.org, and Disanejercito, Direccion de Sanidad del Ejercito Salud Operacional, Leishmaniosis (2008). Available on: http://www.disanejercito.mil.co/Salud_Operacional_Leishmaniosis.htm.).*

resistance with the administration of Miltefosine, with the hope of eradicating the visceral leishmaniosis form [36].

Miltefosine was effective in experimental and clinical trials. Resistance to this drug is possible. Single point mutations in the *Leishmania* putative miltefosine transporter can lead to resistance [25]. This drug has a potent effect with a longer half-life when orally administered; thus it has high potential to induce parasite resistance [17]. Furthermore, Basu et al. [37] reported a gene isolated from *Leishmania infantum* that encodes a putative polypeptide (without similarities to known proteins) that confers resistance not only against antimonials but also against miltefosine. Other drawbacks are that this drug has teratogenicity, severe gastrointestinal side effects, and a narrow therapeutic window that makes its application restrictive owing to the high cost [38].

22.3.4 Paromomycin

Paromomycin (Fig. 22.2C) is an aminoglycoside antibiotic used for various clinical infections. The spectrum of activity of paromomycin encompasses most gram-negative and many gram-positive bacteria, some protozoa, and many cestodes [36]. Paromomycin has been used to treat both VL in a parenteral formulation and CL in both topical and parenteral formulations [12].

This compound showed efficacy and safety in phase IV clinical trials against leishmaniosis [39]. Paromomycin inhibited the in vitro growth of *Leishmania donovani* promastigotes in a dose-dependent manner. Intracellular macrophage-amastigotes were more sensitive. As with Miltefosine, resistance to paromomycin could be induced experimentally in promastigotes in vitro [40]. The developed resistant strain was threefold less sensitive to paromomycin than the wild type.

Paromomycin has undergone extensive clinical trials in Indian kala-azar patients. Because it is an aminoglycoside, acquired resistance is likely to occur when it is used as a monotherapy. To overcome the problem of treatment failure in kala-azar and reduce the length of therapy, the combination of at least two effective leishmanicidal agents is a desirable option [17,30].

22.4 Antileishmanial Drug Development Review

The incidence of millions of new cases of *leishmaniosis* per year worldwide and deficiencies in current treatments point to the urgent need for new drugs to combat these parasitic diseases [41,42]. The massive displacement of populations, weather changes, growing resistance to available drugs, a lack of therapeutic alternatives and long treatments are creating a significant rise in the number of infected population people [43–45,91] with resulting increases in public health problems, mainly regarding poor individuals. Leishmaniosis is an almost neglected disease owing to its prevalence in developing countries. Big pharmaceutical companies are not motivated to search and develop antiparasite compounds with modest economic perspectives.

Nevertheless research for novel therapeutics is desirable because:

1. Parasites develop drug resistance;
2. Patients have bad side effects owing to the high toxicity of current drugs during and after treatment;
3. Treatment is expensive and the market is not supplied market owing to economic factors; and
4. There is undesirable responsiveness in disease recovery and variable efficacy [46,47].

It is unlikely that a single formulation of one or more drugs effective against all forms of leishmaniosis will be developed. Several factors affect this development. Sites of

infection impose different pharmacokinetic requirements and there is intrinsic variation in drug sensitivity for different *Leishmania* species.

There are other emerging problems to overcome:

1. the need for drug activity in regions where resistance to pentavalent antimonials develops; and
2. efficacy in immunosuppressed patients.

The effects of neoplastic diseases and immunologic disorders are often worsened by immunosuppressive drugs such as corticosteroids and cytotoxics [48]. Treatment may be chosen according to disease responsiveness.

Antiparasitic compound availability is usually low in natural products, typically causing difficulties with research in this field. According to Bhuwan [49], practical aspects to consider in active natural substances screening are the low yield and sample representativeness issues, such as only one sample obtained from each source, which makes statistical analyses poor or impossible. In addition, the relative structural complexity of natural products is high and includes the occurrence of multiple stereoisomers. Furthermore, isolated active principles rarely exhibit potent activity themselves, but require follow-up improvement to be effective [49].

22.5 Biotechnological Development of New Antileishmanial Drugs

Considering the drawbacks of these drugs, more attention should be given to the extracts and biologically active compounds isolated from natural sources, such as plant species [50,60] and fungi [51,52] by biotechnological methods. Several promising antileishmanial compounds have been reported over the past few years in various test systems. To identify the most promising compounds, candidate molecules should be compared with one or more standard antileishmanial drugs. If necessary, animal models should be used to assess parasitemia in mice or hamster models.

Many studies have reported the isolation of compounds from plants or by chemical synthesis. One advantage of using microorganisms to obtain active compounds is the possibility of rapidly increasing production by process optimization and genetic manipulation. Many fold increases have been recorded in the production of small metabolites, active principles of new pharmaceutical products. High productivity also facilitates the purification of these substances.

The cell biology of *Leishmania* and mammalian cells differs considerably and this distinctness extends to the biochemical level. This provides the promise that many of the parasite's proteins should be sufficiently different from those in the host to be successfully exploited as drug targets. Consequently, discovering new antileishmanial drugs should be considerably easier than discovering other new drugs, from a scientific perspective.

There is a need to develop new antiprotozoal drugs with novel structures and modes of action [53]. Indeed, over many years, several interesting drugs have been proposed. Unfortunately, few of these compounds have been validated rigorously.

Research into new active principles has been mainly based on these trends: screening of natural products, targeting of biochemical pathways, chemical derivatization of synthetic compounds, molecular modeling, and combination therapies.

A wide variety of products from different sources with leishmanicidal activity are attracting the interest of researchers across the world. Some examples from the past decade are presented.

A series of novel aryloxy benzocycloalkyl azoles prepared from cyclohexanone were highly active in vitro against *L. donovani* and exhibited significant in vivo activity in a hamster model [54]. The evaluation was performed in vitro against transgenic *L. donovani* promastigotes and intracellular amastigotes at various concentrations, taking sodium stibogluconate and pentamidine as a control. Cytotoxicity response was assessed using a mouse macrophage cell lineage. All of the compounds killed promastigotes and amastigotes in a concentration-dependent manner and inhibited parasites.

Hydromethanolic fractions of the aerial parts of the plant *Mitracarpus frigidus* showed potential antioxidant, antimicrobial, and leishmanicidal activity [55]. Phytoconstituents were investigated and the total amount of phenolic compounds and flavonoids was quantified to characterize the extracts.

Crypthophilic acids (from the plant family Scrophulariaceae) and other constituents of *Scrophularia cryptophila* were examined for their in vitro antiprotozoal and antimycobacterial potential [56]. Resin glycosides were studied, which are secondary metabolites exclusive to convolvulaceous plants. With the exception of two compounds, all glycosides tested were active against *L. donovani*; the best was Harpagide.

Derivatives, intermediates, and analogs of several drugs (alkyl-amino-cyclohexanols, cyclohexanediamine, cyclohexanediol, and cyclohexane-fused aziridine) were evaluated in vitro against promastigotes of *Leishmania amazonensis*, *Leishmania Braziliensis*, and *L. donovani*. All types of compounds assayed resulted in highly potent antileishmanials except one unable to form hydrogen bonds [57]. Pentamidine and amphotericin B were used as reference drugs.

An aminoglycoside, neomycin, a structural analog of paromomycin, at 600-μM concentration, resulted in 30% inhibition of *L. donovani* growth for both the wild and resistant strains [40]. Earlier studies also reported a weak effect of neomycin on *L. donovani* growth [58].

Alkaloidal extracts of Brazilian plants (*Aspidosperma ramiflorum*) were active against the promastigote form of *L. amazonensis*. Acid and basic fractions obtained from ethanolic crude extract and pure compounds from the stem bark of this plant were tested. Mainly the basic alkaloidal fractions had good activity and also showing activity against gram-positive bacteria. The basic fraction was further fractionated on silica gel

column chromatography in a bioassay-guided protocol, resulting in individual purified compounds for characterization [59].

Diverse naphtoquinones, which are present on different species of vegetables, have shown some level of activity against *Leishmania*. Benzoquinones, naphtoquinones, and mansonones demonstrated activity against Trypanosomatidae [53]. Diospirine, which showed activity against promastigote forms of *L. donovani*, was chemically modified to reduce its toxic side effects [67].

Nicotinamide (NAm), a form of vitamin B_3, significantly inhibited the intracellular growth of *Leishmania* amastigotes [60]. NAm may thus represent a new leishmanicidal agent that could potentially be used in combination with other drugs during therapy.

The alkaloid renieramycin A from sponges inhibited *L. amazonensis* [61]. Euplotin C, a sesquiterpene obtained from a marine ciliate, was active against *Leishmania major* and *L. infantum* [62]. A peptide derived from defensin of marine mussel inhibited *L. major* [63].

The specialized sterols of *Leishmania* and their close similarities to sterol composition and biosynthesis in yeast led to the assay of activity of a number of antifungal sterol biosynthesis inhibitors against *Leishmania* [64].

Pentamidine activity can be associated with alteration of L-arginine transport, because it inhibits this mechanism in protozoa. Production of nitric oxide (NO•) has a role as a cell signaling agent and its function as an antileishmanial effector molecule. The action of pentamidine isethionate (reference drug) and amidine derivatives (test compound) were studied on the NO• pathway of promastigotes and axenic amastigotes of *L. amazonensis* by measuring nitrite, a by-product of nitric oxide released into culture supernatants. NO• production by infective promastigotes was inhibited by the test compound in about 23% and by pentamidine in only 4%. In axenic amastigotes, the inhibition was 53% for the test compound. This fact is important because L-arginine is the substrate of the NO• pathway [65].

Some lichen galactomannans (polysaccharides) showed leishmanicidal activity in vitro by direct cytotoxicity and immunomodulating stimulation activity of macrophages. This activity was even improved by complexation with vanadium ions [66]. Monomeric and dimeric naphtoquinones were found to have in vitro leishmanicidal activity [67]. Another focus was given to finding oral drugs to minimize injection-associated complications. Many substances, including antifungal ketoconazole, were reported to be effective [68].

Investigations of Atlantic Forest fungi were published, with the report of several active substances: antifungal, cytotoxic, trypanocidal, immunosuppressive, and leishmanicidal compounds [69] from *Agrocybe perfecta* (*Rick*) *Sing.*, *Oudemansiella canarii* (*Jungh.*) *Hohn*, and *Lentinus strigosus* (*Schwein.*). Other studies for the discovery of potential new drugs from natural sources were reported, including the screening of *Basidiomycota* species, using in vitro bioassays to assess the obtained compounds against human cancer cells lines, human peripheral blood mononuclear cells, recombinant *Trypanosoma cruzi*, and amastigote-like forms of *L. amazonensis* [56].

Vitola [51] reported leishmanicidal activities of several extracts obtained from *Perenniporia martiusii*, *Plectania* sp., and *Ganoderma stipitatum*—submerged mycelial cultivation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and tritiated thymidine methodologies. Some assayed extracts showed even more activity than Glucantime, used as positive control.

Fendrich [52] further fractionated *Perenniporia martiusii* extracts by using chromatographic techniques, and detected significant antileishmanial activities by using various in vitro models. More than one fraction exhibited activity. The most active fraction (fraction D) showed more activity than Glucantime in even lower concentrations. The cytotoxicities of the most active extracts were evaluated by assaying Vero cells with MTT. In fact, some active fractions stimulated Vero cell proliferation. This D fraction stimulated macrophages NO• production in vitro, which suggested that this substance had both direct and indirect activity mechanisms.

According to Chang and Miles [70], "only about 20% of all mushroom derived products are based on extracts from cultivated mycelia, and about 2% are from broth used for growing mycelia. Mycelia formed by growing pure cultures in submerged conditions are of constant composition, and this is the best technique for obtaining consistent and safe mushroom products. In addition, increased quality control of the products and year-round production under controlled conditions make mycelial-based products, including exopolymers from culture broth, the wave of the future."

22.6 Methods for Anti-*Leishmania* Drug Screening

Several methodologies (in vitro, in silico, and in vivo) are available to assess antiparasitic activity. Each has its advantages and drawbacks. In vitro tests, for example, are limited for previewing complex interactions that happen naturally in living environments. On the other hand, although in vivo tests on animal models give results more approximated to human reactions, they cannot simply be extrapolated. As better computational models of biochemical structures and even of whole cells or organisms are developed, new in silico assays are becoming more precise and approximate to real biological structures. It is still impossible to substitute in vitro or in vivo tests entirely by in silico assays. However it is a powerful approach for rapidly screening a great number of candidate chemical structures and for predicting the effects of molecular modifications.

Nevertheless it is necessary to reduce, replace, and refine experiments to minimize the suffering of animals. In silico, in vitro, and in vivo models should be used wisely in combination, to obtain the most active and safe new drugs in the fastest, cheapest, and most ethical way possible.

The screening process can be performed in several steps. For each stage, it is necessary to define the experimental model type (in silico, in vitro, or in vivo). Whenever possible, it is a better choice to start with in silico studies, to evaluate candidate substances without the need to spend materials or waste animals' lives. However, it is

unusual to have detailed molecular information about new natural samples. In this case, it may be cheaper and more practical to perform some in vitro tests and even in vivo assays before deciding to deeply analyze the molecular composition of a given unknown sample.

For in vitro methods, it is necessary to choose the species of *Leishmania* and the medium in which to cultivate the parasites. It is possible to work with promastigotes or amastigotes (intracellular form). It is ethical to assay candidate substances in vitro both for their activity and their toxicity before in vivo tests. After a candidate substance passed all preliminary in vitro steps successfully, it is necessary to test it in animal models before prescribing it to humans. Tests with rodent and nonrodent species are required by authorities before allowing clinical studies of new drug candidates with human patients. Various clinical experiment phases are needed before the production, commercialization, and prescription of any given new pharmaceutical substance is authorized. In this process, efficacy and safety must be proven experimentally for the greatest number of cases possible.

In silico methods can also be used to evaluate the effect of chemical modifications of active substances to improve their efficacy further or to reduce side effects.

Fig. 22.3 presents suggested steps for testing metabolites or new drugs.

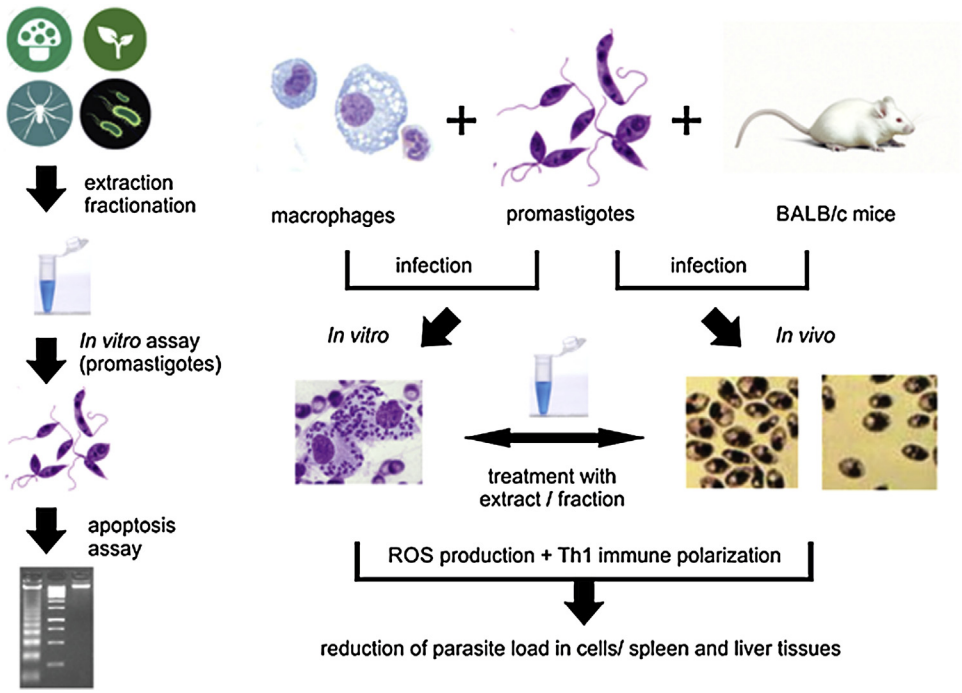

FIGURE 22.3 Flowchart showing an antileishmanial drug screening strategy.

22.6.1 Methods for Working With *Leishmania*

22.6.1.1 Leishmania *Culturing*

Parasite cultivation techniques are necessary for screening substances as drug candidates against leishmaniasis. The first step is to choose among *Leishmania* species. Several media [coeur-cerveau-sang de mouton (CCS), Roswell Park Memorial Institute medium (RPMI), and modified liver infusion tryptose (LIT)] can be used for *Leishmania* cultivation. Media compositions are described in Tables 22.1–22.3.

Other media such as M-199 medium {supplemented with 40 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 100 μM adenosine, 0.5 mg/L hemin, 10% heat-inactivated fetal bovine serum, and 50 μg/mL gentamycin} or RPMI (Sigma) medium, added to 10% fetal calf serum, can be used for *Leishmania* culturing. RPMI and LIT cultures generally are conduced inside Roux bottles, CCS cultures inside 250-mL Erlenmeyer and Novy–MacNeal–Nicolle medium (NNN) in tubes (Table 22.2). The cultures are maintained inside an incubator at $23 \pm 2°C$.

An inverted microscope is used to monitor Roux bottles culture characteristics, including concentration, motility, and contaminations. For Erlenmeyer cultures, samples are taken for observation on a common optical microscope. Whenever necessary, samples are also taken from Roux bottles for better observation on a common optical microscope. Contaminated and old cultures should be adequately sterilized and discarded. Weekly transferences to fresh media are necessary. Only cultures within a concentration range between 10^7 and 10^9 parasites per milliliter are used. An adequate inoculum rate is about 20%.

As described by GOMES et al. [71], "… the different *Leishmania* species are not equally easy to culture. Contamination is a constant problem, and variations in efficacy among different growth medium formulations or even batches may be encountered." For some assays, NNN medium was found to give better results [72].

Table 22.1 Modified Liver Infusion Tryptose (LIT) Medium Composition

Component	Concentration (g/L)
NaCl	5.0
Na_2HPO_4	7.5
Tryptose	5.0
Yeast extract	3.0
Brain heart infusion	3.0
KCl	0.4
Adjust pH to 7.4 with 1 M Na_2HPO_4	
Autoclave at 120°C for 30 min	
Add 2 mL hemine (20 mg/L) for each liter of LIT medium (previously filtered through 0.2 μM micro-pore filters)	
Add 5 mL gentamicin (10 mg/mL) for each liter of LIT	
Add 10% previously decomplemented fetal calf serum	

Table 22.2 Novy–MacNeal–Nicolle Medium Composition

Component	Concentration
NaCl	3.0
Bacto-agar or past-agar	5.0
Boil for 2–3 min	
Distribute in tubes (4 mL/tube)	
Autoclave at 120°C for 30 min	
Add 0.3 mL citrated rabbit blood per tube	
Let medium solidify in inclined position inside tubes	

Table 22.3 CCS Medium Composition

Component	Concentration (g/L)
Brain heart infusion	37.0
Bacto-agar	15.0
Boil for 2–3 min	
Distribute in 125-mL Erlenmeyer flask (10 mL/flask)	
Autoclave at 120°C for 30 min	
Add 1 mL citrated rabbit blood per flask	
Let medium solidify inside flasks	
Add 4 mL physiologic serum	

L. infantum grows properly in RPMI medium and *Leishmania enriettii* is well-adapted to modified LIT medium. On the other hand, *Leishmania braziliensis* does not grow well on blood cell-free media (RPMI and modified LIT), but CCS medium contains rabbit blood cells, which interfere with the MTT assay. To assay this species with MTT, a blood cell disruption procedure and medium transference are necessary before applying the bioactivity evaluation methodology. A cell disruption protocol is described next.

22.6.1.2 Blood Cell Disruption

- Filter about 20 mL of the liquid fraction of a CCS medium previously cultured with *Leishmania* ($\sim 10^7$ cells/mL) through a sterilized cotton cloth and transfer it to a sterilized Falcon tube under aseptic conditions.
- Wash the CCS medium solid matrix and the Erlenmeyer flask originally containing it with 10 mL LIT modified medium; filter it and add it to the remaining suspension in the Falcon tube.
- Add 10 mL of physiologic serum and centrifuge the resulting suspension for 15 min at 5000*g*, at 23 ± 2°C.
- Pipet and discard the supernatant.
- Add 30 mL of physiologic serum to the remaining pellet.
- Centrifuge the resulting suspension for 15 min at 5000*g*, 23 ± 2°C.

- Pipet and discard the supernatant.
- Resuspend the remaining pellet in 25 mL of modified LIT medium (10% fetal calf serum).

22.6.1.3 Leishmania Counting in Thoma Chamber

This technique is used to quantify parasite concentration in a culture's liquid. The Thoma chamber consists of a microscopy slide with a thin well of a precise volume and an imprinted microscopic grid. With the aid of an optical microscope, microorganisms contained in a liquid sample can be counted. The method consists of covering the well with a small glass slide and transferring about 10 μL of the properly stained protozoan cultured liquid to be quantified inside the slide well by capillarity. With an optical microscope, individual parasites inside the grid squares (quadrants) are counted. Because the chamber volume is known (0.004 mm^3), parasite concentration on the sample can be determined.

Leishmania culture samples need to be adequately diluted before counting (one-half, one-fifth, or one-tenth). A fixation/staining solution can be used for the dilution besides its main functions (fixation and staining of the parasites).

A microscope coupled with a video camera linked to a monitor can facilitate counting. A manual counter is also a good tool for high parasite concentrations.

Microorganisms within five quadrants are usually counted and the mean number is divided by the volume over one quadrant. The values are adequately corrected for previously applied dilutions.

22.6.2 Experimental Models for Screening Leishmanicidal Activity

22.6.2.1 In Vitro Antipromastigote Assay

Different species of *Leishmania* can be used for these experiments. In the new world, *L. amazonensis* is generally the model of choice for tests because the parasite grows quickly. In the old world, the most commonly used model is *L. major*. *L. enriettii* is an elegant model because it is not pathogenic for humans, which avoids laboratory accidents. Furthermore, replication of *L. enrietti* promastigotes is fast.

To screen candidate biotechnological metabolites against *Leishmania*, we can use 24- or 96-well plates. Typical protocols suggest properly diluting and distributing parasite suspensions containing 10^6 parasites/mL on multiple microwells and adding the chosen cultivation medium and the drug to be tested. The experiments should be performed with parasites in their logarithmic phase of growth.

After inoculations of the drugs to be tested, the parasites should be incubated at $23 \pm 2°C$ for 5 days without medium replacement. Every 24 h after treatment, aliquots are harvested and the effect on promastigotes growth is evaluated using a chamber for counting, and compared with untreated parasite culture. Cultures should be performed in triplicate. Glucantime can be used as a positive control.

In vitro tests are based on protozoa cultivation and evaluation of their survival or of alterations on their metabolism upon the addition of test substances. One simple

method for assaying protozoa survival is to observe whether their motility is significantly reduced or stopped, using an optical microscope.

Other methods involve the assimilation of radioactive isotopes by protozoa as they grow. Measuring the concentration of incorporated radioactive compounds after an incubation period allows the protozoa growth rate to be inferred. Tritiated thymidine is a current methodology of this kind.

Alternatively, reagents that change color as they are metabolized can be used to measure metabolism rhythm alterations. Examples of these reagents are MTT, XTT {sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium} and WST-1 {4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate}. All of them are tetrazolium salts that change color as they are metabolized. Color intensities can be measured with a spectrophotometer and are proportional to the parasite metabolism rate [69]. Many bioactive compounds screening studies have been performed with this principle. These methods are easy to perform and fast to read. Furthermore, those reagents have the advantage of being nonradioactive. Drawbacks are that sensibility and accuracy are not as high as with DNA-based methods (radiolabeled thymidine or bromodeoxyuridine).

22.6.2.2 In Vitro Antipromastigote Colorimetric Assay

MTT {(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide} is a yellow tetrazolium salt that turns purple when reduced (cleaved) to formazan compounds. It is useful for many bioassays because cellular metabolism can reduce MTT. Cellular metabolism rate can be inferred by the color of the medium after incubation with this reagent [73].

Because formazan salts are water-insoluble, they must be properly solubilized before reading (10% sodium dodecyl sulfate, 50% isopropanol can be used). Color intensity measurement is accomplished with a spectrophotometer. For microassays, a 96-well plate reader is better suited [73,74]. These methodologies are currently used to measure factor-induced cytotoxicity or cell necrosis, but cell viability can also be assayed. Also, because proliferating cells are metabolically more active than nonproliferating cells, these tetrazolium salt–based assays are frequently used to measure cell activation and proliferation [73–75]. Current applications include:

- evaluating growth-inhibitory or cytotoxic effects of compounds,
- analyzing cytotoxic and cytostatic effects of potential drugs,
- analyzing cytopathic effects of viruses and screening compounds with antiviral activity,
- screening antibodies for growth-inhibiting potential, and
- studying the effects of substances over immune system cells (proliferation or suppression).

22.6.2.3 In Vitro Antipromastigote Radiolabeled Thymidine Assay

Cell proliferation can be measured with radiolabeled thymidine methodologies, based on the fact that proliferating cells incorporate free nucleotides such as thymidine when they are present in the culture medium.

Thymidine molecules synthesized with radioactive isotopes (^{3}H or ^{14}C) are added to culture medium. These are called radiolabeled thymidine molecules. As cells multiply, their DNA is replicated. Radiolabeled thymidine molecules are incorporated through this process.

After some incubation time, cells are collected with a cell harvester and transferred to a filter paper; nonincorporated radiolabeled thymidine molecules are washed. Pieces of the filter paper containing cells are transferred to a tube and immersed in a scintillation solution. The radioactivity emission intensity of each tube is measured with a scintillator and compared with the radioactivity emitted by the total concentration of thymidine initially added.

Many research groups have used radiolabeled thymidine methodologies to assess substance bioactivities against tumor cells, immune system cells, and even parasite cells [75]. These methodologies are elegant and have very high sensitivity. The main drawback is that they involve the manipulation of radioactivity.

An alternative methodology to DNA synthesis quantification involves the use of bromodeoxyuridine (BrdU), which is a modified but nonradioactive nucleotide that incorporates to newly synthesized DNA molecules. For BrdU, immunostaining techniques allow the method to be colorimetric or measured by fluorescence [76–78].

Assaying the activity of substances against promastigotes is a practical tool for screening new biotechnological drugs. Promastigotes are also useful as cytotoxicity indicators in the bioassay-guided fractionation of plant products. However there are significant differences between promastigotes and amastigotes in biochemistry and sensitivity to standard and experimental drugs [30].

22.6.2.4 *Antiamastigote Assays Using Macrophages Infected With Axenically Grown Amastigotes*

Adhered peritoneal macrophages can be infected with *Leishmania* sp. promastigotes (stationary growth phase) at a parasite/macrophage ratio of 10:1 and incubated for 3 h at 37°C and 5% CO_2. In the final of experiment phase the cells are washed with saline solution, fixed in methanol, and stained with Giemsa. The number of parasites is determined by examining three coverslips for each treatment. At least 200 infected macrophages are counted and results are expressed as the infectivity index. The positive control is Glucantime (50 mg/mL), and triplicates should be used for each concentration.

The review by Croft and coauthors [30] showed that different protocols were described for *Leishmania* mexicana [76,77], *L. braziliensis* [78], *L. donovani* [79,80], and *Leishmania infantum* [81]. However the amastigotes used must have confirmed biochemical and immunologic markers, and interpretation of data related to the high concentration of serum is required in some systems. Differences in drug sensitivity between axenic *L. donovani* amastigotes and intracellular amastigotes have been observed [30].

Generally, host cells used for amastigote infection are murine peritoneal macrophages or human–monocyte transformed macrophages. These cells can have variation

in drug sensitivity [82]. For these differentiated nondividing macrophages, the rate of amastigote division in host cells and drug activity can be clearly determined. For species of the *donovani* and *infantum* complex, the slow rate of division of amastigotes in this model is a limitation [30]. Assays that use dividing host cells must ensure that the confounding effects of drug activity on both parasite and host cell number are considered. Mouse (J774) [83] and human (THP-1, U937, and HL-60) monocytic cell lines have been used in drug assays [84]. THP-1 cells can form a nondividing monolayer and make an attractive alternative to primary isolated macrophages, and have been used in antibacterial assays [85].

The sensitivity and drug activity are assessed by determining the percentage of infected macrophages and the parasite survival index (PSI), the mean number of intracellular parasites in an infected macrophage.

PSI is calculated as 100 − (number of amastigotes/100 infected macrophages in treated wells)/(number of amastigotes/100 infected macrophages in untreated wells). The cells are counted with an optical microscope [86–88].

If 100% of efficacy is measured for a substance, it is possible to confirm the result further by assaying the infected cells and positive control in NNN medium [66].

22.6.2.5 Antiamastigote Assays Using Automated Screening

Major limitations of the amastigote–macrophage model are the absence of automation and dependence on microscopic evaluation. Promastigote assays using reazzurin (Alamar blue) and transfected parasites have been successful [89–92] but not within the clinically relevant amastigote–macrophage model.

The firefly luciferase gene has been successfully integrated into the genome of an *L. amazonensis* strain and has been evaluated in vitro. It was used in a drug-screening assay in 96-well plates and results correlated with those of microscopic evaluation. Transfected reference strains of *Leishmania*, such as *L. donovani* HU3, would be of further use to the drug screening community; work is under way to achieve this.

22.6.2.5.1 PROPIDIUM IODIDE DYE

Propidium iodide (PI) is a fluorescent dye that is unable to penetrate intact cell membranes, and which is used to detect apoptosis cells on viability assays. PI binds the DNA of cells that lost membrane integrity intercalating between base pairs with low or absence of sequence preference. When PI is bound to nucleic acids, it emits an enhanced red fluorescence with a maximum at 617 nm wavelength. PI can be used in fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, and fluorometry. PI is preferentially used in flow cytometry because it permits a rapid and reliable method. PI has been commonly used in assays to measure the effect of apoptosis by drugs on promastigote plasma membrane. Aliança and coworkers used this methodology with promastigotes of *L. amazonesis*. After staining with PI dye, cultures were incubated with candidate drugs and labeled. The intensity of the stain was

measured in a flow cytometer and then analyzed on software [93]. Researchers also reported the use of PI cell viability assay to measure the apoptotic effect of drugs against amastigote forms [94]. Antileishmanial drug activity was tested against *L. amazonesis* axenic amastigotes. After an incubation period with the drug, the amastigotes were stained with PI at room temperature for 5 min. Then the stain intensity was measured by flow cytometry and analyzed on software [95]. The PI stain method through flow cytometry has the advantages to be accurate, which permits simultaneous evaluation of different drugs and automated reading of results. Other fluorochromes can also be used, such as (2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester), SYTO-17, and a fluorescein conjugated with monoclonal antibodies [95].

22.6.2.5.2 HIGH-THROUGHPUT SCREENING AND HIGH-CONTENT SCREENING

High-throughput screening (HTS) is a technology used to select active compounds in a range of small molecules widely used in the pharmaceutical industry to discovery new drugs. Some research groups have reported the use of this technology in in vitro assays to find new drugs against *Leishmania* [96,97]. The efficiency of HTS can be combined with assay information to provide measures of the molecule effect in the assay system, resulting in high-content screening (HCS) [97].

An HCS protocol was developed for in vitro drug screening for the intracellular amastigote form of *Leishmania* sp. to infect human macrophages [97]. This protocol consists of two principal steps: (1) a 384-well plate containing the host cells (THP-1), the parasites, the testing, drugs and the controls, is imaged on an automated microscope; (2) the images are analyzed using an image analysis algorithm.

The results are stored in a database and can be accessed with software that provides assay features using statistical analysis. The features include, among others, the number of cells, the number of infected cells, the infection ratio, and the total number of parasites per cell [97].

The HCS methodology provides a promising and relevant system for the discovery of new antileishmanial drugs, because it compares and quantifies drug activity against the intracellular form of the parasite.

22.6.2.6 In Vivo Assays

To confirm the results of in vitro experiments, in vivo models should be used. In in vivo models, it is possible to study absorption (route of administration), distribution (different sites of infection), metabolism (pro-drugs or immunomodulators), and excretion of candidate drugs, and even to give an early indication of toxicity. In the literature, the most commonly used animal model is the mouse, because small amounts of test compounds are required, as well as a relatively small number of animals (five animals per group). The BALB/c mouse—*L. major* model has been used for others species such as *L. mexicana* and *L. amazonensis*. Lesions on the back of mice are also used to test topical formulations. To determine drug efficacy, changes in lesion size should be measured during and after the course of treatment. Poor animal models for

L. tropica, *L. aethiopica*, and *L. braziliensis* are a limitation of studies. Work on the *L. braziliensis* hamster model has shown the potential for drug testing [98]. For visceral leishmaniosis, dogs are frequently the model of choice [99].

In animal models, the determination of drug activity upon necropsy or biopsy depends on culture in NNN medium or microscopy to determine the level of infection. Molecular techniques such as real-time polymerase chain reaction are currently used [30,100−104]; however these are better used as preliminary evaluation assays. It is preferable to reisolate the parasite, to avoid false negatives or positives.

Before starting in vivo experiments, it is necessary obtain approval for the clinical trials from the Ethical Committee for Animal Care, following international rules (for details, see the 3R rules [105]). Some examples of animal models, inoculation procedures, and inoculation rates are given in Table 22.4.

22.6.2.7 In Silico Assays

Theoretical knowledge can aid the screening of natural compounds for active molecules. With the proper analytic techniques, it should be possible to predict the potential of a novel natural sample before any in vitro or in vivo experiments.

The interaction of molecules can be predicted by physicochemical models. Computers allow the calculation of the interaction of highly complex molecules, such as those present in biological systems. Evolving knowledge of Leishmania genes, proteins, RNA molecules, and other metabolites, combined with knowledge about humans and vectors molecular profiles, provides a field for virtual experiments that can result in good insight for the development of better new antileishmanial drugs.

It has been 10 years since the first *Leishmania* genome was fully sequenced [121]. Since then many *Leishmania* species and strains have been fully sequenced. Genomic, transcriptomic and proteomic research can greatly contribute to the development of new antileishmanial drugs. New vaccines and chemotherapeutics, for example, could be projected based on molecular differences between humans and parasites. Proteins present exclusively on parasites are good candidates as chemotherapeutic targets or vaccine immunogens.

Moreno et al. [122] identified that tyrosine aminotransferase from *L. infantum* should be a good drug target candidate because it is significantly different from human proteins and is overexpressed during infection.

Khademvatan et al. [123] compared the activity of biurets and Glucantime against *Leishmania* previewed by an in silico model with experimental in vitro results. The most active substance pointed out by the in silico model was confirmed by in vitro results. Roldos et al. [124]. compared in silico, in vitro, and in vivo results of a new leishmanicidal drug (14-hydroxylunularin), with a positive correlation.

Furthermore, resistance development mechanisms may be profoundly comprehended by genomic and proteomic perspectives, thus allowing a rational approach toward developing new pharmaceutical molecules capable of effectively overcoming the parasites' defenses in the long term.

Table 22.4 In Vivo Antileishmanial Assays

Species	Animal Model Choice	Objective of Study	Size of Inoculum/Inoculation Route	References
Leishmania (L.) infantum	BALB/c mice	Assess leishmanicidal effect of series of synthesized compounds containing nitrobenzene and sulfonamido moieties	1×10^7 promastigotes per animal were inoculated by intracardiac route	[106]
L. infantum *Leishmania (V.) braziliensis*	Mice peritoneal macrophages from Balb/c, C57BL/6 and knock-out ($TLR2^{-/-}$, $TLR4^{-/-}$)	Measure production of nitric oxide, cytokines, mitogen-activated protein kinases, and nuclear factor-κB to evaluate macrophage modulation by *Leishmania* lipophosphoglycan (LPG)	Mice macrophages were primed with interferon-gamma and stimulated with purified LPG from *L. infantum* and *L. braziliensis*	[107]
Leishmania (L.) donovani	BALB/c mice (6 weeks old)	Evaluation of protective efficacy of tuzin as DNA vaccine obtained by reverse vaccinology approach using novel high-throughput immunologic screening assay	1×10^8 stationary phase promastigotes suspended in 100 μL phosphate-buffered saline (PBS) (freshly transformed from spleen isolated amastigotes) were intravenously injected using 22-gauge needle	[108]
L.(V.) braziliensis *Leishmania (V.) peruviana*	Golden hamsters (*Mesocricetus auratus*) (4 weeks old)	Create model able to demonstrate reproducibly differences in infectivity among representative strains from *Leishmania* of subgenus *Viannia*	1×10^6 parasites resuspended in 100 μL saline solution were inoculated at right footpad: Group I stationary phase promastigotes and Group II metacyclic promastigotes purified by Ficoll protocol.	[109]
L. (V.) braziliensis	Golden hamsters (*Mesocricetus auratus*)	Evaluation of reproducibility of *L. braziliensis* infection as appropriate model for immunopathogenesis studies of cutaneous leishmaniosis, supporting its use in clinical, vaccine, and chemotherapy experimental protocols	1×10^6 stationary growth phase promastigotes (third in vitro passages in supplemented Schneider's *Drosophila* medium) were inoculated intradermally in dorsal hind paw of hamsters.	[110]
L. (V.) braziliensis	Golden hamster (*Mesocricetus auratus*)	Assess antileishmanial activity of metabolites, mainly flavanones, from *Picramnia gracilis* Tul., motivated by traditional use of this species in skin problems	5×10^8 promastigotes *(EpiR-GFP)* in 100 μL PBS were inoculated in anesthetized hamsters at dorsal skin.	[111]

Continued

Table 22.4 In Vivo Antileishmanial Assays—cont'd

Species	Animal Model Choice	Objective of Study	Size of Inoculum/Inoculation Route	References
L. (V.) braziliensis *Leishmania (V.) guyanensis*	C57BL/6 murine model for LRV dsRNA, that exacerbates disease in dose-dependent manner	Propose refinements of methods for successful *Leishmania* RNA virus detection for use as diagnostic tool relating to cutaneous leishmaniosis complications	1×10^6 stationary phase promastigotes (with and without *Leishmania* RNA virus) were injected subcutaneously into base of hind footpad.	[112]
Leishmania (L.) major	BALB/c mice	Evaluation of in vitro and in vivo antileishmanial activity of *Pistacia khinjuk* stock alcoholic extract compared with reference drug.	2×10^6 stationary growth phase promastigotes were subcutaneously injected into base of tail.	[113]
L. (L.) major	BALB/c mice	Evaluation of value of oleylphosphocholine for treatment of cutaneous leishmaniosis by in vivo tests	1×10^7 stationary growth phase promastigotes were injected into base of tail.	[114]
Leishmania (L.) amazonensis	BALB/c (4–5 weeks old)	Test combination therapy of tamoxifen and amphotericin B in vivo	1×10^6 stationary-phase transgenic promastigotes of *L. amazonensis* expressing luciferase were inoculated at base of tail.	[115]
L.(L.) amazonensis	Experimental model of cutaneous leishmaniosis in BALB/c mice	Test antileishmanial activities of six 2-nitrovinylfurans	5×10^6 stationary phase were injected through intradermal route in footpads.	[116]
L. (L.) amazonensis	C57BL/6 mice	Characterize changes in pattern of immune response during subcutaneous vaccination with Leishvacin	1×10^5 promastigotes from stationary phase cultures (4 days) were inoculated in hind footpad.	[117]
Leishmania (L.) tropica	BALB/c mice	Evaluate effects of ethanolic extract of *Berberis vulgaris* fruits and chloroform extract of *Nigella sativa* (folk medicine)	1×10^6 stationary promastigotes were inoculated in male BALB/c mice macrophages.	[118]
Leishmania (L.) enriettii	Guinea pigs (*Cavia porcellus*) C57BL/6 mice and knockout mice ($TLR2^{-/-}$, $TLR4^{-/-}$)	Characterization of LPGs and glycoinositolphospholipids, two important macromolecules of disease immunopathology model	1×10^5 promastigotes in 100 μL PBS were inoculated by intradermal inoculation.	[119]
L.(L.) major	Mouse models	Review immunologic aspects of disease	—	[120]

22.6.3 Cytotoxicity Assays

Whenever a candidate drug shows activity in in silico or in vitro experimental models, the next logical step is to perform assays with animal models and then clinical tests with human volunteers. However before taking these steps, it is ethical to perform other important experiments to detect substances that would be toxic against human cells, besides their leishmanicidal effect. As with activity assays, there are many possible experimental systems for assaying a substance's cytotoxicity. It is possible to use in silico methods to compare the candidate drug molecule with molecular data banks, seeking for similarities between the sample and known toxic compounds. It is also possible to use computers to solve models predicting the interaction of test substances with human molecular structures.

There are many different in vitro experimental models to assay the cytotoxicity of a given substance. Cells from multiple organ sources (kidney, liver, heart, lung, and nervous system), cell types (epithelial, endothelial, myoskeletal, neural, germ line, leukocyte, etc.) and different species (zebrafish, rodent, sheep, human, etc.) can be cultivated and used for toxicity assays [125]. Most standard methodologies involve simply measuring the proliferation of cells in cultivation with and without the addition of candidate substances. Several methodologies are also available to identify cell death mechanisms, such as apoptosis or necrosis.

Cell proliferation can be measured with colorimetric methods (based on MTT, XTT, and WST-1, for example), or DNA synthesis detection methods (including methods based on tritiated thymidine and BrdU), as described in previous sections. Other assay mechanisms include the absorption or exclusion of dyes by living or dead cells [125].

Cellular components can also be used to assay drug toxicities. For example, DNA and RNA microarrays, immobilized proteins, and antibodies developed with the objective of interacting with known toxic compounds can aid in identifying toxic substances in new or complex samples.

The advent of high-throughput systems to perform in vitro assays allowed the screening of thousands of substances, pointing out an elevated number of molecular candidates for in vivo and clinical experiments. This screening power resulted in problems for choosing among candidates for further experimental phases.

Although in vitro cytotoxicity assay methods are applied extensively in the pharmaceutical industry, at least 10% of all drugs that reach clinical testing stages have toxicity problems and 3% are discontinued because of them [122]. It proves that in vitro or even in vivo assays are limited in predicting a substance's toxicity against whole human organisms; nevertheless these methodologies are fundamental to drug development processes.

22.7 Conclusions and Perspectives

Although leishmaniosis has afflicted humanity for a long time, the pursuit of better antileishmanial drugs continues to be a considerable challenge. Current medications

have serious side effects and parasites continue to evolve, developing resistance and spreading to new areas, and infecting a rising number of individuals.

This situation constitutes a matter of public health concern. Research on new antileishmanial drugs is needed. Many groups within the scientific community are already involved but more work is required to bring about a satisfactory solutions. Governmental contributions are necessary in this process, because the big pharmaceutical companies do not see great economic perspectives in this area.

Public universities currently constitute the major responsibility for developing new therapeutic strategies against leishmaniosis. However, most public university laboratories face restrictive budgets and lack of automation and researchers frequently work independently in a noncollaborative manner. This scene contributes to slow and disorganized technological development.

Countless biomolecules are available for antiparasitic substances screening. The methodologies described here are ready for an evaluation of the activities of natural and synthetic products against *Leishmania* species.

Already discovered natural active extracts must be further fractioned to determine the active principles responsible for the antileishmanial effects observed. This suggests molecular groups for further antileishmanial biocompound screening. Characterization of these molecules and their mechanisms of action can also offer insights useful for antileishmanial drug design.

Biotechnological tools will be necessary to improve the productivity of some natural products. Even molecules from vegetable and animal sources can be expressed in microbial vectors aided by molecular biology tools. As active principles are identified, biotechnological processes parameters, such as process duration, aeration, agitation, temperature, pH, culture medium composition, and extractive procedures, can be optimized to maximize active biomolecule production and recovery.

The effect of the bioactive compounds can be improved by chemical manipulation of the molecules, such as complexation with vanadium ions. Combinations of active substances must be considered as well.

Edible active organisms such as mushrooms and vegetables should be analyzed through other methodologies to evaluate their potential as antiparasitic nutraceuticals. The edibility indicates that their compounds will not have significant toxicity against human cells. This is important because specificity is probably the main goal of drug design, besides effectiveness.

Besides screening new active substances, it is also important to create new methodologies to further select the best candidates for in vivo and clinical studies. Nevertheless molecular data banks have plenty of useful and unexplored information about *Leishmania* and antileishmanial drugs. With the proper in silico methods, this knowledge can result in novel breakthroughs that can lead to intelligent solutions.

Leishmanicidal compounds are not the only alternative to control the dissemination of leishmaniosis. Both vaccines and strategies to control vector insects are effective approaches and should be considered in parallel.

This chapter may inspire researchers and supply experimental tools to contribute to the discovery and invention of new antileishmanial drugs.

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23

Factors/Genes in Maternal Recognition of Pregnancy: An Overview

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23.1 Introduction

Reproduction is a highly complex, luxurious biological process requiring a dialogue between the developing conceptus and maternal uterus that must be established during the periimplantation period for pregnancy recognition signaling and the regulation of gene expression by uterine epithelial and stromal cells. The greatest limitation to reproductive efficiency across mammalian species is early embryonic mortality. Early embryonic mortality denotes the death of fertilized ova and embryos up to the end of implantation [1]. It is a major source of embryonic and economic loss, with a mortality rate of up to 40% in animal production through repeat breeding and the increased cost of artificial insemination [2–4]. Most losses are characterized by asynchrony between conceptus (embryo/fetus and associated membranes) signals and uterine receptivity, resulting in defective implantation and/or placentation. Suboptimal communication, resulting from impairment of conceptus (embryo and associated extraembryonic membranes) development and/or from abnormal uterine receptivity [5], contributes to a high incidence of embryonic mortality. Understanding and unraveling the pregnancy recognition, secrets of implantation, embryo development, and reciprocal signaling networks between the embryo and uterus will lead to alleviation of the problems of infertility. Early embryonic development, including implantation of the fetus and maintenance of pregnancy, is critically influenced by embryo-maternal cross-talk [6]. The survival of the embryo during early embryonic life mostly depends on the efficiency with which the maternal recognition of pregnancy (MRP) is established [7]. Pregnancy recognition signaling, as related to sustaining the functional lifespan of the corpora lutea, is required to sustain the functional lifespan of the corpora lutea for the production of progesterone (P_4), which is essential for uterine functions supportive of

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implantation and placentation required for successful outcomes of pregnancy. In ruminants, the main signal for MRP is identified as interferon-ι (IFNτ) secreted by the trophoectoderm of blastocysts for a limited period during early pregnancy. Oxytocin (OT) triggers the pulsatile release of prostaglandin (PG)$F_{2\alpha}$ by the endometrium at the end of an infertile estrous cycle to induce luteolysis. Interferon-ι abrogates the uterine luteolytic mechanism by suppressing the normal pattern of the pulsatile release of $PGF_{2\alpha}$ in late estrous cycle by a mechanism that involves the down-regulation of estrogen receptor in the uterine epithelium which in turn prevents a rise in OT receptor (OTR) [8]. IFNτ acts in concert with P_4 to induce the expression of genes for transport and/or secretion of histotrophs that includes nutrients such as glucose and arginine that activate the mechanistic target of rapamycin nutrient-sensing cell signaling pathway to stimulate proliferation, migration, differentiation, and translation of messenger RNAs (mRNAs) essential for growth and development of the conceptus. Several factors such as genetic abnormalities, nutrition, environment, hormonal imbalance, thermal stress, impaired maternal–fetal interaction, and infections are responsible for impaired communication between the embryo and the dam. There is an urgent need to unravel the complexities of maternal recognition of pregnancy and implantation to address two contrasting global issues: to improve fertility and develop novel contraceptives. The challenge is to understand the complex molecular dialogue between the conceptus and maternal uterine microenvironments that are characteristic of successful implantation and to use that knowledge to develop optimal reproductive management strategies and paradigms to improve embryo survival. Advances in genomic technologies will certainly enhance our understanding of the underlying biological processes involved in fertilization and the establishment of pregnancy and to explore gene profiles and the potential to identify genes responsible for improved embryo survival.

23.2 Maternal Recognition of Pregnancy

Higher rates of early embryonic mortality are a major cause of reproductive failure during the preimplantation period in livestock. These preimplantation losses are associated with inadequate luteal function during early pregnancy in ruminants [9]. The survival of embryo during early embryonic life mostly depends on the efficiency with which the MRP is established [7]. The term “MRP” was coined by Roger Short and can be defined as a delicate and sensitive process that involves the interaction between a conceptus and the maternal endometrium. It is defined as the uterine recognition of conceptus(es)’ signal and extension of the ovarian corpus luteum (CL) lifespan, which occurs in a coordinated synchronous manner if pregnancy is established. It is critical so that the endometrium does not secrete $PGF_{2\alpha}$, lysing the CL and destroying the source of P_4, the hormone necessary to sustain a pregnancy. There are two categories of mechanism of MRP: luteotrophic and antiluteolytic. Luteotrophic directly promotes luteal function, supporting the CL, and antiluteolytic prevents the uterine release of luteolytic PGF, which causes the lysis of the CL. Humans, nonhuman primates, and rodents

maintain luteal function through luteotrophic mechanisms. In primates the maternal recognition signal is chorionic gonadotropin (CG). The syncytiotrophoblast cells secrete CG from days 8 to 10 postovulation for pregnancy recognition. CG stimulates the CL to produce P_4 until there is a shift between luteal and placental P_4 secretion. Once the shift occurs, CG secretion decreases. Rodents also maintain luteal function through luteotrophic mechanisms; prolactin is the MRP signal. Prolactin is the initial maternal recognition signal until day 12 of pregnancy; after day 12 the conceptus and uterine decidua take over, stimulating P_4 secretion by secreting lactogenic hormones (prolactins). These hormones act on the luteal cells through the prolactin receptors to maintain luteal cell function and the secretion of P_4 (Figs. 23.1 and 23.2).

Sheep, cattle, and goats have estrous cycles of about 17, 21, and 20 days, respectively. An ovulatory surge of luteinizing hormone (LH) occurs near the time of onset of estrus that leads to ovulation about 30 h later. With the development of CL, the concentration of P_4 in peripheral circulation peaks around the mid to late diestrus phase and luteolysis is induced by the pulsatile release of $PGF_{2\alpha}$ from endometrial epithelium on day 15 and/or 16. The luteolytic mechanism that develops in the endometrial luminal epithelium (LE) and surface GE (sGE) requires sequential effects of P_4, estrogen, and oxytocin, acting through their respective receptors [10,11]. At estrus (day 0), estrogens from graafian follicles stimulate increased uterine estrogen receptor alpha (ERα), P_4 receptor (PR), and OTR expression [12,13]. During early diestrus, P_4 from the newly formed CL stimulates accumulation of phospholipids in LE and sGE that can liberate arachidonic acid for the synthesis and secretion of $PGF_{2\alpha}$. During diestrus P_4 levels increase and act via PR to "block" expression of ERα and OTR in the endometrial LE and sGE [14]. Therefore ERα and OTR expression is not detected between days 5 and 11 of the cycle, i.e., during most of diestrus. In sheep and cows endometrial receptors for OT were present between estrus and about day 4 of the cycle; they were low or undetectable

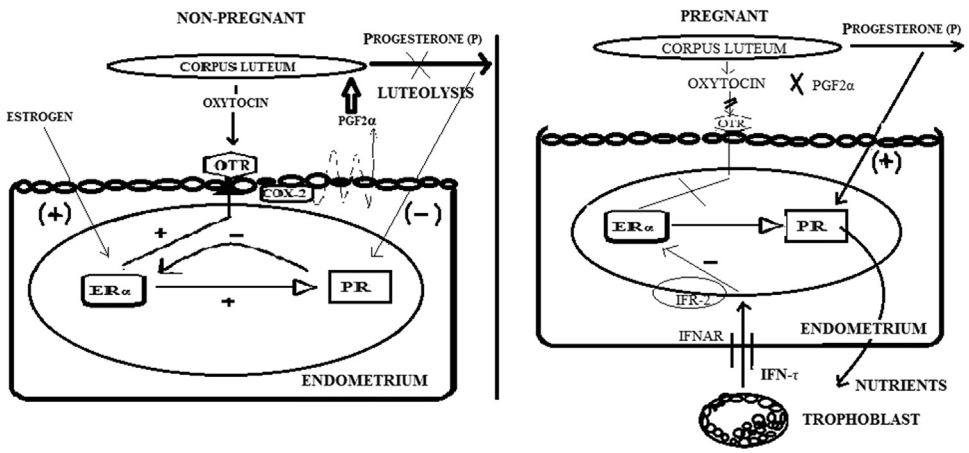

FIGURE 23.1 Events of maternal recognition of pregnancy. *ER*, Estrogen receptor; *IFN-τ*—Interferon tau; *IFNAR*, type 1 IFN receptor; *IRF2*- Interferon regulatory factor two; *OTR*, Oxytocin receptor; *PR*, Progesterone receptor.

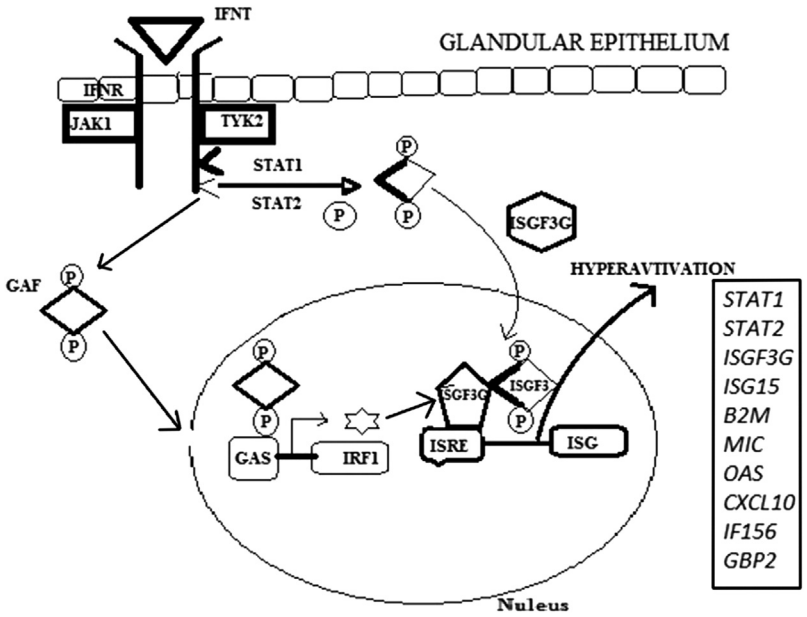

FIGURE 23.2 IFNτ signaling in uterine glandular epithelium. *GAF*, gamma interferon activation factor; *GAS*, gamma interferon activation sequence; *IFNT*, IFNτ; *IFNAR*, type I IFN receptor; *IRF1*, IFN regulatory factor; *ISGF3G-IFN*, stimulated transcription factor-3, gamma 48-k Da; *ISG15*, IFNτ-stimulated gene-15; *ISGF3*, IFN-stimulated transcription factor-3; *JAK1*, janus kinase-1; *STAT*, signal transducers and activators of transcription.

between days 5 and 13 and increased rapidly between days 14 and 16 in sheep [15] or days 17 and 20 in cows [16]. Endometrial ER and PR were highest during the first 10–12 days after onset of estrus in cows, and then both declined to their lowest levels on about day 13. ER then increased between days 14 and 15, with OTR increasing rapidly between days 17 and 21. The cytosolic PR concentrations were highest during fourth stage of estrous cycle (days 18–20 of the estrous cycle) and lowest during the second stage of the estrous cycle (days 5–10 of the cycle) in buffalo endometrium [17]. High P_4 levels during the second phase of the estrous cycle antagonize the ability of estrogen to promote the synthesis and/or replenishment of uterine steroid receptors whereas cytosolic estradiol receptor concentrations were higher in follicular phase compared with luteal phases [18]. Rising concentrations of estradiol in peripheral plasma shortly before estrus may be the physiologic cause of increased concentration of estradiol receptors in the follicular phase.

MRP in ruminants (sheep, cattle, and goats) requires the conceptus elongate to be from a spherical to a tubular and then a filamentous form to produce IFNτ, which is the pregnancy recognition signal that prevents the development of the endometrial luteolytic mechanism [10,19]. During MRP, the mononuclear cells of the conceptus trophectoderm synthesize and secrete IFNτ between days 10 and 21–25 with maximal production on days 14–16 [19]. IFNτ appears to be the sole factor produced by the

conceptus that prevents development of the endometrial luteolytic mechanism. It is a novel member of the type I IFN family. It acts differentially on the endometrial LE, glandular epithelium (GE), and stroma to regulate expression of a number of IFN-stimulated genes (ISGs) that have roles in endometrial differentiation and conceptus implantation. The IFNτ gene has a 595−base pair (bp) open reading frame that encodes a 195−amino acid preprotein containing a 23−amino acid signal sequence and is cleaved to yield the mature protein. The IFNτ genes expressed by ruminant conceptuses share approximately 70% homology with IFNw. It shows remarkable homology with the complementary DNA (cDNA) nucleotide sequence across ruminant species. Bovine, ovine, and caprine IFNτ are more similar to each other in sequence. In the coding region, the nucleotide sequences exhibit approximately 90% identity, and their inferred amino acid sequences about 80% identity. IFNτ is thought to act antiluteolytic by:

1. stabilization or up-regulation of P_4 receptors in the endometrium;
2. direct inhibition of endometrial estrogen receptors;
3. direct inhibition of endometrial oxytocin receptors;
4. initiation of postreceptor mechanisms, which prevent oxytocin-induced release of PGF; and/or
5. induction of the endometrium to synthesize an inhibitor of enzymes necessary for the synthesis of $PGF_{2\alpha}$ [20,21].

In sheep, it is secreted between days 10 and 21 by the mononuclear trophoblast cells. In vitro culture (IVC) of sheep conceptus homogenates and analysis for radiolabeled proteins released into the culture medium indicate that ovine trophoblastic protein-1 (oTP-1) is secreted by mononuclear cells of ovine trophoectoderm. oTP-1 is secreted in two phases: one between days 10 and 21 of pregnancy and the other by chorion between days 25 and 45 of pregnancy. oTP-1 is thought to exert a paracrine antiluteolytic effect on endometrium because there is no evidence that it is transported from the uterus to affect CL directly. Secretion of oTP-1 (ng/uterine flushing) begins on about day 10 and increases with morphologic changes: from spherical (312 ng) to tubular (1380 ng) to filamentous (4455 ng) forms on days 12−13. Goat conceptus secretes caprine IFNτ between days 16 and 21, which is assumed to be an antiluteolytic signal. Intrauterine injections of recombinant ovine IFNτ in cyclic goats extend the CL lifespan in goats. Pulsatile release of oxytocin and $PGF_{2\alpha}$ is suppressed in pregnant compared with cyclic goats between days 10 and 12 and estrus or day 20 of pregnancy, which suggests that the antiluteolytic mechanism mediated by IFNτ is similar for sheep, cows, and goats. The endocrine−exocrine theory of MRP in pigs was studied. In pigs, MRP and antiluteolysis are not mediated by trophoblastic IFNs, but by trophoblast-secreted estrogen from days 10.5 to 11 [20]. A second period of estrogen production occurs between days 15 and 25−30 of pregnancy. Injection of exogenous estrogen on days 11−15 of the estrous cycle results in CL maintenance for a period equivalent to or slightly longer than pregnancy (about 120 days later). This condition is referred to as pseudopregnancy [22], whereas a single injection of estradiol on day 9.5, 11, 12.5, 14, 15.5, or 14−16 results in interestrous

intervals of about 30 days. Estradiol must be administered to gilts on day 11 and days 14−16 or daily from days 11 to 15 to obtain interestrous intervals of greater than 60 days. This suggests that two phases of estradiol, similar to those produced by conceptuses on days 11−13 and days 15 to 25−30, are necessary for prolonged secretion of $PGF_{2\alpha}$ into the uterine lumen. PGF from the uterus is taken up by the mesometrium and transferred to the ovary in arterial blood by a countercurrent system that exists in the broad ligament of the uterus [23]. Estrogen also changes the ratio of PGE_2 to $PGF_{2\alpha}$ [24,25] and maintains LH receptor levels in both CL and the uterus [26]. PGE_2 may protect the CL from the luteolytic effects of $PGF_{2\alpha}$. Estradiol may induce receptors for maternal hormones, e.g., prolactin, or conceptus secretory proteins, which influence the exocrine secretion of $PGF_{2\alpha}$ [27]. The first estrogen signal may induce those receptors and the second estrogen signal may be required to replenish those receptors. Available evidence indicates that estrogens of blastocyst origin are essential for MRP in pigs and that secretory proteins from the conceptus, including IFNs, have other roles during early pregnancy in pigs, including cellular changes that lead to increased polarity of endometrial epithelial cells for exocrine secretion of components of histotrophs, including $PGF_{2\alpha}$ [28].

The IFNτ novel member of the type I IFN family acts differentially on the endometrial LE, GE, and stroma to regulate expression of a number of ISGs that are hypothesized to have roles in endometrial differentiation and conceptus implantation [29]. All type I IFNs bind a common receptor composed of two subunits, IFNAR1 and IFNAR2, to induce cell signaling via the Janus-activated kinase (JAK) and tyrosine kinase 2 (TYK2) pathways, respectively.

IFNτ binds to a common type I IFN receptor, IFNAR1 and IFNAR2, containing tyrosine kinases such as JAK1 and TYK2 to activate the JAK/STAT cell signaling pathway. IFNτ-mediated association of IFNAR subunits facilitates cross-phosphorylation and activation of JAK1 and Tyk2, which in turn phosphorylates the receptor and creates a docking site for STAT2. STAT2 is then phosphorylated, thus creating a docking site for STAT1, which is then phosphorylated. Phosphorylated STAT1 binds phosphorylated STAT2 to form a heterodimer and translocates to the nucleus after forming a heterotrimeric transcriptional complex by binding with ISGF3G, collectively termed ISGF3, which binds to IFN-stimulated response element (ISRE) in promoter regions of ISGs and transactivates genes containing an ISRE(s), such as STAT1, STAT2, IRF9, B2M, ISG15, MIC, and OAS. In addition to STAT1/2 heterodimerization, type I IFN induces the formation of phosphorylated STAT1 homodimers termed GAF, which enter the nucleus and transactivate genes containing a GAS element(s) such as IRF1. IRF1 can also bind and transactivate ISRE-containing genes as well as IRFE-containing genes.

23.3 Gene Regulating Maternal Recognition of Pregnancy Events

Successful embryo development and survival include the formation of the blastocyst, implantation into the uterus, the formation of the placenta, the development of the

heart, and vascularization of both the embryo and fetus to assist nutrient deliverance. Among these developmental events, implantation of the embryo is a crucial step and its success mostly depends on the efficiency with which the MRP is established [7]. The MRP includes a series of events that are synchronized by the endocrine interaction between the mother and the embryo. The genes regulating MRP are discussed here.

23.3.1 Prostaglandin E Synthase

PGs have, important roles in ovulation, luteolysis, implantation, decidualization and parturition. The production of PGs is primarily catalyzed by two rate-limiting enzymes, e.g., cyclooxygenase-1 (COX-1) and -2 (COX-2), also referred to as PG endoperoxidase H synthase-1 (PGHS-1) and -2 (PGHS-2), respectively. PGHS-1 and PGHS-2 are responsible for the conversion of arachidonic acid into PGG_2 and PGG_2 to PGH_2, respectively. The downstream enzymes, PG E synthase (PGES) and PG F synthase (PGFS), catalyze the conversion of PGH_2 to PGE_2 and $PGF_{2\alpha}$, respectively. PGE_2 protects the CL from spontaneous regression and helps maintain pregnancy [30]. It enhances endometrial vascular permeability at the sites of blastocyst apposition and decidualization, blastocyst hatching, and implantation. In contrast, $PGF_{2\alpha}$ acts as the luteolytic agent in ruminants, which regresses the CL and causes termination of the estrous cycle or pregnancy. PGES is a member of the membrane-associated protein in eicosanoid and glutathione metabolism superfamily, which consists of six proteins with divergent functions [31]. The activity of PGES has been detected both in cytosolic and microsomal fractions of various cells, and in most cells it requires glutathione (GSH) as a coenzyme for optimal activity. Cytosolic PGES (cPGES) is constitutively expressed in a wide variety of cells and tissues and is predominantly linked with COX-1 to promote immediate response during which relatively high concentrations of arachidonic acid are released in a short period [32], whereas microsomal PGES (mPGES) is a membrane-associated, inducible perinuclear enzyme with GSH-dependent activity and is expressed in variety of tissues including prostate, testis, and small intestine [31]. There are three isoforms of PGES in bovines: mPGES-1, mPGES-2, and cPGES [33]. Unlike mPGES-1, mPGES-2 does not require GSH for catalytic activity and is activated in the presence of a broad range of reducing agents. Although three forms of PGES have been characterized, mPGES-1 is the main PGES responsible for PGE_2 production in endometrial cells. The PGES gene structure has been evaluated in human [34]. The gene size is 14.8 kb and has been mapped to chromosome 9. The genomic sequences of human PGES (Acc. No. AC 007,936) indicate three exons (136, 83, and 1526 nt) separated by two introns (4.2 and 8.8 kb). The complete cDNA sequences of PGES have been characterized in rats (Acc. No. NM 0,211,583; AF 280,967), cattle (Acc. No. AY 032,727), horses (Acc. No. AY 057,096), and pigs (Acc. No. AY 857,634). Filion et al. [35] reported the complete cDNA sequences of 462 bp of bovine PGES (Acc. No. AY 032,727). Partial PGES gene sequences are available (Acc. No. AY260142) in cattle. The nucleotide sequence of buffalo PGES cDNA encoding the entire mature peptide except the first three amino acids is also available (Acc. No. DQ167808).

Expression of mRNA encoding for the PGES has been evaluated in a variety of tissues such as uterus, CL, placenta, and ovary. Expression of mPGES-1 mRNA was high between days 13 and 21 of the cycle in bovines, which indicated that the bovine endometrium becomes responsive to produce PGE_2 after day 13 of the cycle [36]. However no variation in PGES mRNA levels was observed during late diestrus and early pregnancy in horses [37]. Even infusion of PGE_2 did not extend the lifespan of CL and PGE_2 did not induce a luteotropic effect in cultured luteal cells [38]. Moreover, equine conceptus produced huge amounts of PGE_2 during days 10−16 of the cycle. PGE_2 produced by the endometrium supplemented PGE_2 produced by the conceptus rather than playing a luteotropic role. Waclawik et al. [39] also reported that there was no significant variation in mPGES-1 expression throughout the estrous cycle in pigs. In contrast, abundant levels of mPGES-1 protein were expressed in endometrium during the proliferative phase, whereas a low expression was observed during the late-secretory phase of the menstrual cycle in humans owing to a decrease in PGE_2 production by the endometrium [40]. The expression of mPGES-1 mRNA could not be detected in the cyclic endometrium of buffalo during the first (days 3−5) and second (days 6−15) stages but it was observed in the endometrium during the third stage (days 16−21) of the estrous cycle [41]. Limited reports are available on PGES regulation in uterus in vitro studies using cultured endometrial cells. Tanioka et al. [32] observed that GSH-dependent PGES activity increased in vivo threefold after lipopolysaccharide (LPS) challenge in cytosol of rat brain, but not of other tissues. PGES mRNA expression is also increased in the presence of LPS, tumor necrosis factor-α (TNF-α), and IFNτ in epithelial cells [42]. They also reported that phorbol-12 myristate-13 acetate increased PGES mRNA, COX-2 mRNA, and PGE_2 production, which suggested that the expression of PGES was correlated with that of COX-2 for the production of PGE_2. Increasing production modulated the PGE_2−$PGF_{2\alpha}$ ratio, leading to the establishment of a successful pregnancy.

23.3.2 Prostaglandin F Synthase

PGFS is a monomeric protein with a molecular weight of about 36,000, which converts PGH_2 to $PGF_{2\alpha}$. PGFS is a member of the aldo-keto reductase (AKR) superfamily based on substrate specificity, molecular weight, and amino acid sequence. The AKR superfamily consists of monomeric NAD(P)H-dependent oxido-reductase with a broad specificity of substrates: endogenous substrates such as steroid hormones, PGs, and xenobiotics such as drugs and carcinogens. The PGFS gene consists of nine exons and eight introns. The initiation codon is located at exon 1 and the stop codon at exon 9 and encodes a protein of 323 amino acid residues. The complete cDNA sequence of PGFS has been characterized in cattle (Acc. No. J03570), buffalo (Acc. No. DQ884879), sheep (Acc. No. AY135401), horses (Acc. No. AY304536), and pigs (Acc. No. AY863054). PGFS is known to exist in six isoforms: lung type PGFS (PGFS1), lung type PGFS found in liver (PGFS2), liver type PGFS (DDBX), and three other PGFS isolated from human, sheep, and *Trypanosoma brucei*. All of these isoforms belong to the AKR1C subfamily,

except *Trypanosoma*, which belongs to the AKR5A subfamily. In bovine, six isoforms of PGFS [PGFS1, PGFS2, DDBX, PGFS like 1 (PGFSL-1), PGFSL-2, and 20α-HSD] have been identified [43]. A new isoform, AKR1B5, possessing aldoreductase activity has been implicated as the most probable enzyme responsible for production of $PGF_{2\alpha}$ in bovines [43]. Kuchinke et al. [44] reported the complete cDNA sequence of 972 bps of bovine lung PGFS (Acc. No. M86544). The cDNA sequences of bovine PGFSL-1 protein (Acc. No AY135400) and PGFSL-2 protein (Acc. No. AY135401) are of 972 bp. The nucleotide sequence of buffalo PGFSL-2 cDNA exhibited 98%, 87.6%, 87.3%, 77.2%, 68.9%, 68.7%, 65%, and 77.7% identity with bovine PGFSL-2, bovine lung PGFS, bovine liver PGFS, horses, pigs, monkeys, dogs, and humans, respectively [41].

The expression of PGFS mRNA has been studied in domestic ruminants including cattle, buffalo, sheep, horses, pigs, monkeys, and rats. Madore et al. [43] reported that no known PGFS enzymes belonging to AKR1C family (PGFS1, PGFS2, PGFSL-1, PGFSL-2, and DDBX) were expressed in the bovine endometrium during the estrous cycle. Interestingly, it was observed that AKR1B5 expression was up-regulated in the endometrium during the second half of the estrous cycle. PGFS expression was found to up-regulate around luteolysis in pigs [39]. However expression of PGFS mRNA was not up-regulated during luteolysis and pregnancy in horses, which suggests that PGFS does not have a crucial role in endometrial $PGF_{2\alpha}$ synthesis. They also reported that expression of COX-2 increased around luteolysis (day 15) and this induction was observed in pregnant mares, which indicated that horse conceptus blocks endometrial $PGF_{2\alpha}$ synthesis by down-regulating the expression of COX-2. Palliser et al. [45] demonstrated that the expression of PGFS increased in placentome after dexamethasone induced and spontaneous labor onset in sheep. Mondal et al. [41] reported that the expression of PGFSL-2 mRNA could not be detected in buffalo uterine endometrium at any stage of the estrous cycle. In vitro studies have shown that IFNτ down-regulated PGFS mRNA expression in bovine endometrium, which suggested that conceptus interferes with $PGF_{2\alpha}$ synthesis by targeting PGFS gene expression [46].

23.3.3 Cyclooxygenase-2

PGs are generated via the COX pathway, and COX is the rate-limiting enzyme for the conversion of arachidonic acid into PGH2. COX exists in to two isoforms that are encoded by two separate genes, COX-1 and COX-2, which are also known as PGHS-1 and PGHS-2. To date, three isoforms of COXs have been identified. COX-1 is a glycoprotein of 71 kDa, which is constitutively expressed in different tissues. COX-1 is encoded by a gene on chromosome 9 and has a role in tissue homeostasis by modulating several cellular processes ranging from cell proliferation to angiogenesis or platelet aggregation owing to thromboxane production. Although COX-1−deficient female mice are fertile, they have specific defects in parturition, whereas COX-2−deficient female mice are infertile with abnormalities in ovulation, fertilization, implantation, and decidualization. COX-2 is the inducible isoform, which is regulated by growth factors and different cytokines such as

interleukin (IL)1α, IL6, or TNF-α and overexpressed during inflammation. The COX-2 gene is located on chromosome 1 and its promoter displays a nuclear factor-κB response element as well as other cytokine-dependent (i.e., IL6) response elements. Finally, COX-3 has been identified as a splice variant of COX-1 and it is present mainly in the brain and spinal cord. The role of COX-3 is not known. The requirement of COX-2 for normal blastocyst implantation and decidualization in mice is due to the role of COX-2−derived PGs in the regulation of vascular endothelial growth factor and angiopoietin signaling that influence uterine vascular permeability and angiogenesis. Parent et al. [42] correlated the expression of PGES with that of COX-2, which is an important enzyme for the production of PGE_2 in bovine endometrium. Increasing this production will modulate the PGE2−$PGF_{2\alpha}$ ratio and contribute to the establishment of pregnancy.

23.3.4 Interferon-τ

IFNs are cytokines with antiviral, antiproliferative, and immunomodulatory biological effects critical to immune responses that protect the body against viral infections and malignant cells. In many species of ruminants, IFNτ is the pregnancy recognition signal expressed only by mononuclear trophectoderm cells of ruminant conceptuses (the embryo and its extraembryonic membranes) into the uterine lumen on days 13−21 of pregnancy. It is a subclass of the 172−amino acid type I omega (w) IFNs that compete with IFNα and -β for binding of a common cell surface receptor. IFNι of cattle and sheep arise from multiple mRNAs approximately 1 kb in length, which arise from multiple genes. Like other type I IFN genes, IFNτ genes are intronless and consists of 585 bp ORF coding for a 195−amino acid preprotein containing a 23-signal sequence which after cleavage yields a mature protein of 172 amino acids. In the coding region, the nucleotide sequences of bovine, ovine, and caprine IFNτ exhibit approximately 90% identity and their inferred amino acid sequence about 80% identity [19]. The predicted amino acid identity between IFNτ and $IFN_{\alpha 1}$ and IFNw is 50% and 72%, respectively. IFNτ acts differently on the endometrial LE, GE, and stroma to regulate expression of various ISGs that have crucial roles in endometrial differentiation and implantation of conceptus. The mechanism of action of IFNτ includes inhibition of estradiol receptors, consequent reduction in oxytocin receptors, and activation of a COX inhibitor that abrogates the oxytocin-dependent release of luteolytic pulses of $PGF_{2\alpha}$ by uterine epithelium; therefore the CL continues to produce P_4 required for pregnancy.

23.3.5 Osteopontin

Osteopontin (OPN) is a highly phosphorylated acidic glycoprotein that stimulates cell−cell adhesion, increases cell−extracellular matrix communication, and promotes cell migration. It is a negatively-charged acidic hydrophilic protein and is secreted into all body fluids. It has an arginine−glycine−aspartic acid (RGD) cell binding sequence, a calcium binding site, and two heparin binding domains. In general, OPN is a monomer ranging in length from 264 to 301 amino acids that undergoes extensive posttranslational

modification, including phosphorylation, glycosylation, and cleavage, resulting in molecular mass variants ranging from 25 to 75 kDa. It contains a hydrophobic leader sequence characteristic of a secreted protein, a potential calcium phosphate aspartite binding region of consecutive asparagine residues, a cell attachment GRGDS sequence, a thrombin cleavage site, and two glutamines that are recognized substrates for transglutaminase-supported multimer formation. OPN is hypothesized to serve as a bifunctional bridging ligand that mediates the adhesion between LE and trophoblast essential for implantation and placentation [47]. It binds to integrin heterodimers (αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α4β1, α5β1, and α8β1) via its RGD sequence, and to α4β1 and α9β1 by other sequences to promote cell adhesion, spreading, and migration. During the periimplantation period of pregnancy in sheep, OPN mRNA is expressed only by the endometrial glands, is first detected in some glands of some ewes by day 13, and is present in all glands by day 19 [48]. Multiple integrin receptors for OPN are present on trophoblasts and LE of humans and domestic animals, some of which increase during the periimplantation period [47]. Evidence suggests that secreted OPN binds integrin receptors expressed on conceptus trophoblast and endometrial LE, where it can stimulate changes in proliferation, migration, survival, adhesion, and remodeling of the conceptus as it elongates, opposes, and adheres to the LE. OPN protein is expressed at high levels in the uterine glands and on the apical surface of LE during the midsecretory phase, in decidualizing stroma, and on invading cytotrophoblast in humans [49,50].

23.3.6 Galectin

Galectins belongs to a family of calcium-independent α-galactoside-binding proteins, the lectin superfamily, and is involved in immunomodulation, cell adhesion, and defense against invading microorganisms. In sheep, the size of the LGALS-15 gene transcript is 243 bp, which encodes a protein of 81aa. Ovine and caprine LGALS-15 were highly homologous at the mRNA (95%) and protein (91%) levels, and all contained a conserved carbohydrate recognition domain and RGD recognition sequence for integrin binding. Several forms of galectins [galectin-1 (LGALS-1), galectin-3 (LGALS-3), galectin-5 (LGALS-5), galectin-9 (LGALS-9), and galectin-15 (LGALS-15)] were discovered. Galectin-1 possesses adhesive and antiadhesive roles and is an immunomodulator in maternofetal tolerance as well as embryo implantation. Galectin-3 (LGALS-3) is a galactose-specific lectin, the expression of which increases significantly during the secretory phase of the menstrual cycle [51] endometrium, with expression in glandular and luminal epithelial but not stromal or immune cells [52]. A new galectin family member, galectin-15, was discovered in the endometrium of sheep and has a prospective role in trophectoderm attachment [53]. In sheep, the galectin-15 (LGALS-15) gene is expressed specifically in the endometrial LE. Galectin-9 has been identified in mid- and late-secretory and decidual phases in human and sGE of the uterus in concert with blastocyst elongation during the periimplantation period; it is also expressed in the uterus of cattle, goats, and pigs. Endometrial LGALS-15 mRNA levels increased after day

11 of both the estrous cycle and pregnancy, and were considerably increased after day 15 of pregnancy in goats. In situ hybridization detected abundant LGALS-15 mRNA in endometrial LE and sGE of early pregnant goats [54]. The proposed extracellular role of galectin-15 in the uterine lumen is functionally to bind and cross-link β-galactosides on glycoproteins, such as mucins, integrins, fibronectin, laminin, and other glycoproteins and glycolipids, thereby allowing it to function as a heterophilic cell adhesion molecule bridging the blastocyst and the endometrial LE.

23.3.7 Integrin

Integrins are another family of glycoconjugate-containing members expressed on the uterine luminal epithelium, and have spatial and temporal relationships with blastocyst development and implantation. They also have a major role in cellular differentiation, motility, and adhesion. They comprise a large family of transmembrane heterodimeric glycoprotein receptors that are intimately associated with the cytoskeleton and signaling proteins [55]. Integrin subunits α (v, 4, and 5) and β [1,3,5] are constitutively expressed on the apical surfaces of both conceptus trophoblast and endometrial LE during the periimplantation period of pregnancy [48]. Integrin receptors, composed of á and â subunits, participate in bidirectional signaling involving both "outside-in" (i.e., ligand activation at the cell surface to modify cytoskeletal organization, intracellular signaling, and gene expression) as well as "inside-out" (i.e., cytoplasmic domain transduction of intracellular signals to regulate ligand binding affinity) pathways [56]. Luminal epithelial expression of integrin subunits $á_4$, $á_5$, and $â_1$ increases during the period of MRP, and $á_4$, $á_5$, $á_v$, $â_1$, and $â_3$ are localized to implantation sites [57]. These subunits potentially result in the integrin receptors $á_vâ_1$, $á_vâ_3$, $á_vâ_5$, $á_4â_1$, and $á_5â_1$ at the maternofetal interface during pregnancy and may function as part of an adhesion cascade that serves to generate both stable adhesion between apposing epithelial surfaces and activation of outside-in signal transduction [58]. Altered expression of integrins is correlated with several causes of infertility, null mutations of several integrins leads to periimplantation lethality, and functional blockade of selected integrins reduces the number of implantation sites [59]. In the sheep, receptivity to implantation does not appear to involve changes in either temporal or spatial patterns of integrin expression, but may depend on expression of other glycoproteins and extracellular matrix (ECM) proteins, such as galectin-15, OPN, and fibronectin, which are ligands for heterodimers of these integrins [48]. In species such as pigs, mice, and humans, interactions between specific integrins and ECM proteins frame the putative window of implantation [60].

23.3.8 Serpin

Uterine serpins (SERPINA14) belong to a super family of serine protease inhibitors secreted from the uterine endometrium under the influence of P_4 in various livestock: bovine [61] ovine [62], and porcine [63]. Serpins are a group of proteins with similar

structures that were first identified as a set of proteins able to inhibit proteases. The acronym "serpin" was originally coined because many serpins inhibit chymotrypsin-like serine proteases (serine protease inhibitors) [64]. The SERPINA14 gene consists of five exons and four introns that encode a protein of 446 amino acids. This gene is present in chromosome 21 and 18 in bovines and ovines, respectively. In case of pigs this is known as serpin D (heparin cofactor) and is present on chromosome 14. The size of this is 2098 bp and the protein size is 495 aa. The SERPINA14 protein performs diverse biological functions including direct nutrition to the conceptus, growth control, inhibition of proteolytic activities, and suppression of local invasion of the mouse uterus by distinct trophoblast subtypes. The ability of SERPINA14 to bind with members of the pregnancy-associated glycoproteins [61] and aspartic proteinases [65] and to the growth factor activin suggests its role as a carrier protein during gestation. SERPINA14 has been further implicated as an important GE during days 13−15 of the estrus cycle as well as during days 15−50 of pregnancy in sheep [66] and on day 25 of pregnancy in goats [62]. In bovines, SERPINA14 is reported to be expressed predominantly in the uterine endometrium during pregnancy [67]. UTMP and SPP1 are excellent markers for differentiation and overall secretory capacity of uterine GE during pregnancy in ewes [68].

23.3.9 Fibroblast Growth Factor-2

Fibroblast growth factor-2 (FGF-2) belongs to the member of the fibroblast growth factor (FGF) gene family. FGF-2 is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor expressed at low levels in many tissues and cell types and reaches high concentrations in brain and pituitary. The FGF-2 gene consists of three exons and two introns and encodes a protein of 155 amino acid residues. This gene is located in chromosome 17 in bovines. FGF-2 has been implicated in a multitude of physiologic and pathologic processes, including limb development, angiogenesis, wound healing, and tumor growth [69]. In ruminants, conceptus development beyond the blastocyst state requires input from uterine-derived factors. FGF-2 is expressed by the bovine endometrium throughout the estrus cycle and early pregnancy and stimulates trophectoderm expression of IFN-τ, the MRP factor in ruminants. During MRP, the mononuclear cells of the conceptus trophectoderm synthesize and secrete IFNτ between days 10 and 21−25 with maximal production on days 14−16 [19]. This antiluteolytic effect of IFNτ results in the maintenance of a functional CL and hence secretion of P_4 that is essential to maintain a uterine environment that supports events critical to successful development of the conceptus to term. Okumu et al. [70] reported that the expression of FGFR1 and FGFR4 was higher during early embryo development (days 7−13) but decreased on day 16 whereas FGFR2 expression was similar from days 7 through 13, with a significant increase by day 16 that was maintained until day 19 in cows. However, no pregnancy-dependent changes in endometrial FGF-2 mRNA abundance were detected until placental attachment was well under way in sheep [71].

23.3.10 Insulin-Like Growth Factor-1

Insulin-like growth factor-1 (IGF-1) has been referred to as a "sulfation factor" and its effects were termed "nonsuppressible insulin-like activity" in the 1970s. In bovines, IGF-1 genes are present on chromosome 5 and it has five exons. The IGF-1 gene is conserved in humans, chimpanzees, Rhesus monkeys, dogs, mice, rats, chickens, zebrafish, and frogs. IGF-1 is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/autocrine fashion. Production is stimulated by growth hormone (GH) and can be retarded by undernutrition, GH insensitivity, lack of GH receptors, or failure of the downstream signaling pathway after GH receptor including SHP2 and STAT5B. IGF-1 is expressed by uterine glands of cyclic and pregnant pigs and IGF-1 receptors (IGF-1R) are expressed by cells of the endometrium and conceptuses, which suggest paracrine and autocrine actions of IGF-1. Its primary action is mediated by binding to its specific receptor, IGF-1R, which is present on many cell types in many tissues. Binding to IGF-1R, a receptor tyrosine kinase, initiates intracellular signaling; IGF-1 is one of the most potent natural activators of the AKT signaling pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. It is likely that estrogens produced by the conceptus are important in inducing pregnant-specific uterine proteins and in facilitating uterine contractions. Although uterine proteins are important for embryonic and placental development, their possible role in MRP is not known [72]. Both the embryo and the endometrium produce significant quantities of IGF-1, which is independent of estrogen secretion [73] and has a role in autocrine-paracrine maternal—conceptus signaling.

23.3.11 Mx

Mx belongs to the class of dynamin-like large guanosine triphosphatases known to be involved in intracellular vesicle trafficking and organelle homeostasis. Mx is a protein-coding gene, and proteins encoded by genes are key components of the antiviral state induced by type I IFN in many species [74]. Mx expression in the endometrial epithelium is apparently regulated during the estrous cycle and the conceptus induces Mx expression in the uterine epithelium, stroma, and myometrium. Mx expression may be influenced by P_4 in the cyclic endometrium and may have a role in preparing the uterus to support the developing conceptus. Expression of Mx mRNA is up-regulated during pregnancy in cows and gilts whose conceptuses secrete IFNs during early pregnancy [75]. In cyclic ewes, mRNA abundance in endometrium increased from day 1 to peak levels on day 13 and then declined to day 15. In pregnant ewes, steady-state levels of Mx mRNA were first detected above the level in cyclic ewes on day 13 after mating, were greater than 10-fold higher on day 15, and remained elevated on day 19 [76]. Johnson et al. [77] reported that Mx mRNA continued to be strongly expressed in LE and superficial GE through day 17 of pregnancy in sheep, whereas ISG17 remained low to undetectable in these cells. Both ISG17 and Mx mRNA increased in the stratum compactum stroma (ST) between days 11 and 13, and expression extended into the deep GE and stratum spongiosum ST on days 15—17 in pregnant ewes.

23.4 Causes of Early Embryo Loss

23.4.1 Genetic Abnormalities

Most embryonic loss occurs during the first 18 days of gestation owing to genetic abnormalities affecting cleavage, MRP, attachment, or formation of the placenta [78]. Genetic causes of embryo death include chromosomal defects, individual genes, and genetic interactions [79]. Embryos produced with chromosomal defects are unable to continue development and are progressively lost during early developmental stages. Abnormal segregation during gametogenesis and cleavage of embryos and polyspermy are main reasons for the occurrence of aneuploidy. Sometimes a lethal gene is expressed that causes death of the embryo within the first 5 days of pregnancy. King [80] reported that abnormal chromosome numbers had resulted in abnormal growth of the embryo and usually death within the first trimester of gestation. Cytogenetic studies revealed that gross chromosomal defects account for 3–5% of embryo losses in bovines [81]. Chromosome abnormalities can cause embryonic death owing to the presence of tetraploid cells in one of the eight blastocysts at 12–16 days of age. Deficiency of uridine monophosphate synthase [82], a homozygous recessive condition, had been found to cause fetal death at days 40–50 of gestation [83]. Yadav et al. found about 4.2% and 10.7% of causes of aneuploid and diploids, respectively, in cattle [84].

23.4.2 Nutrition

Nutrition has an important role in the maintenance of pregnancy. Cows will have less embryo mortality if they are gaining condition, whereas those losing condition will tend to have higher losses [85]. Dunne et al. [86] showed that sudden reductions in dry matter intake (DMI) around the time of artifical insemination (AI) adversely affected embryo survival in the heifer dam. When energy intake was reduced from twice maintenance to 0.8 times maintenance for 2 weeks immediately after AI, the embryo survival rate in heifers was consistently less than 40%. When heifers were provided with either a constant level of feed intake or changed from a lower to a higher level feed intake, the embryo survival was 65–71%. Intake of high-protein diets by pregnant cows has been shown to alter pH and the concentration of other ions in uterine secretions only during the luteal phase and not at estrus. The intake of high dietary protein can result in elevated blood concentration of ammonia, urea, or both, depending on the balance of protein fractions present in the rumen and the availability of fermentable carbohydrates. Higher plasma urea concentrations had been observed to interfere with normal inductive actions of P_4 on the microenvironment of uterus and thereby caused suboptimal conditions for the support of embryo development [87].

After parturition, the nutrient demands of the dairy cow had been found to increase dramatically as peak lactation yield was approached and typically exceeded dietary intake, resulting in a state of negative energy balance (NEB). High-producing cows of high genetic merit experience greater NEB in early lactation under grazing conditions

than cows of lower genetic merit, notwithstanding the somewhat higher DMI capacity of the former [88]. Meta-analysis of the combined data from Kennedy et al. [88] and Horan et al. [89] showed that there were no interaction effects on conception rate from service x study x supplementation rate, service x study or study x supplementation rate. The rate of supplementation had no effect on first-service conception rate (56% v. 53%), but cows on the low level of supplementation had a lower second-service conception rate (39% v. 58%) than cows on the high level of supplementation. Morris et al. [90] and Wathes et al. [91] studied gene expression in the uterine tissue and spleen of cows with severe postpartum NEB and reported that these cows had increased expression of many key genes known to be involved in inflammatory responses, which is consistent with a remodeling of the postpartum uterus and the clearance of microbial infections.

23.4.3 Hormonal Imbalance

P_4 is essential for maintaining a suitable endometrial environment for early embryo development, implantation, and the maintenance of pregnancy. During preimplantation embryo development, P_4 increases embryo growth and induces the secretion of IFNτ by acting on the endometrium to stimulate the production of a variety of embryotrophic factors [92]. In addition, P_4 is necessary to maintain uterine quiescence that allows for proper implantation of the embryo within the endometrium and for fetal development [93]. In cows it is believed that intraluteal P_4 is implicated in a survival pathway in the CL by stimulating PGs, OT, and its own production in a stage-specific style and inhibiting apoptosis. The role of P_4 in embryonic loss has been extensively explored, and it is now widely accepted to have a pivotal role in the establishment of pregnancy, embryo survival, and development. Stronge et al. [94] and McNeill et al. [95] found a positive relationship between the probability of embryo survival and milk P_4 concentrations on days 5−7 and 4−6, respectively. Similarly, Franco et al. [96] observed that cows undergoing pregnancy loss had lower milk P_4 concentration. Starbuck et al. [97] found that pregnancy loss before day 45 was greater in cows with the lowest plasma concentration of P_4 on days 28−37 of gestation. However, optimal or threshold blood P_4 concentrations below which embryo mortality may occur are not known. It is possible that because P_4 is transferred locally from the ovary to the uterus, peripheral concentrations of P_4 may be less relevant for embryo survival than local (uterine tissue and lumen) concentrations [98]. There are many potential mechanisms by which low P_4 concentrations may compromise embryo survival because it is essential to regulate the most critical steps of pregnancy establishment and embryo development. Low P_4 may lead to asynchronous uterine environment, lower uterine secretions [99], failure to inhibit uterine immune response against the conceptus [100], excessive concentrations of other hormones that may affect either oocyte quality or embryonic development [101], and lower antiluteolytic signal [102]. Inskeep (2004) pointed out four major periods during embryo development in cows when are susceptible to lower concentrations of P_4, which result in embryonic mortality: the early postovulatory period (when lower P_4 exists during the

preceding luteal phase); days four through 9 after mating; the period of maternal recognition of pregnancy (days 14–17 with a slightly elevated E_2 concentration) and the late embryonic period (days 28–42). In addition, it is well-documented that a delay in the postovulatory P_4 rise is most important for embryo development rather than the concentrations achieved at later stages in cows and ewes [103]. Even though studies reported lower concentrations of P_4 in plasma or milk during the luteal phase or the pregnancy of lactating dairy cows, it is unclear whether this is due to reduced production by CL or increased metabolism of secreted P_4. Supplementation with P4 during early pregnancy has given unequivocal results; most studies reported increased PR in lactating cows but no or reduced effects in heifers [104].

23.4.4 Thermal Stress

Heat stress is an environmental factor that affects the survival of embryos and accounts for approximately 10% of embryonic loss. Whether an embryo can survive the early stages of pregnancy depends, at least in part, on the degree to which the embryo can adjust its own physiology [105]. Embryonic loss occurs when there is disruption in the physiologic regulation of oviductal and uterine function owing to environmental stresses. Conception rates had been found to decline from averages of 40–60% in cooler months to only 10–20% during times of high temperature and humidity [106]. Conception rates in beef heifers exposed for 72 h to an elevated temperature (32°C) or ideal temperature (21°C) immediately after breeding were 0% and 48%, respectively [107]. Interestingly, we observed that higher P_4 levels during hotter months (June and July) in buffalo may be responsible for the suppression of reproductive efficiency associated with high environmental temperatures [108]. Environmental stress has a lethal effect on embryos both in vivo and in vitro. The preimplantation embryo is most susceptible to certain types of stresses very early in its development because its genome is largely repressed. However the embryo possesses cellular adaptive mechanisms to limit adverse effects of stress, as occurs in the case of heat stress. Heat shock induces the disruption of embryonic development by influencing several key events leading to a successful pregnancy. First, follicular dynamics are altered during heat stress: Persistent preovulatory follicles [109], lower steroidogenic capacity [110], and a decline in oocyte competence [111] have been found in cows exposed to a high environmental temperature. Exposure to heat (39.5–41.0°C) during the first 48 h of IVC of bovine zygotes significantly reduced the rate of development to the eight-cell stage at 72 h of IVC and the rate of development to the morula or blastocyst stage at 144 h of IVC [112]. Furthermore, the reduction in the percentage of embryos that developed into blastocysts was especially notable in the heat-treated embryos (41°C for 12 h) at the two-cell [113] and one-cell stages [114]. Under heat stress conditions proteins synthesis is reduced [115] and free radical metabolism is increased [116]. However, as development proceeds, the embryo acquires mechanisms to become resistant to elevated temperatures, which involves heat-shock protein-70 (HSP-70) expression [115], antioxidant systems

activation [116], and the ability to undergo apoptosis [117]. Failure of any of these mechanisms to develop may lead to embryo death. Roth et al. [118] observed that exposure of cows during the early stage of follicular development hampers the oocyte quality and subsequent embryo development. The effect of in vitro heat stress on synthesis and secretion of protein and PG by oocyte conceptus and endometrium has been reported by Putney et al. [119]. The elevation of temperature of tissue incubation from 39°C to 43°C reduces the synthesis and secretion of protein by the conceptus and stimulates the release of $PGF_{2\alpha}$ by pregnant endometrium in cattle. Whenever the embryo is exposed to heat shock, the cell fails to synthesize HSP-70, which ultimately protects the embryo from heat shock. Interestingly, it has been observed that GSH, which removes free radicals from cells, is reduced by heat shock [116].

23.4.5 Infectious Agents

Infectious diseases cause reproductive problems for livestock producers worldwide. With the development of cost-effective vaccination and other methods to control infection and diseases, infectious agents are becoming less of a factor for embryo survival. It is therefore essential to understand the importance of maintaining these agents to a minimum by using vaccination and medication. Bovine viral diarrhea (BVD) is the most prevalent cause of abortion in cattle, with an incidence of infection often in excess of 70% [120]. There are two forms of BVD: cytopathogenic (CP) and noncytopathogenic (NCP). BVD-CP induces apoptotic cell death whereas NCP replicates in cultured cells without inducing cell death and has the ability to cross the placenta. Because NCP can cross the placenta, any vaccination must be able to protect the fetus. Infection with BVD-NCP in the first 40 days of gestation causes early embryonic death, infection between 40 and 125 days of gestation results in persistently infected offspring or later abortion, and infection after 125 days of gestation is nonlethal, because the fetus is able to mount an immune response [121]. Embryos infected with BVD-NCP have a reduced rate of in vitro blastocyst formation when recovered at day 8 postbreeding [122]. Infectious bovine rhinotracheitis causes sporadic abortions throughout gestation. Abortion is most common after the fourth month of gestation; however Miller et al. [123] inoculated heifers on day 14 postbreeding and within 10 days two heifers showed a decline in P_4 to concentrations similar to those seen at estrus, whereas an additional two heifers lost embryos on days 40 and 42 after breeding. Inoculation at day 14 postbreeding resulted in early embryonic death, but heifers inoculated on day 21 or 28 postbreeding calved normally [124]. The presence of *Actinomyces* pyogenes in the reproductive tract is often considered to result from other infections such as endometritis [125]. Visual embryonic decomposition and pus were found after abortion from cows infected between days 27 and 41 of pregnancy compared with cows given PG to terminate the pregnancy. *Cornebacterium pyogenes*, the most common bacteria in cattle uterus, is responsible for abortion and endometritis [126]. Vibrio is also responsible for infertility and causes early embryonic mortality in cows [127]. *Brucella abortus* has more of an effect during mid to

late gestation, and causes abortion. Bovine herpes virus-1 causes early embryonic death, cystic CL, and pathologic changes in the uterus [128].

23.4.6 Impairment of Maternal–Fetal Interactions

The uterine environment provides the medium in which the embryo develops. In cows, the embryo lies free in the medium for 30 days or more, during which time extensive embryonic development takes place before the conceptus becomes firmly attached to the embryonic wall. During this period the composition of the uterine fluid constantly changes and strict synchrony exists between the uterine environment and the developing conceptus. In the early stages of gestation, embryo development has to synchronize with changes in the uterine environment to prevent abnormalities and failure to implant. Natural asynchrony between the embryo and the mother can arise from late onset of the first meiotic division, which leads to a delay in oocyte maturation [129]. Also, delayed fertilization may result in asynchrony, but undoubtedly the main cause of asynchrony is an altered secretory pattern of P_4 [99]. The major cause of preimplantation embryo mortality is thought to be difficulties in signaling between the conceptus and the mother [130]. Failure of the conceptus to communicate its presence at the appropriate time by secreting adequate concentrations of IFNτ leads to pregnancy loss. Inadequate IFNô production may result from intrinsic defects of the embryo (e.g., delayed or retarded trophoblast development) or adverse alterations in the maternal environment (e.g., low P_4 concentration in the uterus) [105]. Thatcher et al. [131] hypothesized that some embryonic losses in cows could be caused by the inability of the embryo to suppress the luteolytic cascade, likely because of a failure of the conceptus to produce luteotropic signals, or perhaps failure of the CL to respond to luteotropins. Failure of the embryo to produce steroids may compromise its own survival. Steroids of conceptus origin could have a local effect on the endometrium to stimulate production of histotrophe or factor(s) necessary for the apposition and attachment phases of implantation [3].

Defects in endometrial adenogenesis or function during the periimplantation period may cause embryo mortality [132]. The unequivocal role of secretions of endometrial glands as primary regulators of conceptus survival and development, onset of pregnancy recognition signals, and implantation is well-established [132]. Uterine secretions are thought to be particularly important for conceptus survival and development in ruminants because of the prolonged period of preimplantation conceptus development that precedes superficial attachment and placentation [133]. Failure of endometrial gland development or the servomechanism supposed to regulate endometrial function in early stages of pregnancy can lead to embryo mortality. Uterine pH is also thought to have an important role in determining whether the embryo might survive and develop. There is abundance of evidence that low pH in the uterine luminal fluid has detrimental effects on embryo survival and reproduction in general [134]. It has been suggested that the decrease in uterine pH caused by an excess of protein supplementation may influence endometrial secretory activity, creating a hostile or suboptimal environment for embryo development and thus compromising the establishment of pregnancy.

23.4.7 Quality of Oocytes and Sperm

An embryo's fate is also dictated by events before fertilization: Factors affecting the health of the gametes ultimately may determine the ability of an embryo to survive and grow. Indeed, embryos formed from incompetent oocytes have a lower probability of successful development than do embryos developing from normal oocytes. Oocyte competence can be compromised by alterations in follicular dynamics. Oocytes belonging to persistent dominant follicles exhibit various morphologic abnormalities [135]; after fertilization they give rise to zygotes whose development is retarded and early embryonic death usually occurs before the 16-cell stage [136]. Cows with persistent preovulatory follicles show low fertility and have altered protein secretory patterns in the oviduct [137]. These events may be because of the larger size and the longer lifespan of a persistent follicle. Oocytes belonging to persistent follicles are at a too-advanced stage of maturation at the time of fertilization, which increases the probability of having poor-quality zygotes [135]. Persistent follicles also produce greater quantities of estrogens than do normal follicles, with detrimental effects on embryo survival [138]. This abnormal follicular dynamic could result from the failure of normal luteal function that affects P_4 production and its regulation of LH secretion. The sequential relationship of low P_4, increased frequency of LH pulses, a persistent dominant follicle, and increased secretion of estradiol-17β is widely accepted as one a cause of early embryonic death [101]. Inadequate follicular development also reduced the ability of fertilized oocytes to develop to the blastocyst stage. Perry et al. [139] observed low pregnancy rates and high embryo losses in cows induced to ovulate prematurely, likely because the P_4 concentration rose at a slower rate than in cows induced to ovulate later. This could have led to poorly developed embryos although, like the oocyte, the male gamete influences fertility by imparting characteristics to the embryo that influence its ability to proceed through development [105]. The main male-derived causes of embryo loss are chromosomal abnormalities, such as nondisjunction in spermatogenesis [140], 1/29 Robertsonian translocation [141], and a defective chromatin structure [142].

23.5 Strategies for Reducing Early Embryonic Wastage

The successful establishment of pregnancy depends on a delicate balance between luteolytic mechanisms inherent in the endometrium at the end of diestrus and anti-luteolytic mechanisms orchestrated by the conceptus to change endometrial function and ultimately, block luteolysis. Several strategies, such as pharmacologic, nutritional, and management manipulations of the process of MRP, have endeavored to increase the probability of successful gestation in livestock.

23.5.1 Development of Timed Insemination Programs

23.5.1.1 Ovsynch

One program that has been extremely successful for inseminating cows at a fixed time for first service without the need to detect estrus is the Ovsynch program, in which

injections of gonadotropin-releasing hormone (GnRH) are given 7 days before and 48 h after an injection of $PGF_{2\alpha}$ and cows are inseminated 12−16 h after the second injection of GnRH. This system synchronizes follicle maturation without regression of the CL before GnRH-induced ovulation and timed insemination. In 1995, Pursley et al. [143] developed the hormonal program Ovsynch to synchronize ovulation in lactating dairy cows. Cows treated with Ovsynch yielded overall conception rates similar to those obtained after breeding to detected estrus (37% versus 39%, respectively) [144]. However a major limitation to Ovsynch is the wide variability in synchronization rates (defined as having a regressed CL and ovulation after the final GnRH) in Ovsynch-treated cows. Variations among dairy cows in synchronization rates to Ovsynch were primarily attributed to the stage of estrous cycle in which Ovsynch was initiated [145]. Cows started on Ovsynch at midcycle (days 5−9 of the cycle) had a greater probability of synchronizing to Ovsynch and therefore had a greater chance of conception than did cows at any other stage of the estrous cycle [146]. The key physiologic reasons for increased synchronization rate in midcycle are: (1) the presence of a functional dominant follicle capable of ovulating to first GnRH of Ovsynch; and (2) the presence of a CL that remained functional during the 7 days between first GnRH and $PGF_{2\alpha}$ of Ovsynch. In contrast, during the early (days 1−4) or late (days >10) estrous cycle, the physiologic follicular and luteal scenarios were not conducive to synchronization to Ovsynch. When cows were started on Ovsynch early in the estrous cycle (days 1−4), ovulation to first GnRH of Ovsynch was impaired by the presence of an emerging follicular wave [145,146]. Moreover, when cows were started on Ovsynch in the late estrous cycle, spontaneous luteolysis was likely to occur before $PGF_{2\alpha}$ of Ovsynch. In cows with spontaneous luteolysis before $PGF_{2\alpha}$, the dominant follicle was likely to trigger spontaneous ovulation before the final GnRH of Ovsynch. Successful synchronization of ovulation is less likely when cows are started on Ovsynch during the early or late estrous cycle compared with midcycle. Because insemination occurs at a fixed time after the final GnRH of Ovsynch, chances of conception to timed AI are lower in nonsynchronized cows. In buffalo [147,148], this protocol was successful in synchronizing ovulation in 70−90% of buffalo, with conception rates ranging from 33% to 60%.

23.5.1.2 Presynch−Ovsynch

This program consists of two injections of $PGF_{2\alpha}$ administered at an interval of 14 days with the second $PGF_{2\alpha}$ administered 12−14 days before an OvSynch program. This program increased pregnancy rates 18% units (i.e., 25−43%) in lactating cyclic cows [146]. This stimulation in pregnancy rates was attributed to manipulation of the estrous cycle such that the OvSynch timed insemination program was initiated at the most favorable stages of the estrous cycle. Several presynchronization strategies were developed based on the idea that controlling ovarian function before the initiation of Ovsynch could improve synchronization rates to Ovsynch [149,150]. The most popular presynchronization strategies were based on the use of $PGF_{2\alpha}$ at specific times before the initiation of Ovsynch. A presynchronization protocol before implementation of the

Ovsynch protocol was developed by giving two injections of $PGF_{2\alpha}$ 14 days apart, with the second injection given 12 days before the first GnRH of the Ovsynch protocol. El-Zarkouny et al. [149] demonstrated that presynchronization before the GnRH-based protocol with a controlled internal drug release insert enhanced pregnancy rate compared with that achieved without presynchronization (46.8% versus 37.5%; $P < .01$), but this effect was not evident in anestrous cows. The success of the OvSynch program depends on whether lactating dairy cows are anestrus or cycling. Pregnancy rates were lower in cows that were not cycling at the time the OvSynch program was initiated (22% versus 42%). If anestrus cows ovulated to the first and second GnRH injections of OvSynch program, pregnancy rates appeared to be normal (39%).

23.5.1.3 Heat Synch

Heat synch is an estrus synchronization protocol that substitutes an injection of estradiol cypionate (ECP) at 24 after the $PGF_{2\alpha}$ for the last GnRH in the OvSynch protocol. Based on the synchronization of ovulation and pregnancy rates, ECP is used as an alternative to inducing ovulation in place of GnRH for timed insemination. Because lactating dairy cows have reduced concentrations of plasma estradiol in the preovulatory period and reduced intensity of estrus, the elevation of estradiol after ECP injection is a supplement for lactational-induced deficiency. If cows are anovulatory (they are anestrus or have not developed positive estradiol feedback), the Heatsynch program may not be as effective as the GnRH-based OvSynch program. This is because GnRH causes the direct secretion of LH.

23.5.2 Nutritional Strategies

Energy status at service affects embryo survival. Sudden reductions in dry matter intake around the time of insemination adversely affect embryo survival in heifers. When energy intake was reduced from a high level twice their maintenance requirement to 0.8 times maintenance for 2 weeks immediately after AI, embryo survival rate in heifers was consistently less than 40%. When heifers were provided with either a constant level of feed intake or were changed from a low to a higher-level feed intake, embryo survival was consistently high at 65−71%. High dietary protein, resulting in high concentrations of urea in plasma and milk, has been associated with decreased fertility in dairy cattle [151]. By-products of nitrogen metabolism, such as ammonium ions, can have detrimental effects on the uterine environment, reducing sperm, ova, or embryo survival. Jordan and Swanson [152] reported that the increased supplementation of crude protein from 12.7% to 19.3% at 4 days postpartum and continuing for 91 days improved the number of service per conception from 1.47 to 2.47. Supplementation of 0.8 kg fishmeal per day substituted for 0.8 kg standard concentrate improved conception rate to all services (0.64 versus 0.44) and reduced the number the number of services per conception (1.62 versus 2.31) [153]. Dietary supplementation of omega-3 fatty acids [eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and linoleic acid] increases the fertility rate and

reduces embryonic mortality by inhibiting uterine secretion of $PGF_{2\alpha}$ [154]. These fatty acids are capable of decreasing the secretion of $PGF_{2\alpha}$ and complement the antiluteolytic action of IFNτ. EPA and DHA are known to have distinct antiinflammatory and immunosuppressive effects that complement the normal immunosuppressive and antiinflammatory effects of P_4 and IFNτ in early pregnancy.

23.5.3 Supplementation of Luteotrophic Agents

One approach to improving embryonic survival in cattle has been direct supplementation with P_4. However, direct P_4 supplementation during the early or midluteal phases of the estrous cycle has not consistently increased pregnancy rates. P_4 treatment seemed to increase fertility in herds with low fertility. However, Macmillan et al. [155] reported that controlled internal drug release-containing P_4 was inserted for 6 or 12 days starting on days 5–7 and a significantly higher pregnancy rate (79%) was achieved than in untreated cattle, even though the fertility of the untreated cattle was high (66%). Treatment with human chorionic gonadotropin (hCG) may slightly improve fertility in some low-fertility herds, such as in heat-stressed cows in which conceptuses are slightly delayed in development [156], and also in embryo transfer recipients [157]. Treatment with GnRH at the time of insemination had a more consistent effect in increasing pregnancy rates in repeat-breeders than in first-service cattle [158]. An alternative strategy to increasing endogenous P_4 concentration is to induce an accessory CL by ovulating the dominant follicle on day 5 of the estrous cycle with hCG [159]. In addition to increasing P_4 concentrations by day 9, hCG injection resulted in all treated heifers having three follicular waves (the dominant follicle of the third wave did not reach 9–10 mm in size until approximately day 20, versus day 14 in the control heifers). Thus hCG treatment would reduce the estrogenic environment during the period of maternal recognition of pregnancy. Similarly, injecting hCG on day 5 after artificial insemination induced the formation of an accessory CL in 86.2% of cows (compared with 23.2% of control cows that had multiple ovulations), increased P_4 concentrations and pregnancy rates [160].

23.5.4 Supplementation With Interferon (IFN)

Species	Dose and Route of IFN Used	Pregnancy Rate (%)	
		Treated	Control
Sheep	**1.** 2 mg of r boIFN-α i/m ly twice a day on days 12–14 after mating	78.3	74.8
	2. 2 mg of r boIFN-α i/m ly twice a day on days 12–15	81.6	76.3
	3. 2 mg of r boIFN-αi/m ly twice a day on day 12–16 Martinod et al. [161]	80.5	73.2
	1. 2 mg of r boIFN-$\alpha_1$1 i/m ly twice a day on days 17–18	79.0	58.0
	2. 2 mg of r boIFN-$\alpha_1$1 i/m ly twice a day on days 11–18 SchalueFrancis et al. [162]	89.0	78.0
Cattle	**1.** 20 mg of boIFN-α i/m ly daily on days 14–17 after estrus	48.0	60.0
	2. A single injection of 40 mg of boIFN-α on day 13 after estrus	38.0	48.0
	3. An increasing dose of 0.01–10 mg, i/m ly, twice a day on days 11–19 Barros et al. [163]	44.0	56.0

r boIFN-a (α), recombinant bovine interferon-(α)

23.5.5 Use of Bovine Somatotropin

Administration of bovine somatotropin (bST) (500 mg Posilac, USA) at the time of the first GnRH injection or during the insemination in cows in a Presynch-OvSynch program increased pregnancy rates (57% compared with 42.6%; 148). Because bST was effective at insemination, it is likely that bST stimulated embryonic development and survival after insemination in lactating dairy cows. bST treatment increased the proportion of transferable embryos, the number of blastocysts present per flushing, and the pregnancy rates of recipients treated with bST or receiving embryos from donors treated with bST. In vitro administration of recombinant bST increased fertilization rates, hastened embryo development, and increased embryo quality [164], which indicated that both maternal and conceptus components were affected by bST treatment.

23.6 Conclusion

MRP is an incredibly useful biological system, a better understanding of which will advance our knowledge in several basic physiologic processes. Unraveling the secrets of MRP will assist biologists in developing a paradigm to reduce early embryonic loss. MRP is initiated at various times in the periimplantation period by the embryologic agent, which is not well-characterized. Until we understand the complex interaction between the conceptus and its maternal environment, it will not be possible to achieve a major reduction in embryonic growth retardation and mortality. Current biotechnological approaches will be of tremendous importance in understanding the complex mechanism of low reproductive efficiency in various livestock species to evolve safer and potent gene-based strategies to enhance production. Promising technologies include the use of adenoviruses, antisense oligodeoxynucleotides, morpholinos, and small inhibitory RNAs that could be used in vivo and ex vivo to perform gain-of-function and loss-of function studies of specific gene(s) in the endometrial epithelia and trophoblast. The sequencing of the genomes of domestic animals is expected to generate knowledge and reagents useful for understanding the basis of enhanced fertility as well as infertility. Understanding the signals that regulate uterine receptivity and implantation can be used to diagnose and identify the cause(s) of recurrent pregnancy loss and improve pregnancy rates in domestic animals and humans. Use of genomic technologies in animal agriculture will definitely bring about exciting changes in livestock production and the tailoring of animals to produce products needed by humanity. The challenge is to understand the complexity of key mechanisms that are characteristic of successful pregnancy in animals and to use that knowledge to enhance fertility and reproductive health of ruminant species in the livestock industry.

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