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New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and Proteins

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New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and Proteins

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An Introduction to „Recent Trends in the Biotechnology Industry: Development and Manufacturing of Recombinant Antibodies and Proteins“

Michael Pohlscheidt, Robert Kiss, and Uwe Gottschalk

Abstract The production of the first therapeutic proteins in the early 1980s heralded the launch of the biopharmaceuticals industry. The number of approved products has grown year on year over the past three decades to now represent a significant share of the entire pharmaceuticals market. More than 200 therapeutic proteins have been approved, approximately a quarter of which are represented by monoclonal antibodies and their derivatives. In 2016, the list of the top 15 best-selling drugs included more than eight biologics and in 2020 the trend will continue, with more than 50% of the top 20 best-selling drugs predicted to be biologics. From 1986 to 2014 several first-in-class, advance-in-class, and breakthrough designated therapeutic options were approved, with advanced therapies such as immuno-oncology and cell-based therapies being approved for several indications.

Keywords Antibody formats and evolution, Biotechnology, Cell culture, PAT, Processing technologies, QbD

Contents

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1 Introduction to this Special Issue

The production of the first therapeutic proteins in the early 1980s heralded the launch of the biopharmaceuticals industry. The number of approved products has grown year on year over the past three decades to now represent a significant share of the entire pharmaceuticals market. More than 200 therapeutic proteins have been approved, approximately a quarter of which are represented by monoclonal antibodies and their derivatives [1–6]. In 2016, the list of the top 15 best-selling drugs included more than eight biologics and in 2020 the trend will continue, with more than 50% of the top 20 best-selling drugs predicted to be biologics. From 1986 to 2014 several first-in-class, advance-in-class, and breakthrough designated therapeutic options were approved, with advanced therapies such as immuno-oncology and cell-based therapies being approved for several indications [1–6].

The first biopharmaceuticals were produced in bacteria, but these relatively simple cells are only suitable for the production of small proteins that do not require complex post-translational modifications [7, 8]. Mammalian cells are required for the expression of proteins that contain multiple disulfide bonds, glycans, and other modifications, because they contain the necessary cellular machinery to achieve the correct modifications efficiently [9, 10]. Since the first mammalian cell fermenters were deployed by the industry, continuous process developments (including the optimization of cell lines, cultivation equipment, media, and downstream processing) have resulted in product titers increasing from less than 0.1 g/L to more than 10 g/L in fed-batch processes [11–13]. Yet this rise in productivity has only served to keep pace with increasing market demand as the number of new products continues to grow [5, 6]. The current state of the art in mammalian cell platforms involves manufacturing scales of up to 25 m^3 in batch, repeated batch, or fed-batch modes, followed by a sequence of filtration, chromatography, and concentration steps that deliver product batch sizes of 50–100 kg [11, 12]. Therapeutic proteins are diverse in terms of size, charge, and solubility, so an equally diverse panel of purification technologies is required. However, monoclonal antibodies have a generic structure that can be purified using a platform approach, typically involving clarification by multistep filtration or centrifugation, followed by Protein A capture chromatography and orthogonal downstream chromatography steps to separate the product from host cell proteins and product-related impurities [13]. More recently, large-scale fed-batch processes have been replaced by perfusion-based processes that require smaller bioreactors (up to 1,000 L, compared with 25,000 L for fed-batch processes) and that last for up to 200 days compared with the typical 5–18 days in fed-batch processes [14–21]. Similarly, the batch-based purification of therapeutic proteins can be accelerated by switching to continuous processing, as is common in food production and other industries [22, 23].

2 Product Evolution of Recombinant Antibodies

Advances in production technology have been matched by innovations in the design of therapeutic proteins to increase their efficacy and reduce off-target effects. Nowhere is this more apparent than in the development of monoclonal antibodies and their derivatives, which benefit from a range of enhanced molecular approaches. The original hybridoma technology described by Kohler and Milstein is rapidly being supplanted by molecular techniques such as phage display that allow the direct selection of heavy and light chains and their transfer to mammalian cells for expression [24]. Hybridomas produce murine antibodies, and various strategies have been developed to humanize them in order to avoid triggering immune responses against the murine components, including the creation of chimeric antibodies with murine antigen-binding regions grafted onto a human antibody framework, through to the production of fully human antibodies isolated directly by phage display or produced in transgenic mice carrying human immunoglobulin genes. Indeed, of the 19 'fully human' therapeutic antibodies currently approved, 13 were isolated from transgenic mice [25]. Other innovations include the exploitation of antibody fragments for their favorable characteristics (such as the small size and absence of effector functions in minimal constructs like the single chain fragment variable), glyco-engineered variants for improved efficacy [26], and the introduction of novel scaffolds based on antibody technology (e.g., bi-specific antibodies) [27]. Initially, antibody-drug conjugates were monoclonal antibodies labeled with radioisotopes, but now these conjugates include a range of products in which antibodies are conjugated to various effector molecules such as anticancer drugs [28], as well as protein-only versions (fusion proteins known as immunotoxins), in which antibodies are directly fused at the genetic level to peptides, protein toxins, and effector enzymes that induce apoptosis in target cells [29]. In principle, existing technologies and combinations of existing unit operations are suitable for producing these new molecular formats. Therefore no specific chapter in this book is dedicated to these new formats.

For today's and tomorrow's new molecular formats, the major challenges for the pharmaceutical industry include maintaining research and development (R&D) success rates to ensure that therapeutic options remain accessible for patients around the world. Although the biologics market continues to grow, patent expiry is a major threat to developers and manufacturers, particularly given the high R&D costs associated with bringing these products to market. In the small-molecule drugs market, the impact of patent expiry has been felt, with several manufacturers losing 80–90% of sales to so-called generics in the first year off patent. A similar landscape is likely to emerge in the biopharmaceuticals industry within the next few years, with many best-selling drugs coming off patent and many antibodies not far behind. The global biosimilars market was worth US$ 1.3 billion in 2013 and could reach US$ 35 billion by 2020 [30]. The European Medicines Agency has approved so-called biosimilars or bio-betters of several products. Some examples include a version of human growth hormone (Omnitrope®) in Europe in 2006, followed more recently by

Remicade® (infliximab), Enbrel® (etanercept), MabThera® (e.g., Rixathon®, Rituximab®), and other biosimilars that are under review or approved. Patents expire on seven other major biologics before 2020. In addition, increasing safety requirements, the transfer of growth to emerging markets, restricted market access, increased development costs, and declining R&D productivity will put more pressure on the value and supply chain of innovation-driven companies [31]. However, this pressure has encouraged process and product development, resulting in technology and process maturation, higher degrees of automation, increased process robustness, and the application of lean principles in biopharmaceutical operations.

3 Process and Technology Improvements

Although most significant process and technology improvements to date have been based on conventional cell-line/media optimization, high-throughput robotics for both upstream and downstream process development have allowed extensive miniaturization for screening, leading to rapid improvements in cell lines, growth media, and chromatography resins, ultimately increasing product titers. Advances have also resulted from the introduction of omics technologies, including genomics (e.g., sequencing of the Chinese hamster ovary [CHO] cell genome), transcriptomics, proteomics, and metabolomics, to characterize how cells respond to different growth conditions and products [32].

Another advance that has increased process efficiency by enabling the rapid installation of new manufacturing facilities and rapid switching between production campaigns is the development and implementation of disposable or single-use technologies early in process development [33]. The first disposable modules were filters and media bags, but nearly all unit operations are now available in a disposable format, including disposable bioreactors, chromatography columns/membranes, and all the connectors and tubing in between. The smart integration of these technologies has led to fully disposable facilities, which can be scaled up and down in response to demand. Furthermore, the use of disposables is economically attractive compared with stainless steel at increasingly large scales, and the product yields are comparable, e.g., DSM reported titers in their XD technology® of up to 25 g/L in a fully disposable system, based on integrated perfusion PERC6® cell culture and product concentration by ultrafiltration. Titers in more traditional fed-batch cultures are up to 8 g/L in disposable bioreactors of up to 2 m^3. One batch from a fully disposable system can therefore deliver ~16 kg of product with a much smaller footprint than a stainless steel system. The disposable products currently on the market are compliant with current good manufacturing practice while avoiding the need for cleaning and validation, but one drawback is the need to ensure supplier consistency and a sufficient inventory of disposable modules for each campaign.

Reduction of the risk of virus contamination is another important trend in biomanufacturing, particularly following some high-profile contamination cases at production facilities [34]. The risks of adventitious contamination can never be entirely eliminated, but the presence of endogenous viruses can be avoided by using alternatives to mammalian cells. As stated above, bacteria are used for the production of many simple proteins, and a small number of commercial products are manufactured in yeast or insect cells, including several vaccines and recombinant proteins, such as erythropoietin. These platforms are unlikely to replace CHO cells or other specific mammalian cell lines, such as the PERC6 human cell line that is used to produce recombinant antibodies, owing to the ability of mammalian cells to produce correctly glycosylated products and their unparalleled regulatory track record. Emerging platforms based on transgenic animals, transgenic plants, transient expression in plants, or cultured plant cells currently appear to be preferred for a small number of niche products, but have yet to make a significant impact on the industry as a whole, despite the potential for plants to be cultivated on an agricultural scale [35]. The risk of contamination can also be reduced by eliminating animal-derived raw materials both upstream (e.g., by using chemically defined serum/peptone-free media) and downstream (e.g., by using „Veggie“ protein A affinity resin). Additional risk mitigation is achieved by using viral clearance barriers during manufacturing (e.g., heat treatment, virus filters, ultraviolet inactivation). To increase efficiency and mitigate risks, most companies have developed and implemented platform technologies, beginning with a host cell line and medium translated into a manufacturing process based on standardized bioreactor platforms and purification processes (two or three chromatography steps followed by formulation and filtration). Pilot and large-scale manufacturing facilities are also equipped with sophisticated sensors and data analysis systems, and are highly automated to support process monitoring and continuous improvement, based on process analytical technology (PAT), in order to support the quality by design (QbD) initiative by the United States Food and Drug Administration (FDA) [36]. Partnership with major suppliers – as in other industries – has also ensured significant gains in process throughput and robustness. The processing options gained through such collaborations help the industry to accelerate development, share validation principles, and mitigate risks during scale up and technology transfer.

Although the discussion to this point has been focused on bioprocess development, advances and trends in pharmaceutical R&D have also driven improvements in the ability to formulate, manufacture, and deliver biological drug products. The benefits of subcutaneous delivery for protein products have driven the development of stable high-concentration formulations (100–200 mg/mL), but such products are extremely viscous, which necessitates parallel advances in formulation ingredients and delivery devices. Significant growth has occurred in the use of pre-filled syringe devices and sophisticated auto-injectors that support targeted self-administration.

4 The Next Decade: Visible Trends for New Holistic Processing Strategies

The next decade will see the development of new molecular formats to address unmet medical needs. Combination products will be pursued as one means to improve patient convenience while reducing healthcare costs. Further increases in automation and paperless systems for manufacturing will also help to reduce costs and improve efficiency. Two types of biopharmaceutical manufacturing facilities are likely to dominate the landscape: platform-based large-scale manufacturing facilities in large-market regions, and small regional disposable plants in emerging markets. Given that large-scale (>10 m^3) plants can produce huge quantities of proteins for blockbuster markets, and that more targeted (and smaller) patient populations can be addressed by moderate-scale disposable systems (up to 2 m^3), cell culture titers will not necessarily need to be increased. The trend towards continuous manufacturing will increase to avoid capital spending and, in some cases, to reduce manufacturing costs. Recently, perfusion technology has been applied to seed trains for the intermediate storage of large-volume starter cultures and to increase inoculation cell densities in production-scale bioreactors in order to shorten operation cycles and boost productivity in existing manufacturing plants [37].

It is likely that resources will target the improvement of product quality attributes and increase of downstream processing efficiency in order to drive costs down further.

5 The Structure of this Special Issue

This introductory article has set the scene for current and future trends in biopharmaceutical manufacturing. This special issue is divided into three sections: the first looking at novel aspects of mammalian cell cultivation, including a guide to recent mammalian production systems; innovations in cell banking, expansion, and culture; and finally an overview of strategies to mitigate the risk of contamination with adventitious viruses and other agents. The second section switches to downstream processing, considering recent innovations in harvesting technology and the unit operations required for protein purification. The final section features a series of specially commissioned articles that discuss emerging topics, including the concepts of fully disposable manufacturing; PAT for process monitoring and control; formulation and fill/finish technology; process integration and continuous manufacturing; cell therapy; gene therapy; and integrated high-throughput process development.

Finally, we ask a question from a different perspective: How much innovation is really needed for antibody production using mammalian cells?

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New Mammalian Expression Systems

Jie Zhu and Diane Hatton

Abstract There are an increasing number of recombinant antibodies and proteins in preclinical and clinical development for therapeutic applications. Mammalian expression systems are key to enabling the production of these molecules, and Chinese hamster ovary (CHO) cell platforms continue to be central to delivery of the stable cell lines required for large-scale production. Increasing pressure on timelines and efficiency, further innovation of molecular formats and the shift to new production systems are driving developments of these CHO cell line platforms. The availability of genome and transcriptome data coupled with advancing gene editing tools are increasing the ability to design and engineer CHO cell lines to meet these challenges. This chapter aims to give an overview of the developments in CHO expression systems and some of the associated technologies over the past few years.

Keywords Cell engineering, Cell line development, Chinese hamster ovary cells, Gene editing, Therapeutic protein production

Contents

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1 Introduction

1.1 Why Mammalian Cells?

Mammalian cells are used for the production of recombinant monoclonal antibodies (mAbs) and complex proteins because they have the capacity to assemble and fold complex polypeptides and to perform post-translational modifications (PTMs) which are important for therapeutic bioactivity and bioavailability. Production processes using mammalian cell cultures in bioreactors are high yielding (up to 10 g/L for mAbs) and scalable (up to tens of thousands of litres), making them compatible with large-scale manufacture for clinical supply of therapeutic proteins. Transgenic animal systems can also produce complex proteins and offer some advantages in terms of cost and scale of supply over mammalian cell culture systems [1]. However, the timelines to establish transgenic herds or colonies are significantly longer than those for establishing cell culture systems and there are concerns regarding the theoretical transmission of xenotropic viruses to humans.

Mammalian cell culture expression systems rely on the introduction of vector DNA encoding the recombinant protein into a host cell line and harnessing the

synthetic capacity of the cell to express and secrete the encoded protein into the cell culture medium. Systems for large-scale production of therapeutic proteins are generally based on stable recombinant cell lines created by integration of linearized plasmid DNA encoding the therapeutic protein into the host genome, so that the transgenes are transmitted to each daughter cell at cell division. Traditionally, the production process from a stable cell line is performed using the controlled culture conditions in a bioreactor using a fed-batch mode, with additional nutrients being "fed" into the bioreactor to sustain cell growth and productivity for the duration of the culture period. The recombinant protein is then recovered from the cell culture medium.

2 Choice of Mammalian Host Cell Lines

Mammalian host cell lines are able to perform PTMs including glycosylation, carboxylation, hydroxylation, sulfation and amidation, which can be important for biological activity [2]. A number of different mammalian host cell lines are used for large-scale production of complex therapeutic proteins (reviewed by Butler and Spearman [3]). Historically, these have been based on rodent cell lines – mouse myeloma (NS0 and Sp2/0), baby hamster kidney and CHO cells. Although these cell hosts are able to produce glycoproteins with human-like glycosylation profiles, they also produce non-human glycoform structures which can impact in vivo clearance and immunogenicity [4, 5].

A number of human host cell lines can be used for the production of recombinant proteins with fully human PTMs, as reviewed by Dumont et al. [6] and Swiech et al. [7]. Cell lines generated from the human embryonic kidney cell line HEK-293 are used for the production of approved therapeutic proteins, such as recombinant clotting factors and fusion proteins, where additional PTMs such as gamma-carboxylation and sulfation are required for bioactivity. The human fibrosarcoma cell line HT-1080 is used for the manufacture of approved enzyme therapies – iduronate-2-sulfatase, agalsidase alfa and velaglucerase alfa. The PER.C6 cell line, derived from human embryonic retinal cells [8], and the CAP-T cell line, derived from human amniocytes [9], have been employed to produce therapeutic proteins currently in preclinical and clinical development. Engineered human leukemic cell lines have been developed for the production of therapeutic proteins with fully human and optimised glycosylation [10, 11]. In addition, a human neuronal cell line, AGE1.HN, is being used for production of proteins with complex glycosylation profiles [12].

3 CHO Host Cells

Over several decades, the CHO host cell line has become established as an industry-standard expression platform with a strong regulatory track record, and it accounts for the production of >70% of approved therapeutic proteins [13]. Owing to the rodent origin of CHO cells, there is a species barrier to the production of viruses able to infect humans, and studies have confirmed that CHO cells are resistant to infection by many viruses that can infect humans [14]. Significant advances have been made in the productivity of CHO bioreactor processes through upstream process development, particularly with respect to the development of media and feed formulations [15]. This optimisation has resulted in robust and scalable bioreactor processes, achieving high cell densities and product yields, with titres of the order of 10 g/L for mAbs being attainable in fed-batch culture at scales of tens of thousands of litres. Importantly, not only can CHO cells be engineered with genes encoding therapeutic proteins but also further cell and genetic engineering can be used to modify cell line characteristics, such as growth and metabolism, as well as product quality attributes (reviewed by Fischer et al. [16]). Therefore, CHO cells provide a flexible expression platform that can be engineered to fit both process and product requirements. This engineering approach for CHO cells has been facilitated by the availability of genome sequences for CHO host cell lines [17, 18] and the recent advances in genome editing [16]. In light of the central importance of CHO cell systems to the biopharmaceutical industry, the remainder of this review focuses on the recent developments to CHO production systems.

3.1 Development of CHO Expression Systems

There are a variety of drivers for further developing CHO expression platforms for therapeutic protein production:

- *Efficiency and timelines*. Discovery platforms are becoming more efficient in identifying multiple leads with different modes of action, and at the same time there is pressure to advance projects rapidly into the clinic. As the creation of the stable manufacturing cell line is a pre-requisite for the production of clinical material, it is desirable to reduce timelines for cell line development and even parallel track cell line development for multiple molecules to enable project acceleration to critical-path GLP toxicology and to the clinic (reviewed by Estes and Melville [19]).
- *Innovation of novel therapeutic proteins*. Following the success of engineered antibodies, proteins and fusion proteins as therapeutics, biological activities are now being combined to create novel bispecific molecules. These non-natural molecules can pose challenges for development because of their often low expression yield, need for more-complex PTMs and other product quality attributes such as aggregation. Although it is preferable that these undesirable

characteristics are screened out during the discovery process, this is not always possible. Therefore, engineering of the production cell line and/or process is needed to improve the ability to manufacture these molecules.

- *Manufacturing processes*. The pressure to reduce cost of goods and to maximise the efficiency of production capacity and facilities is driving manufacturing processes towards new process paradigms such as continuous processing [20, 21]. Continuous upstream processes involve higher cell densities in the bioreactor and longer culture times, creating unique demands on the performance parameters of the production cell line, such as cell metabolism and production stability, compared with those for traditional fed-batch processes.

3.2 CHO Cell Line Diversity and Evolution

There are a number of different CHO host cell lines, as reviewed by Wurm [22] and Lewis [17]. The first CHO cell line was derived from the ovary of an adult Chinese hamster [23] and later underwent cloning and other manipulations to generate different cell lines, including CHO-K1, CHO DG44, CHO-S and CHO DUXB11. These CHO cell lines were originally cultured in media containing animal serum, but, because of concerns about the cost of serum, batch-to-batch variation in serum performance in culture media and the potential for adventitious agent contamination, these cell lines have been adapted to grow in culture media that are free from serum or any other animal-derived components. The choice of a CHO host cell line is partly driven by the compatibility with the expression plasmid selection system used for recombinant protein production. The CHO DG44 cell line is deficient in dihydrofolate reductase (DHFR) and so is typically used with the DHFR selectable marker that can complement this deficiency. The other commonly used CHO expression system is based on using glutamine synthetase (GS) as the selectable marker and is generally used with host cell lines derived from CHO-K1.

Different CHO host cell lines can exhibit differences in productivity. Hu and co-workers demonstrated that recombinant cell lines from a CHOK1 host showed higher productivities for two difficult-to-express (DTE) mAbs compared with cell lines constructed using a DUXB11 host [24]. Similarly, auditioning of DG44 and CHOK1 cell lines with an artificial chromosome carrying copies of genes for a recombinant mAb showed differences in performance, with cell lines derived from the CHOK1 host showing higher productivity [25]. However, it is difficult to make direct comparisons between different hosts as the performance of the cell line is also strongly affected by the process conditions, including media and feed composition, which can be optimised to improve individual cell line performance. In addition, the CHO host cell lines are themselves heterogeneous, containing a population of cells that show variation in growth, metabolism, biosynthetic capacity and ability to perform PTMs [26, 27].

The phenotypic variation of CHO cells results from the underlying genetic and epigenetic diversity. The genetic heterogeneity can be observed at a gross level as the

varied karyology profiles of individual cells in a host population with different chromosomal structures [22, 28]. This chromosomal variation arises from dynamic genome restructuring which occurs during continuous subculture and is characteristic of immortalized cell lines. It is the combination of genomic and epigenetic remodelling at cell division that contributes to the versatility of CHO as a host cell line with the ability to adapt to different culture media and conditions, and to generate recombinant cell lines that express proteins with varying product quality profiles.

The phenotypic and genotypic variation within CHO cell populations can be exploited to isolate host cells with more desirable characteristics by serially sub-culturing cells in the presence of physical or chemical stresses that can select for desired properties. A striking example of this "directed evolution" approach is the use of plant cytotoxic lectins that recognise specific glycoform structures to select for host cells with modified glycosylation pathways – the Lec mutants (reviewed by Patnaik and Stanley [29]). On binding to specific glycoproteins at the cell surface, the lectins are internalized, whereupon they can exert their toxic effects, resulting in cell death. Cells that do not display the reciprocal glycoform structures, because of mutations caused naturally or by treatment with mutagenic agents, are able to survive the lectin treatment. The use of lectins with different specificities has allowed the identification of cell lines with different glycosylation mutations, which in turn have contributed to the elucidation of glycosylation pathways and associated genes as well as glycosylation engineering [29]. An example where a desirable bioprocessing characteristic was selected is described by Bort and colleagues [30], in which CHO cells were sequentially cultured in medium containing stepwise-reduced levels of glutamine. The cells able to survive each reduction in glutamine were recovered by fluorescence-activated cell sorting (FACS), and the final population of selected cells was able to grow in glutamine-free medium. This follows on from the work of Prentice and co-workers [31] where DG44 host cells were selected for their ability to survive in bioreactor conditions, leading to increases in peak cell density and the ability to grow in the absence of growth factors. Similarly, bioreactor evolution and selection may provide a strategy to generate host cell lines that are more suited for continuous upstream processes. To be able to take advantage of an evolved phenotype in the host cell line, it must be maintained over the timescales needed for cell line development and manufacture. This can require continued application of the selective pressure used to derive the phenotype or the screening of individual cell lines for stability of the desired characteristic without selection.

4 Vector Engineering

Currently, conventional non-viral expression plasmids containing transgenes are still the major vector platform for cell line development. These plasmids contain multiple expression cassettes, each consisting of a promoter and associated regulatory elements to drive transcription, the coding sequences of the recombinant

protein and selectable marker, and a sequence for transcript termination and polyadenylation. The recombinant protein gene encodes a homologous or heterologous N-terminal secretory leader peptide to direct the protein for secretion via the endoplasmic reticulum (ER) and the Golgi, where PTMs, such as glycosylation, take place. Following transfection of the plasmid DNA into the host cell line, stable transfectants are generated through the application of drug to select for the expression of the selectable marker gene. Standard plasmid transfection processes result in random integration of the vector into the host genome, and the site of integration along with copy number of the vector influence the level of transgene expression. Therefore, extensive transfectant screening needs to be performed to identify high-expressing cell lines. Expression vectors have been optimised to increase the productivity and stability of cell lines and to improve the efficiency of the cell line generation process. These vector optimisation approaches have included manipulation of selection markers, promoter engineering, incorporation of new DNA regulatory elements, the usage of different codons to regulate translation, and modulation of the order and ratio of expression of different gene cassettes, some of which are described in more detail below. Meanwhile, novel vector platforms for targeted integration and transposon-based vector systems have been developed to increase integration efficiency.

4.1 Manipulation of Selection Markers

There are two main selection systems used to generate production CHO cell lines, and these are based on the metabolic genes encoding DHFR and GS that are typically selected by the respective addition of the inhibitors methotrexate (MTX) and methionine sulfoximine (MSX) to the cell culture medium [32, 33]. As the selectable marker and the gene(s) encoding the recombinant protein are usually combined on the same vector, integration of the vector into a genome location favourable for selectable marker transcription is also generally beneficial for the expression of the linked recombinant protein genes. Therefore, a high stringency of selection facilitates obtaining cell lines possessing a high level of transcription from integration of the expression vector at an active locus in the genome and also for removing any low producers.

The CHO-DG44 and DUKXB11 hosts are DHFR deficient and require addition of glycine, hypoxanthine and thymidine (GHT) to the culture medium for cell growth. Integration and expression of the DHFR gene complements the DHFR deficiency of the CHO host cell line, allowing growth in the absence of GHT. Furthermore, higher levels of DHFR expression and the linked transgenes can be selected by stepwise increases in the levels of MTX, which is a highly selective competitive inhibitor of DHFR. Gene amplification resulting from chromosomal remodelling is a naturally occurring phenomenon in CHO cells, and the increased level of MTX selects for cells that have undergone amplification of the copies of the DHFR marker gene loci, which can also include the recombinant protein genes.

However, this amplification process is laborious and time-consuming, and the multiple tandem vector repeats that result from the amplification process can be unstable, leading to a loss of productivity over time [34]. An alternative approach to increasing gene copy number to enhance transgene transcription is to attenuate the expression level of the DHFR selectable marker. Thus, only cells from the most transcriptionally active loci survive the selection. Marker attenuation can be achieved in a number of ways. It has been reported that codon de-optimisation that decreased the translation efficiency of the DHFR gene resulted in approximately threefold higher production of an Fc fusion protein [35]. The addition of the AU-rich elements in the 3′ untranslated region (3′ UTR) of the DHFR gene to reduce mRNA half-life and/or the inclusion of the murine ornithine decarboxylase (MODC) PEST amino acid sequence to promote DHFR protein degradation were shown to improve the specific productivities for recombinant human interferon gamma in DG44 cells [36]. Another approach to de-optimising DHFR expression by placing the DHFR gene downstream of an attenuated internal ribosome entry (IRES) element allowed the production of high levels of the small soluble glycoprotein Dectin-1 [37]. By combining the engineered PEST motif with an attenuated IRES sequence, the DHFR protein level was further reduced and resulted in increased recombinant alpha-1 antitrypsin production [38].

The GS gene encodes glutamine synthetase, which catalyses the conversion of glutamate to glutamine. As glutamine is an essential amino acid, GS expression is required for cells to grow in glutamine-free medium. However, CHO cells naturally express GS in glutamine-free medium, so the use of GS as a selectable marker requires the addition of the competitive inhibitor MSX to the cell culture medium. The addition of MSX ensures that only those cells producing higher levels of GS resulting from expression of the GS selectable marker on the plasmid vector can survive in the selective conditions [32]. Efforts to increase the selection strength of the GS gene have mostly focussed on cell line engineering and optimisation of the transfection and selection processes. It has been shown that knocking out the endogenous GS genes in the CHO host with zinc finger nuclease (ZFN) technology resulted in multiple cells lines with higher sensitivity to MSX selection and yielded a sixfold increase in the frequency of high producers for a recombinant antibody, thereby providing the potential to improve the efficiency of the cell line development screening process [39]. Suppression of endogenous GS gene expression by increasing the glutamine concentration in the cell culture medium before transfection has also proved to be an efficient way to increase the strength of selection with the same concentration of MSX [40].

4.2 Multi-Gene Expression with Novel Promoters and Elements

Production of mAbs requires the co-expression of the heavy- and light-chain genes along with the selection marker. Often, all three genes are incorporated into a single vector to ensure co-expression of the physically linked genes. However, the development of novel multi-unit bispecific antibodies, as well as large enzyme complexes and DTE proteins that require co-expression of genes encoding specific chaperones and PTM enzymes, necessitates the co-expression of multiple transgenes. Incorporating multi-gene expression cassettes into a single plasmid is technically challenging because of size restrictions of standard plasmids, both in terms of plasmid construction and propagation in *Escherichia coli* as well as the efficient transfection and integration of larger plasmids into the CHO host cell line. In addition, repeated use of the same promoter for the expression of multiple genes on a single plasmid can cause promoter interference [41], which may limit expression.

One approach to avoid repeated promoter sequences is to utilize different promoters for each gene cassette. In addition to the commonly used human cytomegalovirus immediate early (hCMVIE) promoter, there is a range of viral and housekeeping promoters, such as those derived from the simian virus 40 (SV40) and the human elongation factor 1 alpha gene (EF1α), which can be used for protein production. Further CHO endogenous promoters with desirable expression profiles have been identified by utilizing transcriptomics data [42]. To expand the search beyond natural promoters, a synthetic-biology approach has been applied to construct a library of synthetic promoters by combining different transcription factor regulatory elements (TFREs) from powerful viral promoters [43]. Screening of the synthetic promoters from this library by evaluation in transient transfections has identified promoters with transcriptional activity ranging over two orders of magnitude, some significantly exceeding that of the hCMVIE promoter. The use of a strong synthetic promoter has the potential to improve gene expression and the use of multiple promoters of varying strength could more precisely control the relative expression of different genes encoding multi-subunit proteins, which might be advantageous for protein expression and product quality [44–47]. Synthetic promoters are also shorter than conventional promoters, reducing the size of vectors with multi-gene cassettes and thereby improving vector handling and transfection efficiency.

Another approach for removing repeated promoters is to drive transcription of linked multiple genes as a single transcript from a single promoter. The insertion of an IRES element between each coding region, or cistron, allows ribosomes to initiate translation at multiple points along the transcript and so different polypeptides can be translated from the same transcript. As the translation of the gene downstream of an IRES sequence is through a weaker CAP-independent mechanism, it usually results in a lower level of expression of the second and any subsequent cistron. This can create an imbalance in the production of two linked subunits

which might not be desirable for some molecules [48]. However, for other molecules, changing the proportion of the different subunits can be beneficial [44–47]. In the 2A technology, multiple linked genes are translated as a single open reading frame. The coding sequences of the different genes are separated by motifs encoding the self-cleaving viral 2A peptide sequence. This enables the production of equimolar ratios of component subunits from the single precursor polypeptide. The 2A self-processing peptide system has been used for antibody production and has shown a twofold increase in transient expression compared with the equivalent IRES-linked construct for the same antibody [44, 45]. Viral 2A peptides from different viruses, such as foot-and-mouth disease virus, equine rhinitis A virus, porcine teschovirus-1 and Thosea asigna virus, have been used for mAb production [49]. None of these 2A peptides produced complete cleavage, but adding a glycine–serine linker provided more flexibility at the boundary between two linked chains and thus enhanced the cleavage [49]. The insertion of a furin recognition site upstream of the 2A peptide sequence allowed additional sequence-specific protein cleavage and the removal of 2A residues that otherwise remained attached to the upstream heavy chain protein [49].

5 Vector Integration

Despite efforts to optimise plasmid-based vector systems, reliable and efficient integration of transgenes into transcriptionally active genomic loci still remains a major challenge for stable protein expression. As productive integration events are rare, extensive cell line screening is required to identify and characterise the desired high producers. In recent years, the frequency and/or efficiency of productive integration events has been increased by including chromosomal elements on the expression plasmid vector or by combining with transposon or targeted integration technologies. Viral-based integration systems such as lenti- and baculovirus-mediated gene delivery technologies are also being developed for the efficient expression of secreted proteases and membrane glycoproteins [50, 51].

5.1 Incorporation of Chromosomal Elements

A number of chromosomal elements that have a positive effect on promoting high-level and stable gene expression have been incorporated into plasmid vectors. These include nuclear scaffold/matrix attachment regions (S/MARs) [52] and ubiquitous chromatin opening elements (UCOEs) [53]. These chromosomal architectural elements affect the adjacent chromatin structure once the plasmid vector has been integrated into the genome to maintain accessibility of the vector DNA for transcription and prevent gene silencing. Recent work has suggested that CHO cell

lines generated with UCOE-containing vectors not only showed resistance to chromosomal position effects with increased mRNA production per copy of transgene but also grew to a higher cell density [54]. The UCOE system is versatile having been used in combination with multiple selection and amplification platforms, in different CHO host cell lines and with the high-throughput Clonepix screening process [54–56]. Its beneficial effect on the frequency of higher-expressing cell lines and robustness of cell growth allows the rapid generation of stable transfectant pools and has been used to replace transient transfection for the rapid production of cytokines and other recombinant proteins [57]. Saunders and co-workers [58] compared a number of chromatin structural elements including UCOE, MAR, STAR (Stabilising Anti Repressor) and cHS4 (an insulator from the chicken beta-globin locus control region) for their ability to confer resistance to insertional position effects that could decrease mAb expression. UCOE had the most beneficial effect of all the elements tested, maintaining a high level of expression and showing reduced promoter methylation, which is one cause of gene silencing.

5.2 Transposon-Based Vector Systems

Transposons are a class of naturally occurring non-viral mobile genetic elements that have the ability to integrate single copies of DNA sequences with high frequency at multiple loci within the host genome [59]. Transposon DNA vectors have been developed for a variety of purposes, including insertional mutagenesis as well as gene transfer and therapy. Typically, these transposon systems have two components: a donor plasmid with the cargo transgenes flanked by the transposon inverted repeat sequences and a helper plasmid or mRNA encoding a transposase. The transposase is transiently expressed from the helper plasmid or mRNA and then catalyses the excision of the inverted repeat sequence flanked region of the donor plasmid and facilitates its integration into the host genome. Transposon vectors have been deployed with CHO cell lines for the production of recombinant proteins including a gamma-secretase integral membrane protease complex [60]. The Piggy-back (PB) transposon, a class II transposable element originally derived from the cabbage looper moth, has been favoured in the bio-production field because of ease of handling and its capability for mobilizing very large DNA sequences, such as bacterial artificial chromosomes [61]. However, the frequency of transposition decreases as the size of the artificial transposon increases beyond 14 kb. In a side-by-side comparison, Matasci and colleagues [62] showed that the PB transposon system resulted in 15–20 times more recombinant cells in the transfectant population and that the derived clonal cell lines had higher average volumetric productivity and greater production stability than cell lines originating from the standard plasmid vector. Based on this result, the group utilized the PB transposon system for rapid transfectant pool generation to produce high titres of an antibody and a human tumour necrosis factor receptor-Fc (TNFR-Fc) fusion protein [63].

The PB transposon pools expressing TNFR-Fc fusion protein had a constant volumetric productivity for up to 3 months in the absence of selection. Further optimisation of the PB transposon system has been performed by incorporating a human MAR sequence into the PB vector, which significantly increased transgene integration and transcription [64]. This study also showed that, with the PB transposon system, transfectants can readily be generated without selection, and high levels of expression could be obtained from as few as 2–4 genomic copies of the MAR-containing transposon vector. The attributes of low transgene copy number and stability in the absence of selection that are conferred by the PB transposon system are highly desirable for production cell lines as they are associated with transgene stability over long-term culture. Moreover, the higher productivity and the increased frequency of productive cell lines are highly beneficial for the efficiency of the cell line development process.

5.3 Targeted Integration

Integration at a predefined chromosomal locus that gives homogeneous, high expression is advantageous for protein production and the efficiency of cell line generation. In addition, because cells with the same isogenic background are expected to have similar and predictable growth and metabolism, this approach is also beneficial for upstream process development [65]. Site-specific recombinase systems such as Cre-Lox and Flp-FRT have been the common tools used for targeted integration (reviewed by Bode et al. [66] and Turan et al. [67]). These systems are usually operated in two steps: first, following random integration, screening and tagging expression hotspots using a reporter gene vector that also contains a recombination-specific sequence tag (Lox or FRT) and then, second, targeting integration of a vector containing the cargo gene(s) and complementary recombination-sequences to the pre-tagged locus using transient expression of the recombination enzyme. Several groups have successfully demonstrated the generation of homogeneous cell lines expressing recombinant protein with good productivity and long-term production stability by using this approach [68, 69]. However, this is a lengthy process, and, as only a single copy of the transgenes is integrated, the resulting cell lines tend to have lower titres than the best cell lines derived from a random integration approach. In addition, as fluorescent reporter genes are not secreted, the tagged cell lines that are selected are not necessarily proficient in the production of secreted proteins.

To speed up the hotspot screening step and integrate an increased number of transgene copies, two technologies, ϕC31 integrase and CRE-Lox recombinase systems, have been combined [70]. The ϕC31 integrase mediates attB-specific DNA integration into the CHO genome at pseudo-attP sites. As there is a limited number (100–1,000) of pseudo-attP sites in the CHO genome, the scale of the first step of searching and tagging (with LoxP integration sites) of transcriptionally active pseudo attP spots is manageable. Moreover, it has been shown that targeted

integration of two copies of antibody genes doubled the titres compared with targeted integration of one copy of the genes. Meanwhile, Zhang and colleagues [71] have used a vector containing mAb genes flanked with recombination sequences for large-scale screening to identify cell lines with good productivity, long-term production stability and possession of low-copy number transgenes. After removing the mAb genes through recombinase-mediated cassette exchange (RMCE) using an Flp-FRT-containing null cassette, the resulting host was used for the efficient and consistent construction of cell lines possessing high mAb productivity (2–2.5 g/L in fed-batch shake flasks) and stability of expression for more than 100 generations. This approach not only identified transcriptional hotspots for integration but also generated host cells with intrinsic production capability and stability that was inherited from the progenitor cell lines.

In addition to naturally occurring site-specific enzymes, such as Cre, Flp and ϕC31, which are capable of recognising specific sequences and then promoting the interchange between two recombination sites, a number of programmable sequence-specific nucleases that generate double-stranded breaks (DSBs) have been applied to genome editing in mammalian cells. The first of these programmable reagents were the ZFNs where protein engineering is used to enable targeting of double-strand DNA cleavage adjacent to chosen DNA sequences (reviewed by Chandrasegaran and Carroll [72]). Typically, the chromosomal DSBs introduced by ZFNs are repaired by a non-homologous end joining (NHEJ) repair pathway, in which the DSBs are ligated without the use of a homologous template. In some instances, the DNA joining repair is imprecise and leads to a deletion or insertion of nucleotides, causing a frameshift that can result in gene disruption. By designing and engineering a nuclease target site into the donor plasmid vector, Cristea and colleagues [73] showed that a ZFN can cleave both donor and chromosome DNA to produce efficient integration of the donor plasmid into the CHO genome through a non-homologous end joining (NHEJ) pathway.

The transcription activator-like effector nucleases (TALENs) are another class of programmable site-specific nucleases. TALENs consist of two domains, an engineered TALE that binds to a specific DNA sequence and a DNA cleavage domain. It has been demonstrated that a large expression cassette that includes a gene encoding a single-chain Fv-Fc (scFv-Fc) can be knocked in at a predefined locus in the CHO genome mediated by a TALEN with micro-homology to the targeted locus [74, 75]. The simplicity of the vector construction that requires no large regions of homology along with the efficiency of the process for isolating knock-in cell lines is advantageous for the generation of production cell lines.

More recently, RNA-guided nucleases, based on the CRISPR–Cas9 system from prokaryotes, have been developed and are being widely used for genome editing, including for CHO cells (reviewed by Lee et al. [76, 77]). Cas9 is an endonuclease that uses a guide RNA to target specifically cleavage of DNA sequences that are complementary to the guide RNA. Unlike ZFNs and TALENs, which require complex protein engineering to cleave new DNA target sequences, the CRISPR–Cas9 system uses a universal DNA endonuclease and cleavage specificity is engineered by simply modifying the sequence of the guide RNAs. Therefore, the

CRISPR–Cas9 system significantly increases the efficiency and reduces the cost of the design and generation of the reagents for genome engineering. Furthermore, engineering of a CRISPR–Cas9 recognition site into a donor plasmid can promote NHEJ-based integration of transgenes into a predefined locus, albeit at a low efficiency [78]. The efficiency of NHEJ targeted integration remains low even with the aid of promoter trapping (HEK293) or phenotypic screening (*HPRT*$^-$ in CHO) strategies at 0.17% and 0.45%, respectively. However, using a transgene cassette flanked by homology arms in the presence of locus-specific guide RNAs and Cas9 protein enables more efficient integration (10.2–27.8%) into multiple pre-defined loci in the CHO genome through a homology-directed repair mechanism [76, 77]. The application of the CRISPR–Cas9 system for targeted integration shows benefits in terms of the consistency of transgene expression in the resulting cell lines. In addition, the insert capacity for multiple gene cassettes (~5 kb) and the increasing targeting efficiency mediated by the CRISPR–Cas9 system advocate its development as a targeted integration platform for production cell line generation. However, challenges remain as off-target effects, presumably because of non-targeted integration, have the potential to affect cellular functions in the engineered cells.

Targeted integration technologies provide advantages for the rapidity of cell line development and also the potential homogeneity and consistency of cell line productivity. These inherent benefits are exploited by the deployment of targeted integration as a research tool and for rapid supply of early preclinical and clinical supply of therapeutic proteins. However, a key drawback of targeted integration systems for commercial manufacturing is the lower productivity compared with that of cell lines derived from conventional random integration and screening approaches. The lower expression results from the lower transgene copy number and also the lack of epigenetic selection for high expression for targeted integration compared with random integration. These factors are being addressed by ongoing developments to enable targeted integration of multiple transgene cassettes and also by "reusing" a high-yielding, stable, random-integration production cell line by removing and replacing the product genes with a suitable targeting cassette, as demonstrated with an RMCE system by Zhang et al. [71]. However, there is a concern as to whether a single clonal targeted integration host can possess all the intrinsic properties, genetic and epigenetic, to generate the desired product quality characteristics for all molecular formats. Therefore, a toolbox of host clones might be required, with different host clones for different products.

6 Glycoengineering

Glycosylation is the enzymatic addition of carbohydrate (glycans) and is one of the most important PTMs for therapeutic proteins as it can affect biological activity, stability, pharmacokinetics and immunogenicity. N-linked and O-linked glycosylation are the most common types of protein glycosylation, with the pathways for

N-linked glycosylation being the best characterised in mammalian cells (reviewed by Hossler et al. [79]). The sites for glycosylation are determined by the structure of the protein, and the protein conformation can also affect glycan structures. The host cell line and the cell culture conditions also influence the glycan structures and the glycan homogeneity [79]. This can result in heterogeneous glycan profiles and, because of the potential impact on biological activity, there can be a need to demonstrate consistent lot-to-lot glycosylation depending on the mode of action of the molecule [80]. Although CHO cell lines generally produce human-like glycans, they can also produce some non-human glycoform structures (NGNA and non-human alpha-gal) which are undesirable from an immunogenicity perspective. The natural N-glycosylation profiles from CHO cells can be improved by cell line engineering to produce more-homogeneous and more-desirable glycosylation profiles (reviewed by Dicker and Strasser [81]). A "glycodelete engineering" strategy to produce simplified and homogeneous glycan structures has been developed in HEK 293 cells [82]. This approach could be beneficial for production of mAbs from CHO in which the mode of action is antigen neutralization without effector function. Recent work on engineering the N-linked glycoforms in the CHO cells most relevant for therapeutic recombinant proteins is described below.

6.1 Terminal Sialylation

Sialylation plays important roles in the half-life of therapeutic proteins. The hepatic asialoglycoprotein receptor (ASPR) can recognise terminal galactose residues and mediates serum protein degradation. Terminal sialylation can hide the galactose from recognition; thus sialylated proteins are cleared more slowly than those that are asialylated. Consequently, it is highly desirable to control and increase the level of sialylation, but recombinant proteins from CHO cells tend not to be fully sialylated. Therefore, sialylation pathways are genetic engineering targets for improving the extent and/or consistency of recombinant protein sialylation profiles.

There are different ways to attach sialic acid to galactose: human proteins predominantly use the α2,6-linkage, whereas CHO cells have incomplete α2,3-linked sialic acid. This difference is because of the lack of significant expression of the α2,6-sialyltransferase gene in CHO cell lines [17]. Early work showed the feasibility of using genetic engineering approaches to enhance sialylation of recombinant glycoproteins secreted from CHO cell lines by co-expressing α2,6-sialyltransferase or α2,3-sialyltransferase in recombinant CHO cell lines [83, 84]. More recently, a CHO-K1 host cell line has been engineered to express the hamster *ST6GAL1* gene, which encodes α2,6-sialyltransferase [85]. Antibody produced in the engineered host not only showed the human-like α2,6-linked terminal sialic acid, but also a twofold increase in the overall sialylation level compared with that of the unmodified host. Similarly, recent work conducted by Yin and co-workers [86] showed that overexpression of the human *ST6GAL1* gene in CHO cell lines producing human erythropoietin (EPO) resulted in increased

sialylation. Furthermore, co-expression of two additional glycosyltransferases, α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase (GnTIV/Mgat4) and UDP-*N*-acetylglucosamine: α1,6-D-mannoside β1,6-*N*-acetylglucosaminyl transferase (GnTV/Mgat5) in the *ST6GAL1*-modified CHO cells, produced further enhancement of the terminal branching. As a result, tri- and tetra-antennary N-glycans represented approximately 92% of the total N-glycans on the resulting EPO protein. RNAi knock-down experiments have been conducted to investigate further which of the six CHO α2,3-sialyltransferases (ST3GAL1-ST3GAL6) with their different substrate specificities are critical for alpha 2,3-sialyation linkage of CHO glycoproteins [87]. Results indicated that ST3GAL3, ST3GAL4 and ST3GAL6 are involved in N-linked sialylation and ST3GAL4 may play a vital role in glycoprotein sialylation of complex glycoproteins such as EPO. This study demonstrated the power of RNAi as a screening tool to identify individual and combinatorial effects of multiple genes in the glycosylation pathway and to provide targets for successful glycoengineering.

6.2 High-Mannose Glycans

High-mannose glycans are known to increase antibody immunogenicity and decrease half-life, making them undesirable for therapeutic proteins [88]. Nevertheless, proteins with mannose-only glycans are advantageous for X-ray crystallography studies because of the simple and homogeneous glycan structure [89, 90]. The *MGAT1* gene product, also called GnTI, catalyses the transfer of *N*-acetylglucosamine to the Man5GlcNAc2 (Man5) N-glycan structure as part of complex N-glycan synthesis. Disruption of the *MGAT1* gene either by chemical mutagenesis followed by lectin selection [91] or ZFN-mediated targeted knock-out (KO) technology [92] in multiple CHO cell lines resulted in the production of protein with Man5 as the predominant N-linked glycosylation species. Unlike the chemical mutagenesis method, ZFN mediates precise genomic modifications, so that the growth and productivity of the resulting KO cell lines are not adversely affected by random mutagenesis throughout the genome. The *MGAT1* KO host is also useful in the production of mannose-terminated enzymes, such as recombinant glucocerebrosidase to treat patients with Gaucher disease, as the terminal mannose residues bind with better efficiency to the mannose receptor on the surface of the target macrophage cells [93]. Interestingly, by re-introducing the *MGAT1* gene into the mgat1 mutant or KO cell lines, two independent groups have shown that the sialylation levels of IgG1 molecules were improved as well [85, 94–96]. This phenomenon was not observed when the *MGAT1* transgene was expressed in wild-type CHO K1 cells. Although the exact mechanism is not well understood, this strategy of restoring the *MGAT1* function in deficient cells has been applied in CHO cells from transient expression through to stable and large-scale perfusion systems to produce EPO with a greater proportion of tri- and tetra-antennary sialylation [94, 95].

6.3 *Afucosylation for Increased Antibody-Dependent Cell-Mediated Cytotoxicity*

Fucosylation remains a major target for glycoengineering as afucosylated mAbs have enhanced ADCC activities and an increased anti-tumour activity. Knocking out the *FUT8* transferase gene through traditional homology-based recombination approaches [97] or ZFN-mediated gene disruption [98] has been shown to produce completely afucosylated antibodies. The recent development of the CRISPR–Cas9 technology has significantly increased the efficiency of gene editing, and it has been reported that the triple gene targets *FUT8*, *BAK* and *BAX* can be knocked out in a one-step manipulation to produce a FUT8-deficient host with anti-apoptotic properties [99]. This work demonstrated the multiplexing capability of the CRISPR–Cas9 system for genome editing with high efficiency.

Besides the *FUT8* gene, many other genes in the fucosylation pathway have become engineering targets. Haryadi and colleagues [100] used a ZFN to inactivate the GDP-fucose transporter gene (*Slc35c1*) in a cell line with an existing mutation in the CMP-sialic-acid transporter gene (*Slc35a1*). This resulted in a cell line (CHO-gmt5) that produced afucosylated and asialylated mAbs. These investigators also compared ZFN, TALEN and CRISPR–Cas9 technologies for the modification of the *slc35c1* gene locus and found changes in mAb titre in cell lines engineered with ZFN and CRISPR–Cas9, but not with TALEN, suggesting that TALEN might have fewer off-target effects.

A novel approach to engineering the fucosylation pathway is that of "biosynthetic deflection". Von Horsten and colleagues [101] described the expression of a bacterial enzyme, GDP-4-keto-6-deoxymannose, to divert an intermediate substrate from the fucose synthesis pathway. This resulted in the production of afucosylated mAbs even when the expression levels of the bacterial gene were relatively low.

6.4 *O-Glycoengineering*

In contrast to N-linked glycosylation, the capabilities of CHO cells for O-linked glycosylation of proteins are less well understood. However, work using a "SimpleCell" strategy has been used to increase knowledge of O-glycoproteins and sites of O-glycan attachments in the CHO proteome [102]. This approach used a ZFN to knock out a component of the O-glycan pathway, leading to homogenous and truncated O-glycans and allowing enrichment of O-linked glycan proteins for identification by mass spectrometry. Data analysis from the study indicates that CHO cells have a limited capacity for O-glycosylation, which supports transcriptome studies also showing expression of a limited number of O-glycosylation GalNAc transferases [18]. Consequently, cell engineering approaches have the potential to improve O-linked glycosylation, which is important for PTMs in molecules such EPO and Etanercept (TNF alpha receptor-Fc-fusion).

7 New Formats and "Difficult-to-Express" Proteins

More recently, new classes of proteins such as multi-specific antibody and fusion proteins have been designed as therapeutic proteins for unmet medical needs. These novel formats can pose more challenges to mammalian cell expression systems compared with conventional mAbs. They can be poorly expressed and show undesirable levels of aggregation because of a combination of the intrinsic properties of these proteins and the limited biosynthetic capacity of the host cell lines for these heterologous proteins. In addition, there are some mAbs and "natural" molecules that also fall into this class of DTE proteins. Although high levels of transcription are required for high levels of protein expression, steps downstream from transcription are also important in regulating protein secretion from mammalian cells. These post-transcriptional steps include mRNA translation, translocation of polypeptides from the cytosol into the ER, polypeptide folding and assembly, addition of PTMs and secretion. Depending on the individual recombinant protein, limitations in these steps can result in aggregation and low productivity of the desired product. Investigations have been focussed on understanding the bottlenecks that underlie the poor levels of expression of these proteins and addressing these bottlenecks with cell line and vector engineering tools. A summary of some of the successful approaches is given below and in Table 1.

7.1 Protein Trafficking, Assembly and Secretion

Secretory proteins have an N-terminal secretory peptide that targets the polypeptide for processing through the secretory machinery of the cell. As the nascent polypeptide emerges from the ribosome, the signal peptide binds to the signal recognition particle (SRP) and the resulting complex is targeted to the translocon on the ER membrane. As the polypeptide is translocated into the ER, the signal peptide is cleaved off by the signal peptidase so that the signal peptide is not part of the mature protein. Newly synthesized proteins are folded and assembled in the ER, before addition of PTMs and progression through the Golgi and final secretion. If the ER capacity for protein folding is exceeded, then the resulting unfolded or misfolded proteins accumulate in the ER, and this is detected and induces the unfolded protein response (UPR). The UPR aims to maintain protein homeostasis by shutting down translation or increasing the level of chaperones to aid folding (reviewed by Chakrabarti et al. [111]). At the same time, misfolded proteins are removed by upregulation of ER degradative enzymes. If ER stress is sustained, this can result in apoptosis and cell death. Where the production of unfolded or misfolded recombinant protein is contributing to significant ER stress then this naturally selects for low levels of productivity of the recombinant protein. The UPR is a dynamic and complex process, and two groups have developed UPR-responsive reporter systems in order to monitor and understand better the factors that can contribute to UPR

Table 1 Summary of the approaches to increase the productivity and product quality of "difficult-to-express" proteins

Attribute	Recombinant protein	Cellular target or pathway	Approaches	Result	Reference
Aggregation and product quality	ActRIIB-Fc	Transcription rate	Reduced input DNA and reduced culture temperature	80% reduction in plasmid DNA in 293-EBNA1 cells resulted in decreased aggregation from 36 to 14% at 37°C, and to 4% at 32°C Also showed increased signal peptide cleavage and N-linked glycosylation profiles	[103]
Aggregation and glycoprofile	mAb	Kinetics of mAb assembly	Increased relative level of LC: HC expression using attenuated IRES for HC gene or by re-transfection of LC	Increased LC:HC ratio >1 resulted in increase in monomer level to >97.5%. Increase in LC: HC ratio from 0.32 to 3.43 decreased level of high-mannose glycan from 33 to 14%	[44–47]
Low productivity	mAb	Signal peptide processing	Overexpression of signal receptor protein SRP14	Up to twofold increase in specific productivity rate (SPR)	[104]
	mAb	Rate or cellular capacity for mAb folding and assembly	Changed LC:HC gene ratio and co-expression of BiP, CypB, PDI, and active forms of ATF6 and XBP1	Up to twofold titre increase by increasing LC:HC ratio or co-expressing CypB, PDI, ATF6 or XBP1s	[105]
	Fc fusion protein Sp35Fc	Cell growth and protein folding	CypB co-expression and addition of chemical chaperones	CypB and chaperones increased SPR by 1.5-fold; CypB reduced aggregation by 40%; reduced temperature decreased aggregation significantly (5 to 1%)	[106]
	mAb	UPR pathway	Inducible expression of XBP1s Reduced culture temperature	Fourfold increase in SPR when XBP1s is induced with DOX and temperature reduced to 30°C	[107]

(continued)

Table 1 (continued)

Attribute	Recombinant protein	Cellular target or pathway	Approaches	Result	Reference
Aggregation and product-induced toxicity leading to cell line instability	mAb	Regulated target gene expression	Turned on target gene expression with "Tet on" inducible system only during production period	Inducible expression maintained at high levels for up to 145 days of culture	[108]
Product-related cell toxicity causing low productivity	Human IGF-1	IGF-1 receptor	IGF-1 receptor gene was knocked down with shRNA or KO with ZFN	Tenfold increase in titre from 0.1–0.2 to 1.3 g/L	[109]
Low productivity	Small positively charged protein	Recombinant protein binding to cell surface causing growth inhibition	Used multiple host cells; selection of host cells "resistant" to target protein by adapting the host cells in the presence of positively charged protein; increased screening scale and media development	Tenfold improvement in productivity from 50 to 500 mg/L	[110]

stress [112, 113]. As highlighted below and in Table 1, components of the UPR, including chaperones, present potential engineering targets to improve cell line productivity for DTE proteins, as do strategies to reduce the levels of unfolded or misfolded protein.

Using an empirical modelling system to compare the transient expression of a panel of eight IgG1 molecules with a fourfold variation in volumetric productivity, Pybus and colleagues [105] determined that the mAb-specific expression limitation can be at the folding and assembly step. The DTE mAbs showed an induction of UPR in host CHO and a decrease in cell growth. By changing the ratio of heavy-chain (HC) and light-chain (LC) expression, and by co-expression of a variety of molecular chaperones, foldases or UPR transactivators (Table 1), the expression level of the DTE mAbs was significantly improved. A similar strategy and screening platform was used by Johari and colleagues [106] to investigate successfully the low productivity of an Fc-fusion protein (Sp35Fc), which was associated with the formation of intracellular oligomeric aggregates. By screening a panel of cellular and chemical chaperones and UPR transactivators, specific productivity and cell growth were manipulated and the productivity was increased by combinatorial approaches with reduced culture temperature (Table 1). An inducible system to express the spliced form of human X-box binding protein (XBPs) in combination with reduced temperature has also been used to increase mAb productivity [107]. Besides the manipulation of chaperones and the UPR pathway, the work from Le Fourn and co-workers [104] identified light-chain signal-peptide processing as the limiting step for the expression of a DTE antibody. The low level of mAb secretion was associated with an intracellular accumulation of unprocessed light chain that had retained the signal peptide. Overexpressing a human signal receptor protein, SRP14, and other components of the secretory pathway improved both the processing of the LC signal peptide and levels of mAb secretion. Other studies have shown that changing the secretory leader sequence can improve expression, although the mechanism underlying this effect is not well understood [114].

7.2 Aggregation

Protein aggregates are a concern for recombinant therapeutic proteins as they can impact efficacy as well as induce immunogenic responses and cause adverse events upon administration to patients. Therefore, there is a desire to minimize and control protein aggregation. By testing a tenfold range of ratios of LC to HC in stable CHO pools, Ho and colleagues [44, 45] have found that a higher ratio (>1) of LC:HC resulted in higher mAb titres and higher levels of monomer (Table 1). They also found that high-mannose-type N-glycans increased, whereas fucosylated and galactosylated glycans decreased significantly at the lowest LC:HC ratio. Further work by this group [46, 47] showed that the antibody aggregates consisted mostly of HC polypeptide, and, if cell pools producing higher levels of aggregate were re-transfected with the LC gene, then the level of aggregate was reduced.

Overexpression of the BiP chaperone also reduced the level of aggregate, although the effect was less dramatic. It was found that the level of aggregation of an Fc-fusion protein was proportional to the gene dose in a HEK293 transient system [103]. As shown in Table 1, reducing input vector DNA and lowering the temperature significantly reduced mAb aggregation. It also increased the cleavage efficiency of a signal peptide, presumably because the reduced transcription rate allowed more time for cells to translate and process polypeptide. In a separate study of 28 individual mAb-expressing cell lines, the level of aggregate had an inverse correlation with intracellular and secreted light chain [115]. Another study of a bispecific antibody suggested a relationship between N-glycans and aggregation, with aggregate present in the cell culture medium containing antibody with reduced levels of N-glycan fucose and galactose residues [116]. Culture process development, such as optimisation of osmolarity and temperature, can also be used to reduce protein aggregation [117, 118]. Together these studies suggest that, depending on an individual recombinant protein, optimisation of the protein expression rate, the balance of expression of different subunits and the extent of glycosylation might be beneficial in reducing aggregation. High-throughput expression systems, such as that described by Hansen and colleagues [119], would potentially be useful in evaluating and optimising factors that influence these processes for an individual recombinant protein to improve expression and reduce aggregation.

7.3 Product-Related Cell Toxicity

There are several examples of recombinant proteins showing toxic effects in CHO cells, including reduced cell growth. Not surprisingly these proteins are difficult to express as the selection pressure generated by such toxicity leads to low productivity or productivity loss during cell expansion. Depending on the nature of the underlying interaction of these recombinant proteins with the CHO cell line, different mitigations have been identified to allow improved expression of these "toxic" products.

Misaghi et al. [108] observed up to a fourfold decrease in the expression of a mAb by clonal cell lines over 45 days of expansion, and the reduction was not associated with a decrease in heavy- and light-chain gene transcription. An inducible expression system was established that reduced the exposure of cells to the product, and, as a result, the stability of mAb productivity was maintained. In another example, cell line engineering proved to be a powerful tool in down-regulating a specific receptor to avoid the toxicity produced by expression of a bioactive ligand product. Romand and co-workers demonstrated that expression of human IGF-1 variants resulted in both poor growth and low productivity in CHO cells [109]. The negative effect of the IGF-1 product on cell growth was found to be mediated through the CHO IGF-1 receptor (IGF-1R). Consequently, by knocking out or knocking down the *IGF-1R* receptor gene in the CHO host cell lines, the productivity of recombinant IGF-1 in CHO cell lines was increased up to tenfold. In

a cell line and process development case-study for another recombinant protein that bound to the cell surface and inhibited cell growth, one approach included selecting a host cell population that was able to grow in the presence of the recombinant protein in the culture medium [110]. Transfectant pools generated from the adapted host produced levels of the recombinant protein that were approximately threefold higher than those from the non-adapted host. Other approaches included using multiple host cell lines with diverse genetic composition, significantly increasing the bioreactor screen size during cell line development by using micro-bioreactors and also developing a modified cell culture medium to improve cell growth in the presence of the recombinant protein. This integrated approach resulted in a tenfold titre improvement.

8 Operating Existing Systems in New Ways

With the increasing emphasis on reducing timelines from lead discovery to clinical studies, there is a desire to accelerate the preclinical development phases, including toxicology studies. The availability of product of the appropriate quality is a limiting step for preclinical studies. To produce representative material, it is desirable to use the final cell line clone in the manufacturing bioreactor process. Progression to this stage of clonal cell line and process development can be expedited by the use of standard production platforms and processes, but it is still time consuming – in the order of at least 8 months from transfection to final clone and process. However, material can be made much earlier using transients or pools of cells, as outlined below. In considering this approach it is important to recognise the risks and impact if the product quality from the final clone and process is significantly different from that of the preclinical material. However, this risk can be mitigated by screening final cell lines and processes for product quality that matches that of the early material used for preclinical studies.

8.1 Transients

In contrast to stable expression, transient gene expression (TGE) does not require integration of the expression plasmid into the host cell genome and so no selection pressure is applied. The expression plasmid DNA is transfected into host cells; the DNA that reaches the nucleus is transcribed and the resulting transcript translated into protein, which is then secreted into the cell culture medium. The cells express the recombinant proteins encoded by the plasmid over a period of a few days to a few weeks. TGE has traditionally been used for the rapid production of recombinant proteins for use during discovery as research reagents as well as for the production of candidate molecules for evaluation and early characterisation. Although the capacity for high TGE yields from HEK 293 cells has been well

established for many years [120], expression in CHO cells was limited until relatively recently. The product quality of recombinant proteins obtained from HEK and CHO cells can be different because of the differences in PTMs [121–124]. The desire to generate early material that is representative of later-stage processes that use stable CHO cell lines has driven the development of improved CHO transient expression systems. The strategies for enhancing transient expression have involved engineering the CHO host cell line, and developing the transient transfection and production processes with either wild-type or engineered CHO host cell lines. The various approaches for CHO transient expression development that have been documented in the literature have recently been reviewed by Jager and colleagues [125] and are summarised in Table 2. However, as a key driver for CHO transient system development is producing representative material of stable cell lines, industrialists have tended to set up optimised systems that are bespoke to their individual CHO host cell lines and production platforms, and consequently this information is not necessarily in the public domain. With the advent of the "Expi CHO" system there is now a commercially available CHO transient expression kit that is widely accessible (Table 2).

8.2 Transient Scale-Up

The strategies to improve CHO transient expression systems have culminated in the ability to express mAbs at 2 g/L in 21 days from transfection to harvest at a 6-L culture volume in a wave bioreactor using a process that is amenable to further scale-up [128]. Such high productivities at scale mean that it is now feasible to produce sufficiently large yields of recombinant proteins by transient expression to enable preclinical development and potentially even clinical development. However, sourcing the large amounts of plasmid DNA of the appropriate quality required for transient expression at large-scale is challenging and represents a different potential bottleneck for recombinant protein production compared with stable cell line systems. The inherent variability of transient transfections can also make the reproducibility of transient batches technically challenging.

8.3 Expression Predictability

Although it is now feasible to perform CHO transient expression at scale, the typical development process is to use a clonal cell line for the manufacture of clinical products. It typically takes several weeks to obtain readouts on expression from the stable cell line development process, which can cause project delays if there are issues with expression. However, the recent performance improvements in the CHO transient system allow prediction of the productivities in stable CHO cell lines as there is a correlation between CHO expression of mAbs in transient and

Table 2 Summary of the development and capabilities of CHO transient systems for recombinant protein production

Host cell line	Transfection reagent/method	Engineering target (s)	Rationale	Process yield, scale, production time	Reference
Engineered CHO-K1	Lipofectamine	Expression of Mouse polyomavirus large T antigen (CHO-T)	Host cell line is engineered to express polyomavirus large antigen (PyLT) and combined with a transient expression plasmid carrying the polyomavirus origin of replication (PyOri) allowing episomal replication. Transient expression plasmid also carries the Epstein-Barr virus antigen 1 gene (EBNA1) and the EBV origin of replication, OriP, for plasmid maintenance and segregation	140 mg/L IgG, 50 mL, 20 days Includes a temperature shift Batch process	[126, 127]
Engineered CHO-K1	PEI	Co-expression of GS and EBNA1	Host cell line expresses EBNA1 and exogenous glutamine synthetase whilst expression plasmid has OriP. Enhanced transient expression thought to be caused by improved nuclear transport of DNA and transcription enhancement	2 g/L IgG, 0.5 L (shake flask), 14 days 1.9 g/L IgG, 6 L (wave bioreactor), 21 days Fed-batch process Temperature shift	[128]
		Transfection and process optimisation	[DNA], PEI:DNA, cell density, culture media and feed, temperature shift to 34°C		
Engineered CHO-S	Electroporation	Co-expression of human XBP-1S and human ERO1-L alpha	Engineered host has increased secretory capacity because of enlarged ER and improved recovery from oxidative stress.	875 mg/L IgG, 200 mL, 14 days Fed-batch process Temperature shift	[129]
		Transfection and process optimisation	Media and feed optimisation, addition of sodium butyrate, temperature shift to 32°C		
Engineered CHO-K1	PEI	GS-knock-out	System designed to be compatible with stable cell line development manufacturing platform that uses GS-KO	350 mg/L IgG, 2 L, 7 days 1 g/L IgG, 2 L, 16 days Fed-batch process Temperature shift	[124]
		Transfection and process optimisation	Optimised ratio of DNA:PEI, addition of *N, N*-dimethylacetamide (DMA), temperature shift to 32°C		

(continued)

Table 2 (continued)

Host cell line	Transfection reagent/method	Engineering target (s)	Rationale	Process yield, scale, production time	Reference
CHO-DG44	PEI	Transfection and process optimisation	High cell density, optimisation of [DNA] and PEI, reduced [glutamine] (reduces ammonia accumulation), addition of DMA polar solvent (gives increased transcription rate), temperature shift to 31°C	500 mg/L IgG, 5 mL, 7 days	[130, 131, 123, 124]
CHO-S	ExpiFectamine	Transfection and process optimisation	High cell density culture, optimised transfection reagent, medium and feed, temperature shift	2.5 g/L IgG, 1 L, 14 days	ExpiCHO system from ThermoFisher

stable expression formats. Therefore, CHO transient expression is also a valuable tool in screening different molecules during the discovery process to predict expression titres rapidly as well as producing batches of representative product for developability studies.

8.4 Stable Pools for Rapid Large-Scale Supply

In contrast to transient transfections at scale, the process for generating clonal stable cell lines requires much less plasmid DNA, but is both time and resource intensive as individual clones are recovered from single cells and then expanded for screening. An intermediate solution for more rapid production is to use transfectant pools of cells. Instead of screening individual transfectants, multiple transfectants are selected and recovered together as a pool, allowing a more rapid recovery of cell populations and therefore a more rapid scale-up for production. However, a transfectant pool is heterogeneous, containing a mixed population of cells with different levels of productivity that gives lower overall productivity than can be obtained from the best individual clonal cell lines. Furthermore, transfectant pools can show instability of expression over time. There are some technologies that promote more homogeneous transfectant expression, improving expression levels and expression stability; these include incorporation of UCOE sequences into expression vectors along with targeted integration and transposon-based systems, which were described in Sect. 5. These pool approaches are also compatible with the use of platform bioreactor processes that are typically used for clonal cell lines, which helps to ensure that the pool-derived product is representative of product from later-stage clonal cell lines.

8.5 Development of Cloning Technologies

Although feasible to make high yields of clinical grade recombinant proteins rapidly through scaled-up transients and transfectant pools, clonal production cell lines are central for commercial supply strategies because of their higher productivity and robustness for scale-up. Critical to the cell line development process is the regulatory guidance to isolate production cell lines from single progenitor cells [132] to ensure consistency of product quality. A number of different strategies and technologies are used to isolate clonal cell lines, with more recent developments focussing on reducing timelines and improving efficiency for cell line development. In limiting-dilution cloning, dilute cell suspensions are dispensed into multi-well plates at less than one cell per well and then cell lines are recovered from the single colonies that grow in individual wells. A statistical analysis of data on the recovery of colonies in wells and multiple rounds of cloning are used to support the clonality of the derived cell lines. More recently, the development of high-content plate

imaging systems has allowed the generation of detailed images of the originating single cell in a well at the time of plating and can reduce the number of rounds of cloning required to support clonality. Another approach uses the ClonePix technology, which involves introducing low concentrations of cells into a semi-solid medium, allowing single cells to grow into colonies and then using the automated imaging and picking capabilities of the robot to transfer single, well-separated colonies into the individual wells of a multi-well plate [133]. Typically, ClonePix methods use two rounds of cloning to derive cell lines with a suitable assurance of clonality. Fluorescent detection reagents can be added to the semi-solid medium to allow identification of the colonies that are secreting recombinant product. FACS is an efficient technique for sorting cell suspensions and depositing single cells into individual wells of a multi-well plate. In combination with multi-well plate imaging of the deposited cells, FACS-based cloning requires only a single round of cloning [134] and is therefore more rapid for cell line development than methods requiring two rounds of cloning. In addition, the sorting capability of the FACS instrument can be harnessed by using fluorescently labelled detection reagents to bind either the product or a surrogate present on the cell surface, and then sorting on the basis of the bound fluorescence signal. New microfluidics technologies are also being applied to single-cell cloning [135]. Cell suspensions can be emulsified in oil, creating picodroplets that can then be imaged on microfluidic chips, with those containing a single cell being sorted and subsequently dispensed into plates. In addition, microfluidics provides the prospect of being able to couple isolation of single cells with performing assays on the picodroplet for secreted product to assess yield or product quality [136]. Single-cell cloning technologies based on "cell printing", which involve microfluidic dispensing integrated with cell imaging and analysis, also show potential for cell line development applications [137, 138].

8.6 Improved Cell Line Screening

Stable transfection generates cell lines that show variation in productivity, growth and product quality. This diversity of characteristics arises from a combination of the random integration of the expression vector into the host genome, variation in transgene copy number and also from the phenotypic variation in individual cells in the host cell population, as discussed in Sect. 3.2 of this chapter [22, 27]. With the resulting recombinant cell line heterogeneity, it is important to incorporate the appropriate screens during the cell line development process to ensure the selection of candidate production cell lines with the appropriate growth, productivity and product quality attributes. Many of the recent advances in cell line screening are oriented towards increasing the efficiency of the cell line development process, often through automation, and enhancing the predictability of the cell line screening data of the performance of cell lines in bioreactors.

Key to the cell line development screening strategy is that cell lines are tested in a process representative of the platform bioreactor process using production

medium and feed. Hence, cell lines are selected to "fit-to-process", and this reduces the need for upstream process development before scale-up and clinical manufacture. During cell line development, multiple cell lines are evaluated to find those with suitable characteristics. To handle the large numbers of cell lines involved, this evaluation process involves a screening cascade with a series of cell line assessment steps where the numbers of cell lines reduce at each stage and at the same time the amount of characterisation data for each cell line increases. The first step identifies those cell lines expressing the recombinant protein usually by detecting product secreted into the culture medium. Expressing cell lines are then advanced to the next step that involves evaluating cell lines in fed-batch culture to assess growth and productivity. This was traditionally performed using shake flask cultures, but the laborious manual handling involved limits the number of cell lines that can be evaluated in parallel to a few tens. The development of high-throughput, small-scale, fed-batch culture processes using 24- or 96-well plates enables hundreds of cell lines to be assessed in parallel [139], with automation further reducing the manual handling effort required. Once the numbers of cell lines have been reduced to the top 24–48, scaled-down bioreactor systems that control pH and dissolved oxygen can be used to generate data that are predictive of larger-scale fed-batch bioreactors in terms of cell growth, productivity and metabolism [140, 141]. These microscale bioreactor systems are also being adapted to operate in a simulated perfusion mode, enabling the screening and identification of cell lines that are compatible with continuous upstream processes. Importantly, microscale bioreactors provide not only predictive bioreactor performance data but also product for the generation of representative product quality data; together these data are analysed to identify candidate production cell lines for further process characterisation, including cell line stability, before selecting the final clone for the creation of the master cell bank that is used for manufacture.

8.7 Product Characterisation During Cell Line Screening

Both the cell line and the upstream process used for therapeutic protein expression influence product quality attributes such as aggregation, fragmentation and PTMs. Product quality screening therefore needs to be incorporated into the cell line development process to ensure selection of cell lines that express product with suitable characteristics. The product quality attributes that are characterised are determined by the properties of the product itself, but typically include an assessment of glycosylation, aggregation, fragmentation and amino acid sequence integrity. The generation of analytical data for product from multiple cell lines during cell line development is facilitated by high-throughput analysis of product within the cell culture medium, for example for aggregation [142], or by integration with high-throughput purification methods, for example glycosylation assays

[143]. Mass spectrometry and peptide mapping methods [144] are used to confirm that the product has the expected amino acid sequence. Product sequence variants that contain one or more amino acid substitutions can result from mutations in the encoding DNA or misincorporation of amino acids during translation [144–147]. As these sequence variants are cell line specific, they can be screened out during clone selection. Sequencing of cDNA can be used to characterise and confirm that the correct transcript sequence is expressed. However, this might not be sufficiently sensitive to identify low levels of a sequence variant, whereas next-generation sequencing (NGS) is more sensitive and can also provide additional data on transcript integrity [148–150].

8.8 Next-Generation Sequencing for Cell Line Characterisation

The level of annotation of the CHO and Chinese hamster genomes along with bioinformatics tools relating to the analysis of CHO omics data are continuing to be developed [151]. This facilitates the use of NGS to characterise the genomes of CHO cell lines. NGS allows detailed analysis of the genome following transgene integration or gene editing and can be used to assess transgene sequence, copy number, integrity and integration site. NGS is a very powerful technology, producing vast amounts of sequence data and it is essential to have the appropriate bioinformatics capabilities to process and analyse these data. Multiple targeted massive parallel sequencing (MPS) approaches have also been developed to focus on particular genomic regions defined by primers to reduce the scale of the data [152]. Other applications for NGS are to compare the transgene structure and integration at different times over long-term culture to assess the genetic stability of stable cell lines. Next-generation nucleic acid sequencing technology also provides an additional potential method for testing and investigating incidences of contamination [153]. As next-generation sequencing technology gains regulatory acceptance, it also has the potential to reduce the need for the in vivo testing that forms part of the traditional program of virus testing. Collaborative efforts involving regulators and cross-industry representatives are under way to investigate the sensitivity, robustness and validation of NGS methodologies for safety testing and to establish a framework for implementation [154, 155].

9 Perspectives on CHO Expression System Development

Traditionally, CHO cell line development for recombinant protein production has been a screening-led process using a sequential cascade of assays to identify cell lines with suitable characteristics in terms of growth, productivity and product

quality for large-scale production. These screening processes mine the variation in cell line performance that arises from a combination of heterogeneity of cells in the host cell population and the heterogeneity resulting from random integration of the plasmid expression vector into the host cell genome. Many of the developments for CHO cell line generation have focussed on improving the predictability and efficiency of the screening processes, as described above. These systematic approaches have been very successful, leading to significant improvements in the timelines and the resources needed for cell line development, and in concert with intensive media development and bioreactor optimisation have delivered cell lines with higher productivities, achieving up to 10 g/L for mAbs. Although highly effective, these approaches have treated CHO cells as a "blackbox" with limited molecular understanding of the integrated networks of CHO biosynthetic processes. However, new challenges with increased requirements for efficiency in cell line development, expression of innovative molecular formats and new production processes require additional, more-rational design-led strategies to achieve the required optimisation and develop the next generation of CHO cell line development platforms.

The first publication of the genome sequence of CHO-K1 in 2011 [18] marked a shift in the level of molecular understanding in CHO cells and has stimulated more-rational engineering approaches to develop CHO production cell lines. Expression profiling of CHO cells and their responses to bioprocessing conditions have enabled a greater understanding of cellular processes and identified engineering targets to make improvements [156]. Furthermore, CHO cells can now be evaluated with multiple omics tools to describe the proteome and metabolome as well as the genome and the transcriptome, allowing the application of systems biology modelling [157]. Data from these omics approaches can be integrated with metabolic networks into computational "genome scale" metabolic models for specific pathways (reviewed by Gutierrez and Lewis [158]). These models are potentially very powerful, generating the ability to perform in silico experiments to predict the outcomes of changing components within biochemical pathways. Reported applications for these models to CHO cells have included media development and understanding the impact of different culture conditions [158]. Further integration of omics data to refine and build more-extensive models to incorporate biosynthetic processes is computationally challenging, but will enable more detailed and accurate predictions and further help to define engineering targets. Meanwhile, the collection and mapping of data from the CHO glycoproteome and phosphoproteome are improving the understanding of PTMs, which are important for the product quality of therapeutic proteins.

Having identified potential gene engineering targets, tools such as shRNA for gene knock-down and genome editing for gene knock-out along with standard plasmid vectors for gene knock-in or overexpression have been essential in validating and exploiting these gene targets. High-throughput screening expression systems such as that described by Hansen et al. [119] are potentially useful tools to explore rapidly the impact of combinations of genes on the expression of recombinant proteins and on product quality to validate potential targets.

Programmable sequence-specific nucleases based on ZFN and TALEN technologies have shown great utility for knocking out single genes and in a few cases a combination of a small number of genes. However, the advent of the CRISPR–Cas9 system with its simpler and more rapid target engineering capacity means that multi-gene genome editing has become more feasible. Additional targeted genome editing applications for CRISPR–Cas9 include gene insertion and gene activation or repression as well as gene knock-out [76, 77], which are also important tools for creating optimal production lines.

Standard expression plasmid vectors and gene editing technologies offer a way to modulate individual or small numbers of genes involved in CHO cellular pathways. However, engineering of miRNAs can allow simultaneous modification of multiple genes across multiple pathways. MicroRNAs are small non-coding RNAs that are involved in regulating many cellular processes by a mechanism based on anti-sense recognition of specific sequences in target RNAs. The knowledge of the function of miRNAs is still developing, but they have been characterised as being involved in cell growth, apoptosis and cell death, hypoxia and oxidative stress as well as protein production (reviewed in Jadhav et al. [159]). Therefore, miRNAs are promising engineering targets to improve CHO production cell lines, and this is borne out by studies where overexpression of miR-17 and miR-30 produced higher expression levels of recombinant proteins from CHO cells [160, 161], although the molecular basis for these effects is not yet understood.

CHO cells can be conventionally engineered by accessing the natural diversity of genetic sequences from CHO cells or other organisms. However, synthetic biology approaches can devise and develop novel combinations of biological components and have the potential to redesign and improve CHO cellular processes radically for recombinant protein production (reviewed by Lienert et al. [162] and Xie and Fussenegger [163]). Through precise control of new gene networks, the resulting "designer cells" have the potential to improve production efficiency and robustness (e.g. through engineering metabolic and biosynthetic pathways), improve product homogeneity (e.g. through engineering pathways for PTMs) and enable the production of innovative and new molecular formats that are currently challenging to express. The building blocks for synthetic CHO-based systems are under development with the availability of libraries of new synthetic promoters to regulate transcription in CHO cells [164] and the development of new multi-gene engineering vectors to introduce new multi-gene synthetic networks into mammalian cells [165].

With the advantages of regulatory provenance, increasing knowledge of, and ability to manipulate, biosynthetic pathways and compatibility with new continuous manufacturing processes, CHO expression systems are set to become an even more flexible platform and are expected to continue to be central for delivery of increasingly complex therapeutic proteins. In future, it is envisioned that the data from omics technologies and integration with systems biology approaches will "open" the CHO "blackbox" and should enable a step change in the understanding and modelling of cellular processes in CHO cells to identify new rational engineering targets to improve recombinant protein production [166]. At the same time, genome

editing in combination with synthetic biology technologies will provide the translational tools required to re-engineer CHO cells and exploit these targets. Together, these approaches should allow implementation of a more rational engineering and design-led approach to develop the next generation of CHO production cell lines tailored according to the product and process requirements.

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Innovation in Cell Banking, Expansion, and Production Culture

Rashmi Kshirsagar and Thomas Ryll

Abstract Cell culture-based production processes enable the development and commercial supply of recombinant protein products. Such processes consist of the following elements: thaw and initiation of culture, seed expansion, and production culture. A robust cell source storage system in the form of a cell bank is developed and cells are thawed to initiate the cell culture process. Seed culture expansion generates sufficient cell mass to initiate the production culture. The production culture provides an environment where the cells can synthesize the product and is optimized to deliver the highest possible product concentration with acceptable product quality. This chapter describes the significant innovations made in these process elements and the resulting improvements in the overall efficiency, robustness, and safety of the processes and products.

Keywords Cell banking, Fed-batch, Innovation, Mammalian cell culture, Productivity

Contents

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1 Introduction

The productivities of modern mammalian cell culture-based production processes that can be executed at industrial scales have reached ranges that in the past were possible only in microbial systems [1–7]. Although microbial systems offer high productivities for a limited number of protein products, mammalian cell culture has become the dominant production system for recombinant proteins. This can be attributed to its flexibility to enable many product moieties commercially and its ability to offer mammalian-like post translational modifications that are important for activity, efficacy, or efficiency of the drug product [8–11].

Cell culture unit operations are an integral part of any biologics manufacturing process. The overall productivity and efficiency of the recombinant protein production process and the product quality (PQ) features of the drug substance are defined in this group of unit operations. The key enablers of a good cell culture process are the cell line used and its specific productivity, the ability to grow and maintain high cell mass, and the physiological environment in a bioreactor to ensure that consistent PQ is generated and a consistent feedstock is provided to downstream operations. Such a cell culture process invokes the need for biology and engineering to work hand in hand to generate continued innovation. Although the improvements achieved over the last three decades have been enormous (see Fig. 2 for example) it would be naive to think that no further improvements can be expected or are desired. Quite the opposite – the cell culture field continues to demonstrate process innovations toward improved productivity, PQ control, robustness, and efficient use of capital investment. The rapid development and adaptation of disposable bioreactor systems for bench scale high throughput optimization and for more flexible manufacturing solutions is one example of innovation that is impacting the cell culture field and overall efficiencies. The surge of biosimilar products with their unique requirements for PQ management to achieve comparability and cost reduction is another example of needs and opportunities leading to further innovation and improvements. These in turn benefit innovator products and production processes.

A typical mammalian cell culture process starts with thawing a vial of cryopreserved cells into cell culture medium. The cells are expanded in suspension in flasks, spinners, or bags at controlled temperature and under carbon dioxide. The cells are routinely diluted in fresh medium to keep them in an exponential growth phase and to create sufficient cell mass to initiate the production culture. The production culture can be run in batch, fed-batch, or continuous perfusion mode and involves supplying the cells with sufficient nutrients to maximize product formation (Fig. 1). In this chapter we discuss the main objectives of cell culture

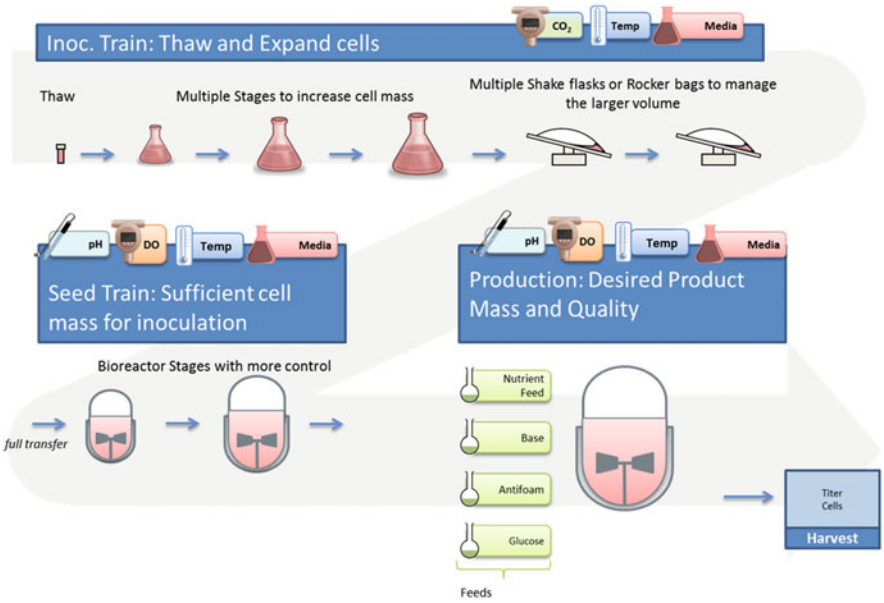

Fig. 1 Cell culture process flow diagram showing thaw, seed expansion, and production culture stages

processes, the innovation and progress that has been made over the last decades, and what we can expect going forward. For the production culture we focus on fed-batch technology and only refer to continuous cultures where necessary or useful. Innovations being made toward continuous processing are described in detail in [12].

This chapter is organized following the three main elements of a cell culture process, namely thaw and initiation of culture, seed expansion, and production culture. The subsequent harvest operation is discussed in [13]. Significant innovations have been made in all three main elements of the cell culture production process, resulting in enormous improvements in the overall efficiency, robustness, and safety of the processes and products. Driving forces behind these improvements include the desires for:

1. Enhanced patient safety
2. Improved process robustness and consistency
3. Enhanced product concentration (titer)
4. Manipulation of PQ
5. Reduced processing time
6. Reduced process scale
7. Easement of downstream operation
8. Ease of technology transfer
9. Overall cost reduction (cost per gram of product produced)
10. Facility throughput (volumetric productivity, grams of product produced per culture volume and time)

Some of the innovations and improvements to cell culture processes target very specific needs, whereas others impact many of the desires listed above. For example, the use of a specific additive in production culture to manipulate a specific PQ feature may not have any other impact on the process overall (specific manipulation of glycan features are examples [14]). Other innovations impact many of the needs listed above. The development and implementation of chemically defined media and feeds, for example, leads to improvements in patient safety and process consistency, offers better options for PQ management, and eases downstream operations.

2 Cell Banking and Culture Expansion

One of the key requirements for sustained commercial success of a biotherapeutic is the availability of the cell source to initiate its production. As a product may be developed and then commercially manufactured over many years, a robust cell source storage system is essential. Cryopreservation is routinely used to establish a cell bank of the producer cell line. A two-tier cell banking system consisting of a master cell bank (MCB) and a working cell bank (WCB) is widely accepted as it was originally proposed in 1963 during a workshop of the Cell Culture Committee [15, 16]. This process also enables the safety and quality testing of the cell source ahead of time and is one of the ways we can assure safe, reliable, and reproducible supply of a biotherapeutic to the patients.

Cryopreservation has evolved over the last two decades. Today, serum-free cell banks made with cell culture medium and 5–15% DMSO as the cryoprotectant are the norm in the industry. However, in the past, cell banking medium that contained serum in addition to DMSO was used. As the industry has matured, there have been significant advances in adapting cells to grow serum free and development of chemically defined medium to support the nutritional needs of cells. This has helped move away from the use of serum in cell banking medium [17–19].

The main purpose of culture expansion in a biotherapeutic production process is to create sufficient cell mass for the production culture. The additional benefit is to condition the cells in a culture environment so that they enter the right physiological state to enter the production phase. Typically, a seed train is initiated from a vial of the WCB and the cells are serially expanded and further scaled up via an inoculum train to enable inoculation of a production bioreactor. Typically, the initial stages operated at smaller working volumes in shake flasks, spinners, or bags are called the seed train. The later stages in bioreactors are called the inoculum train. The nomenclature may vary from company to company, where some may call all of it the seed train or the inoculum train.

In one approach, each batch is initiated from a new WCB vial (one vial – one batch). There is also an option to take some of the cells from the end of the seed train that was used for inoculum train initiation for the first batch and “roll” a seed train bioreactor for the next batch and continue this process for several batches. This

Table 1 Pros and cons for different seed expansion approaches

	Advantages	Disadvantages
One vial one batch approach	• Batch to Batch identical seed train • Limited population doubling per batch • Identical cell age for all batches	• More WCB vials used • More labor intensive • Plan redundant thaws
Rolling approach	• Less labor intensive • Less WCB vials used • Reduced number of seed train stages per manufacturing campaign	• Cell line stability limits the length of seed train • Each batch has difference cell age

approach is called a rolling seed and inoculum train. There are pros and cons to both approaches, as captured in Table 1.

The production bioreactor performance has been tied to the physiological state of the cells in the seed train. Hence, it is important to avoid overgrowing cells in any stage, as it may result in lag in the next stage. It is ideal to have a consistent growth rate from stage to stage [20]. In addition, it is important to model the effect of pH, temperature, seed density, and culture length in the last few stages of the inoculum train on the inoculum train and production bioreactor performance. This ensures that the most optimal parameters are selected for the inoculum train bioreactor used to inoculate the production bioreactor [21].

2.1 Innovations in Cell Banking and the Culture Expansion Process

Recent innovations in cell banking have involved moving away from cell banks made at moderate cell densities of 10–25 million cells/mL and banking vials with 1 mL culture volume to higher cell density and larger banking volumes in vials or cell bags. Some of the key advantages include reduction in processing time in seed trains to generate sufficient cell mass for the production bioreactor, reduction in labor in cGMP facilities, and increased flexibility. High density and volume banks also help reduce or completely eliminate open aseptic manipulations in shake flasks or spinners at the initiation of a seed train. Some of the challenges to generate high density and volume banks are that the cell banking process needs to generate large volume of culture for cryopreservation, the cryopreservation process in the larger volume containers needs to be optimized, and the overall process needs to be robust, easily reproducible, and amenable to be a platform. There have been reports in the literature of the different approaches taken to achieve these cell banks.

The first approach was presented by Heidemann et al. where they used CHO or BHK perfusion reactor cultures at 20–30 million cells/mL to prepare cryobags with 50- to 100-mL aliquots in 250- to 500-mL bags [22]. Cultures were frozen without concentration in the 100-mL aliquot and a 50% concentration to reach 40 million cells/mL for the 50-mL aliquot was used. The authors relied on previous success in

use of these cryobags for blood cell banking at similar cell densities. Frozen cell bank stability had also been reported for more than 7 years when used to store umbilical cord blood. The rationale for the volumes and cell density was the ability to thaw these banks directly into an inoculation reactor at 2 L working volume and 1 million cells/mL and be the starting point of the seed train. This approach had all the key advantages mentioned above. In addition, the seed train included pH and DO control from thaw in addition to temperature and carbon dioxide control, which is not possible in shake flasks, spinners, or wave bags. It was demonstrated that the bags could be frozen in a –40 °C freezer or a controlled rate freezer before storing them in the vapor phase of liquid nitrogen. DMSO was not removed at thaw and the cells were directly cultured.

A slight variation of the above approach is presented by Seth et al. for which they coined the acronym FASTEC [23, 24]. The FASTEC (Frozen Accelerated Seed Train for Execution of a Campaign) approach uses an ATF perfusion system to concentrate cells in the seed train to higher than 70 million cells/mL and freeze the cells after further concentration at about 110 million cells/mL in 150-mL cryopreservation bags called FROSTIs (FROzen Seed Train Intermediates). The FROSTI bags can be thawed and used to initiate an inoculum train in an 80-L bioreactor for production batch in a campaign. These bags are not cell banks and are prepared and used within a manufacturing campaign. This is an alternative approach to rolling the cells in a seed train bioreactor which mitigates the cell-age related variability as well as saves on labor for maintaining the cells in the seed train.

Another approach also used the perfusion system to generate a large cell mass for cell banking. However, a wave bioreactor perfusion system was used to grow the cells to more than 20 million cells/mL and the cell mass was further concentrated by centrifugation to make cell banks with 5-mL vials at 90–100 million cells/mL [25]. These cell banks would be thawed into wave bioreactors at 2 L working volume, similar to the first approach, eliminating all the open manipulations in shake flasks or spinners. However, pH and DO is not controlled in the wave bioreactors and you do have to undergo the traditional thaw of a vial in a biosafety cabinet, which can be avoided with the previous approaches that use bags. However, the advantage was that high density working cell banks could be made, frozen, and stored using the same equipment used to make 1-mL vials.

Finally, another group used wave bioreactor and ATF perfusion for cell banking. The cells were frozen at cell concentrations of 50–100 million cells/mL by adding 10% DMSO. They observed high cell growth and viability on thaw [26].

Overall, the cell cultivation to generate the large volume of culture for cryopreservation seems straightforward as long as the cells are amenable to perfusion culture operations. The centrifugation and cell freezing process may need to be fine-tuned for cell lines and the cryopreservation containers (vials or bags) [23, 24, 27]. The use of disposable equipment is highly desirable to enable ease of transition from one cell bank to another. However, one has to be cautious when using disposable equipment. Several groups have reported growth inhibition resulting from the use of disposable bags. A recent report with independent data from four companies, using several different cell lines and growth media, captures the issue

and suggests a method that can be implemented for quality control at disposable-bag vendors [28]. Another report reported challenges associated with cholesterol–polymer interactions, which suppress cholesterol-dependent NS0 myeloma cell growth, where cholesterol had to be supplemented into the process to achieve high cell density in a disposable bioreactor perfusion system [25, 29].

Innovations in cell banking have enabled shorter and robust culture expansion schemes. This enables maximizing the productivity in the facility as seed and inoculum train design can impact facility productivity. For example, culture expansion can be designed using a fixed time or fixed cell mass approach. In the fixed cell mass approach, each stage is run for variable time but to achieve a certain cell mass and then all the cells are transferred to the next stage. This approach eliminates any cell wastage but requires built in flexibility and scheduling batches to allow for variable length stages, and can result in downtime where the production bioreactor is not being utilized, as the cells take longer to progress through the expansion stages. In the fixed time approach, a fixed length is designated for every stage and the cells are diluted to the next stage. This approach requires excess cells to be maintained and some of the additional culture is discarded. However, it allows back-to-back production cultures to be scheduled and reduces downtime in the facility, thereby maximizing the facility output.

Another approach that has been particularly successful in increasing further the facility volumetric output is improvements in the culture expansion where the cells were expanded to significantly higher cell mass, whereby the production bioreactor was seeded at a high enough seed density to shorten the growth phase in the production bioreactor and reduce the length of the production bioreactor, the rate-limiting step. This approach has been reported to be successful by multiple teams in the literature [6, 30, 31]. All reports propose running the last inoculum bioreactor, typically called the N-1 bioreactor, in perfusion mode. Two reports have used a filtration system (ATF, Refine Technology, USA) for cell retention [6, 30] and a third report used inclined cell settler [31]. The N-1 final cell density ranged from 16 to 60 million cells/mL. This was dependent on the cell line and its growth rate, the medium used for batch and perfusion, and the ability to retain cells in the devices without impacting the cell physiology and hence their growth. Depending on the final N-1 cell density, the production bioreactor could be seeded using a 1:4 to 1:5 split with 4–10 million cells/mL instead of the traditional 0.2–1 million cells/mL. This effectively moved the unproductive growth phase from production bioreactor to N-1 bioreactor. The different reports translate this to a 12–30% improvement in facility output as they cut the production bioreactor time down by 2–6 days. Having further applied the strategy reported by Yang et al. in combination with a well-established platform process and some project specific optimization, we have shown that a volumetric productivity of above 700 mg/L/day and a titer of above 10 g/L can be achieved at a full manufacturing scale of 17,000 L operating volume (see Fig. 2). Assuming 10% packed cell volume and 95% harvest yield, this production culture can deliver about 150 kg of product to downstream operations. Such a large batch size is unique and can to serve new indications that require high

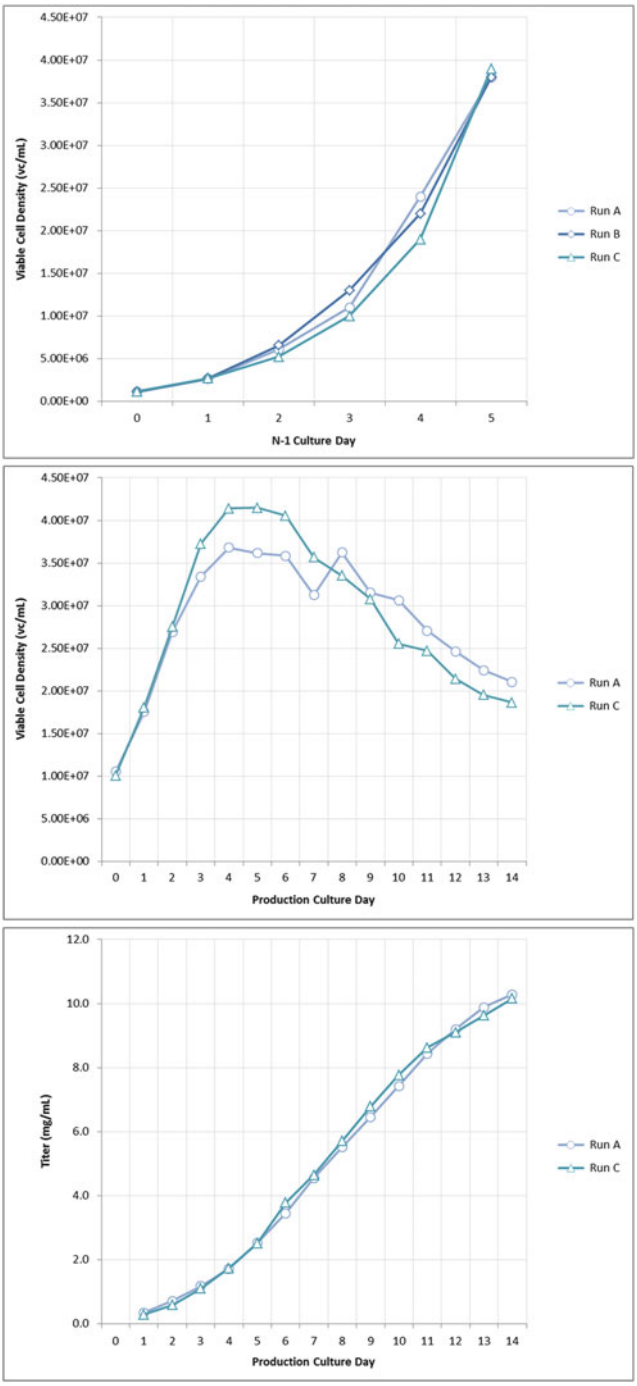

Fig. 2 Optimized production culture using N-1 perfusion and 17,000 L production culture operating volume. Maximal cell concentration can be achieved much faster cutting out low

dose chronic treatments with large patient populations, such as Alzheimer's disease, for example.

An important point to consider as we intensify seed expansion and inoculum processes is work to define optimal aeration, CO_2 removal, DO, and media optimization typically conducted only in the production phase which need to be conducted in the culture expansion and production stages to ensure consistent process performance.

3 Production Culture

The purpose of the production culture is to provide an environment where the cells can synthesize the product most efficiently, resulting in the highest product concentration possible (product titer) with acceptable PQ. Overall, a combination of product titer (g/L) and space time yield (volumetric productivity, g/L/day) are important when optimizing production cultures. Recombinant protein production processes are first approximation water treatment operations: products are very dilute and during most unit operations large volumes need to be processed. Increasing the product concentration at the end of a few-batch production culture from 5 to 10 g/L, for example, reduces the amount of culture suspension that needs to be handled by a factor of 2. On the other hand, volumetric productivity per established reactor volume is important as well because it reflects the total amount of product that can be produced in a facility per unit time.

Beyond productivity the production culture is the stage where the product is formed and therefore its purity and initial impurity profiles are determined. Control of PQ is as important as productivity of the production culture. Performance of production cultures in the end is often a compromise between reaching the highest productivity and assuring acceptable PQ. The main objective of downstream operations is to control the impurity profile (process and product related impurities that are not part of the active product) and, where possible, manipulate the purity profile (isoform composition that forms the product) of the drug substance. Significant manipulation of the purity profile during downstream processing typically reduces downstream yields significantly and consequently manipulation of the purity profile is best achieved during the production culture. Innovations around improved productivity and enhanced PQ control are discussed in the following sections.

Fig. 2 (continued) productive days from the production culture. This strategy can significantly improve volumetric productivity and thus facility output

3.1 A Brief History of Production Cultures

Cell culture-based recombinant protein production at industrial scales has been around for about the last 30 years. A flurry of different reactor systems and designs have been developed and tested in the early days. In the end the stirred tank reactor has quickly become the basic reactor design used today for cell culture operation. We experienced a similar renaissance of different reactor designs within the field of disposable reactor systems in the last 10 years or so [32–35]. However, convergence toward the stirred tank reactor format has also been taking hold with disposable reactor systems [36, 37] and we can expect the stirred tank to maintain the basic reactor design for cell culture operations in years to come (for examples look up single use bioreactors from Sartorius, GE, ThermoFisher, Millipore or others).

Looking back 20–30 years, continuous cell culture (perfusion mode) coupled with batch-wise downstreaming was a common approach. Perfusion systems developed in the 1980s and 1990s spanned a wide range of technologies including internal and external membrane systems, centrifugation, different types of settlers, acoustic filtration, use of microcarriers, fixed beds, and more [38–41]. Hollow fiber bioreactors were trendy in the early days and offered very high cell mass concentrated in the extracellular space of a cartridge, maintaining nutrient supply and waste product removal through hollow fiber ultrafiltration membranes. Such systems offered multiple benefits including the reduced use of serum or growth factors that could be maintained in the cellular compartment and omitted from the feed medium. The product was concentrated in the cellular compartment and could be harvested with high concentration and high purity [42, 43]. A harvest concentration of 16 g/L for an antibody was reported using a hollow fiber production system [42]. However, hollow fiber bioreactors have poor mass transfer and scalability and have all but disappeared as bioreactor systems for recombinant protein production. Nonetheless, the fundamental principle of retaining the product behind an ultrafiltration membrane to concentrate when exchanging larger volumes of nutrients and waste products is getting a renaissance in the form of a perfused or concentrated fed-batch (we get to this later).

The lack of efficient culture media made the operation of perfusion systems a challenge at industrial scales. Production scales were typically restricted to a few hundred liters only. Maintaining a quasi-steady state with stable productivity and stable PQ has also been difficult. Therefore fed-batch technology has matured into the dominant production mode in the 1990s with the emergence of ever-improving productivities at industrial scales (typically in the 2,000–20,000 L reactor scale range). Figure 3 shows the author's personal experience of fed-batch culture productivities over the last 22 years. Listed are production culture titers for antibodies and Fc-fusion molecules. Processes included in this figure were executed at either production or pilot scales or are judged to be scalable. Although this reflects our personal experience, we think that this reflects well the improvements made in the cell culture community during this period of time. Interesting to note is that titers

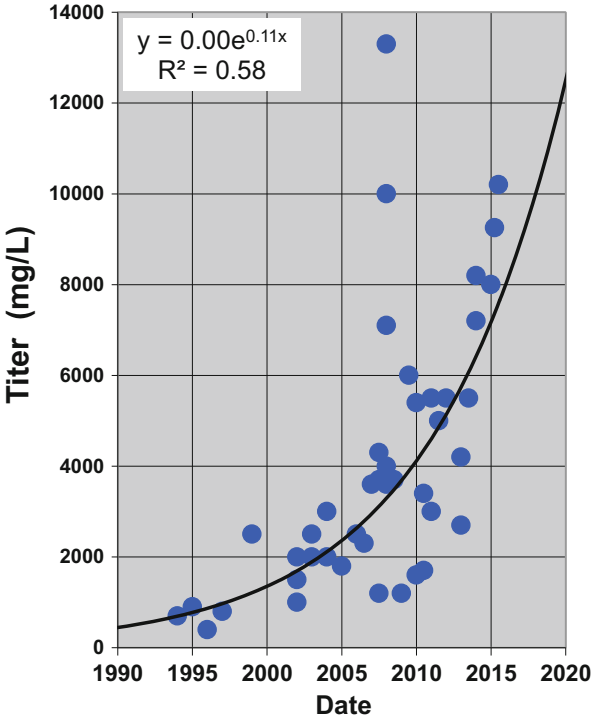

Fig. 3 Snapshot of the author's personal experience with fed-batch-based production culture titers for antibodies and Fc-fusion molecules over the last 22 years. Depicted are titers from manufacturing or pilot scales as well as bench scale processes that are judged to be scalable. The growth rate of 0.11 corresponds to a 6.3-year doubling time

have increased exponentially with a doubling time of approximately 6–7 years. It is obviously questionable for how long this trend can continue but we believe that it is reasonable to assume that we are likely to see another doubling over the coming years. It should also be noted that during the last 20 years not only have titers increase enormously but also, at the same time, the cell culture community has accomplished the replacement of serum with animal-derived hydrolysates, followed by plant- or yeast-derived hydrolysates, and, finally, chemically defined media and feeds, resulting in enhanced control, improved consistency, and improved product safety.

Today, fed-batch cultivation is still the dominant modus of operation for industrial protein production [44–46]. Potent platform process formats have been developed and typically include the use of an adapted host cell line and associated expression system, proprietary culture media, feeds, and process formats that can easily be scaled up and transferred to new manufacturing sites. Using such platforms enables the cell culture scientist to establish highly productive processes with titers in the 5 g/L range or higher and without the need for any optimization work early in the process life cycle.

3.2 Advances in Culture Productivity

The product titer of a fed-batch culture process is determined by two variables. These are the specific productivity of the cell mass (q_p) used and the amount of cell mass that can be generated and maintained in the bioreactor (integral of cell mass over time). The relationship can be described using the following equation, where Titer = final product concentration (g/L), q_p = cell specific productivity (productivity per cell and time unit), and $\int X\,dt$ = integral of cell mass (X) over time:

$$\text{Titer} = q_p * \int X\,dt$$

Average volumetric productivity can be estimated by dividing the final product concentration (titer) by the run duration to yield a term with mass per volume per time unit (g/L/day); volume is typically the reactor working volume, thus volumetric productivity is typically expressed per reactor volume. For perfusion culture titer and volumetric productivity are also determined by cell mass and specific productivity and need to factor in the dilution or perfusion rate. Optimization of culture productivity has therefor focused on increasing cell mass and increasing specific productivity. For best efforts such optimizations have been done using a specific host cell line and process format. The resulting "platform process" makes repeated use of a well-adapted host cell line and process format and offers high productivity with low investment at the beginning of a product's life cycle.

3.2.1 Culture Physiology

For superior and consistent performance it is important to reduce excessive metabolic waste product formation such as lactate and ammonia to maintain the cell mass in a healthy state and insure longevity. Cell cultures have been known to switch metabolic profiles and a switch to lactate production may indicate damage of the oxidative phosphorylation processes in mitochondria and a decline in specific productivity and cell mass accumulation [47–51]. When developing highly productive fed-batch cultures we have notice that overfeeding cultures can lead to access production of ammonia and hydrogen sulfide, for example, and associated reduced performance and impact on PQ [52–55] .Several strategies have been developed to improve feeding of fed-batch cultures. Bolus feeding that results in large variations in nutrient concentrations has been replaced by daily nutrient feeds or by continuous feeding approaches (Fig. 4). Alternatively, physiological parameters such as culture pH can be used to control feeding rates [56]. Using capacitance sensors, feeding can also be automated and tailored to the actual present cell mass in culture and can thus stabilize feed per cell and the physiological environment for improved consistency [4, 57]. Capacitance-based perfusion has also been used to improve perfusion rate consistency in perfusion culture.

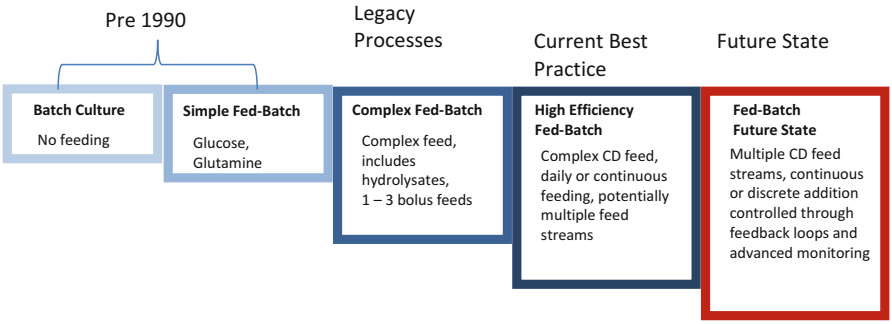

Fig. 4 Evolution of fed-batch production culture strategies. *CD* chemically defined

3.2.2 Specific Productivity

Specific productivity is mainly a function of the cell line generated to produce the product. Typical manufacturing cell lines for antibody products, for example, feature specific productivities in the 10–40-pg/cell/day range (pcd). Cell line generation and advances in this field are discussed in [58] and are not be further discussed here. Although the cell line determines the range of specific productivity that can be achieved, process conditions do impact q_p and have been subject to intensive optimization. A shift to lower temperature, increased osmolality, or the addition of histone acetylase inhibitors can in some cases lead to enhanced specific productivity because of enlargement of the intracellular mRNA pool or other factors [59–62]. This typically happens at a loss of cell mass and thus needs to be carefully optimized for a net benefit.

Limitations of amino acids or other key media ingredients can reduce specific productivity [63, 64]. Consequently, the development of a balanced media and feed formulation and a feeding scheme that avoid access production of byproducts such as lactate and ammonia has proven to be key to highly productive manufacturing processes [2, 63]. Using a well-developed platform process we have noticed that specific productivity increases when the growth rate of the cell population ceases. This results in a higher productive cell mass after the growth phase and consequently maintenance of this highly productive cell mass has additional benefits. However, this behavior is not observed with all process platforms in our hands.

3.2.3 Cell Mass

Given the use of a certain production cell line, the cell mass that a process can achieve and maintain is the variable that determines the titer and volumetric productivity of the culture process. The achievable cell mass determines productivity of the production culture using fed-batch, perfusion, or perfused fed-batch approaches. Providing additional nutrients and disposing of waste products by using media perfusion enhances the cell mass that can be accumulated and

maintained. Consequently, perfusion cultures and perfused fed-batch cultures have the potential for higher productivity. We discuss the authors view on how productivity of culture systems could increase in the future briefly in a later section. Here we focus on advancements achieved to increase cell mass in fed-batch mode.

3.2.4 Power of Platform Processes

We have already mentioned the importance of developing a platform approach to achieve high productivity with low labor and time investment. Developing a platform process requires several elements that ultimately result in a host cell line and process format achieving high cell mass and high specific productivity. First a host cell line and expression system needs to be selected that fits the purpose (high antibody expression for example). Then the host cell line needs to be adapted to the media and feed combination to assure good and robust cell growth after a final cell clone has been chosen. The fed-batch process needs to be optimized in terms of media composition and feeding regime to insure that the selected clone can grow to high cell mass without the need for any additional adaptation. High cell mass here means cell concentrations above 20 million/mL. The feeding regime also needs to be optimized for longevity of the culture. After maximal cell mass is achieved, typically 4–7 days after inoculation, the culture needs to be maintained at high viability for about 10–15 days to maximize productivity. If a host cell line is well-adapted to such a process format and delivers the targeted cell mass, production clones produced from this host typically follow this performance without the need for extensive optimization. Using such optimization approaches, the cell mass integral in fed-batch production cultures has increased from about 50–100 million/mL 10–15 years ago to 200–400 million/mL in today's production cultures. Elements of this approach can be found elsewhere [2, 7, 56, 64–69].

3.2.5 Perfusion Applications

Perfusion culture offers the opportunity to grow larger cell mass and can increase the volumetric productivity of a reactor system [39, 40, 69, 70]. This is achieved by using large medium volumes leading to dilution of the product concentration. Significant improvements have been made in generating continuous production schemes using perfusion culture coupled with continuous downstream operations. These advances are discussed in [12].

More recently a combination of perfusion and fed-batch operation has been explored. When replacing the typical microfiltration membrane used in perfusion culture with an ultrafiltration membrane, a fed-batch culture can be provided with additional nutrients for a few days, resulting in very high cell mass accumulation. At the same time, the ultrafiltration membrane maintains the product in the culture space, resulting in very high titers and increased volumetric productivities. This approach is called a perfused fed-batch or concentrated fed-batch culture and has

been explored for about the last 10 years [26, 71–73]. This strategy is similar to what was used with hollow fiber reactors 30 years ago but combines the scalability and mass transfer of a fed-batch culture in a stirred tank reactor with concentration of the product in the culture volume. Initially, titers in the 10 g/L region were achieved and have been improved, reaching as high as 40 g/L [73] . However, using this approach the cell mass generated is very high and can reach as high as >150 million cells/mL and 50% packed cell volume [73]. Beyond the harvest challenges this strategy offers, the measured titer has to be normalized, taking the cell mass into consideration. Thus a measured titer of 40 g/L with 50% packed cell volume maybe equivalent to a 20–25 g/L titer when comparing with a standard fed-batch culture of lower cell mass. Nonetheless, this approach offers an opportunity to improve further fed-batch titers and volumetric productivity. Challenges are the large media volumes required, the harvest operation, and robustness and consistency of this very intensive operation. Impact on PQ has to be evaluated carefully when growing such enormous cell mass. The authors estimate that this strategy offers volumetric productivities that can compete with high density perfusion culture and thus are attractive to explore further. In particular, further optimization of specific productivity can have significant positive impact on this culture mode.

3.3 Advances in Controlling PQ

Beyond cellular productivity and cell mass accumulation, a third and no less important factor is PQ and its consistency. When developing a production process for an innovator product, the PQ fingerprint is determined by the initial process used to provide clinical material. Any changes to the PQ profile (purity and impurity profiles) have to be complemented by an extensive comparability data set and, if necessary, pre-clinical or clinical data have to support a change to the quality profile. Achieving biochemical comparability and improving process productivity late in the life cycle of a product is challenging but typically attainable. The challenge becomes significantly larger if the cell line has to be changed. Consequently, the strategy of generating a cell line that can support commercial demands at the beginning of a product's life cycle has enormous value. Similarly, using a platform process format that can support commercial demand from the start is highly desirable (cell mass and run duration mainly). Thus investing in platform process technology that can be applied from the pre-IND stage onward has great value, not just for eliminating the need for major process changes targeting productivity improvements but, more importantly, insuring a consistent PQ profile throughout the life cycle of a recombinant protein product.

3.3.1 Biosimilars

The era of biosimilars has attracted substantial resources into optimizing processes toward lower cost and comparable PQ. Talking about strategies to develop biosimilar products could take up a whole chapter by itself. Here we briefly mention strategies that have been developed over the last few years to match PQ. Copying the PQ fingerprint of an innovator product is much more difficult than optimizing an existing process for improved productivity when maintaining comparability, for example. The reason is that a new cell line has to be generated and only limited knowledge of the innovator's manufacturing process is available. A typical strategy to develop a biosimilar product would include the following stages:

1. Intensive analysis of the innovator product to define the PQ target range. This is important because changes made to the innovator manufacturing process over the product's life cycle typically have shifted the PQ profile slightly and thus may offer a larger target PQ profile for a biosimilar product [74, 75].
2. Selection of a host cell line that is similar to what is used by the innovator. Using a similar host cell line can bring the initial PQ profile closer to the innovator. This is useful but not necessarily mandatory. In the case where the innovator product is made in a cell substrate that is sub-optimal for patient safety (mouse cell lines for example, Erbitux) [11, 76], a CHO cell line can be developed to produce a highly similar product and improve the safety aspects of the product. Access to a cell line toolbox can provide a diverse host cell line background, enabling selection of the best starting point [68].
3. Although selection of the best host cell line is very useful, extensive PQ screening of a large number of cell clones is mandatory to achieve biosimilarity. Accordingly, access to high throughput analytical support during cell line selection is essential.
4. Cell clone candidate screening under production conditions. This can be achieved using high throughout small-scale models [77]. At this stage, a lead cell line would be chosen.
5. PQ modulation using process parameters and process additives. Depending on by how much the PQ fingerprint differs from the innovator product modulation of pH, temperature, and run duration, for example, it may suffice or a more integrated modulation involving media and feed composition may be necessary [78, 79]. Specific additives that target PQ aspects may also be explored [14, 80, 81].
6. Scale-up and PQ verification.

Particular challenging may be the achieving of a highly similar effector function profile if effector functions are part of the mechanism of action of the innovator product. Thus specific attention needs to be paid to the glycosylation profile of the biosimilar product and selection toward the desired profile needs to happen early in the development process and needs to be monitored closely. Metabolic engineering of cell lines is also an option to manipulate PQ features [8, 10, 82–85]. However, if

faced with tight development time lines, metabolic engineering may not be able to eliminate a problem without delays. Consequently, metabolic engineering may be best applied when developing a cell line tool box that can be applied early in the development process.

3.3.2 Advances in Process Control for Improved PQ

Significant advancements have been made in the field of advanced process control. This topic is discussed in detail in a later chapter. Here we point out some developments that target improved control and consistency of PQ.

Fundamental to consistent performance of production cultures are consistent growth profiles. We can assume that, if cultures follow the same growth profile, the overall physiological environment is consistent, resulting in constant PQ. This assumption mostly holds true but raw material variation has been shown to be able to manipulate PQ features without necessarily impacting the growth profile of a culture (personal experience). Ensuring a constant growth profile, however, is the first step toward controlling consistent performance and PQ of a cell culture production process. For fed-batch cultures the feeding regime becomes important in this context. Throughout the intensification of production processes we have seen less frequent bolus feeds being replaced by daily feeding and, in some cases, continuous feeding to maintain a more homogeneous environment, at the same time insuring that cells have all the nutrients they need for best performance (Fig. 3). Very intensive feeding regimes can maximize achievable cell mass but also feature the risk of overfeeding in the case where the cell mass in the bioreactor is slightly below the target value. We have seen changes to PQ and performance in cases of overfeeding. Examples are the elevated formation of trisulfides and glycated species [53, 54, 86, 87] and a change in the physiological state leading to high lactate and/or ammonia production [47]. To mitigate such risk we have developed a strategy using a capacitance sensor and feedback loop. Using this tool, the feeding is adjusted to the actual present biomass and thus reduces the risk of overfeeding, improving process performance and PQ consistency [57]. A similar approach can be used for perfusion culture to control perfusion and bleeding rates.

Many advances have also been made in monitoring and controlling production cultures using spectroscopical sensors. The use of a Raman spectroscopy sensor, for example, has enabled the development of process models for a number of culture metabolites and antibody titer [86, 88–90]. Such models can be used to control feeding or addition of specific metabolites. A low-hanging fruit is the control of glucose feeding and lactate production. It has been shown that automated glucose control using a Raman spectroscopy approach can lead to reduced glycation of an antibody, for example [86].

3.4 Where Is the Field Moving?

We expect advanced process control and PAT (Process Analytical Technology) aspects to take hold in future cell culture processes and, as a result, we expect improved raw material control, better performance consistency, and improved control of PQ. Biosimilar development and the advancement of new modalities, such as antibody drug conjugates (ADCs), can also offer opportunities to maintain a high degree of scientific challenge in the coming years and decades.

We envision a future standard fed-batch antibody production culture to feature the following elements:

1. An optimized seed expansion process initiated using a large amount of frozen cells can reduce seed expansion times and improve consistency by avoiding less controlled culture vessels. The duration of the whole seed expansion process is managed based on fixed time for optimal facility output.
2. N-1 perfusion or intensive feeding to deliver high production culture inoculation density.
3. The production culture should feature high cell mass, automated feeding tailored to the actual requirements of the culture, and a number of metabolites, as well as antibody concentration measured in situ using a spectroscopical sensor and process models.
4. Process models and in situ measurements can be used to tune PQ aspects either through feedback or feed forward loops that include downstream operations.
5. The whole production process is supported by rigorous control of raw materials that reduces disturbance entering the process.
6. Process modeling, monitoring, and increased off-line, on-line, and in-situ data collection build the backbone for Real Time Product Release approaches of the future.
7. The whole culture process can be performed in disposable systems of intermediate scale because of enhanced productivity, reduced capital investment needs, and improved flexibility. Exceptions may be products that need high doses in large patient populations, such as Alzheimer disease.
8. Cost and volumetric productivity considerations may drive the choice of fed-batch, perfusion or perfused fed-batch and the degree of continuous processing and coupling to downstream operations

Each of these elements offers a wide range of opportunities for further optimization and innovation for the cell culture scientist and engineer.

Current best fed-batch processes conducted at industrial scales reach volumetric productivities in the 500 mg/L/day region. Among the benefits of perfusion culture is the ability to grow higher cell masses. In first approximation, perfusion culture offers the ability to increase cell mass by a factor of about 3–5 over fed-batch operation and thus the opportunity to increase volumetric productivity by the same

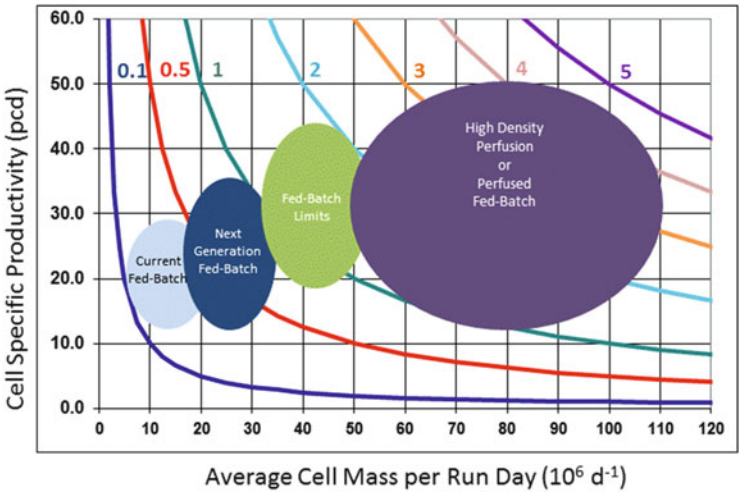

Fig. 5 Conceptual volumetric productivity space for fed-batch, perfused fed-batch and high density perfusion cultures. Cellular productivity in pg/cell/day (pcd) is plotted over average cell mass per run day. Average cell mass per run day depicts the integral of cell mass over time divided by run duration. *Curves* demonstrate the iso-productivity lines with g/L/day given in the number next to the lines

factor. This enhancement in volumetric productivity (g/L/day) needs to be recognized with greater process complexity, lower product concentrations, and restriction to smaller reactor scales. Following the factor of 3–5, an optimized 2,000-L perfusion reactor system can be considered equivalent to the output of an optimized 6,000–10,000-L fed-batch system. To compare volumetric productivities of fed-batch, perfusion, and perfused fed-batch strategies, we have created a figure depicting a volumetric productivity space governed by cellular productivity (pcd) and the average cell mass achieved under these different operating conditions. This is presented in Fig. 5. Average cell mass per run day depicts the integral of cell mass over time divided by run duration. If cellular productivity is plotted over this term, the space in this diagram portrays a Volumetric Productivity Space for the different production culture modes. The authors expect fed-batch volumetric productivity limits to be reached around 2 g/L/day. High density perfusion and perfused fed-batch offer opportunities to enhance volumetric productivity toward 5 g/L/day. The figure is based on the author's opinions. The improvements made to productivity of our production cultures have enabled us to scale down the volume requirements for future products. We expect disposable reactor systems of intermediate scale (500–3,000 L) to become more center stage for future processes. Exceptions may be products that entertain large patient populations and high doses, as expected for the Alzheimer's market, for example.

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Risk Mitigation in Preventing Adventitious Agent Contamination of Mammalian Cell Cultures

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Abstract Industrial-scale mammalian cell culture processes have been contaminated by viruses during the culturing phase. Although the historical frequency of such events has been quite low, the impact of contamination can be significant for the manufacturing company and for the supply of the product to patients. This chapter discusses sources of adventitious agent contamination risk in a cell culture process, provides a semiquantitative assessment of such risks, and describes potential process barriers that can be used to reduce contamination risk. High-temperature, short-time (HTST) heat treatment is recommended as the process barrier of choice, when compatible with the process. A case study assessing the compatibility of HTST heat treatment with a cell culture medium is presented, and lessons learned are shared from our experiences over many years of developing and implementing virus barriers in mammalian cell culture processes.

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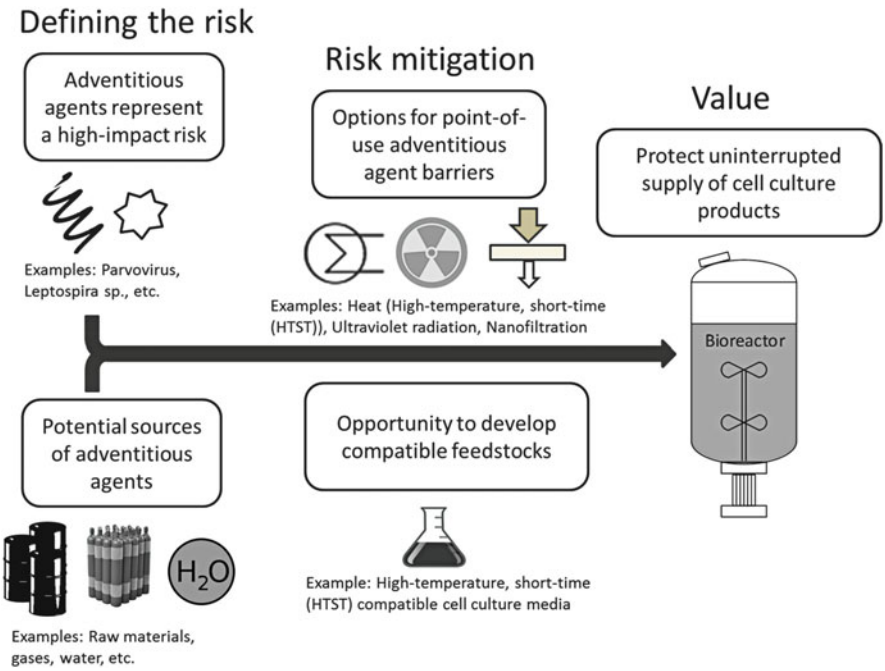
Graphical Abstract



Keywords Contamination risk assessment, HTST heat treatment, Nanofiltration, UV radiation, Virus barriers, Virus contamination

Contents

1 Introduction

Health authorities have provided regulatory guidance for manufacturers to ensure the safety of their products, requiring them to be free of detectable adventitious agents such as viruses [1]. Adventitious agents are microorganisms, including viruses, bacteria, fungi, mycoplasma, and transmissible spongiform encephalopathy agents, that are unintentionally introduced into a biological product during the manufacturing process. Health authorities expect manufacturers to have control systems in place at various stages of drug manufacture to ensure product safety. Key aspects of control systems include (1) selecting and testing source materials for adventitious agents, (2) testing the capacity of the production process to remove or inactivate adventitious agents, and (3) testing the product to demonstrate it is free from detectable adventitious agents.

For the purposes of this chapter, we assume that sufficient manufacturing controls are in place to prevent the introduction of nonviral adventitious agents into the process, as is typically provided by rigorous application of industry-standard sterilizing-grade filtration (typically a 0.1-μm pore size rating for mammalian cell culture processes). In particular, the potential for a contaminating virus to enter a manufacturing process or facility is an ongoing concern; sterilizing-grade filtration is not capable of removing many viruses because their size is typically similar to or smaller than the filter pore size. A virus acts as an obligate parasite and highjacks the transcriptional and translational machinery of a host cell to rapidly generate many progeny [2]. The viral process of multiplication means that even with a small amount of infectious virus particles per cell (multiplicity of infection), an infection event could propagate into a widespread infection in a cell culture environment because the kinetics for virus multiplication are typically very fast relative to cell culture manufacturing durations. It has been described, for example, that even 1 virus particle/L cell culture medium may be sufficient to initiate an infection [3]. The virus may enter the biological product manufacturing process either endogenously, from the organisms used to produce the biologic, or unintentionally, as an adventitious agent. For the former, regulatory guidance is provided to evaluate the viral safety of biological products from cell lines of animal origin [4]. For the latter, guidelines and expectations require that the biologics manufacturer's process demonstrate significant clearance capabilities should an adventitious agent be introduced into the manufacturing process [5]. The guidance relates to the capacity of a biological manufacturer's process to clear potential adventitious agents in order to ensure patient safety. This includes clearance of any endogenous retrovirus or retrovirus-like particles derived from the host cell line, as well as adventitious agents introduced during manufacture. Clearance potential, due to either inactivation or removal, is frequently described as the ratio of initial viral titer in the starting material to the viral titer in the relevant product fraction after a clearance (or series of clearance) step(s). The ratio can be expressed as the $\log_{10}$ reduction value (LRV) or the $\log_{10}$ reduction factor.

It is important for biologics manufacturers to implement strategies to minimize the risk of a contamination event in addition to a robust product and process monitoring approach. In practice, quality control systems for biologics manufacturing are binary, such that any batch showing detectable virus is rejected, while batches that pass the test are released. The ability to detect virus to a level where patient safety is assured is limited by the sensitivity of the assays available to detect viruses and other adventitious agents. And it is not possible to prove the absence of an existing adventitious agent or one that has not yet been identified, even with improved assay sensitivities, and they may not be detected by current testing methodologies.

It is helpful to define first the risk for a potential undetectable, low-level adventitious agent contamination in upstream manufacturing processes. The risk of contamination by an adventitious agent at a biologics manufacturing facility is low, but if it occurs, would have a large impact or high severity [3, 6]. If an adventitious agent makes its way into a biologics manufacturing process, the potential impacts include (1) risk to patient safety (if the agent is undetectable by existing control measures); (2) direct impact on the product being made, which in turn could negatively affect supply of the product to patients in need (e.g., the adventitious agent infects the cells producing the product or cells as a product); and (3) indirect impact on all products scheduled to be produced using the same equipment and in the same facility, which in turn could negatively affect the supply of all products made in that facility to patients in need (for facilities that manufacture multiple products).

As one can imagine, a company that experiences a contamination event and does not meet patient demand for critical products (e.g., oncology indications) and/or generates a negative impact to patient safety will suffer financial losses in both the near- and long-term (e.g., the trust of consumers and health authorities in the company could also be affected long-term). In the near-term, the response needed to investigate and remediate the contamination may be costly and complicated, depending on the extent of the potential contamination within a given facility. Examples are publically available of adventitious agent contamination within the biotechnology industry, wherein the adventitious agents reported include viruses and bacteria that are able to pass through the industry-standard sterilizing-grade filters with a 0.1-μm pore size [6–11]. For the Genzyme manufacturing crisis caused by contamination with Vesivirus 2117, the estimated loss in sales was US$100 to $300 million, which may not include all the associated costs of the cleanup or lost opportunities (opportunity cost) [7].

The theoretical risk mitigation strategy for a low-frequency, high-impact event is to avoid or transfer the risk (e.g., insurance). Unfortunately, as we have discussed, no clear solution exists to completely avoid or practically transfer the risk of the high-impact scenario of contamination by an adventitious agent in a biotech manufacturing facility. It can be argued that a rare event with severe consequences requires more robust measures when uncertainty surrounds the predictability of the event (e.g., a black swan event) [12]. One way to decrease the potential for the

presence of undetectable, low-level adventitious agents (e.g., viruses) is to include additional barriers at key points within the process.

Examples of additional barriers to adventitious agents that can be used during the product purification process (i.e., downstream processing) include (1) adventitious agent inactivation steps (e.g., extreme pH holds, use of detergents) and (2) adventitious agent removal steps (e.g., anion exchange chromatography, nanofiltration) [13, 14]. In this chapter, we focus on adventitious agent barriers to improve process robustness and reduce the risk of contamination during the product expression process (i.e., upstream processing).

2 Sources of Risk for Adventitious Agents Entering Upstream Bioprocessing

Adventitious agents can enter a typical upstream manufacturing process at several potential points, and mitigation strategies can be implemented to address these potential sources and entry points. The main source of potential adventitious agents is from raw materials that directly enter the process stream. Raw materials used in upstream processing include the cell source (i.e., the cell bank and its contents), the process gasses (e.g., air), water used to prepare cell culture media, and process media and supplement components [3].

Animal cell cultures from cell banks are rigorously tested for the presence of adventitious agents per regulatory guidelines (e.g., compliance with 21 CFR 610.18, FDA Points to Consider, and ICH Q5A, Q5D).

For process air, aerosol is a potential adventitious agent, and some viral aerosols can travel thousands of kilometers and remain infectious for several days [15]. The risk of viral particles entering a Chinese hamster ovary (CHO) cell culture process via process gases can be addressed both at the source of the gas supply and at the point of use. The production of process gasses (e.g., air, oxygen, carbon dioxide) can leverage conditions that are likely to inactivate viruses and other adventitious agents (high temperature and low absolute humidity). For this reason, biologics manufacturing facilities often generate their compressed air on site. This also addresses the fact that the source air used to generate compressed air on site is derived from the local environment around the production facility. Process gasses are typically filtered before use, and sterilizing-grade hydrophobic filters have been shown to remove particles as small as 10 nm when the filters are dry. Nanofiltration could also be considered to increase the likelihood of virus particles being removed, although costs would be significantly higher.

Process water for Good Manufacturing Practices applications (e.g., for preparing culture media, cleaning equipment) is typically highly purified using high-temperature treatment (e.g., use of distillation in producing water for injection), deionization, filtration, and ultrafiltration. Process water used for bioprocessing is also typically stored at high temperatures (>80°C). Therefore,

when the process water is exposed to the aforementioned barriers before use, it is considered a low risk for introducing adventitious agents into upstream processes. However, the most environmentally resistant viruses, such as parvoviruses, may not be fully inactivated by water temperatures below 90–100°C. The source of process water should also be considered; starting water should be selected to ensure a low risk for bioburden, undesirable chemicals, and adventitious agents. The chemical constituents of cell culture media and other process solutions and supplements are considered the highest risk for introduction of adventitious agents into upstream processes through raw materials and therefore deserve additional attention.

Cell culture media and process supplements are necessary to support a cell culture process from the cell bank through the final production stage (e.g., thaw medium, seed/scaleup media, production medium, production culture feeds, supplements added at any stage). Each raw material that comprises a given cell culture medium or supplement has its own inherent risk of containing an adventitious agent. A given raw material can be qualitatively assessed as a potential source of an adventitious agent in order to encourage selectivity when choosing raw materials, evaluating key aspects for raw material qualification, and determining which raw materials and feed stocks would benefit most from adventitious agent barriers. Table 1 provides a qualitative risk assessment of raw materials as a guide.

It is important to keep in mind that supply chain transparency is not a given. Many processing steps and exchanges may occur between primary source and receipt at a biologics manufacturing facility. Some processing steps may actually act as adventitious barriers (e.g., prolonged heating, drying, exposure to extreme pH, exposure to organic solvents), and some events may increase the risk (e.g., poor segregation of raw and finished goods, storage conditions that may enable contact with unintended adventitious agent vectors such as rodents).

Table 1 Qualitative risk assessment for cell culture raw materials

Raw material characteristics	Level of risk for infection by adventitious agent		
	High	Medium	Low
Source	Animal-derived	Biological origin (e.g., plant, yeast)	Microbial fermentation, synthetic, other
Manufacturing process	Crude material	Some purification	Inactivating/clearance conditions during purification/formulation (e.g., heat, prolonged exposure to caustic or acidic conditions)
Supply chain	Limited segregation, nonvalidated CIP/SIP, rodent food source, multiple handlers	Some segregation, CIP/SIP	Raw vs finished segregation, validated CIP/SIP
Amount used in biotechnological process	Large	Moderate	Small

CIP/*SIP* clean in place/sterilization in place

3 Available Point-of-Use Adventitious Agent Barriers

Given the variety of potential raw material risk levels and uncertainty in consistency of aspects of the supply chain or some raw materials (e.g., unplanned or vendor-initiated changes in sources, processing, storage, and handling for a given raw material), it makes sense to pursue a general and effective adventitious barrier at the point of use (point of entry) of raw materials in upstream processes. A few reasonably well-defined point-of-use options are available as adventitious agent barriers in upstream processes and encompass filtration, ionizing radiation exposure, or heat exposure. These options have unique advantages and disadvantages for both virus clearance capacity and large-scale processing suitability (Table 2).

When assessing the potential adventitious agent risk for a biologics manufacturer, one can think about "worst-case" model agents. A particularly difficult adventitious agent may be very small (i.e., able to circumvent sterilizing-grade filters of 0.1-μm pore size typically used as the final barrier in upstream biologics manufacturing), be highly resistant to potential processing conditions it would encounter (e.g., pH, chemicals, light, heat), have the ability to multiply quickly such that an undetectable level of the agent could multiply and affect a culture or product, have the ability to remain undetectable but potentially affect the culture or product, and/or be highly likely to be present in one or more of the aforementioned raw materials typically entering a biologics manufacturing process. One such adventitious agent relevant for upstream biologics manufacture is mouse minute virus (MMV; formerly known as minute virus of mice). MMV is a single-stranded,

Table 2 Upstream virus agent barrier options

Technology option	Virus clearance capacity[a]	Large-scale processing suitability
Heat[b]	Broad spectrum of viruses; very effective (>3–8 LRV for MMV)	Demonstrated/in use Point-of-use barrier
UVC[c]	Virus-dependent (>4–8 LRV observed in serum, some lower)	Demonstrated for water treatment Use with media demonstrated Point-of-use barrier
Gamma irradiation[d]	Virus-dependent (>3–7 LRV reported, some report >1 LRV)	Not suitable Not a point-of-use barrier
Filtration[e]	>3–7 LRV reported	Flexible (e.g., used in single-use facility) Point of use May be cost prohibitive for some applications and situations

MMV mouse minute virus, *UVC* ultraviolet C

[a]Log_{10} reduction value (LRV) data summarized from a variety of experimental conditions and multiple sources

[b]High-temperature, short-time treatment (e.g., 102°C for 10 s)

[c]Wavelength 254 nm at 100 mJ/cm^2

[d]25–40 cGy

[e]Ability to filter small viruses (~20 nm in diameter)

nonenveloped DNA virus and a representative of the Parvoviridae family. Parvoviruses are among the smallest known adventitious agents, with diameters of 18–28 nm (symmetrical icosahedral shape). The protein capsid of the Parvoviridae family confers high stability to the virus particles, allowing for resistance to inactivation by pH, solvents, and heat (exposure to heat up to 50°C on the order of hours, depending on the parvovirus, may be completely ineffective). References are available describing MMV inactivation via multiple inactivation methods, including pH [16–18], heat [3, 16, 18–21], ionizing radiation (e.g., ultraviolet [UV]-C, gamma) [18, 19, 22, 23], and chemical treatments [18, 19]. And, MMV is a specific risk for entry into processes in the biological manufacturing of CHO cell culture, as it is commonly harbored by mice that may potentially come in contact with raw materials at multiple points between the initial source and the point of use. MMV also has historically been shown to be a problematic adventitious agent in biotechnology processes [3, 9, 10].

For upstream manufacturing processes, it is important to consider the potential impact the choice of adventitious agent barrier may have on the ability to produce the desired quantity of a quality product (e.g., CHO cell culture performance and resulting product yield and quality). Therefore, it makes sense to pursue an adventitious agent barrier that has the following attributes: (1) low potential for undesirable leachates entering the process or otherwise negatively affecting the performance of the treated fluid/medium, and (2) is suitable for the process (e.g., meets the desired level of clearance, is appropriate for the process fluid being treated, meets the desired processing times, the format fits the relevant equipment and facility, is cost-effective).

4 Nanofiltration (e.g., Viral Filtration)

One general approach in adventitious agent barriers is to remove adventitious agents by physiochemically partitioning (e.g., filtration, chromatography) the adventitious agent from the desired process stream. To remove a virus by filtration, the primary method of partitioning within a process stream is the physical retention of the virus or other adventitious agents. A few manufacturers use commercially available virus-retentive filters. The variety of available filters may have various membrane chemistries, target retention sizes (e.g., potential to retain larger or smaller virus particles), membrane formats (e.g., membrane and support differences, layers, devices), membrane symmetries, and filtration modes (e.g., direct flow vs tangential flow filtration). One valuable review describes key factors, filter attributes, and process considerations of virus-retentive filters that influence virus clearance [13], though it focuses primarily on removing viruses from purified materials downstream of the cell culture step. Point-of-use application of virus filters should be assessed before implementation. The assessment should include testing of potentially viable filters with a representative process application (e.g., process-specific cell culture media and desired filtration process). Virus filtration of

cell culture media is not trivial, and some media components may affect the filtration process, including the presence or absence of serum, hydrolysates, and surfactants such as poloxamers, Pluronics, Kolliphor (e.g., poloxamer 188). It is recommended that manufacturers work closely with a desired vendor(s) to determine the appropriate filter and filter size for a given application. Currently, we are aware of two established filter manufactures that have or will soon have commercially available filters specifically for cell culture media applications. The first commercially available virus filter specifically designed for cell culture media applications is the Virosart® Media filter (Sartorius AG, Gottingen, Germany), with claims of >4 LRV for small, nonenveloped viruses and >7 LRV for *Mycoplasma orale* and *Leptospira licerasiae*. In general, filters that are already available or are coming to the market from various suppliers are targeting an average throughput capacity of 1,000 L/m^2 for a processing duration not to exceed 4 h (media dependent), for roughly not more than $5/L. From an end-user perspective, this cost target seems reasonable for a first-generation technology, but it is expected that this cost will need to come down to support user adoption for any moderate- to large-scale applications. Virus filter manufacturers should consider three desirable features of virus removal filters for the cell culture industry: (1) enabling a sterilizing-grade filter so the virus filter can be used to replace existing 0.1-μm sterilizing-grade filters (thereby avoiding the need for double-filtration and the costs for additional goods required by the biologics manufacturer), (2) enabling multiple sterilization options (e.g., autoclave and/or sterilize in place, gamma irradiation) to allow cost-effective implementation in a manufacturing facility, and (3) enabling multiple device options (e.g., cartridge, capsule) to meet various manufacturing facility needs. Filter manufacturers may also continue to improve their filtration technology, allowing for lower cost and higher flux without sacrificing virus clearance capacity (ideally at least >4 LRV for relevant small viruses such as MMV).

5 Ionizing Radiation

Another general adventitious agent barrier approach is to inactivate adventitious agents entering at the point of use or within the desired process stream. For ionizing radiation, gamma and short-wavelength UV (UVC, e.g., 254 nm) have been used as adventitious agent barriers to provide a germicidal/sterilization treatment for multiple materials, ranging from sterilizing disposables (e.g., gamma) to purifying drinking water (e.g., UVC).

Gamma irradiation has been used in upstream biologics manufacturing to inactivate virus and mollicutes (*Mycoplasma* sp. and *Acholeplasma* sp.) in animal sera [22]. Gamma irradiation acts through direct interaction to significantly disrupt genetic material (e.g., nucleic acids) of organisms by inducing mutations, cross-linking, and strand breakage. Furthermore, the ionizing radiation can also generate free radicals, leading to free radical damage to organisms. This approach works

very well for sterilization of prepackaged materials but is not practical as a point-of-use barrier in a biologics manufacturing facility.

UV irradiation has been used in upstream biologics manufacturing to inactivate bacteria and viruses in cell culture media. Laboratory-scale UVC systems are available that claim >4 LRV inactivation for small nonenveloped and large enveloped viruses. Some adventitious agents, such as bacteria, are more resistant to UVC and require high doses (>200 mJ/cm^2) for effective inactivation [24]. The literature describes the UVC doses for a desired LRV of various bacteria, protozoa, and viruses [18, 19, 24]. For upstream processes, it is strongly recommended to assess the potential for a negative impact on the process (e.g., cell culture media, supplements, and performance, and product quality) when using UVC as a point-of-use adventitious agent barrier to achieve a desired LRV. In particular, direct exposure to UVC and UVC-induced free radical reactive species has the potential to negatively affect a cell culture medium. For example, seven chemicals (tryptophan, tyrosine, lysine, pyridoxine, pyruvate, acetate, and formate) were found to be reduced or generated in a CHO cell culture medium as a result of exposure to UVC in a dose-dependent manner; these were statistically significant even at 100 mJ/cm^2, a common dose to enable a relatively broad range virus inactivation [23].

6 Heat and High-Temperature, Short-Time Treatment

In general, heat inactivation is the most effective option currently available for virus clearance [3, 18, 21]. Heat inactivation has been demonstrated to show significant virus clearance even for challenging viruses such as parvovirus (e.g., MMV). In addition, heat inactivation is effective for other adventitious agents such as some bacteria (e.g., *Leptospira licerasiae*) and *Mycoplasma* sp. that can pass through sterile-grade filtration [8, 25]. Therefore, it is recommended to consider heat inactivation first because of its broad spectrum effectiveness and robustness as an adventitious agent barrier.

Similar to the previously discussed inactivation methods (e.g., gamma irradiation and UVC), development work is recommended to determine the potential impact to a given biologics manufacturing process before implementing a heat inactivation strategy. Rigorous heat treatments such as steam sterilization under pressure (e.g., autoclave treatment) are ideal for some pieces of equipment, some supplements (e.g., trace elements in aqueous solutions), and some simple media components in single stock solutions (e.g., kept from reacting with other components upon heating). However, autoclaving complete liquid cell culture media is not typically feasible, as many of the components react with or are degraded by the heat (e.g., vitamin degradation, glutamine degradation, Maillard reaction product formation), and other important properties of the media can be perturbed (e.g., solubility/concentrations, pH, osmolality, free radical potential, redox potential), rendering the cell culture media unusable for the process. Therefore, applying heat as an adventitious agent barrier for cell culture media requires short exposure to

heat in order to balance effective inactivation of adventitious agents while maintaining an acceptable cell culture media for the process. This is commonly referred to as high-temperature, short-time (HTST) treatment.

7 Use of HTST Treatment as an Adventitious Agent Barrier in Upstream Biologics Manufacturing: A Case Study

HTST units designed for bioprocessing applications can be standard shell and tube heat exchangers using steam to input heat into the shell side of the exchanger. Figure 1 shows a schematic of an HTST skid used in upstream bioprocessing. Many of the components of an HTST skid can be procured off the shelf. However, the HTST skid design and operation should be carefully considered. These are important to ensure that HTST treatment temperatures and process fluid (e.g., cell culture media) residence time are rigorously controlled, thus maintaining the desired clearance of adventitious agents. For typical processes, the HTST treatment temperature and residence time are controlled at 102°C ± 2°C for 10 ± 2 s to ensure significant log reduction of potential adventitious agents [3]. Pilot HTST skids based on manufacturing-scale HTST skids have been designed and implemented for process and media development efforts. These pilot systems can be designed to match the desired hold temperature, residence time, and heating and cooling

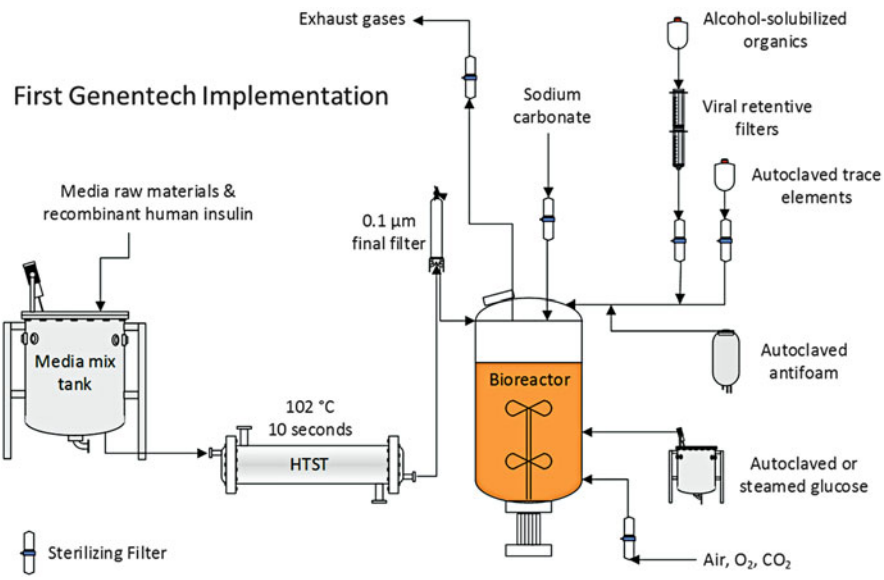


Fig. 1 Manufacturing-scale high-temperature, short-time (HTST) treatment schematic

profiles of the manufacturing-scale systems. In addition, pilot systems can provide flexible hold temperatures and residence times, thereby enabling options for development studies and providing data to set acceptable operating ranges for the implementation of HTST.

Bench-scale model systems have also been developed to study heat treatment of cell culture media and to predict media and HTST treatment compatibility. We have experience with bench-scale model systems using both an oil bath and a fluidized sand bath heat source. During the development of the bench-scale systems, the fluidized sand bath method was found to be the most practical for use in the laboratory and was used to identify conditions to minimize degradation of media components and/or precipitate formation after being subjected to high-temperature treatments [26]. The fluidized sand bath system seen in Fig. 2 is considered the worst case with respect to heating time and heat exposure of the cell culture media tested. This is because of a slower heating and cooling rate for the heat exchange relative to the true manufacturing-scale and pilot-scale HTST systems, resulting in a greater area under the heat exposure curve for the sand bath system. In practice, the worst-case heat exposure using the sand bath method provides a safety factor for scaleup and implementation when executing experimental designs with higher throughput (e.g., design of experiments (DOE) methodology) and/or generating compatibility assessments quickly.

As already discussed for heat treatment of cell culture media in general, the main risk when implementing HTST technology is the compatibility of the cell culture medium with the HTST treatment. The expected mechanisms for HTST and media incompatibility are thermal degradation of media components and/or thermally induced precipitation and loss of components from the soluble phase [26–28].

Although HTST by definition is a short operation, it entails a risk for some component degradation (e.g., degradation of potentially heat-sensitive components such as certain vitamins). HTST treatment of a Roche/Genentech proprietary cell culture media at the prescribed conditions was demonstrated to have no significant

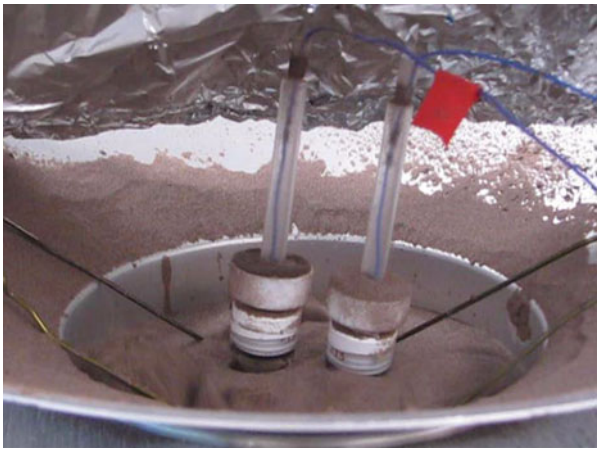


Fig. 2 Fluidized sand bath with test media in pressure vessels

effect on typical cell culture media amino acids, a majority of the vitamins, selection agents (e.g., methotrexate, methionine sulfoximine, puromycin), some growth factors, glucose, and salts, among others. When component losses were observed (e.g., ~10–20% loss of recombinant human insulin or ~10–20% loss of highly heat-labile vitamins), no negative effects were seen on the cell culture performance or product quality when using the treated media [3]. The level of loss relative to the component concentration may be negligible, but this risk should be assessed for a given medium and process. Under more extreme conditions of heat exposure in a sand bath model system, we have observed up to 60% loss of some heat-labile vitamins (e.g., vitamin B12) from related cell culture media formulations.

For the risk of thermally induced precipitation, the key factors for HTST treatment compatibility are temperature, pH, calcium concentration, phosphate concentration, and the presence of trace elements [28]. Exposure of the media to heat is a risk for media destabilization through the formation of compounds containing calcium and phosphate (hereafter referred to as calcium phosphate) and loss of trace elements (e.g., iron) likely associated with calcium phosphate and/or iron phosphate formation [27, 28]. The potential for calcium phosphate precipitation is an operational risk because the precipitate may foul the surfaces of the HTST flow path and disrupt temperature control. If this happens, the heat input to the heat exchanger will need to be increased because of reduced heat transfer efficiency, which leads to increased temperature at the walls of the heating tubes, which can further exacerbate the degree of precipitation. This behavior potentially affects the medium quality, the level of adventitious agent inactivation, and/or the desired processing throughput. In addition, calcium phosphate precipitates may negatively affect operations downstream of the HTST unit, such as fouling of downstream filters. Figure 3 shows an example of precipitation fouling HTST equipment surfaces following HTST treatment of a cell culture medium.

The precipitate shown in Fig. 3 that formed from HTST treatment of cell culture media at 102°C for 10 s were analyzed by energy-dispersive X-ray spectroscopy and determined to be compounds highly enriched with calcium and phosphate, such as whitlockite, a member of the hydroxyapatite ($Ca_5(PO_4)_3OH(s)$) family of

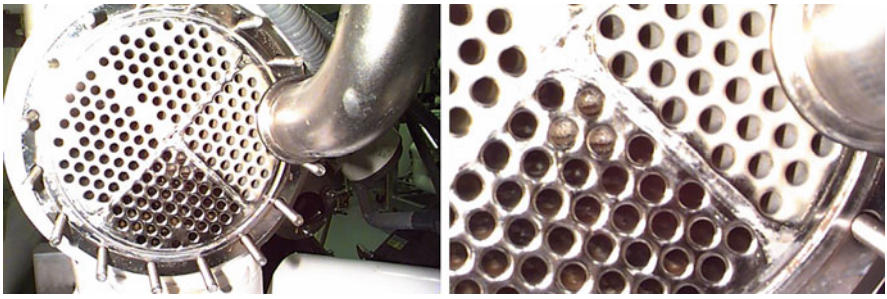


Fig. 3 Example of calcium phosphate precipitate from HTST unit following media treatment

compounds. Cell culture medium commonly contains calcium and phosphate because both components are important nutrients for cell culture. Calcium phosphate precipitate formation is known to be a function of pH and temperature [29]. In general, calcium phosphate solids dissolve under acidic conditions, whereas calcium phosphate solids with a pH above neutral are more likely to form and be stable [29]. This knowledge is important for at least two reasons: (1) the ability to clean (e.g., clean in place) the HTST skid between processes with an acidic cleaning solution (typically requires a high-temperature [>65°C] acid wash to be effective), and (2) the information suggests that pH can be used as a lever for processing media that may have a high risk for thermally induced calcium phosphate precipitation. For example, a medium could receive HTST treatment at a lower pH (without destabilizing the media as a result of the low pH) and then adjusted to the desired pH after HTST treatment [28]. In addition, knowing the components and mechanism involved in the thermally induced precipitation, one can also manipulate the calcium and phosphate in a given cell culture medium by removing one of the two components and adding it to the process outside of the main medium through an alternative adventitious agent barrier [26]. Finally, the potential for iron phosphates to form upon HTST treatment can be a concern for loss of trace-level components. Trace-level components in cell culture media tend to be biologically relevant metals such as iron, copper, and manganese. These components are present in trace amounts (approximately nanomoles to hundreds of micromoles, which are very low relative to typical calcium and phosphate concentrations in cell culture media), and their amounts may need to be accurately controlled, as some metals are closely linked to cell culture and product quality attributes (e.g., glycosylation [30], charge variants [31], drug substance color [32]). Among the media components that could be lost upon precipitation, the risk of losing trace metals upon heat treatment is potentially the highest risk that could negatively affect bioprocessing. Therefore, it is recommended to add trace elements to the process outside of the main medium through an alternative barrier, such as an autoclave heat treatment or virus-retentive filter [3].

Based on an understanding of the mechanism and the key parameters leading to precipitation formation when processing cell culture media using HTST treatment, cell culture media can be designed as HTST compatible when media formulations can be developed (e.g., platform media development and implementation). Our approach to HTST treatment–compatible media design leveraged the sand bath system to evaluate visual and turbidity changes as a function of the key input parameters: temperature, pH, calcium concentration, phosphate concentration, and trace metal concentrations. Note that magnesium concentration can also play a role depending on the amount of magnesium in the cell culture media relative to the calcium concentration. The sand bath system allowed us to examine the precipitation risk for various media formulations. The sand bath studies provided results that showed a strong correlation among the sand bath data and the pilot and manufacturing-scale HTST treatment data, thus supporting the use of the sand bath as a predictive tool. With confidence in the sand bath method established, we performed a full factorial statistically designed experiment (DOE) with center points including pH, calcium concentration, and phosphate concentration for

multivariate analysis of a proprietary cell culture media formulation; we generated a multidimensional response "surface" (design space model) to find optimal calcium and phosphate concentrations in order to enable HTST processing. Another goal of the effort was to find calcium and phosphate concentrations that enabled HTST compatibility at the desired pH (i.e., to alleviate the need to adjust pH after HTST treatment) without sacrificing cell culture process performance, product yield, and product quality. A visual example of the resulting multidimensional model for two of the parameters versus turbidity (precipitation) is provided in Fig. 4. In Fig. 4, when gridlines are visible, after heat treatment the medium was below the turbidity (and visual) limits set for predicting precipitation at the pilot and Good Manufacturing Practices–scale HTST treatment operations (HTST compatibility), whereas the solid green areas with no gridlines visible are regions of phosphate concentrations and media pH before heat treatment that led to precipitation (HTST incompatibility). In addition, Fig. 4 maps the response of a preexisting medium to the surface (red dot) and demonstrates some potential changes that could be implemented, such as lowering (1) the phosphate concentration, (2) the media pH, or (3) both the pH and phosphate concentration before HTST treatment (red arrows).

The model based on the sand bath data enabled the development of an HTST treatment–compatible, chemically-defined cell culture platform media. The model prediction was verified for the new platform media based on results with specific combinations of calcium and phosphate concentrations and pH in pilot-scale HTST treatment studies, and with the desired locked concentrations and pH for manufacturing-scale HTST treatment operations. This verification included cell

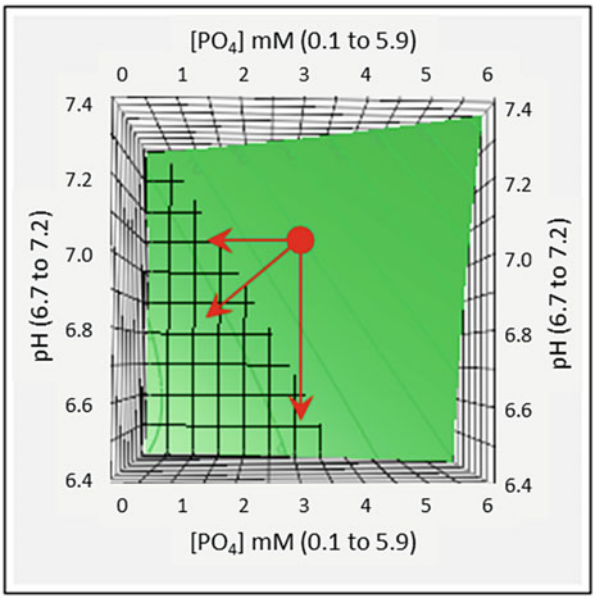


Fig. 4 Two-dimensional projection of phosphate concentration (millimoles per liter) and pH vs turbidity Nephelometric Turbidity Units (NTU) from a design space model for high-temperature, short-time (HTST) treatment compatibility based on data from the sand bath system

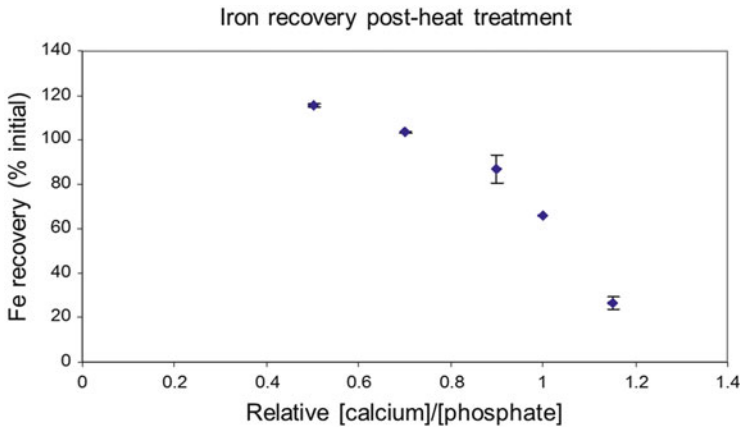


Fig. 5 Example of iron recovery as a function of calcium-to-phosphate concentration ratio in a medium formulation

culture testing and analysis of the cell culture performance, product titer, and product quality.

During the course of our chemically-defined media development studies, trace metal losses were observed upon sand bath heat treatment under some media conditions, in particular those conditions where precipitate comprising calcium phosphate was formed (e.g., higher concentrations of calcium and/or phosphate and/or higher pH). Of particular interest was the observation that conditions that did not lead to problematic calcium phosphate precipitation levels in the sand bath method, and the pilot and manufacturing-scale operations still showed some metal losses (specifically iron) upon HTST treatment. As a result of this key knowledge, it was recommended that trace elements (e.g., iron, manganese) be added separately to the process from an autoclaved, concentrated stock solution (or other viable adventitious agent barrier option, such as a virus filter) to maintain the desired trace element concentrations. Additional testing for elemental concentrations before and after heat treatment of various media formulations demonstrated (1) no significant difference in the potential iron loss due to heat treatment of iron-containing media when using ferric citrate or ferrous sulfate as the iron source, (2) pH dependence of iron recovery after heat treatment, and (3) calcium and phosphate concentration dependence of iron recovery after heat treatment (Fig. 5).

8 Lessons Learned and Conclusions

As a result of our experiences in developing and implementing additional virus barriers beyond 0.1-μm filtration for mammalian cell culture processes, the following key lessons learned were identified:

1. Upstream processing is vulnerable to a low-frequency but high-impact scenario of contamination by an undetected adventitious agent that could threaten the safety and supply of multiple products (at a multiproduct facility).
2. The absence of an adventitious agent cannot be proven by detection methods.
3. Raw materials for upstream processes (in particular cell culture media components) represent a significant vulnerability to the success of cell cultures, particularly when significant quantities are required for large-scale manufacturing.
4. Testing of raw materials alone is not likely an effective and robust barrier to viral contamination.
5. An understanding of the raw material supply chain can enable smart risk mitigation strategies.
6. Multiple options for adventitious agent barriers exist, and each has its advantages and disadvantages for specific applications.

Based on the risks, lessons learned, and the desire to take reasonable measures to ensure product supplies are available to patients when needed, point-of-use adventitious agent barriers are recommended as a preventative measure in upstream processes. In addition, performing an assessment is recommended when implementing any adventitious agent barrier in order to demonstrate no impact on existing processes upon implementation. There is value in understanding the adventitious agent barrier methodology and compatibility with a given application to enable smart implementation (i.e., by design). We advocate the use of HTST treatment in upstream applications as a broadly effective and economical adventitious agent barrier. Although capital expense for HTST implementation is not inconsequential, those costs are effectively depreciated over the lifetime of a typical biotechnology facility (likely >10 years), resulting in a minimal increase in overall manufacturing costs. Biotech manufacturers should carefully consider the value provided by this low-frequency and high-impact risk scenario as a form of process and product insurance. Based on technical studies such as those briefly summarized here, HTST compatibility can likely be designed or enabled for new or existing cell culture processes. To our knowledge, HTST treatment for cell culture media has been successfully implemented in at least 10 manufacturing sites across the industry and evaluated by at least 4 other major biologics manufacturing companies.

Acknowledgments We acknowledge many individuals too numerous to specifically name who, over the course of many years since the initial decision to develop and implement additional virus barriers, have contributed significant time and effort to ensuring success. It has truly taken a village.

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Manufacturing of Proteins and Antibodies: Chapter Downstream Processing Technologies

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Abstract Cell harvesting is the separation or retention of cells and cellular debris from the supernatant containing the target molecule Selection of harvest method strongly depends on the type of cells, mode of bioreactor operation, process scale, and characteristics of the product and cell culture fluid. Most traditional harvesting methods use some form of filtration, centrifugation, or a combination of both for cell separation and/or retention. Filtration methods include normal flow depth filtration and tangential flow microfiltration. The ability to scale down predictably the selected harvest method helps to ensure successful production and is critical for conducting small-scale characterization studies for confirming parameter targets and ranges. In this chapter we describe centrifugation and depth filtration harvesting methods, share strategies for harvest optimization, present recent developments in centrifugation scale-down models, and review alternative harvesting technologies.

Keywords Capillary shear, Centrifugation, Continuous centrifugation, Depth filter, Disk-stack centrifuge, Filter capacity, Harvest, Mammalian cell, Primary recovery, Scale-down, Sigma factor, Single-use centrifuge, Tangential flow filtration

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Contents

1 Introduction

Cell harvesting is the separation or retention of cells and cellular debris from the supernatant containing the target molecule. Selection of the harvest method greatly depends on the type of cells, mode of bioreactor operation, process scale and characteristics of the product and cell culture fluid. Whether the bioreactor is operated in continuous (perfusion), batch or fed-batch mode, the optimal harvest method preserves cell viability to avoid release of intracellular enzymes and impurities that could negatively impact product quality and complicate the purification process. Harvest techniques used for perfusion cell culture must also be designed to maintain sterility of the bioreactor throughout the duration of the perfusion operation which may be as long as several weeks. Most traditional harvest methods use some form of filtration, centrifugation or a combination of both for cell separation and/or retention. Filtration methods include normal flow depth filtration and tangential flow microfiltration and achieve separation based on particle size differences. Centrifugation methods achieve primary separation based on density differences and utilize depth and sterilizing-grade filtration for removal of small debris from the centrate. Flocculation has also been used to improve separation via filtration or centrifugation by increasing the particle size of the solids to be removed. Centrifugation and depth filtration harvest methods have become the harvest methods currently preferred over tangential flow filtration (TFF). More information on TFF harvest methods can be found in Van Reis and Zydney [25], Kompala and Ozturk [16] and Voisard et al. [26]. In this chapter we describe centrifugation and depth filtration harvest methods, share strategies for harvest optimization, present recent developments in centrifugation scale-down models and review alternative harvest technologies.

2 Centrifugation

Continuous centrifugation followed by depth and sterilizing-grade filtration is widely used for the recovery of biological products from cell culture fluid [2, 11, 20]. Efficient separation, continuous discharge mechanisms and lower-shear designs have led to a preference of disk-stack centrifuges over other centrifuge types for most biopharmaceutical harvest operations. Because disk-stack centrifuges are capable of separating particles as small as 0.5 μm, depth and sterilizing-grade filters are typically used downstream of the centrifuge to capture the smaller debris. Given that disk-stack centrifuges require significant capital investment and are used in multi-product facilities, it is important to evaluate the design options available to adjust harvest conditions from product to product. Although mammalian cells are fairly easy to separate using a disk-stack centrifuge, they may also be susceptible to lysis when exposed to the high shear rates within the separator. Cell lysis during harvest can generate additional quantities of host cell proteins (HCP) and host cell DNA which must be removed in downstream process steps. Ideally, cell lysis during harvest should be minimized or avoided entirely. Operational conditions and centrifuge design options can be selected to reduce the levels of lysis. Minimizing cell lysis may also be important for antibody products that are susceptible to aggregation and reduction caused by release of additional enzymes during harvest. Measurement of antibody monomer, aggregate and fragment levels may also be included in centrifuge harvest optimization studies.

2.1 Centrifuge Separation Theory

Centrifugation takes advantage of density differences between cellular solid particles and cell culture fluid to achieve separation. Particles that differ in density settle at different rates in response to an applied gravitational force. When the gravitational force (g) is replaced by centrifugal force ($\omega^2 r$), the settling velocity of the particle is significantly increased, enabling efficient separation of very small cells (<20 μm) and cellular debris (<5 μm) from cell culture fluid. Assuming laminar flow and approximating cellular particles as spheres enables application of Stoke's law which defines particle settling velocity as

$$V_t = \frac{\omega^2 r\left(\rho_p - \rho_l\right) d^2}{18\mu} \tag{1}$$

where V_t = settling velocity, ω^2 = angular velocity, r is the distance of the axis of rotation, ρ_p is the density of the particle, ρ_l is the density of the liquid and μ is the viscosity of the liquid.

The particle settling velocity is related to the flow rate and settling area of a centrifuge using

$$V_t = \frac{Q}{\Sigma} \tag{2}$$

where Σ is the sigma factor and Q is feed flow rate.

The sigma factor is a relationship Ambler derived for various types of centrifuges [1]. For a disk-stack centrifuge, Ambler defined Σ as

$$\Sigma = \frac{2\pi N\omega^2}{3g} \cot\alpha\left({r_2}^3 - {r_1}^3\right) \tag{3}$$

where N = number of disks, ω^2 = angular velocity, r_2 = maximum disk radius, r_1 = minimum disk radius, cot = cotangent and α = half cone angle of the disk.

With these relationships, the development scientist can experimentally determine the settling velocity of the cellular solid particles using bench-scale centrifuges. As sigma factors are known for each centrifuge, the scientist can solve for feed flow rate in (2). In theory, operation of the centrifuge at the determined flow rate should yield a centrate with similar clarity as that found in the bench-scale centrifuge experiment. However, in reality, the centrifuge system is more complicated and additional experimentation is needed to predict fully the separation performance. Prediction of performance using the sigma factor relationship is more reliable when comparing Q/Σ from similarly designed centrifuges. For example, a pilot-scale disk-stack centrifuge may be used to predict the flow rate range needed to achieve the same separation as a production-scale disk-stack centrifuge via

$$\left(\frac{Q}{\Sigma}\right)_{\text{pilot}} = \left(\frac{Q}{\Sigma}\right)_{\text{production}} \tag{4}$$

Theoretically, the sigma factor can also be utilized to compare centrifuges of different types: see (5). The correction factor (c) accounts for the deviation from the assumptions of Stoke's law. Lower values of c indicate a larger deviation from Stoke's law. Different centrifuges have different c values. Tubular bowl, multichamber and disk-stack centrifuges have been quoted to have c values of 0.9, 0.8 and 0.4, respectively, and the c value for the laboratory-scale centrifuge is 1 [18]. Equation (5) describes the performance of a disk-stack centrifuge based on the equivalent settling area of a laboratory-scale centrifuge (Σ_{lab}):

$$\frac{Q_{\text{ds}}}{c_{\text{ds}}\Sigma_{\text{ds}}} = \frac{V_{\text{lab}}}{t_{\text{lab}}c_{\text{lab}}\Sigma_{\text{lab}}} \tag{5}$$

where Q_{ds} = volumetric flow rate into the disk-stack centrifuge, V_{lab} = volume of material used in the laboratory centrifuge, t_{lab} = centrifugation time and c_{ds} and c_{lab} = correction factors that account for the non-ideal flow properties in the disk-stack and laboratory-scale centrifuges, respectively.

Even when using a scale-down version of the production centrifuge or laboratory-scale centrifuges, scientists often need to apply a safety factor to account for the effects of differing particle size distributions, shear-induced cell lysis, higher discharge pressures and variable solids volumes from batch to batch. Finding an appropriate scale-down model to predict accurately separation performance for a centrifugation harvest has been an active field of study which is addressed in this chapter.

2.2 Centrifuge Harvest Parameters

In the absence of a reliable scale-down model system, development of a centrifuge harvest step usually requires multiple experiments with a pilot-scale centrifuge and multiple representative feedstocks. Bench-scale centrifuges are generally unreliable for predicting separation performance in a continuous disk-stack centrifuge because they do not incorporate any shear effects that affect cell lysis (and particle diameters). Using a small-scale disk-stack centrifuge, experiments are conducted by adjusting operating parameters until the desired clarity and filtration capacity are achieved. The key operational parameters to be established for an intermittent discharge disk-stack centrifuge are discharge frequency, bowl speed and feed flow rate. Measured outputs include centrate turbidity, cell lysis, centrate particle size distribution and downstream filter capacity. Product quality measurements for host cell protein, host cell DNA and product aggregates may also be included in harvest experiments.

Discharge frequency is based on the packed cell volume (PCV) of the cell culture fluid, the solids holding space within the centrifuge and the optimized feed flow rate. Discharge frequency can be varied for each harvest batch if PCV measurements are known, or can be set at a conservative limit based on experimental data. If PCVs are too high (conservatively above 15%), then dilution of the feedstock may be required to achieve acceptable centrate clarity and to avoid discharging the bowl too often. Typically, bowl discharges should be spaced at least 1–2 min apart to allow the bowl to return to full operating speed after each discharge.

The two key harvest system parameters which can be optimized to handle various feedstocks are bowl speed and residence time. Separation efficiency and cell lysis can be impacted by both bowl speed and residence time within the disk stack. Residence time of the cell culture fluid is increased by operating at lower feed flow rates, either via reduction of the feed pump speed or by lowering of the pressure on the bioreactor. In general, lower feed flow rates lead to better separation of cell debris, but at the cost of longer processing times. The optimum feed flow rate is often established by the optimum flux through downstream depth and sterilizing-grade filters. The second key parameter to evaluate is the centrifuge bowl speed. Inclusion of a variable frequency drive for the centrifuge motor enables operation at bowl speeds of 40–100% of maximum speed. Operation at lower rotational speeds

may be useful for cultures containing cells that are both relatively easy to settle and more sensitive to breakage caused by shear. Depending on the inlet configuration, the required centrate back pressure and shear forces created in the inlet zone generally decrease with decreasing bowl speed. Lower shear forces may generate lower levels of cell lysis. However, operating at higher bowl speeds provides the greatest separation capacity for cell cultures that are less impacted by potential shear-induced cell lysis. Additionally, higher feed flow rates are achieved at the highest bowl speed, reducing overall process time.

Harvest characterization studies typically evaluate centrate turbidity and filter capacity at a range of centrifuge bowl speeds and flow rates. Process purity measurements for HCP, DNA and monomer levels may also be included to demonstrate that the centrifuge harvest conditions do not impact product quality or produce HCP and DNA levels that are challenging to downstream chromatography steps. For example, Fig. 1 shows results from a harvest characterization study conducted at pilot-scale with a mammalian cell culture product. The range of bowl speed and flow rates were determined based on previous studies. The data show that

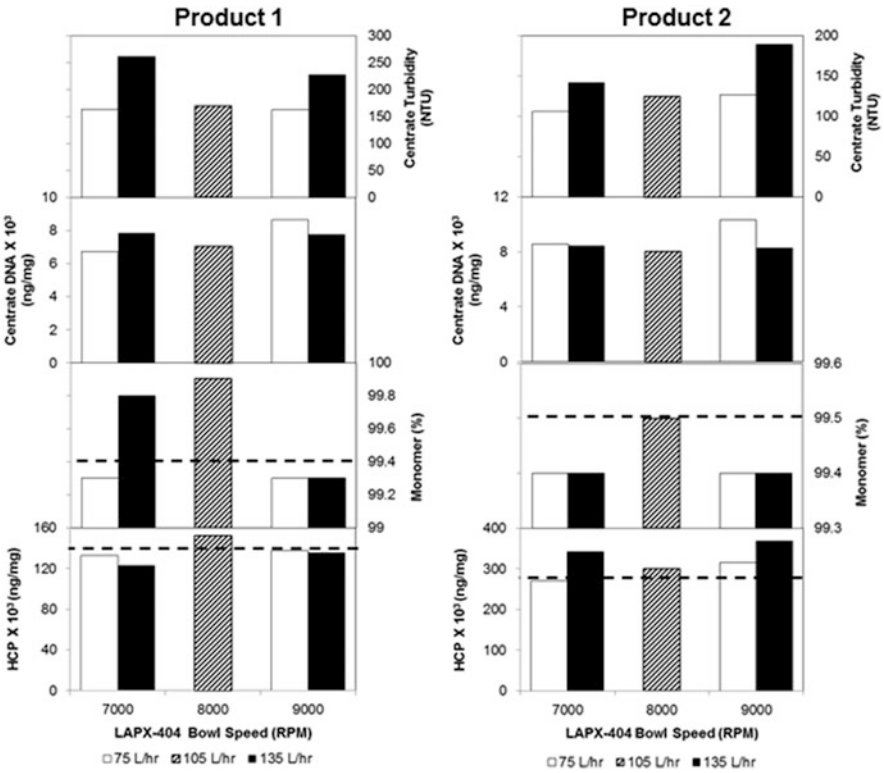

Fig. 1 Study identifying centrate quality attributes for two mammalian cell culture processes (Products 1 and 2) during harvest characterization. The *dotted line* represents the monomer (%) and HCP concentrations (ng/mg) observed for cell culture material pre-harvest

bowl speeds of 7,000–9,000 rpm and flow rates of 75–135 L/h generate centrates of comparable quality and purity. Depth and sterilizing-grade filter turbidities and DNA levels were also comparable across these centrifuge conditions. The depth and sterilizing-grade filter capacities were all within the target capacity (data not shown).

2.3 Low Shear Designs

Changes in centrifuge designs in response to early studies showing the negative impact of high shear on mammalian cells during harvest have significantly reduced shear to acceptable levels with no loss in separation efficiency [23]. Centrifuge inlets can be designed as open or hermetic, referring to the inclusion of air within the feed zone. It has been shown that air–liquid interfaces in contact with the high shear regions created by the rotating bowl can damage mammalian cells. However, hermetic and hydro-hermetic inlet designs and re-designed feed inlets in non-hermetic centrifuges are now able to provide an air-free zone for acceleration of the feed and a larger volume for dissipation of the kinetic energy that would otherwise be translated into shear. For example, Fig. 2 shows the Culturefuge 400 (Alfa Laval, Inc., Lund, Sweden) which contains a hermetically-designed

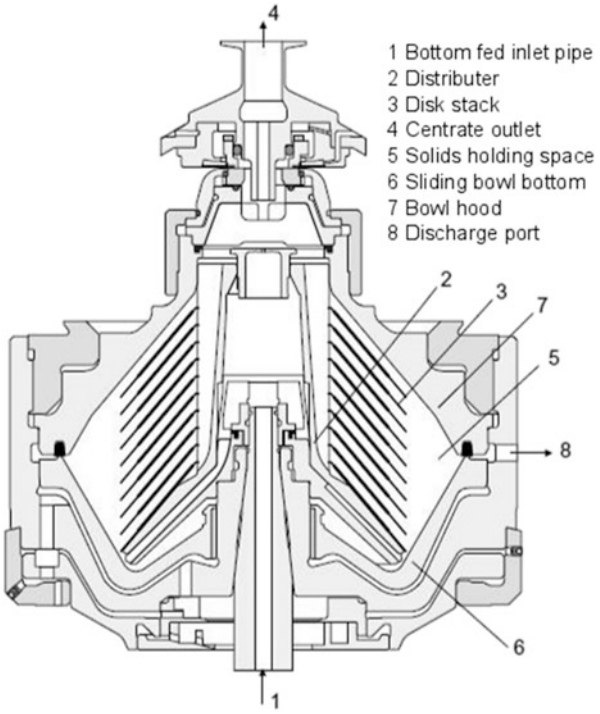

Fig. 2 Schematic of the Culturefuge bottom-fed hermetic centrifuge (Courtesy of Alfa Laval Inc., Lund, Sweden)

hollow spindle to accelerate the cells gently, effectively reducing the overall shear imposed on the cells. The hollow spindle design eliminates the air–liquid interface which has been thought to produce foam and lyse cells. Another key benefit of a hermetic centrifuge is the ability to deliver cell culture fluid by pressurizing the bioreactor and to control the feed flow rate at the centrate outlet. This simplifies the inlet flow path and overall system complexity by removing the need for a feed pump and associated controllers. Removal of the feed pump, flow control valves and inlet piping leads to fewer opportunities for the cells to be exposed to shear created by flow through reduced piping segments such as valve diaphragms and pump rotors.

2.4 Stainless Steel Centrifuges

Stainless steel centrifuges are available in a variety of sizes and styles, ranging from 1,000 m^2 to >300,000 m^2. Because of their strength of materials, they are capable of achieving g-forces of 1,000–15,000 g, providing powerful separation of cells and cellular debris. As with most stainless steel unit operations, centrifuges require a significant capital investment, supporting utilities and a trained staff for operation and preventive maintenance. Stainless steel centrifuge manufactures offer many features tailored to meet biotechnology requirements such as clean-in-place and steam-in-place operation, highly polished stainless steel finishes, recipe-based control systems with data acquisition, high quality Class VI tested elastomers and comprehensive validation packages. Cleaning protocols for stainless steel centrifuges typically use similar cleaning solutions as used for bioreactors. Generally, combinations of basic and acidic solutions are sufficient to remove residual cell culture material completely. Stainless steel centrifuges currently have a large installed base in both pilot-scale and commercial-scale manufacturing facilities.

As cell culture productivity continues to increase, many biotechnology manufacturers are able to meet the projected commercial demand for therapeutic medicine with bioreactor volumes closer to 2,000 L rather than 10,000 L. This downwards trend in bioreactor volume has triggered a greater emphasis on disposable, or single-use, technologies. Single-use bioreactors are now available at volumes of 200–2,000 L. The gaining popularity of creating an entire manufacturing facility in which every operation is single-use has led to a renewed focus on harvest via depth filtration and on the introduction of single-use centrifuges.

2.5 Single Use Centrifuges

Currently, two single-use centrifuge technologies have been implemented for cell harvest applications. The CARR UniFuge® (Pneumatic Scale Angelus, Stow, OH), shown in Fig. 3, is a tubular bowl centrifuge with a disposable insert and disposable flow path. The cell culture fluid is pumped into the rotating tubular bowl where cells

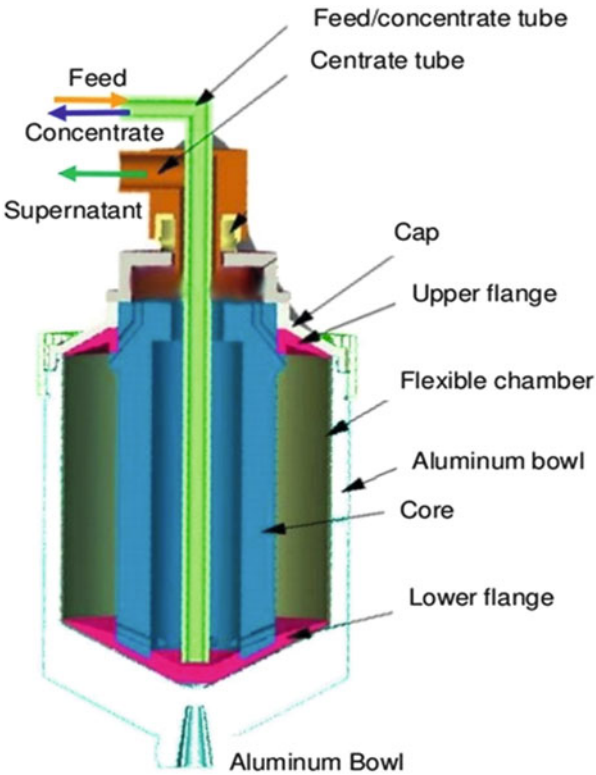

Fig. 3 The CARR UniFuge single-use centrifuge (Courtesy of Pneumatic Scale Angelus, Stow, OH)

and cellular debris are separated at low centrifugal force along the periphery of the bowl. The clarified liquid is continuously discharged. Once the solids space is filled the feed is stopped and the bowl slows down to discharge the solids. Because the solids are gently removed from the bowl under low pressure, cell viability is maintained and cell lysis during harvest is minimized. Because of the low shear solids discharge, the UniFuge® can be used to recover cells as well as clarified supernatant. This system is ideally sized for smaller production bioreactors given its flow rate range of 0.1–4 L/min and solids holding capacity of 1.5 L. As with stainless steel disk-stack centrifuges, depth and sterilizing-grade filters can be used downstream of the UniFuge® to clarify the centrate further.

A second single-use centrifuge option is the kSep system (kSep, Systems, Morrisville, NC), shown in Fig. 4. The kSep system uses a rotor containing multiple conical chambers [17]. Cell culture fluid is pumped into the chamber where two forces act simultaneously on the solids. Centrifugal force, created by rotation, drives the solids from the base to the top of the conical chamber. Additionally, the velocity of a second fluid entering at the top of the chamber drives the solids from the top to the base of the chamber. These forces balance to create a fluidized bed of solids that remain in suspension and the clarified liquid is pumped out of the

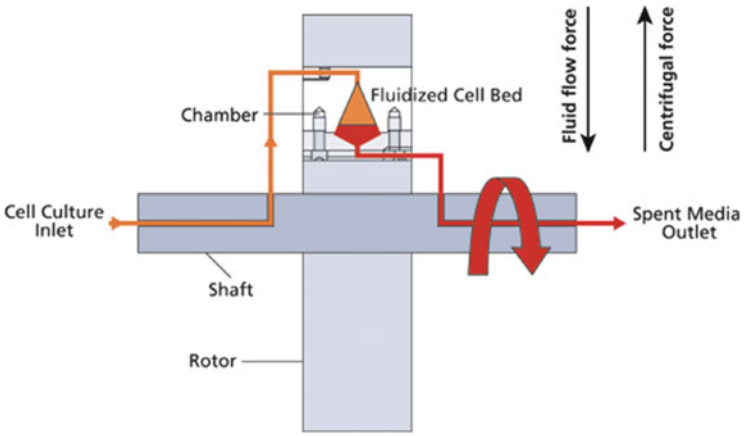

Fig. 4 The kSep second single-use centrifuge (Courtesy of kSep Systems, Morrisville, NC)

chamber. Once the solids space is filled, the solids are pumped out of the chamber. Because this system features cell retention with washing capabilities, high product recoveries are possible. Additionally, the low-shear separation of cells also enables this system to be used for recovery of cells with minimal loss in viability. Ko and Bhatia [15] used the kSep400 system to harvest CHO cells at high (>90%) and low (<50%) viabilities and demonstrated clarification efficiencies of 88–93% with no increase in cell lysis as measured by lactate dehydrogenase (LDH) levels in the centrate. Also reported was the significant reduction in turbidity from the cell culture feed (33–64 NTU) to the centrate (2.5–6.9 NTU), illustrating effective separation of cells and cellular debris.

The UniFuge® and kSep systems offer single-use alternatives to depth filtration for cell harvest operations. As with other single-use unit operations, the key advantages of single-use centrifuges include reduction in the following: equipment lead time, installation time, cleaning validation, water and buffer usage, utility requirements, maintenance and equipment turn-around time. Disadvantages of the currently available single-use centrifuges are the relatively low g-forces and the smaller range of harvest volumes that can be processed. However, for lower volume, low-shear applications, and for recovery of intact cells, these units may provide advantages over stainless steel disk-stack centrifuges.

2.6 Centrifuge Scaling

Achieving process characterization of production-scale centrifuges through the use of pilot-scale machines is a material-intensive and time-consuming process. Centrifuge characterization studies often only encompass a narrow design space and hence the quality of information generated from these studies is limited. By

contrast, high throughput scale-down techniques now enable the rapid generation of extensive experimental data representative of production-scale performance, both in the upstream and downstream manufacturing process. Such large experimental data sets generated through these techniques allow for better identification of the effects and interactions of the input parameters on the process performance and product quality [22].

Typically, Sigma theory is used to scale centrifuges irrespective of size, geometry and type [1]. However, Sigma theory does not take into account the generation of small particles through cell damage in the high-shear regions of centrifuge feed zones. To capture these effects accurately at the laboratory scale, the shear generated in the feed zone needs to be mimicked [3]. A Rotating Shear Device (RSD) has been developed to reproduce the prevailing shear conditions in such feed zones [3]. Operated in combination with a bench-top centrifuge, this has been shown to predict successfully the clarification efficiency of a pilot-scale disk-stack centrifuge for the processing of mammalian cell cultures [10]. The RSD has also been used in conjunction with microwell plates using sub-milliliter volumes of cell culture to model successfully pilot-scale centrifugation performance [21]. Several publications have shown the utility of the RSD to assess the impact of exposure to various levels of shear as might be experienced in the centrifugal step at pilot to production scales [3, 10, 21]. The Capillary Shear Device (CSD) has also been shown to be a preparative device with the ability to mimic the levels of shear present in disk-stack centrifuge feed zones. Flow through the capillary enables the generation of Energy Dissipation Rates (EDRs) equivalent to those found in disk-stack centrifuges [27]. Furthermore, this methodology has shown that it can generate centrates with a particle size distribution equivalent to that from a pilot-scale centrifuge [27].

Figure 5 shows a case study where a correlation was developed connecting the levels of an enzyme released during cell rupture, lactate dehydrogenase (LDH) to a range of EDRs (1.20×10^5–10.7×10^5 W/kg), when operating the CSD to quantify the levels of LDH released in the Westfalia SO1-06-107. Regression analysis of the dataset showed that LDH release from the centrifuge was equivalent to an EDR of 2.40×10^5 (W/kg) created operating the CSD. The values found in this case study closely match earlier published data from Boychyn et al. [4] where CFD analysis of a non-hydrohermetic Westfalia CSA-1 (Westfalia AG, Oelde, Germany) reported an EDR of 2.0×10^5 (W/kg).

Shear devices such as the CSD that mimic the shear stresses in the disk-stack centrifuge feed zone can be utilized in conjunction with a bench-top centrifuge to generate a set of $Q/c\Sigma$ conditions in the Alfa Laval LAPX-404 and BTPX-305 (Alfa Laval, Lund, Sweden) machines using as little as 20 mL of cell culture material. The solids remaining [12] and particle size distribution of centrates generated using the CSD along with the bench top centrifuge closely matched those generated by the pilot-scale centrifuges (Figs. 6 and 7). In a typical mAb harvest process the unit operation subsequent to centrifugation is depth filtration. Any accurate centrate mimic would have to show filtration properties equivalent to those of the centrate obtained at pilot-scale. Figure 8 shows the pressure and turbidity profiles generated during the operation of the X0HC depth filter (EMD Millipore, Billerica, MA)

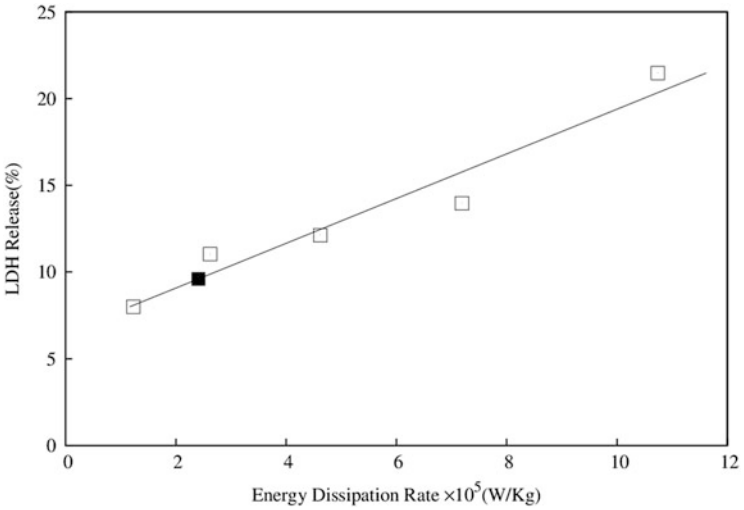

Fig. 5 LDH release for the modelling datasets (*white squares*) generated by shearing cell culture using the preparatory CSD to identify the levels of shear generated in the SO1-06-107 centrate (*black square*). Regression analysis (*line*) was used to determine the relationship between LDH release and energy dissipation rates

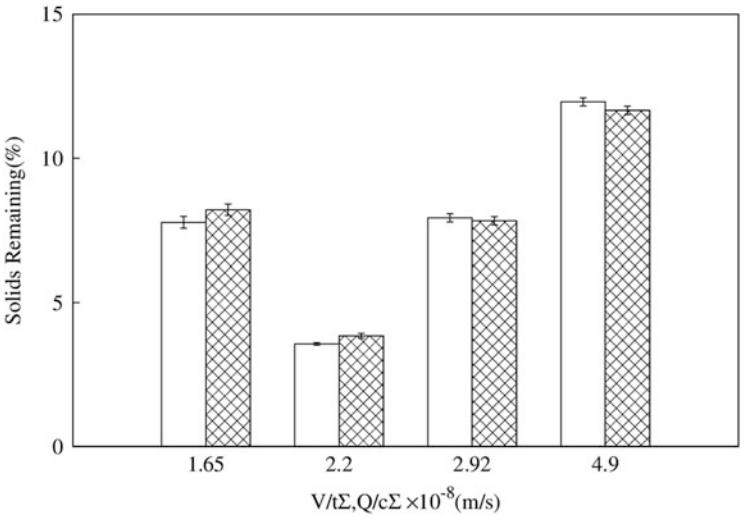

Fig. 6 Comparison of solids remaining in centrate generated using the preparative CSD (*hatched rectangles*) mimicking LAPX-404 and BTPX-305 at a range of $Q/c\Sigma$ conditions and solids remaining from pilot-scale centrifuge runs (*white rectangles*). The values plotted are shown as mean $\pm$ SD ($n = 3$)

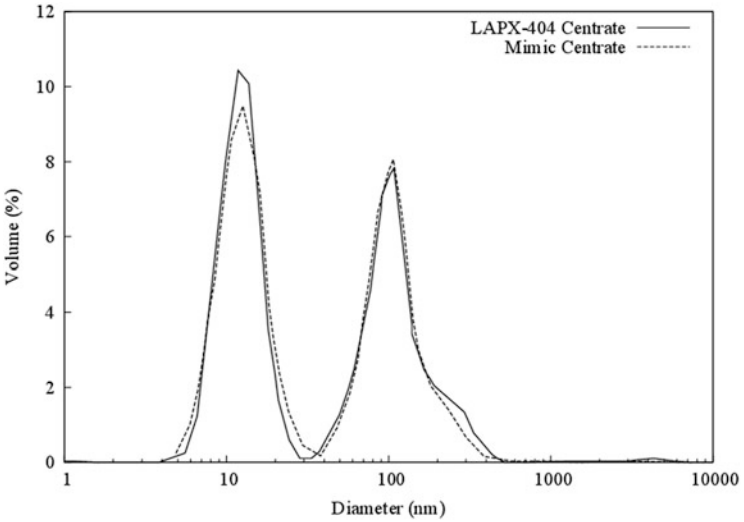

Fig. 7 Comparison of particle size distributions between centrate from the pilot-scale LAPX-404 operated at 7,900 g and its respective scale-down mimic generated through the use of the CSD and lab-scale centrifuge

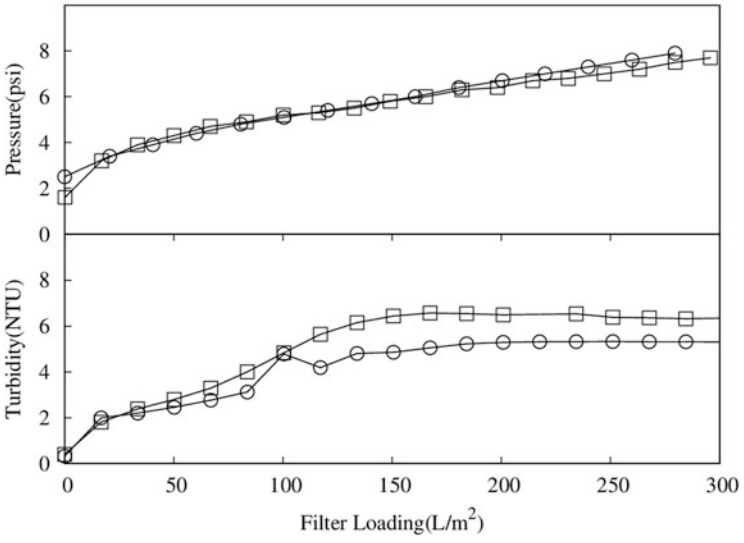

Fig. 8 Comparison of pressure and turbidity profiles for 0.1–2.0-μm X0HC depth filters when filtering centrate from the BTPX-305 machine (*circles*) and the mimic centrate (*squares*) generated applying the preparative CSD methodology. BTPX-305 centrate for this study was generated at 12,500 g. Preparatory CSD centrate was processed to mimic the large-scale centrifuge. The materials for this experiment were filtered at 200 LMH

when challenged with two feed streams: the BTPX-305 centrate and the centrate from the preparative CSD approach. The CSD centrate showed pressure and turbidity profiles that closely matched those of the BTPX-305 [12]. This indicated that the CSD centrate had filtration properties similar to those of the BTPX-305 centrate, confirming the utility of scale-down methodologies to provide a prediction of pilot-scale equipment. This adds to the toolbox of available scale-down models enabling high throughput characterization of harvest operations.

3 Depth Filtration

Prior to chromatography, the feed stream must be sufficiently clarified to enable it to be filtered through a sterilizing-grade filter. Sterilizing-grade membranes exist in a number of configurations and materials with pore sizes ranging from approximately 0.1 to 1.0 μm and can be used to reduce effectively bioburden and prevent fouling of subsequent purification steps. Before economic and practical application of this filtration can occur, centrifugation and/or a series of depth filters are required to reduce particle numbers to a sufficient level. Depth filters are an extremely useful filter type, comprised of a complex porous matrix of materials that enable cellular debris and other contaminants to be retained at both the surface and the internal layers of the medium [6, 7, 28].

Depth filters typically contain multiple, graded layers of cellulose or polypropylene fibres. In addition, this class of filters often contains charged components and other filter aids such as diatomaceous earth [14]. Currently there are a large selection of depth filters of varying configuration and composition. Each of the main vendors of depth filters provides a number of potential options and sizes (Table 1). The basic constituents of depth filters typically used in clarification are similar; however, their performance can vary significantly and often it is only possible to establish the optimal filter type and sequence through rigorous and empirical evaluation.

The use of fibrous filter mats configured and packed alongside inorganic filter aids such as diatomaceous earth (DE) is an industry standard approach for clarification of cell culture supernatants. Other industries that use depth filters, such as the blood fractionation industry, add the DE filter aid directly to the feed stream before filtration [24]. The principle behind this approach is, rather than accumulate cell

Table 1 Examples of depth filters used in clarification of mammalian cell culture fluid and centrates

Vendor	Example filter	Description
3M	Zeta plus SP	Composed of inorganic filter aid and cellulose fibres
Millipore	Millistak D0 HC	Composed of inorganic filter aid and cellulose fibres
Pall	Supradisc HP PD	Composed of inorganic filter aid, perlite and cellulose fibres
Sartorius	Sartoclear P	Composed of inorganic filter aid, perlite and cellulose

debris at the surface of the filter media, the dynamic body feed principle allows a more homogeneous cell debris and filter aid accumulation prior to the more conventional filtration. This approach can potentially enable higher flux and more economic filter capacity (Fig. 9). This approach is very attractive, especially for high cell density culture supernatants where centrifugation is not an option because of cost or equipment availability.

In routine process-scale operation the typical depth filter is provided in the form of either a disposable capsule or cartridge. Filter holders and housings are provided by the filter manufacturer to contain the filter setup (Fig. 10). The use of capsule filters is becoming increasingly popular in manufacturing as they enable set up and use of the system without the requirement for cleaning or cleaning validation. Once used, the filter assembly is dismantled and both filters – and often the other wetted parts of the filter assembly – are discarded, thereby minimizing additional validation.

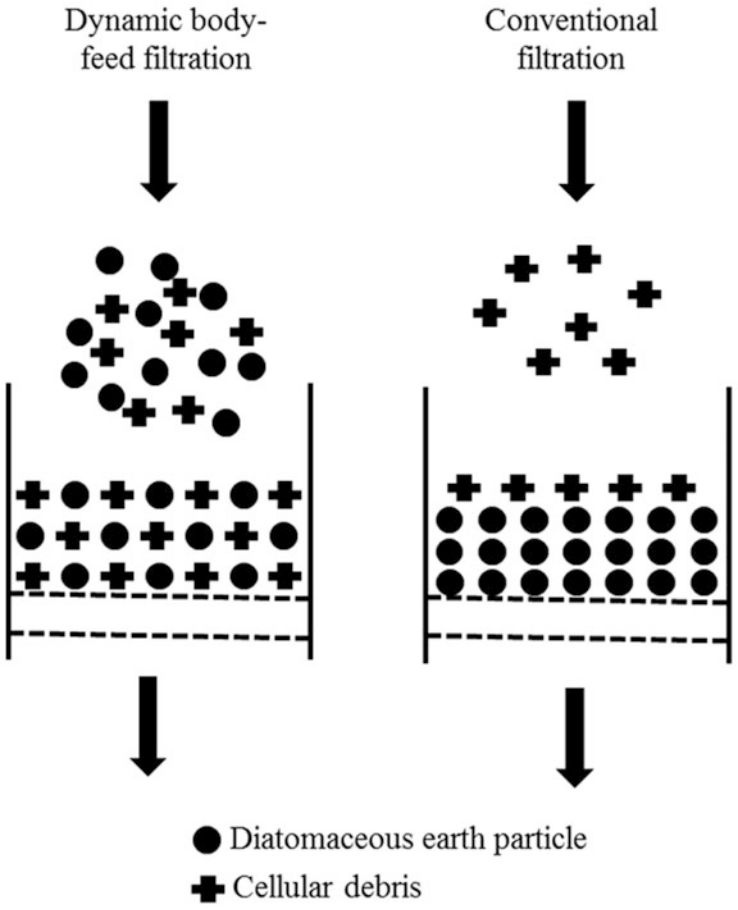

Fig. 9 The basic principle of conventional and dynamic body-feed filtration

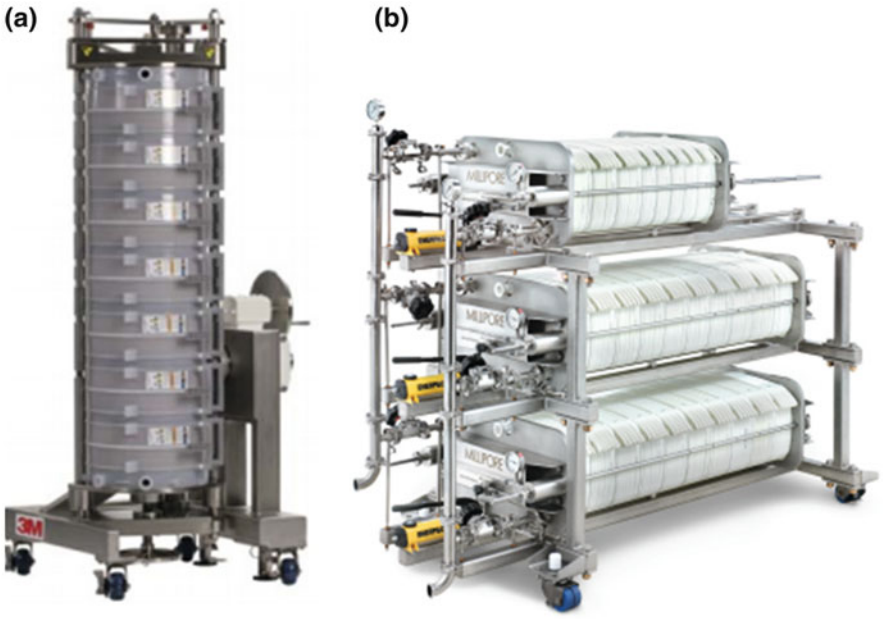

Fig. 10 Example of depth filter and holders. (a) Zeta Plus encapsulated system, 3M. (b) Millistak POD system, EMD Millipore, Billerica, MA

4 Alternative Harvest Technologies

Centrifugation and depth filtration technologies, separately or in combination, have been shown to be very effective at removing cells and cellular debris across a wide range of feedstock conditions. However, when these techniques are not capable of providing the desired clarity or when the filtration areas increase beyond what is practical for manufacturing, the development scientist must look to alternative technologies or other ways to enhance the harvest performance. Alternative technologies reported for harvest of mammalian cells include precipitation/flocculation, acoustic settling and body fed filtration.

Precipitation and flocculation are techniques aimed at decreasing the solubility and/or increasing the particle diameter of the solids to enable a more efficient separation. Precipitation of the target product is typically not used for harvest applications. However, precipitation during harvest may significantly reduce the levels of host cell protein and DNA that have to be purified downstream. For example, Glyn evaluated precipitation agents such as ammonium sulphate, cationic detergents and caprylic acid for their ability to reduce HCP and DNA levels prior to downstream chromatography steps [8]. The study at pH 6 with 500 mM caprylic acid showed the best results compared to ammonium sulphate and cationic detergents. Precipitation step yield was 92%, with 6,475-fold reduction in DNA and 650-fold reduction in HCP. As a primary harvest step, precipitation of cells and

cellular debris has not been successfully demonstrated as a robust harvesting method. However, when optimized, precipitation as part of the centrifugation or filtration harvest could reduce the overall harvest time and downstream filtration area and provide some purification advantages.

Flocculation is the addition of a component that causes cells and cellular debris to form larger particles which are easier to separate via centrifugation or filtration. Flocculants reported for use in harvest operations include polymers such as polyethylene amine and dextrans, filter aid components such as diatomaceous earth, chitosan and calcium phosphate [9, 13, 19]. For example, Kang et al. [13] studied a novel stimulus-responsive polymer which was defined as a "partially benzylated poly(allylamine)" for flocculation of mammalian cells prior to separation by depth filtration (Jaber et al. 2011). The polymer is designed to transition from a soluble to an insoluble form when in the presence of multivalent anions. The insolubility of the polymer is probably results from aggregation of the polymer chains because of strong interactions between its amine group and the multivalent anions. This unique transition promotes binding of DNA and HCP as well as removal of cellular debris. In this study, under flocculation conditions of 10–40 mM polymer at pH 6–7, harvest yields were greater than 90% and downstream depth filter capacity increased from 70 to >250 L/m^2. Following purification by Protein A chromatography, Kang et al. [13] demonstrated that this flocculation step removed residual HCP and DNA to levels consistent with those required for drug substances. Additionally, the study showed that the residual polymer was cleared to less than 0.1 ppm after purification by Protein A chromatography.

5 Perfusion Harvest

Although fed-batch processes are still the most widely used production systems for mammalian cell cultures, perfusion is used commercially for several products. Recent requirements to improve process performance and productivity and to cope with more labile products has pushed the advancement in cell separation technologies, during fermentation, to enable economical and routine use of perfusion bioreactors [5]. Perfusion processes are those where the cells are retained in the bioreactor and are continuously or semi-continuously fed with fresh media. The spent media, containing the protein of interest, are collected and further processed and purified either in batch mode or continuously. Several methods for cell separation in perfusion are available based on different physical principles. Filtration by cross-flow filter, for example, hollow fibre filter, vortex-flow filter, spin-filter and perfusion floating filter can be performed. Cell separation by acceleration-based settlers such as inclined settlers, acoustic settlers and centrifuge is also carried out. Because perfusion requires continual cell removal over a period of several days to weeks, the selected harvest method must be able to ensure sterility throughout this time. Further details on cell retention techniques for perfusion can be found in the review by Voisard et al. [26].

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Downstream Processing Technologies/ Capturing and Final Purification

Opportunities for Innovation, Change, and Improvement. A Review of Downstream Processing Developments in Protein Purification

Nripen Singh and Sibylle Herzer

Abstract Increased pressure on upstream processes to maximize productivity has been crowned with great success, although at the cost of shifting the bottleneck to purification. As drivers were economical, focus is on now on debottlenecking downstream processes as the main drivers of high manufacturing cost. Devising a holistically efficient and economical process remains a key challenge. Traditional and emerging protein purification strategies with particular emphasis on methodologies implemented for the production of recombinant proteins of biopharmaceutical importance are reviewed. The breadth of innovation is addressed, as well as the challenges the industry faces today, with an eye to remaining impartial, fair, and balanced. In addition, the scope encompasses both chromatographic and non-chromatographic separations directed at the purification of proteins, with a strong emphasis on antibodies. Complete solutions such as integrated USP/DSP strategies (i.e., continuous processing) are discussed as well as gains in data quantity and quality arising from automation and high-throughput screening (HTS). Best practices and advantages through design of experiments (DOE) to access a complex design space such as multi-modal chromatography are reviewed with an outlook on potential future trends. A discussion of single-use technology,

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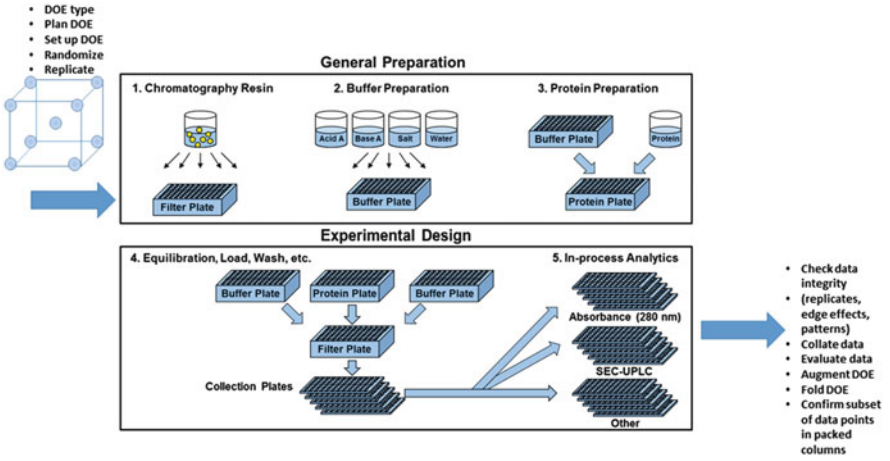
its impact and opportunities for further growth, and the exciting developments in modeling and simulation of DSP rounds out the overview. Lastly, emerging trends such as 3D printing and nanotechnology are covered.



Graphical Abstract Workflow of high-throughput screening, design of experiments, and high-throughput analytics to understand design space and design space boundaries quickly. (Reproduced with permission from Gregory Barker, Process Development, Bristol-Myers Squibb)

Keywords Bioprocessing, Downstream, High-throughput processing, Modeling, Process improvements, Purification

Contents

1 Introduction

Downstream processing (DSP) is engaged in the separation and refinement of mixtures of components. In its simplest definition, DSP encompasses a tool box of separation techniques designed to achieve mass transfer phenomena, converting mixtures of substances into subsets of mixtures or fractions [1]. At its onset, industrial DSP of proteins considered many of the traditional chemical unit operations, such as aqueous two phase systems (ATPS), precipitation, crystallization, and extraction. Although these chemical techniques still have a stronghold in sister industries such as plasma fractionation [2] and vaccine manufacture [3–7], the lack of general utility, emergence of higher yielding, less harsh techniques, and scale-up limitations in cell culture-based protein DSP stifled popularity of these techniques. Novelty in the context of these unit operations therefore mostly derives from improvements in experimental design, high-throughput screening (HTS), equipment, and systems. A deepened understanding of protein surface mapping going hand-in-hand with improved protein engineering contributes as well. Advances in separation sciences over the last five decades have enabled DSP as it stands today, where process chromatography and filtration have evolved into the pillars of downstream processing of protein biologics for the last three decades. A brief review of their development and capabilities serves here to lay the framework as to what targets need to be surpassed to achieve similar success in DSP for alternate unit operations.

The ubiquity of filtration unit operations in DSP arises from its separation prowess and versatility. From removal of cells and particulates, sterilizing grade filtration, over-concentration and buffer exchange to single-use chromatography alternatives, there are virtually no biologics processes which do not encompass multiple forms of filtration. Industrial research of membrane technology began with the discovery of nitrocellulose in 1846 [8]. Although research continued with the first mention of ultrafiltration by Bechold in 1906 [8], it was the discovery of semi-synthetic and synthetic anisotropic membranes with the invention of the Loeb–Sourirajan membrane in the late 1950s and 1960s that began the success story of industrial membrane applications in the pharmaceutical and eventually biotechnology sectors [8]. As one can imagine, such a long history has driven an extensive portfolio. A profusion of filter materials and filter types are available from simple natural materials, such as ceramics, to synthetics, such as hydrophilic polyether sulfone (PES). Filters are much more amenable to single use, a feature which has ensured an even stronger foothold in the industry in the last decade. In spite of its versatility and simplicity, filtration technologies have not been able to meet the resolution of traditional chromatography steps to date. Significant improvements have been made and a thorough review can be found later in this chapter.

Protein process chromatography's foundation was laid in the 1950s and 1960s with the invention of macroporous base matrices. Typically, base matrices were sufficiently hydrophilic and chemistries such as cellulose [9], dextran [10], polyacrylamide [11], polystyrene [12], and agarose [13, 14] were utilized. Early prototypes were too soft, which severely limited both throughput and scale-up because of flow rate and bed stability limitations, respectively. Although early resins were

initially only modified with charged or hydrophobic moieties, significant improvements in coupling chemistry allowed addition of affinity resins to the portfolio [15]. From the advent of process chromatography, researchers clearly understood that porosity, linker, and coupling chemistry significantly impact the performance of chromatography resins [16]. Affinity steps were and still are hampered by high cost and lack of universal application. Ion exchange chromatography (IEC), on the other hand, emerged as an early favorite in process chromatography, driven by its general applicability and simplicity. Mixed mode (MMC) and hydrophobic interaction chromatography (HIC) were described as early as the 1970s [17–20] without gaining the same popularity as affinity, size exclusion, and IEC. Both MMC and HIC can therefore serve as examples of inventions which have not yet lived up to their innovation potential. Adsorptive resins exploit specific physicochemical properties of a protein to enable separation. Although true affinity resins offer unique selectivity with purification factors in excess of 100- to 1,000-fold, their specific interactions are driven by the same physicochemical properties as the much less selective IEC and HIC. To optimize truly any process, a foundational understanding of each step, including affinity chromatography, should be considered imperative to design in process robustness. As the understanding of main drivers of high selectivity purifications grows, it has become evident that the natural selection of proteinaceous ligands is difficult to match with small, synthetic ligands even if underlying mechanisms are the same. However, the advent of HTS, combined with molecular modeling and improved synthesis of larger ligands to mimic native binding, has enabled identification of more promising chromatography media.

Many of the downstream processing reviews of the last decade have stressed the tremendous improvements in upstream processing although frequently lamenting the lack of corresponding innovation in downstream processing [21–26] with few opposing viewpoints [27]. Review papers have consistently referred to the same set of solutions to overcome the bottleneck of downstream processing of high titer cell culture [28]. To stimulate this discussion on the innovation of bioprocess technologies, one could raise three fundamental questions to determine why they have not taken root in the industry:

1. What are the main barriers for successful implementation of new unit operations or innovations in DSP?
2. At what point in its lifetime should an innovation no longer be considered a viable mainstream option?
3. How well have predictions of success served us in the past and how well do they serve us at present?

Some excellent reviews on innovation barriers are available [29–34] and are not discussed here in any great detail. The intention is to provide a more realistic outlook on new technologies and the potential implications of being either an early adopter of an unsuccessful innovation or a late adopter of a game-changing innovation. The reader is directed to the seminal work of Everett Rogers now in its fifth edition. His model of innovation diffusion and adoption is briefly summarized here (Fig. 1).

Premature implementation of unsuccessful innovation, often referred to as "bleeding edge technology," is discussed less often, although the impact on

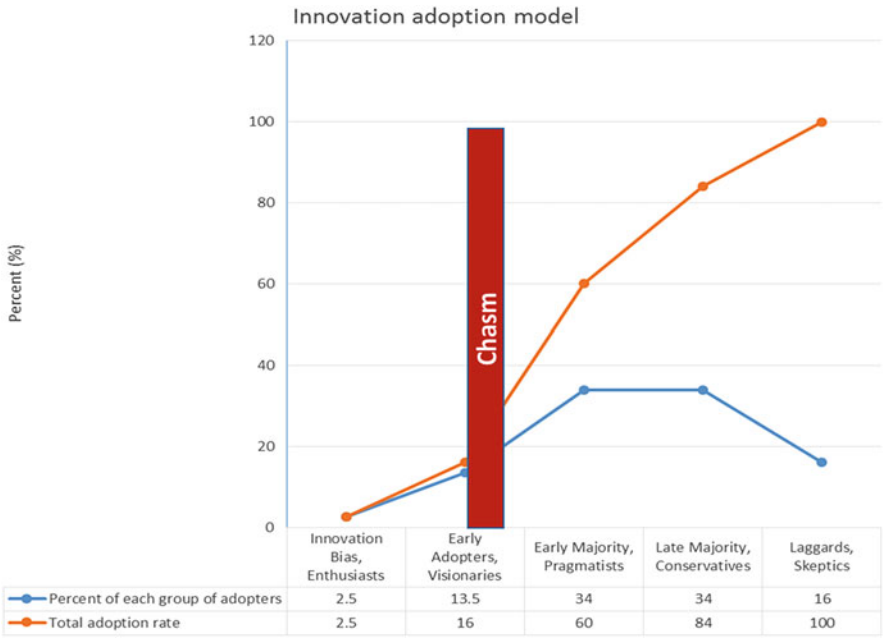


Fig. 1 Overview of the innovation adoption curve: *x*-axis, time scale, *y*-axis, percent (%) of group of adopters and adoption rate. The threshold for adoption is shown as a *bar* (chasm)

corporate culture and resulting reluctance to adopt new technology in the future can be significant. One relevant example to DSP is chromatography itself. Since chromatography's first inception in 1903 [35], more than half a century passed before it started to find utility in the industrial separation of proteins [36]. For an excellent review of the tortuous path the discovery of chromatography took from its outset to first successes please see Guiochon et al. [37]. Another, more recent example is expanded bed adsorption chromatography (EBA). EBA is built upon the concept of fluidized beds, a concept employed industrially since 1922 [38] and successfully used for antibiotics since the 1950s [39]. The utility of fluidized beds for protein adsorption was more limited but expanded in the 1980s and early 1990s with the advent of EBA [40–43]. EBA offers a more stratified bed with very low back mixing through use of particles with a density and size distribution [44]. At its zenith, EBA even warranted its own set of annual meetings [45] as it promised to bundle three unit operations into one, namely centrifugation, filtration, and chromatography. Unfortunately, the technology experienced significant growing pains and failed to deliver for a multitude of reasons. The most prominent hurdle was and is the significant interaction of many feed streams with the resin. EBA's requirements for large bead size to offer sufficient weight led to significant mass transfer challenges impacting both capacity and recovery. Initial hardware designs were costly, lacking robustness with limited scale-up options. Many of these challenges have been overcome. Hardware improvements have been impressive and column diameter is no longer a limitation with columns up to 1.5 m in diameter.

Chromatography media improvements have also been made to minimize biomass interaction and improving density to enable higher flow rates. Tungsten carbide-based beads offer high density, at a cost. The main limitation, however, remains the significant biomass interaction and appropriate bioburden control produced by improper hardware that creates additional problem in GMP operation which has yet to be overcome, leaving EBA as a niche application [46]. With the sobering example of EBA in mind, it may be prudent to ask vendors of any new technology poignant questions about commitment and market share expectations. Ideally, departments focused on assessment of innovations should build models to forecast costs which may reduce or increase over a 10- to 20-year time frame. In the case of EBA, unless some breakthrough discoveries are made on minimizing biomass interactions and significantly reducing media cost and hardware investments, it seems unlikely that this invention can ever enter the realm of disruptive technology.

This brings about the last question as to the general competency to predict success or failure. Unfortunately, research has taught us that human ability to predict future trends and developments accurately is poor regardless of discipline [47]. Non-model-based predictions of future trends in the sciences favor personal preferences and experiences of the author(s), ensuring an unwavering, subjective bias. Any team tasked with assessing the utility of new technologies should therefore consider investing in models to distinguish an interesting academic invention from a true market innovation. Committing to innovations simply based on one's view point or personal preference is unlikely to be successful in a very competitive, highly regulated market.

Having framed expectations around fallacies in innovation prediction and implementation, the reader is advised that this review does not attempt to predict success or failure of a particular innovation. Instead, it tries to cover the breadth of innovations aimed at addressing challenges the industry faces today, remaining impartial, fair, and balanced. In addition, there is more emphasis on chromatographic and non-chromatographic separations directed at the purification of proteins, with a heavier weighting on antibodies. All-encompassing integrated USP/DSP strategies such as continuous processing are discussed as well as gains in data quantity and quality because of automation and high-throughput screening (HTS). Best practices and advantages in experimental design through design of experiments (DOE) to gain access to more complex design spaces such as multi-modal chromatography are reviewed with an outlook on potential future trends. A discussion of single-use technology, its impact and opportunities for further growth, and the exciting developments in modeling and simulation of DSP rounds out the overview. Lastly, an outlook on more interesting forays such as 3D printing [48] and nanotechnology is presented.

2 Traditional Chromatography Methods

Traditional chromatography methods in industrial processes encompass affinity (AC), ion exchange (IEX), and hydrophobic interaction (HIC) chromatography. Size exclusion chromatography (SEC) is found in older and/or smaller scale industrial processes and is not further discussed here because of its throughput

limitations and column packing challenges. Both limitations ensure that size exclusion chromatography remains a poor choice for an industrial process. AC, and IEX in particular, are factotums in the industry because of their robustness, simplicity, and, of course, familiarity [27]. With that said, poor mass transfer and back pressure are inherent limitations of traditional chromatography. Mass transfer in traditional chromatography media is intraparticle pore diffusion controlled, and pore concentration is inversely proportional to bead radius and pressure drop is inversely proportional to the bead radius squared [49]. As improved mass transfer is pitted against an increase in back pressure (cost consideration of smaller bead size aside), traditional bead based chromatography always operates within these constraints.

Downstream processing is challenged with developing robust, fast, productive processes to enable purification high titer upstream processes [46, 50–54]. Traditional chromatography is perceived as a bottleneck and its future has been called into question for over a decade [27, 55, 56]. Juxtaposed to this expectation of the imminent demise of traditional chromatography is the entrenched platform process for antibodies [28, 57–63]. As an antibody platform process shortens process development, simplifies manufacturing, and even enables platform analytics without jeopardizing commercial success, finding an equally robust and easy to use process can be hard to envision, though some approaches are described in other sections of this chapter.

2.1 Improved Selectivity Through Phase Modulation and Displacement

Phase modulators can help to improve selectivity and productivity. Their universal applicability for all modes of chromatography and the availability of economical, compendial displacers have made displacers an attractive option for difficult-to-remove impurities even for platform processes. Neither phase modulators nor displacers are particularly novel, but their accessibility has been bolstered through the use of HTS approaches. The patent landscape should be taken into consideration for both [64–77]. Commonly found phase modulators are buffer and/or pH divalent cations [78], salts [79], organics [80], chelating agents [81], and amino acids [82]. The use of displacers has a longstanding history in chromatography for difficult-to-separate proteins. Either histidine or phosphorylated sugar displacers offered the only means for effective recovery of antibodies from Drimarene Rubine R/K-5BL [83]. Divalent cations can be useful phase modulators for the reduction in electrostatically-bound DNA. More problematic from a scale-up perspective are organics. Alkane diols are preferred over alkyl alcohols. Although alkene diols are inherently more viscous, they do not pose costly operational and safety challenges such as explosion proof operation, and have also been found effective in the reduction of lipopolysaccharides [84]. The number of extensively studied phase modulators and displacers is small as industrial processes require compendial grade materials. The short chain alkane diols (polyethylene glycol and propylene glycol), amino acids, and chaotropes have been studied for IEX, MMC, and AC

[85]. Mechanistically, alkane diols weaken hydrophobic interactions. The chaotropic phase modulator urea weakens hydrophobic and hydrogen bonding interactions and alters protein solvation states, simultaneously slightly reducing electrostatic interactions. Arginine interacts with protein side chains by hydrogen bonding and π-electron interactions. In addition, arginine interacts with aromatic moieties of HIC and MMC ligands. Out of all the modulators, arginine's interactions are the most complex, but its overall impact is a reduction in hydrophobic, hydrogen, and electrostatic interactions. Arginine has been particularly helpful in modulating MMC selectivity [86–88]. Medium chain length fatty acids such as sodium caprylate have also been evaluated [88–90]. Sodium caprylate had a dramatic effect for some proteins but was generally less impactful as compared to the other additives in one study [88]. Whenever inherently "sticky" reagents such as detergents or fatty acids are used, an impact on resin reuse and lifetime must be taken into consideration. This is especially important for displacers where high affinity for the ligand is the means of elution. Development of regeneration and cleaning-in-place protocols to preserve resin lifetime can become very challenging.

Amino acids have been investigated [91] as either phase modulators or buffer systems, as driven by an amino acids pKa and buffering capacity. Both Protein A and IEX have been evaluated and amino acid-based systems compared favorable in HMW and HCP clearance to the baseline buffer system. Histidine-based buffer systems are frequently used as a final formulation buffer, and histidine buffer use especially in the final chromatography step may seem advantageous. However, the use of histidine as a process buffer can be problematic. Diafiltration of histidine can impact final targeted buffer concentration and pH because of preferential sieving and/or retention of some histidine charge variants, also described as the Donnan effect [92–94]. Histidine can be problematic for a CEX step as it binds and hence titrates on the column, leading to undesirable pH transitions and requirements for long step holds to reach a stable pH baseline. The role of arginine in Protein A binding and elution was also studied [95]. Molecular computation served to explore the underlying mechanism in arginine's impact on Protein A purification of IgGs. As arginine weakens hydrophobic, electrostatic, and hydrogen bond interactions, it facilitates the dissociation of IgG from the Protein A ligand. The impact of arginine was compared to the kosmotropic citrate buffer system. The citrate system strengthened the binding of antibodies and led to more formation of aggregate during elution. This coincides well with internal data obtained through HTS pH salt mapping studies of antibodies where aggregation is most pronounced in citrate, followed by succinate, acetate, and glycine (Bristol Myers Squibb (BMS), unpublished results). This is in good agreement with the expanded Hofmeister series. The effect of arginine and citrate was juxtaposed in their solvation behavior as well. Although the number of arginine molecules around the proteins in its dissociated state was higher than in the associated state, citrate had a higher concentration around the protein in its associated state as compared to the dissociated state. It was concluded that this impact was caused by the differences in how the two phase modulators interacted with protein surfaces. Although arginine preferentially interacts with charged, aromatic, and polar amino acids, citrate associates preferentially with basic and aliphatic amino acids.

Both MMC and Protein A [96] steps were recently shown to benefit from high salt wash steps for increased viral clearance at levels of greater than five logs. The impact of this strategy on resin life has not yet been investigated and the duration of the total required wash steps was extensive. However, the benefits of high viral clearance for a Protein A or MMC capture step could very well offset the drawbacks of a potential reduction in resin lifetime and increase in total WFI consumption and processing time. Use of pH modulation for narrow range pH responsive resins can be advantageous. Protein A offers sustained binding at pH 6.0–11 with minimal salt requirements in the alkaline range. The presence of higher concentrations of salt may be required from pH 3.0–6.0 to suppress electrostatic repulsion [96]. Exploration of pH-based washes which break up electrostatic interactions can prove beneficial and have long been exploited for high pH wash steps on Protein A [96, 97]. As indicated, arginine and its analogues are phase modulators of high versatility with a multitude of benefits from suppression of HMW formation over increased viral clearance to desirable shifts in elution behavior. The main drawback of some phase modulators, such as arginine, is the intellectual property landscape [73, 98, 99].

Salt washes can reduce HMW formation during elution if impurities are removed. Depending on a protein's solubility, sometimes addition of moderate amounts of a mildly kosmotropic salt, such as sodium chloride, can also prove beneficial. Lastly, recent years have also seen more and more use of dual salt buffers to improve HIC selectivity and reduce overall salt concentration. For more information on utilizing a dual salt system, please refer to the HIC section of this chapter for more detail. In summary, high-throughput screening and improved understanding of binding mechanisms and selectivity modulation have led to a wealth of publications on simple, effective methodologies to improve impurity clearance, recovery, and capacity. Combinatorial approaches of surface modeling, first principles, and HTS experiments are an avenue to explore further in the future to optimize processes more specifically and quickly. Additional points concerning the modeling aspects of this approach can also be found in subsequent sections of this chapter. Displacers can offer significant advantages over traditional elution techniques. Although there have been few publications concerning use of displacers in industry, patents are still being filed as evidenced by a recent patent application [77]. A comprehensive review of displacers was provided by Srajer Gajdosik et al. [100].

2.2 *Use of Traditional Protein A Purification; Improvements in Capacity and Selectivity*

The recent patent expiry of Protein A led to the addition of new Protein A vendors to the market. Some of the new Protein A resins are presently offered at reduced cost ($6,000–7,000/L). This reduction in cost weakens concerns about the viability of the Protein A-based platform process. Nevertheless, even at this reduced cost it is hard to envision a disposable Protein A option, one of several reasons why alternative capture steps are investigated. As it is likely that Protein capture persists

in the industry in at least the near term, a basic understanding of the underlying binding mechanisms and implied opportunities and limitations is warranted.

Antibodies and Fc fusion proteins bind to Protein A predominantly hydrophobically [101], a concept long exploited for weak binders [102]. Studies have shown that a gain in enthalpy, not entropy, drives binding to Protein A [103]. Although the addition of salt to condition a bioreactor harvest for a weak binder is commonly practiced in research, this is not desirable at the large scale. If addition of salt should be required, the addition of kosmotropic salts such as sodium citrate or potassium phosphate should be considered. To date, no comprehensive study has been published regarding the impact of dual salts on capacity and selectivity, a phenomenon studied for HIC [104–106]. Although the addition of salt can increase in capacity, this gain is offset by a commensurate reduction in selectivity and the potential for increased HMW formation during elution if salt concentration during wash steps needs to be maintained to reduce product loss. The use of sodium salts of carboxylic acids to strengthen Protein A affinity was recently described [81]. EDTA and sodium citrate offered comparable levels of binding capacity improvements. The use of EDTA rather than salt during wash steps can circumvent the formation of HMW during elution, simultaneously maintaining good binding strength and capacity [107]. The benefits of pH optimization to maximize wash step efficiency during process development has been described earlier in this chapter. Most antibodies elute from Protein A effectively in a pH range of 3–4, and therefore the elution buffer and pH should also be carefully considered to maintain product quality [108]. Glycosylation pattern [109], IgG class [103, 110], and variable regions all impact not only binding affinity but also elution pH. The impact of the VH3 domain [111] has led to the development of Protein A analogues engineered to eliminate the electrostatically driven interaction, which begins to weaken in the range of pH 2.8–4.0. Current native, recombinant and sequence engineered Protein A ligands have isoelectric points from 4–5. Electrostatic repulsion is the driving mechanism of Protein A elution; the presence of salt during elution can interfere with elution and induce aggregate formation. However, Fc fusion proteins and IgG4 antibodies, which often have lower isoelectric points, may require addition of some salt, or "salting in," to prevent aggregate formation.

The process-related impurity profile in the Protein A eluate is dominated by the physicochemical properties of the antibody [112–117]. For all adsorptive resins, linker, coupling chemistry, and ligand impact the impurity profile [118, 119]. Selectivity is driven by initial resin choice as well as product surface properties. Bench marking publications, extolling the properties of one particular resin, yet utilizing optimal conditions for just one already established resin, do not offer an unbiased assessment [120]. At a minimum, baseline conditions for each resin should be employed in the comparison as per vendor recommendation unless the goal is specifically to replace the current resin with minimal change. Conversely, changing to a completely different base matrix, linker, or ligand chemistry for a platform process should not be an endeavor taken on lightly as the time investment to optimize mobile phases to ensure robust performance is not trivial, as differences in resin characteristics are significant.

The ligand itself has of course also been subject to intense scrutiny in an effort to maximize its desirable properties and minimize its shortcomings. Protein A, a cell wall

protein of *Staphylococcus aureus*, consists of five, highly homologous domains. Recombinant Protein A ligands often consist of repeats of just one of the five domains. Domains are chosen and/or often mutated to increase alkaline resistance, reduce VH3 domain-based interaction [121] (depending on domain choice) and increase ease of expression. Each of the five highly homologous domains could theoretically bind one IgG antibody [122], although stoichiometries are significantly lower than 5:1. For standard IgGs, ratios of 1.6–2.5 apply [103, 123–125]. As reported previously [123], Fc fusion proteins can offer improved stoichiometry. Internal data for an Fc-fusion protein demonstrated ratios as high as 4.5:1 with a corresponding dynamic binding capacity of 60 mg/mL at 5% breakthrough (DBC5%) at 3–4 min residence time (RT). The VH3 domain of the Fc-fusion protein contributed to the more favorable stoichiometry as the ratio dropped from 4.5 to 3.2 when switching from the recombinant Protein A to the Z-domain-based MabSelect SuRe ligand. Although steric hindrance is clearly an effector in the ratio of antibody to Protein A, one can envision several approaches to boost this ratio. One obvious solution is an increase in the number of domains. A commensurate increase in binding capacity when increasing the number of repeats from four to six has recently been described [126]. This approach seems most simplistic, but as prokaryotic expression is currently used to generate recombinant Protein A, the limitations of the microbial host for large proteins and the potential increase in ligand cost must be considered. Fortunately, recombinant Protein A ligands have already been reduced in size as compared to native Protein A at 42 kDa. However, there is an upper limit of domain multiplicity at which effectiveness and cost of expression becomes prohibitive. Another potential option would be a further reduction in functional domain to the bare minimum and use of an inert binding spacer or linker arm to maximize flexibility and distance between the Protein A domains to increase capacity. This approach has not been utilized for commercial resins, which seems to indicate that its practical application is not trivial. One could also envision a more flexible network of Protein A ligand display through grafted polymers. However, given some of the recovery issues observed for other grafted polymer IEX resins, this also seems less attractive. Lastly, the dual flow loading regime initially proposed by GE Lifesciences [127] has recently been revisited with reported capacities of up to 65 g/L [128].

To enhance selectivity further, pegylation of the Protein A molecule has also been explored [129]. Considering the increase in cost that this approach would likely incur, in combination with the availability of simple wash regimes to accomplish similar impurity levels, it is questionable how attractive this approach would be commercially. In addition, more work with industrially relevant feed streams would be required to demonstrate its utility. Several publications have also explored systematic mutations of Protein A to achieve milder elution conditions [130–133]. Aggregate formation during Protein A elution has been a well-studied phenomenon and recent findings indicate that an antibody's tertiary and partially secondary structure can unravel because of Protein A association, as well as the low pH elution and viral inactivation hold [134–137].

To maximize the value of Protein A, resin re-use is critical. Development of a good regeneration, cleaning, and sanitization protocol can be time consuming.

Recent advances in studying both the mechanism for fouling [117, 138] and ligand degradation [139] should be helpful in devising more effective cleaning-in-place protocols in the future. Differences in resin chemistry and ligand stability must be taken into account to ensure that appropriate buffers are used. It is a current fallacy in the industry to use silica optimal buffers [140] interchangeably for agarose- or cellulose-based resins [141]. The use of low pH for the acid-labile agarose and cellulose-based resins is not recommended. Effective sanitization is of the utmost importance for campaign-based production schedules where intermittent periods of prolonged column storage put columns at an increased risk of bioburden. Resin lifetime studies in plate HTS format have also been described [142]. Although these should be considered excellent starting points for a first read on best cleaning agents, cleaning effectiveness is best studied or at least modeled in a packed bed. The impact of column packing quality on cleaning effectiveness should also be considered. Contact times should be based on the lower end of a column's packing quality and a change in flow distribution also needs to be considered. Although cleaning regimes have been published [141, 143], none of them have harnessed the power of DoE and predictive modeling. The fouling mechanism and exploration for potential remedial actions provided interesting insights [117, 138, 143] and serve well in designing better cleaning protocols. A recent study also showed the greater promise of sodium citrate in Protein A ligand stabilization as compared to sodium sulfate [143].

2.2.1 Advances in Custom Affinity Media

Current understanding of an antibody platform process relies on the use of a primary, universally applicable, high selectivity step. Although some arguments have been made for its replacement, the distinct advantage of purification factors well above 1,000-fold have also led to publications making the case for staying with an affinity-based platform as a sustainable, multi-decade solution [27, 55]. Recent advances in ligand identification and development (ProMetic, Affilogic, Sterogene, SomaLogic, VersaMatrix. BAC, Thermo, Avitide) have considerably dropped the price point and lead times [144]. These developments, in combination with advances in high-throughput screening on both the chromatography and analytical sides, could lead to a shift in the development of difficult-to-purify proteins. One could indeed imagine a workflow where a custom affinity resin may be utilized, which then enables a recovery and polishing step platform. As platform processes for antibodies are almost exclusively built around the use of Protein A, the wide span of potential isoelectric points, and the lower selectivity of alternative capture steps, competing approaches never gained the market share it initially hoped for affinity purification. Ligand stability, cleanability, leaching, cost, and toxicity, as well as linker chemistry, are important consideration for affinity chromatography. For any chromatography resin, to ensure a commercially viable processes, security of supply, single sourcing, and customer support are important considerations.

Detailed risk assessments of commercial viability through security of supply and vendor management are advisable.

2.2.2 Advances in Traditional Ion Exchange Chromatography

Although IEX continues to be ubiquitous in the industry, traditional ion exchange chromatography requires feed adjustments for most upstream feed streams. Either dilution or buffer exchange can be used for feed adjustments, but this is not particularly attractive at large scale. As constraints around buffer holding capacity and dilution have already been anticipated for standard platform processes [145], moving to an IEX-based capture does little to overcome these issues. For most antibody processes, disposable membrane adsorbers have become a first choice for flow-through steps after Protein A processing, although comparison for at least CEX steps did not always indicated better impurity clearance at comparable capacities [146]. Membrane exchangers are discussed further elsewhere (single-use systems). AEX provides robust viral clearance [147–155] with well-known boundaries of pH and conductivity. It is difficult to envision future antibody processes limited to the use of CEX in a two-step platform.

Significant improvements in binding capacity have been made over the last decades. The main driver for increases in binding capacity is pore size distribution as it drives available surface area [156]. Grafted polymer display of ligands and hydrogel coatings of bead pores, as well as convective through-pores, has been a more recent addition to the resin portfolio. Differences in protein uptake driven by changes to pore dimensions and phase ratios were observed early on [157]. Although the available pore space was shown to be reduced, uptake rates were nevertheless increased [158]. Further study of the underlying binding mechanism indicated that binding was different from traditional chromatography media and that a shrinking-core diffusion model should be adopted [159]. It was also proposed that grafted polymer binding surfaces should be considered and modeled as a three- rather than two-dimensional surface [160]. An impact on uptake rates through a “hopping” mechanism which could be enhanced by the choice of counter ion confirmed the three-dimensional nature of polymer grafted ligand surfaces [161]. Although uptake could be accelerated, depending on chosen elution conditions, recovery could be impaired as well [162]. CEX offers distinct advantages compared to AEX in resolving product-related impurities such as high molecular weight species and charge variants [163]. Well-established commercially viable IEX ligands are limited and range from tertiary and quaternary amine ligands for anion exchange to sulfopropyl and carboxymethyl ligands for cation exchange. To overcome some of the conductivity limitations, “salt tolerant” media have been developed. Salt tolerance can be achieved by either changing ligand type [164] or through a higher ligand density. Increases in ligand density are derived from either grafted polymer ligand display, polymeric ligands [165], or simply through an increase in ligand density. Although ligand density alone does not determine binding capacity [156, 166], the use of grafted polymers can increase accessible

surface area and ligand density at the same time. Until recently, there has been little concerted effort to understand better the underlying mechanisms for salt tolerance, as studies as early as 1996 indicated significant differences [167]. Recently, studies have provided insight on the molecular properties of these resin types through exploration of macroscopically observable adsorption equilibria and kinetic properties paired with microscopic evidence of transport phenomena. A mechanism for the formation of high molecular weight species was proposed in extended contact with the highly charged surface which plays a key role in this effect. One of the studies showed that the grafted polymer resin induced HMW for antibodies through denaturation of a region associated with the Fc tail [168]. This observation seems to suggest a high propensity to denature for any antibody depending on processing conditions. Further experiments revealed that this effect could also be observed for resins with a bimodal pore size distribution, in particular resins with large through and smaller binding pores [169]. These observations seem to imply an upper boundary for capacity and/or binding strength which is driven not only by steric hindrance but also by the deleterious side effects such as the formation of high molecular weight species. This effect has been well-known in hydrophobic interaction chromatography, but now seems to be more universally applicable to other modes of chromatography. As with HIC, the severity of the impact relates to an antibody's inherent stability. Similar effects were shown for BSA and effects could be attributed to increased charge density rather than hydrophobic interactions [170].

2.2.3 Traditional Chromatography: Hydrophobic Interaction Chromatography (HIC)

As IEX is suboptimal for capture because of conductivity constraints, HIC may be a viable alternative. HIC, however, frequently requires the addition of a kosmotropic salt. As most feed streams, especially clarified cell lysates, are rich in hydrophobic impurities, these impurities limit both capacity and resin re-use. HIC can be extremely powerful for removal of not only cell culture impurities but also product-related impurities [58, 171–174]. For strongly hydrophobic proteins, even short contact times in flow-through mode can be sufficient to induce HMW formation [170, 175–179]. HIC has therefore been difficult to adopt for a platform process but can be included as part of a platform toolbox. A more recent review of hydrophobic interaction chromatography is provided here [180]. Although no final conclusive mechanistic model for HIC has been established, significant progress has been made in building a better understanding of key metrics. The use of the second virial coefficient and high adiabatic compressibility has enabled more predictive models [181, 182]. Proteins with higher structural flexibility were also found to be more likely to denature on HIC surfaces and required consideration of the adsorptive properties of these denatured subsets to more accurately model HIC binding [183]. As in IEX, the protein-accessible surface was found to be the main driver for HIC binding capacity [184]. A more recent attempt to model the behavior

deterministically employed a genetic algorithm with good success for a single component system model protein [185].

One development in HIC is the use of dual salts to improve capacity and selectivity [186]. The benefits of the use of dual salts were first explored in the purification of plasmid DNA [187], but have since translated to protein purification. The concept was first introduced to enhance binding capacity at increased solubility of a model antibody [188]. The concept was then extended further to include ternary mixtures of salt and a mathematical model built based on the synergistic effects of ions rather than salts [105]. As first proposed by Melander and Horváth [189], surface tension measurements have been used in the past to compare lyo- or kosmotropic salt strength and predict DCB. This concept could not be transferred to dual salt mixtures [104]. Instead, it was found that the aggregation temperature provided a better metric for the degree of hydrophobic forces and correlation to the DBCs. A better foundational understanding of HIC is critical not only for robust processes in the industry but also for the improved adoption of mixed mode chromatography, where the complexity increases further as ionic, hydrogen bonding, hydrophobic, and hydrophilic interactions need to be modeled and predicted accurately.

3 New Purification Strategies

As stated above, most commonly used chromatography techniques used in the manufacturing of biological products comprise affinity (AC), ion exchange (IEX), and hydrophobic interaction (HIC) chromatography. However, production costs for drugs generally have shifted from upstream cell culture to downstream recovery and purification, pushing for the development of new separations technologies or improved use of traditional ion exchange chromatography (IEX) for downstream processing.

3.1 Improved Use of Traditional Ion Exchange Chromatography (IEX) for Downstream Processing

Incremental improvements can drive efficiency significantly in downstream purification. Prominent examples are overloading chromatography and weak partitioning chromatography (WPC) [190]. WPC, first introduced in 2008 [190], operates by exploiting differences in the distribution or partition coefficient of the product and impurity under weak binding conditions and does not necessarily require high loadings for effective recovery as shown in Fig. 2. Conditions are sought where the partition coefficient is >0 for both products and impurities, and the partition coefficient of impurities is ideally still significantly larger than that of the product.

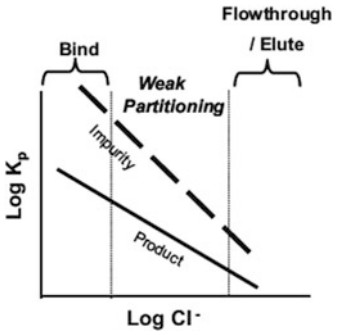


Fig. 2 Schematic diagram of anion exchange chromatography, showing the log of the distribution coefficient, *K*p, for both the product mAb and an impurity as a function of the log of the counterion concentration (Reproduced from Kelley et al. [190])

Overload ion exchange chromatography, on the other hand, was defined as a type of frontal chromatography where overloading eventually leads to preferential displacement of product and retention of impurities as opposed to flow-through frontal chromatography, where the product remains unbound. Overload chromatography therefore implies operation in a stronger partitioning regime where very high loadings are required to ensure product recovery through displacement by impurities. Although overload chromatography claims stronger binding or higher partition coefficients, based on patents filed, it would be hard to distinguish the two techniques as the ranges for effective partition coefficients claimed would also cover strong binding at modest loadings (US Patent No. 9,144,755, 2015; US Patent No. 8,067,182, 2011; USA Patent No. 20070060741, 2007; US Patent No. 20070054390, 2007). Both techniques can be implemented in existing manufacturing facilities without significant capital. In addition, integration of multiple unit operations into a single fluid system can eliminate the need for in-process hold tanks and minimize validation work.

Cation exchange chromatography (CEX) is typically operated in bind and elute mode with capacities $<$100 g/L. This step can be a bottleneck in downstream processing of high product titer in cell culture fluids. Overload chromatography of a cation exchange steps using a perfusive resin or membrane favorable partition conditions and very high loadings enables product displacement by impurities at high loading, flow rates, and consequently throughput. Liu et al. demonstrated that the performance of diffusive and perfusive cation exchange resins (SP-Sepharose FF (SPSFF) and Poros 50HS) can be operated using isocratic overloaded conditions to reduce HCP, DNA, HMW, leached protein A, and gentamicin, allowing monoclonal antibody to break or flow through the matrices after the resin reaches its DBC for the antibody. Contrasting the different matrices when used in the overloaded mode, the conventional perfusive CEX matrix provides a higher binding capacity than convective matrices and better mass transport than the diffusive matrix; it therefore provides a higher capacity to retain HMW and to remove other process-related impurities. As fewer steps and a smaller volume of buffer are needed for overloaded processing, buffer tank capacity also becomes less of an issue. Finally, the use of CEX in the overloaded mode could enable a more streamlined downstream process through the coupling together of multiple steps by simply

incorporating in-line pH adjustment capabilities. Weak partitioning chromatography (WPC) also operates under isocratic conditions. As mentioned earlier, weaker interactions are expected and loadings are generally more comparable to traditional chromatography. Selectivity can be finely tuned, albeit at the cost of recovery, a frequently observed phenomenon in traditional process chromatography. Similar to overloaded chromatography, yield improves with increased column loading. A short wash at the end of the load stage serves to improve yield further. Under conditions that favor very strong product binding, competitive effects from product binding can give rise to a reduction in column loading capacity. More recent work also studied the impact of different base matrices for anion exchange resins in weak partitioning mode [117]. Fractogel® EMD TMAE HiCap, Q Sepharose FF, and POROS 50 HQ were compared in WPC mode by transmission electron microscopy and inverse size exclusion chromatography, along with characterization of WPC-specific protein interactions. The results show that, for each resin, salt and pH conditions can be found for high molecular weight aggregates using the information of product-specific Kp values. However, isocratic elution and adsorption mechanisms are different for each resin and for the different components.

The robust performance of WPC anion exchange chromatography has been demonstrated in multiple cGMP mAb purification processes. Excellent clearance of host cell proteins, leached Protein A, DNA, high molecular weight species, and model virus has been achieved. Optimization of the AEX-WPC step requires tuning of the pH and counterion concentration for each mAb, which can easily be obtained through generating adsorption isotherms in high-throughput batch chromatography. The governing variable used in the design of a WPC step is the product partition coefficient, Kp, which is a fundamental descriptor of the strength of the protein–resin interaction. The improvement in impurity clearance can be correlated to the product Kp using either impurity LRVs or Kp. The product Kp is also used to define the operating window based on allowable ranges of product recovery and load dilution, and the minimum desired column loading. Iskra et al. have also demonstrated the potential of WPC to be included as part of the modular viral clearance approach ([147], p. 73).

Both strategies significantly improve facility utilization through enhancement of throughput for traditional IEX chromatography. Column volume reduction of as much as 90%, reduction in both buffer volumes and total number of buffers, and reduced operational complexity all illustrate the power of both of these options. This can be further enhanced through the use of disposable, pre-packed columns. Single use of traditional chromatography columns is usually challenging because of the relatively low upper limit of available pre-packed columns as well as the cost of single use. In commercial facilities, where columns are re-used up to a pre-established end of life cycle number, a reduction in column volume and hence cost can enable the use of disposable columns. Additional savings are garnered from avoidance of column packing, testing, cleaning, and life cycle studies. Validation cost savings can be significant, as can avoidance of potential investigation of bioburden, a phenomenon more readily preventable for disposable options. This can be a real boon for facilities which operate in campaigns and have older less hygienic column designs.

3.2 *Improved Use of Multimodal Chromatography (MMC) for Downstream Processing*

Multimodal chromatography consists of stationary phases that combine more than one mechanism of interaction into the design of their ligand, including coulombic interactions, hydrophobic interactions, and hydrogen bonding [106, 191, 192]. Multimodal ligands consist of ionic (amino, carboxyl, or sulfonic groups), hydrogen bonding (hydroxyl, thiol, or amide groups), and hydrophobic (aliphatic chain, thiol, or aromatic ring) groups. Electrostatic interactions play a significant role at low to moderate ionic strength, and hydrophobic interactions along with hydrogen bonding play a significant role at high ionic strength. The additional flexibility in designing purification strategies that employ multimodal materials makes them attractive candidates to face emerging challenges in industry, such as the need for more selective chromatography media, improved resolution, and tolerance of feedstock conditions. MMC resins provide additional flexibility in designing chromatography operations and purification processes because the multifunctional ligand can interact with both protein and aggregate species via multiple, simultaneous, and independent intermolecular mechanisms [193, 194]. Given the significant interest in evolving the downstream process platform for monoclonal antibodies, a number of applications for MMC have emerged, particularly modes involving a combination of hydrophobic and electrostatic interactions. The salt-independent binding behavior of MMC resins has been shown to bind mAb cell culture supernatant directly to resins that combine hydrophobic and electrostatic interactions [194, 195]. The hydrophobic nature of MMC resins has been exploited for aggregate removal in post-Protein A polishing steps [193, 196].

Although MMC offers significant opportunities for enhanced selectivity, methods development in these systems can be more complex than single mode of interaction, such as ion exchange or hydrophobic interaction chromatography. The fundamental understanding of how proteins interact with multimodal resins is still improving [106, 197–201]. A connection is needed between some of the fundamental characterizations of protein binding and the development of practical separation steps for preparative protein purification. Several mechanistic models have also been proposed in the literature. Modeling of the retention profile with a salt-dependent Langmuir isotherm provided promising results [200]. Mechanics of protein adsorption in a multimodal system were studied and similarly good agreement with the Langmuir isotherm in the electrostatic attraction regime was found, although the binding behavior becomes more complex when operated near the pI [197]. It was speculated that a multilayer or rearrangement effect when transitioning from electrostatic to hydrophobic interactions increased complexity near the pI. A thermodynamic model based on steric mass action (SMA) included interaction parameters, and could be applied to both hydrophobic interaction and ion exchange chromatography [198]. Multimodal adsorption isotherms were also developed as an extension of Mollerup's generalized thermodynamic framework to

account for the combined hydrophobic and ion exchange effects in multimodal systems [198, 199, 202].

Recent efforts in the development of multimodal membranes (MMM) have focused on addressing the limitations of traditional chromatography's inverse relationship of binding capacity and throughput for multi-modal chromatography [201, 203, 204]. A newly designed multimodal membrane (MMM) with high protein binding capacities and high load productivity was recently introduced [201]. A thermodynamic model [199] provided insights on the nature of protein–MMM interactions and predicted binding capacities under non-test conditions. This in turn is of importance in limiting the number of experiments. It was shown that the rate-limiting step of mAb adsorption on the MMM is the reaction rate of mAb binding with the multimodal ligands rather than the mass transport of protein molecules. Thus, although high load productivities were achieved, improvements in membrane design leading to faster adsorption kinetics would enable still higher productivities.

Numerous studies have shown the impact of mobile phase modulators on protein binding. An overview of the mechanism, recent reviews, and driving forces has been provided earlier in this chapter. Recent research using protein libraries in both multimodal anion and cation exchange systems has demonstrated that mobile phase modifiers such as arginine also play an important role in creating enhanced selectivity when designing multimodal chromatographic processes.

3.3 Hybrid Technologies for Downstream Processing

Alternate approaches have been evaluated for purification of biologics based on functionalized fibrous media and/or composites. Most rely on incorporating a porous gel into the fiber matrix, providing the necessary surface area gain to ensure reasonable binding capacities. However, in such constructions, poor uniformity in gel location and mass generally leads to poor efficiencies (shallow breakthrough and elution fronts). In addition, resistance to flow can be high even for short bed depths, a problem often aggravated by gel compression under modest pressures. The incorporation of activated carbon or silica gel particulates within the fiber matrix has been pursued as an alternate approach. The particulates are often porous, can offer a large surface area, and possess a native adsorptive functionality that separate proteins based on differences in their size and effective charge [205]. Activated carbon was applied to the purification of larger mAbs, where it was found that lower molecular weight proteinaceous impurities are most efficiently removed at their isoelectric point. The most efficient recovery of a small protein from activated carbon occurs at a solution pH further away from the protein's isoelectric point, where it is strongly charged [205].

Recently, fiber-based chromatography media have been produced for biologics purification applications that utilizes a surface-functionalized winged fiber as the adsorptive media in both bind-elute and flow through modes [206–208]. The winged projections on the fiber surface afford a much higher surface area than ordinary round fibers of similar dimensions. The surface-functionalized fiber media also have a much higher protein binding capacity than similarly functionalized fibers which lack such winged projections. The fiber-based stationary phase is non-porous and displays a convoluted surface structure that provides a sufficient surface area for high binding capacity. As the protein binding occurs only on the surface of the fiber, there are no size exclusion issues with binding as seen in the case of porous bead-based bind/elute systems. Furthermore, because the particles can be transported directly to the ligand site by convection, there are no diffusion limitations in the system and the feed streams may be processed at much higher flow rates or shorter residence times. These fiber-based chromatographic media can be combined with a high frequency simulated moving bed (SMB) design to provide superior productivity in a simple bioseparation. Electrospun polymeric nanofiber adsorbents offer an alternative ligand support surface for bioseparations. Their non-woven fiber structure with diameters in the sub-micron region creates a remarkably high surface area material that allows for rapid convective flow operations. Proof of concept experiments demonstrated the performance of an anion exchange nanofiber adsorbent based on criteria including flow and mass transfer properties, binding capacity, reproducibility, and life-cycle performance [209]. Binding capacities of the DEAE ligand surface modified adsorbents appear low at 10 mg/mL, but in the context of throughput this is a significant achievement. Suitable packing into a flow distribution device has allowed for reproducible bind-elute operations at flow rates of 2,400 cm/h, many times greater than those used in typical beaded systems. These characteristics make them ideal candidates for continuous chromatography systems. An SMB system was developed and optimized to demonstrate the productivity of nanofiber adsorbents through rapid bind and elute cycle times of 7 s which resulted in a 15-fold increase in productivity compared with packed bed resins. The integrity of nanofiber adsorbents used in the system would be an important criterion of the validation process.

3.4 Continuous Processing for Biologics Manufacturing

There is a growing interest in realizing the benefits of continuous processing in biologics manufacturing, which is reflected by the significant number of industrial and academic researchers who are actively involved in the development of continuous bioprocessing systems [210–213]. Although at least some regulatory agencies responded positively to the potential of continuous processing in biologics, regulatory guidance would have to be adapted considerably to allow a clear understanding of lot definition and testing requirements. The advantages of continuous manufacturing include sustained operation with consistent product quality, reduced

equipment size, high-volume productivity, streamlined process flow, low process cycle times, and reduced capital and operating costs. This technology, however, poses challenges that need to be addressed before routine implementation is considered. Historically, conversion from batch to continuous processing has resulted in lean, fully automated, and agile manufacturing, regardless of the nature of the industry [214–220]. This conversion has enabled these industries to overcome key business continuity challenges by reducing high manufacturing capital expenditure (CAPEX), important operational expenses (OPEX), and enhancing portability [213]. The technology has been in use for many years in the chemical industry, originally developed for difficult petrochemical separations [221]. Its use in the pharmaceutical industry quickly grew because of its superior performance in chiral separations. Following the first US Food and Drug Administration's approved drug manufactured by SMB technology reaching the market in 2002, efforts have been focused on systems that yielded poor productivity using batch chromatography. The biologics industry is facing similar challenges following the emergence of competition from biosimilars, the need for decreased COGs, and the desire to gain access to emerging markets through standardization and portability [213]. To leverage the lessons learned from other industries, there has been significant interest in the implementation of continuous bioprocessing [222]. This strategic shift is a result of the convergence of both the business drivers and advances in downstream technology. Although, the biologics industry has extensive experience in continuous (perfusion) upstream processing, there are limited examples of downstream continuous processing because of the lack of enabling disruptive technologies [222]. However, the past few years have seen maturation of separation technologies such as simulated moving bed (SMB), periodic countercurrent chromatography (PCC), multi-column chromatography (MCC) [210, 212, 213, 222, 223], continuous countercurrent tangential chromatography (CCTC) [224–226], aqueous two-phase systems (ATPS), single pass TFF [227, 228], high capacity membrane absorbers [229–231], and continuous viral inactivation [232, 233]. At present, the confluence of these emerging technologies and business needs to innovate, driven not least of all by the fact that high titer cell culture is catalyzing the development of continuous bioprocessing.

Several groups have examined the use of multi-column chromatography for fully integrated continuous production of mAbs from a perfusion bioreactor [212, 213, 222, 225, 233]. This integrated system can provide five to ten times higher volumetric productivity than a fed-batch process with comparable yield and purity with a smaller column size [213]. The design and operation of semi-continuous affinity chromatography using a PCC-based chromatography system have been demonstrated for clinical and commercial manufacturing [234], and multi-column counter-current solvent gradient purification (MCSGP) chromatography has been demonstrated with anion and cation exchange resins [212]. Although multi-column chromatography has been successfully integrated with perfusion bioreactors, these systems require column packing and complex valve switching, causing them to

operate in a cyclic rather than true steady-state mode. Recent work has demonstrated that continuous countercurrent tangential chromatography (CCTC) can overcome many of the key limitations of conventional batch and multi-column chromatography, providing opportunities for truly continuous product capture [224, 226, 235]. The CCTC process utilizes the resin in the form of a slurry which flows through a series of static mixers and hollow fiber membrane modules as shown in Fig. 3. The micro-porous hollow fiber membranes retain the large resin particles, simultaneously letting all dissolved and unbound species, including proteins and buffer components, pass through the membrane and into the permeate. The buffers used in the binding, washing, elution, stripping, and equilibration steps flow countercurrent to the resin slurry in a multi-stage configuration, enabling high resolution separations and reducing the amount of buffer needed for protein purification. In contrast to other continuous bioprocessing systems, the CCTC system provides the opportunity to run at steady state without having to switch any valves after system start up, allowing the users to abandon completely all activities related to handling packed beds, including packing, cleaning, validation, and storage.

Although the technical feasibility of a continuously operated platform production process for mAbs has been successfully proven, there are still some challenges to overcome on the way to an industrial application [233, 236–238]. A high potential for simplification and cost savings can be identified in the field of assembly preparation and delivery. Validated sterility testing is also not available

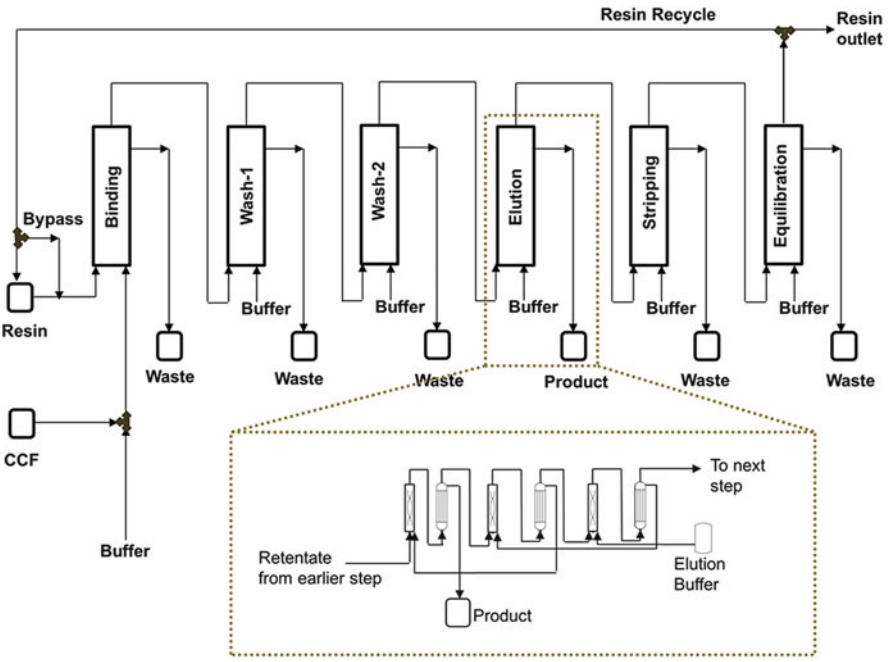


Fig. 3 Schematic of continuous countercurrent tangential chromatography (CCTC)

for chromatography columns to date. Low bioburden equipment in combination with gamma irradiation without validated sterility could be a concept to pass this aspect, although the design of low bioburden equipment is not trivial. Another aspect is the implementation of SUS in continuously operated processes operated for several weeks. The SU equipment, which is available today, is mainly designed for applications in batch processes. Consequently, the question of whether this equipment can be used for weeks instead of days has not been answered yet. Analytics to evaluate extractables and leachables have yet to be applied. Regarding continuous processing of antibodies, additional gaps have been identified. Because of very low flow rates in continuously operated antibody processes, commercial, GMP compliant, production scale equipment is not available. Furthermore, concepts for the implementation of in-line and at-line analytics to improve process understanding and enable pooled batch release have to be found. Assessment of virus safety of a continuously operated process is still a gap as well. Viral safety is always assessed holistically across the entire process. The concept of a continuous viral inactivation at low pH or through detergents still requires a re-thinking of traditional virus reduction studies to prove its effectiveness. Apart from that, solutions for the regulatory acceptance of continuously operated unit operations (UOs) and processes have to be developed as well (Fig. 3).

4 Design of Experiments (DoE) and High-Throughput Screening (HTS)

To provide an overview of the many different types of design of experiments (DoE) or a thorough explanation goes beyond the scope of this chapter. Excellent guidance can be found in the seminal works of Box et al. [239] as well as Montgomery [240]. This brief overview delves into implementation hurdles, potential areas for future growth and expansion, and synergies of DoE and HTS. The pitfalls and frequency of poor statistical experimental set-up and analysis have been reviewed and discussed in other fields [241–243]. One can speculate that a comparable deep dive into set-up and analysis of DSP DoEs would find similar misinterpretation of data caused by improper experimental set-up, execution, and analysis.

Adoption of DoE in biologics, in spite of quality by design (QbD) aspirations, has been slow, although publication output in the last 15 years has risen considerably [244, 245]. Several factors have hindered DoE adoption, not least of all a lack of training and understanding of its utility and knowledge of its practical application, although recent publications have started to fill that gap [59, 246]. The advent of HTS has significantly lessened two obstacles. Biological assays are now available in HTS format, enabling replication to reduce variability and debottlenecking analytics, and high-throughput process development (HTPD) on robotic systems has accelerated process development (PD). Throughput for assay staples in early

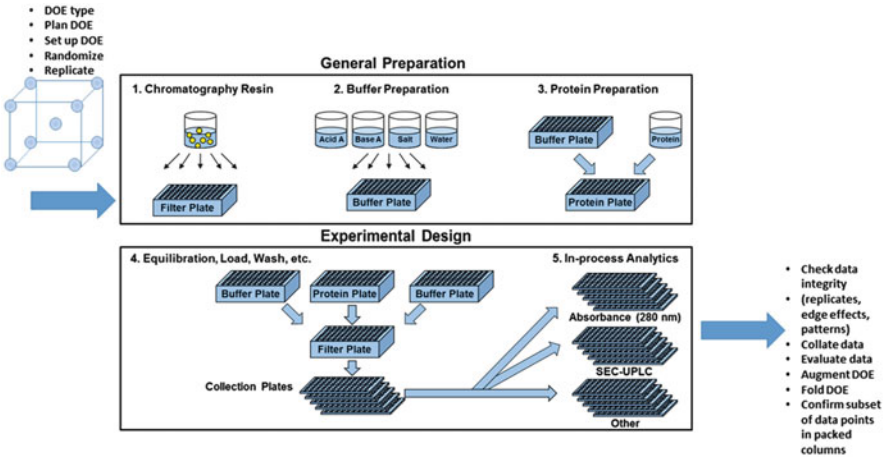


Fig. 4 Workflow of high-throughput screening, design of experiments, and high-throughput analytics to understand design space and design space boundaries quickly. (Reproduced with permission from Gregory Barker, Process Development, Bristol-Myers Squibb)

PD, namely protein concentration, size exclusion chromatography (SEC), and host cell protein (HCP), has been significantly improved. Ultra-performance liquid chromatography (UPLC) offers shortened run times with a minimal loss in resolution [247], whereas high-throughput HCP, DLS, and even residual DNA assays [248, 249] broaden the accessible range further. The low variability of SEC assays [250] as compared to host cell protein assays [251] can make SEC data an excellent internal control of overall experimental variability as long as factor ranges are chosen to show an impact on HMW and LMW levels (Fig. 4).

DoE has distinct advantages over one factor at a time (OFAT) experiments [252] but caution should be used before the untrained delves into the use of statistical design of experiments unassisted. Working with a statistician is beneficial but still requires a solid understanding of the fundamentals of statistics [253]. Even the best statistician is unlikely to interpret data correctly if common ground and language is not available. Ideally, PD scientists and engineers understand both the basics of DoE and how to test designs for pitfalls. It is presumed that the limitations and issues of the experiment are recognized even before executing a design as well as data analysis and the appropriate model selection [254–256]. Model optimization and selection are also not trivial [257–259]. Familiarity of most scientists and engineers with linear regression for one variable can also lead to an over-emphasis on driving the coefficient of determination close to 100%. Models should always be checked for variance inflation factors (VIFs), a measure of overfitting of the data with rules on multicollinearity in mind [260]. Focus should be on the factors that are strong drivers of the model in agreement with the mechanistic understanding of a process step. Recursive partitioning tools, often built into a model, are used to test a model's validity and should be employed to assess different models [261]. This is not to say that data cannot be in disagreement with the current understanding of the

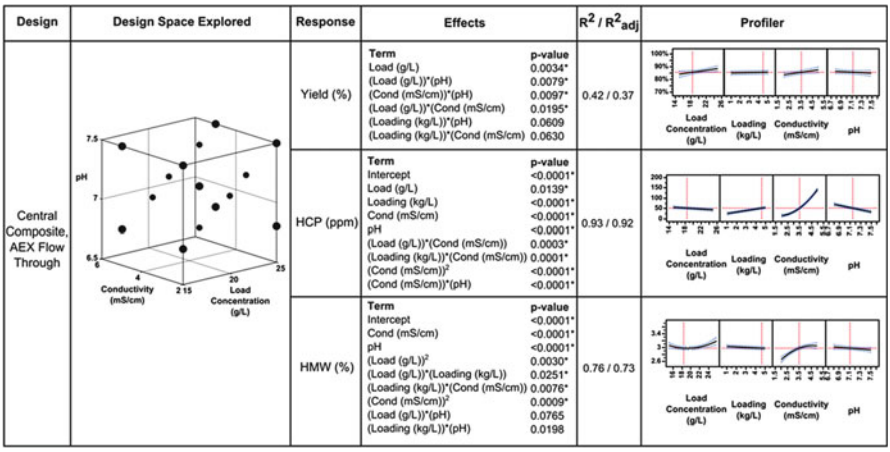


Fig. 5 Design space and observed curvature for both HCP and HMW removal in a flow-through anion exchange membrane chromatography platform process [267]

underlying principles. However, artifacts or overfitting are the more likely root causes of discrepancies. Overfitting of data is very common [262, 263] and this topic is also revisited in the modeling section of this chapter. Many researchers do not consider the significance, of the p-value but are fixated on coefficients of determination [264, 265]. Model cross-validation, built into many of the available software packages, should be used rigorously. Lastly, if fitting for curvature, care must be taken to evaluate the impact of each quadratic term carefully. Assessment of the relative importance of a quadratic term by its contribution to fit through its t-ratio, the fraction of its estimate and standard error, can help to evaluate its overall importance in the model. The prudent researcher also remembers that use of center points in fractional or full-factorial designs, other than definitive screening designs, allows testing for, but not assignment of, curvature. Although most software packages offer automated model analysis, it is always prudent to remove interaction and curvature terms manually to determine how much they actually impact the coefficient of determination. Terms that contribute less than 2% to the overall fit of the model are best eliminated [255, 266].

The utility of a screening or main effects designs should be carefully weighed. A good example is the response variable HCP clearance in an AEX FT step. Curvature in the HCP clearance response is observed in about 80% of cases (unpublished data). An example of assigned curvature in an AEX flow-through design space is shown in Fig. 5.

This curvature can be driven by pH, conductivity, and protein concentration. When screening the design space of an AEX FT step it is therefore prudent to build an augmentable design to assign curvature appropriately in a follow-up augmented DOE if curvature should contribute significantly to fit. This implies that screening designs are only recommended where main effects are expected to be dominant [268]. In the case of HCP curvature for AEX FT steps, curvature in the design space

stems from impurity breakthrough. The breakthrough response of HCP impurities is low at low conductivities (1–3 mS/cm) but begins to slope up from 3–6 mS/cm. Depending on the HCP profile, the response either flattens or sees an even sharper rise in breakthrough above 6 mS/cm. A screening design including main effects pH, conductivity, and protein concentration would have little to offer in gaining understanding of this design space [269]. At a minimum, a fractional factorial with center points must be chosen and care must be taken to avoid confounding of known key interactions. Multicollinearity, as observed, for example, for pH, conductivity (or salt concentration), and protein concentration should be taken into consideration in the planning stages of a DoE as well as its evaluation [260, 270]. The appropriate design based on prior knowledge, minimal confounding of known key factors and interactions, and, not least, a degree of uncertainty one is willing to accept should be chosen. Higher variance inflation factors (VIF) can to some extent be ignored if they are driven by interaction or quadratic terms [271]. Design evaluation which is built into most commercially available DoE software should always be conducted to understand aliasing and bias in the design [272].

Definitive screening designs are a more recent addition to DoEs and offer some distinct advantages [273–275]. Definitive screening designs minimize experimental effort yet still allow exploration of quadratic terms and interactions at minimal confounding. In addition, recent advances have also made it possible to augment these designs. In the past, a DoE was executed over the course of days or weeks using small-scale equipment [276]. Most adsorptive techniques require 4–8 h per experiment on small-scale chromatography equipment. Aside from ensuring correct set-up where lines and columns are returned to the same state between experiments, it is critical to block for equipment (if more than one system is used), day of execution, and potential starting material [277]. Although degrees of freedom (DF) are lost [278], they may be regained if they are shown not to impact both model fit and interpretability. Omitting blocking where necessary can waste time and resources [279]. In its worst outcome, over-interpretation of data can lead to selecting conditions far less robust than assumed.

Although traditional chromatography or filtration experiments necessitate blocking for day, equipment used, or both, screening in a 96-well format allows parallel rather than sequential execution of the experiments. Randomization across the plate is still critical to avoid impact of edge effects for both separation and assays [280]. Experiments in a 96-well format have distinct disconnects from a column-based separation. The ability to replicate and avoid blocking in combination with an increased understanding of the disconnects has made this an attractive, rapid, and extremely useful option as a first step toward design space exploration. Although some publications have found this approach material prohibitive [281], this is rarely the case in the industrial setting.

Currently, HTS chromatography spans several formats, including 96-well batch chromatography, 96-tip packed filter tips, and miniature columns. It is well understood that each of these systems has its own benefits and shortcomings in terms of applications, and this has already been well-summarized [282–284]. With respect to throughput and versatility, batch chromatography offers some distinct advantages

as long as a subset of resin types is excluded [284]. For many of the resins utilized in platform antibody processes, batch chromatography can serve for both foundational work, such as batch uptake and isotherm studies, and design space assessment for batch chromatography. HTS can reduce variability through replication and increased precision by means of automation and robotics. HTS is also critical in reducing material [285] and resource and time requirements. Just as in traditional column chromatography on small-scale chromatography equipment, highly material consumptive HTS experiments can be planned to minimize sample consumption. A typical AEX FT DoE could consume up to 10 g of purified antibody in a 96-well DoE. When set up properly though, only a small fraction of each sample or product pool is used for analysis. This in turn allows use of the remaining material for optimization of subsequent unit operations, such as viral filtration or TFF. Another benefit of HTS is its availability for prediction of an entire dynamic binding capacity design space [267], collecting foundational information for prediction of elution behavior [286], thus significantly narrowing the design space to explore in a DoE [89]. HTS serves to lay the groundwork for detailed process understanding, overcoming resource constraints on the analytical side. Lastly, the advantage of in-house custom resin plates should not be underestimated. The ability to analyze resins from different vendors in the same plate allows the narrowing of resin choices very quickly. In addition, it ensures interpretable results through eliminating the need for blocking between plates, which makes comparison of resins difficult even in HTS mode.

One distinct disconnect in batch chromatography is found in the longer incubation times needed to mimic column residence times [287], especially as loading increases. Another offset stems from the much lower concentration of elution pools. Both effects can lead to disconnects in the design space to understand impurity clearance and yield. Although trends are comparable, confirmation of DoE center and star and/or corner points with a subset of column experiments is not only prudent but highly recommended. Worst case studies are routinely conducted to confirm process robustness. Execution of confirmatory experiments can serve to fulfill this role as well if points are selected appropriately. The combinatorial approach of HTS and DoE enables easy augmentation [288, 289] if a design should fracture because of factor settings that lead to complete failure of the experiment. A fractured DoE becomes uninterpretable and always leads to additional experiments, as a subset of the data drives fits entirely. This is true for all responses, even if the design should only be fractured for one response. For example, if yield at very high loading drops to very low values because of either breakthrough during loading or precipitation during elution, impurity levels at high loadings are also no longer interpretable. Part of the design space used can still be assessed in combination with the newly accessed folded or augmented design which needs to have sufficient overlap to ensure interpretability. At this point, blocking is again necessary. A visual check of the newly generated data points in the previously executed experiment and analysis of a shift in the fit is recommended as well.

The data gap seen between 10 mL and 100 or 200 L process columns widens further when utilizing batch and miniature chromatography. In batch

chromatography, where resin is packed in plates during phase exchange, resolution is lost as each separation is based on one equilibrium or one plate during each stage from adsorption, wash, and elution. Models and bridging to packed columns have, however, been described [287]. Miniaturized columns are dominated by wall and capillary effects and are unlikely to represent flow distribution systems of process columns. Caution and prudence should go hand in hand with careful monitoring and comparison of scale-up data. In addition, the use of at least some miniature column on HTS equipment is limited to execution of eight experiments at a time. This in turn requires blocking for each set of experiments. In spite of some of these limitations, the benefits far outweigh the drawbacks. Automation continues to replace traditional small-scale chromatography (1–5 mL column volumes). Time, cost, material, and resource savings, in combination with improved data interpretability for DoE, are necessary to remain competitive. DoE is likely to be utilized more as it becomes more widely taught at Universities and in companies. Recent years have seen a wealth of publications using HTS for both chromatographic experiments and analytics [58, 172, 246, 285, 290–292]. Improvements have been made in the understanding of translation of residence times to incubation time [293], use of a combination of batch uptake data and static capacity to predict dynamic binding capacities over a wide range of residence times [294], and combination of factors previously difficult to explore [267]. With better understanding of similarities and differences between batch and small column chromatography, the translation to large scale model predictions should improve. The understanding of the complete design space could take just a matter of days. Ideally, a combination of computational fluid dynamics with empirical data, taking advantage of recent exploration of the use of 3D printing [295] to understand better the impact of bed and column geometry on macroscopic flow properties in the bed and HTS data, can enable process development in the future.

4.1 Single-Use Disposable Options

Multi-purpose facilities, the high cost of validation for resin re-use, and steam in place (SIP) systems have all driven quick adoption of single-use systems (SUS). Although existing, older facilities may struggle with adopting SUS completely, new facilities can be designed for the flexibility of SUS. Although not yet available [296], recycling of single-use materials may also eventually make this an even more attractive solution, cost savings for WFI for SIP and CIP processes aside. It is beyond the scope of this chapter to highlight all the advantages of single-use technologies which have led to their pervasive use. For two recent reviews, please see [297, 298]. Disposable tubing and filters as well as buffer and media storage bags have been the most readily adopted, although other disposable options, such as equipment, are catching on more slowly. Fully disposable manufacturing suites are also available [299], which could be an attractive option for a global footprint with minimal rework. The implementation of fully disposable manufacturing does not

take into account any regional differences and regulations that one may encounter. Although different options for disposable mixing units, chromatography, and tangential flow filtration skids are commercially available, centrifugation, though available, suffers from a sparsity of options. In this context, although not a truly disposable option, acoustic wave separation for the clarification of high density cell cultures may also be mentioned. Under the paradigm of high titers and 2,000- or 5,000-L bioreactors, microfiltration or microfiltration in combination with precipitation or flocculation may became the predominant recovery option [61, 300]. If 5,000-L disposable bioreactors become a reality in the future, stainless facilities could become obsolete, which should incentivize more vendors to widen or build a disposable equipment inventory. One main drawback of disposable technologies compared to stainless facilities is the lack of interchangeability of connections and technologies. Unfortunately, most vendors have taken it upon themselves to develop their own flavor of patented, aseptic connectors and connections. The lack of compatibility is not desirable from a security of supply perspective and is not as practical as traditional and interchangeable stainless connections which have been standardized Disposable sensors also offer opportunities for both growth and improvement. Although some gaps were filled very quickly, such as disposable conductivity probes and UV cells, no truly disposable, accurate pH probes are available. The high cost of disposable flow paths, supply delays, and lack of alternative sources of supply may limit their utility. Although it may be painful for a pilot-scale facility to experience delays in the delivery of disposable components, it would not only jeopardize plant operation for commercial facilities but also potentially put patient lives at risk if the necessary disposable parts cannot be delivered for extended periods of time. Although inventory stockpiling can help to offset these issues to some extent, this in turn increases costs and overheads. Another consideration is the long term impact of a fully disposable biologics process. As all of the synthetic process components contribute leachates and extractables to varying degrees, more holistic studies are needed to assess quantities and impacts of leachables and extractables, according to ICH Q7 guidelines, in the final drug product. It seems unlikely that the overall burden of trace synthetics would not be increased and that chronic effects in at least part of the patient populations would emerge over time. The potential, long term side effects implications are still to be evaluated, especially for biologics which are not curative, but sustaining, lifelong therapies.

4.2 Improved Throughput, Disposable Chromatography Options Such as Membranes and Monoliths

The use of membrane and monolith chromatography is now ubiquitous in the industry, yet to date both technologies have only gained a foothold in niche applications such as anion exchange flow-through polishing steps [301, 302],

purification of large biologics such as DNA [303–305], virus-like particles (VLP), viruses [290, 306, 307], and vaccines [308, 309]. In spite of their superior mass transfer properties, the lack of comparable capacities for proteins in adsorptive techniques [36] has led to slow universal adoption. This section provides a brief overview of some of the approaches taken to address these drawbacks and reviews more recent findings in the field.

Membrane chromatography was first introduced as a means to overcome pore diffusion, the main limitation in mass transfer of traditional chromatography resins [310]. The surface of microfiltration membranes is used to attach ligands so that mass transport was driven through convective flow. As microfiltration membranes offer very little pressure drop because of short bed heights, they also offer much better pressure flow properties. Resolution is often comparable to traditional chromatography, albeit at the cost of dilution, which has been mostly attributed to inlet and outlet manifold design [311].

Scale-down models for membrane adsorbers are available, although some disconnects should be taken into consideration [312]. More recent models indicated high confidence in scale-up based on small scale data bolstered with computational fluid dynamics (CFD) [313] where the combination of CFD and scale-down modelling allowed a 15,000-fold scale-up. An in-depth study of membrane pore structure has also been published for at least some of the currently commercially available membrane chromatography media [314]. By design, membrane chromatography always outperforms traditional chromatography media in throughput for flow-through steps in terms of mass of product processed per liter of resin per hour. In addition, membranes do not require packing and testing prior to use, nor do they need to be regenerated or sanitized at the end of processing. At flow rates of 10–15 membrane volumes per minute (MV/min) for membrane exchangers compared to 0.25–0.33 column volumes per minute for traditional chromatography media, the productivity difference in flow rate is as high as 60-fold, not accounting for WFI/buffer, time, and resource savings. Simplicity and robustness drive continued growth in market share. Although membrane adsorbers were initially only available as anion and cation exchangers, HIC [58], MMC [164], immobilized metal chelating (IMC) [315, 316], and AC [317–320] options have also become available. HIC steps frequently offer higher capacity and better selectivity for removal of aggregate. The use of a phenyl-based HIC membrane showed moderate capacity [321], which was offset by much higher throughput. HTS approaches to assess capacity and design space have also been described as being comparable to traditional chromatography techniques [322, 323].

The cost savings of a disposable step which requires neither cleaning nor resin lifetime validation ensure AEX membranes remain a staple in many platform processes. This is advantageous as two-step platform processes often suffer from shortened lifetimes of traditional AEX steps [324, 325]. Most membrane anion exchangers offer robust performance at loadings up to 5 kg of product per liter of membrane and 10 kg/L of membrane for two- and three-step platform processes, respectively. Flow-through anion exchange steps are considered to be a robust viral clearance step. A set of publications [301] explored the impact of ligand type, pH,

and conductivity on viral and impurity clearance. It was found that the quaternary amine ligand offered a narrower design space in terms of pH and conductivity as compared to a primary amine which provided sufficient viral and impurity clearance at conductivities up to 20 mS/cm. The primary amine studies demonstrated that buffer species impacted clearance and phosphate buffer should be avoided. Improved clearance over a wider pH and conductivity range was attributed to secondary, hydrophilic interactions, which were not ablated by competing electrostatic interactions, the primary drivers for binding to quaternary amines. However, a trade-off in antibody yield was also found as product could be more strongly retained. With the slow yet constant improvements made in molecular imprinting, it is also feasible to imagine advancements for new membrane adsorbers [326]. A recent publication assessed the potential of a CEX membrane capture followed by an AEX polishing step for an antibody process [319]. Universal applicability aside, because of the wider range of isoelectric points in antibodies, the advantages in throughput, cost, and disposability would still make this an attractive option for a subset of antibodies. Capacity improvements as demonstrated in this recent publication can be assigned to improvements made in membrane design. Impregnation of membranes with different hydrogels, grafted polymers, or nanoparticles have been assessed to improve capacity [314, 327–332]. Similar approaches have been taken to boost the capacity of monoliths.

The concept of monoliths dates back to the 1950s [333]. The concept stagnated after less successful attempts until a renewed interest in the late 1980s revived the field. Today, monoliths are successful in both the analytical and the process arenas. Monoliths do not contain interparticular voids as they are cast as one block of separation media [334, 335]. This forces all mobile phases to flow through the pores, resulting in convective flow which in turn greatly improves mass transfer. Monoliths also offer distinct advantages over traditional chromatography resins in pressure flow properties. However, as with traditional chromatography columns, increased bed height results in pressure increases. Monoliths, such as membrane and traditional chromatography media, are available in a wide variety of base matrices and ligand and linker chemistries. For recent reviews of monoliths please see [336, 337]. Unlike diffusion-limited traditional bead-based chromatography media, monoliths offer capacity independent of flow rate. As larger through-pores are required to enable convective flow, the available binding surface area per unit volume reduces. This does not come into play for larger biomolecules such as DNA, virions, and IgM, which are diffusion limited [338, 339]. For most proteins, however, capacities are significantly lower. The lower capacities are a distinct disadvantage for binding steps, but of lesser concern for flow-through steps where impurity levels are low after affinity-based capture steps. Recent studies have revisited the use of monoliths for Protein A purification [340]. Although capacities are at least four- to eightfold lower as compared to modern traditional chromatography media, this lack of capacity may be overcome through similar, albeit more costly, solutions explored for traditional chromatography media. Optimization of the linker chemistry, ligand structure, and domain repeat number could serve to close the gap. The use of synthetic biomimetic ligands for a more cost-effective

disposable solution was also re-evaluated [341]. Although dye-based affinity chromatography has a longstanding history in the purification of biologics [342], considerations of ligand leachate [343] and lower affinity and selectivity have resulted in a lower market share of this type of ligand.

As discussed earlier, monoliths and membrane chromatography media have had greater success in establishing a presence in the domain of large proteins, large protein complexes, DNA, virus-like particles (VLP), and virion purification. A distinct advantage of membranes and monoliths is the low shear rate in spite of high velocities. As vaccines and viruses are often less stable, the improvement in throughput is also advantageous. Lastly, the manufacturing scale for large biologics is often smaller as well. Some of the concerns and limitations around scale-up and reproducibility of monolithic stationary phases are lessened if the final scale is relatively small because of the high potency of these products. Scale-up should not be taken lightly when considering a monolith process step. Parallel operation of monoliths has been described as a means to overcome this limitation [336]. Although capacities in flow-through mode are satisfactory, large-scale processes require bed volumes of 20–50 L. To obtain well-defined, interconnected pores within the monolithic structure and reproduce the same structure at large scale are far from trivial [344]. Although many studies have been conducted to manufacture large-volume monolithic adsorbents effectively, to date none have been found to be robust and economical. Aside from concerns about pore size distribution and high production cost, the risk of fractures should also be a consideration. The largest commercially available monolith currently produced is an 8-L tubelike monolith with a thickness of less than 5 cm produced by BIA separation. Tube-type monoliths are commercially available whereas rod-type monoliths are not, though they offer distinct advantages. A rod shape enables longer retention and enhanced interactions as compared to short or thin monoliths. The largest rod-shaped monolith described was less than 1 L in volume. Improvements in analytical techniques and real-time monitoring of polymer casting may eventually improve upon the sparsity of studies on the structural homogeneity of the monolithic bed [345]. In addition, the previously cited work on 3D printing could also prove helpful in overcoming some of these issues [48]. Such 3D printing has not been as extensively explored in DSP as in other industries. Applications have been explored in sensor technology [346–349], scaffolding [350–354], material property studies [355–359], and enrichment and purification of cells and viruses [353, 360–362]. Improvements to the throughput, resolution, and capabilities of 3D printing [355] have been made, and cost continues to decrease [363–365]. As significant improvements lead to cost reduction, a better understanding of scale-up and resolution improvements are needed to envision a future state where monoliths are simply printed rather than cast.

Integration of nanoparticles into monoliths and membranes could overcome some of the capacity limitations [345, 366, 367]. Because nanoparticles can move more freely in either monoliths or membranes, their small bead size does not cause back pressure constraints. Polymer grafting may offer another attractive approach to overcome capacity limitations [368–373]. Lastly, merging of the two

technologies may be even more advantageous, although since its prediction [374], prototypes have yet to emerge. One obvious advantage of both membranes and monoliths is that they do not require column packing and packing quality verification. Minimal preparation time to turn around a production suite between campaigns is of distinct advantage to improve time and throughput. Another potential solution to overcome the limitations of monoliths, membranes, and traditional bead-based chromatography media has also been described [375]. Near-colloidal suspensions of loose particles could be flow-packed in traditional chromatography columns. This approach offered much higher hydraulic permeability with porosity values similar to monoliths.

Lastly, large polymer based systems have also been explored [376–378]. Although many large pharmaceutical companies have shifted their focus from traditional small molecules to biologics [379], the difficulties of producing a biological consistently as compared to a synthetic molecule are far from trivial. Similarly, in the purification of biologics, true affinity ligands with high selectivity and low dissociation constants are usually biologics. Similar to biopharmaceuticals, biological ligands suffer from some of the same issues around production complexity, robustness, and cost. Ever since the eventual displacement of small molecules with biologics has been predicted, organic chemists have sought solutions to overcome some of the limitations of small molecules. Assessment of "druggability" of proteins with small molecules is a similar challenge to the concept of high affinity ligands for a specific class of proteins, as in both instances it is desirable to target cavities small enough to be accessible to small ligands, yet ideally surface bound with minimal or only temporary impact to the tertiary structure of the product [380–382]. The use of large polymers has been considered as a solution to mimic better the complex interaction of biologics. One prominent example is the synthesis and imprint of an anti-bee venom fully synthetic polymer antibody analogue [376]. This approach was effective in the neutralization of bee venom in mice. Polymeric binding systems better suited to cover larger surface areas of protein targets have been employed for products larger than bee venom. In one instance, a flexible polymeric system with high affinity for IgG was described [383]. One distinct advantage of the development of this system was the capture at slightly acidic pH and release at neutral pH. Initial variants were only available as free polymer and two-phase systems did show some promise (BMS internal results). Recent developments with polymer immobilized on chromatography beads also showed promise for more traditional purification approaches [384]. This opens the door to a fully disposable affinity capture with polymeric ligands either linked to or contained in monoliths.

4.3 Modeling in Downstream Processing (DSP)

Modeling can be an incredibly useful tool if used appropriately. As stated by George Box [385]: "Since all models are wrong the scientist cannot obtain a

'correct' one by excessive elaboration. On the contrary, following William of Occam, he should seek an economical description of natural phenomena. Just as the ability to devise simple but evocative models is the signature of the great scientist so overelaboration and over parameterization is often the mark of mediocrity." The reverse of the coin is, of course, oversimplification. As stated by Alfred North Whitehead "Seek simplicity but distrust it" [386]. A good model, whether statistical (empirical) or mechanistic, seeks balance and critical review to ensure relevance.

This section attempts to summarize the current state of modeling and its various applications in downstream processing, referencing several excellent resources for further reading. A few brief definitions are provided for clarity. A mechanistic model is a set of equations, based on known physical and chemical relationships. A mechanistic model is used to predict the behavior of a system. This approach is also sometimes referred to as a white box or first principles approach [387]. Mechanistic models are superior to statistical models, as the latter do not attempt to explain the why or how of the relationship of an input with an output (black box approach) [388]. Statistical models are more adaptable and can be foundational in understanding an underlying mechanism. In addition, because of their wide use, vast improvements in robustness have been made. Two points to consider when using statistical models: (1) large amount of data is needed for neural networks ($n > 100$) and (2) model predictions may be restricted to the observation space of the training data (i.e., extrapolation can be challenging, depending on the variation in the data used to calibrate the model). An example is the Bayesian regularization of neural networks (BRANN) [389]. This approach improves robustness while reducing the need for lengthy cross-validation. This approach can be used in QSAR modeling. It is difficult both to overfit and to overtrain when using BRANN. Models must be useful and practical; it is most practical to think of model types as tool sets in a tool box when it comes to problem solving. Recent approaches have made strides in combining both approaches with success which indicates that a tool box approach is warranted [390–393]. An excellent perspective on a knowledge-based approach and the integration of both mechanistic and deterministic approaches in process development has recently been published [394]. Although modeling has a longstanding history in industry, use of predictive modeling in process development is still the exception rather than the rule. It has been a work of decades to close the gap from primary protein sequence to predictive modeling of both stability profiles and process behavior and, as of yet, there is no closure. Black box approaches, such as evolutionary computing algorithms, have been successfully employed in chromatography optimization [50, 395, 396]. The implementation of genetic algorithms and neural networks, tools which are part of the evolutionary computing algorithm repertoire [397], mimic natural evolution to grasp trends and patterns in complex information.

Models focusing on the optimization and prediction of individual unit operations are most abundant. In chromatography, models are system-dependent but go back to a common theme. Current models focus on describing concentration in three phases: interstitial, pore, and stationary. To simplify, independence of

concentration transport from its cross-sectional position is assumed. The interstitial concentration is then dependent on time and axial position alone and affected by convection, diffusion, and interaction with the pore or stationary phase. The pore phase concentration is defined through an induced velocity, an axial dispersion coefficient, bead porosity, and the chromatography bead radius. An effective mass transfer coefficient can be derived from these two terms which models both pore and film mass transfer. A lumped model distributes concentration depending on porosity, although a general rate model splits the mass transfer coefficient into a pore diffusion coefficient and a film transfer coefficient. Lastly, to complete the model, an isotherm is added to model adsorption or desorption. Different models may be used, such as kinetic, multi-component Langmuir isotherms, or kinetic steric mass action (SMA) isotherms. For greater detail on these concepts, please refer to previously published work [398–401].

Because of their simplicity, the earliest publications focus on model protein systems. In one of the earlier publications, the impact of column regeneration on equilibrium and kinetic characteristics of the adsorption of albumin on AEX was studied [402]. Early on, it was clear that models require experimental calibration [403]. Productivity factors, such as yield and throughput, were often targeted, although conditions considered at the times were not as relevant to preparative chromatography because of small bead size and low titer feed streams. Principal component analysis (PCA), a deterministic statistical model, was found to be useful for the modeling of the separation of four model proteins by CEX [404]. A recent publication compared mechanistic and deterministic models for a three-model protein system [393]. The statistical model was not necessarily set up for success. Both the mechanistic and deterministic approaches showed distinct advantages and disadvantages. Speed and good prediction for simple correlations were the hallmark of the deterministic approach. In addition, the relative importance of each input for the response could be determined. The statistical model failed, however, in its prediction of linear gradient elution (LGE). However, some of the perceived failure is a misconception around the capabilities of deterministics of statistical models. It is well understood that predictions outside the explored design space are not feasible with DoEs. Even at the edges of the design space, predictability decreases as uncertainty increases. The mechanistic model was predictive outside the training data set, albeit at the cost of much higher experimental input. As a design space can easily be augmented, a bias in the comparison is evident The authors proposed a combinatorial approach moving forward as the empirical data were useful inputs of the mechanistic model validation as well. However, the authors believe that, if you choose a good set of informative experiments, the experimental effort can be very low using mechanistic models.

A desire for both robustness and a better understanding of HIC steps has led to several publications. The HIC step was tasked with the separation of HMW species from the monomeric product [173, 174, 405]. It was necessary to apply a binary, irreversible adsorption isotherm as a subset of the aggregate species bound irreversibly to the chromatography medium. Inclusion of this irreversible binding was critical in obtaining a predictive model as the irreversible adsorption strongly

affected selectivity and separation. One of the distinct advantages of this model was the ability to extend important relevant parameters, such as column packing quality, column scale, and adsorbent ligand density. Ligand density effects were only evident when explored in a weak binding regime at the point of product elution. Adsorption isotherm measurements were proposed as a tool to predict performance of new batches of chromatography resin tested under weakly bound conditions. Different models have been employed to describe and predict IEX. A series of publications focused on building a simple model to allow scale-up of linear gradients in CEX [406–408]. A lumped kinetic model was used to evaluate different CEX stationary phases [409]. The model was developed from measured retention factors, mass transfer parameters, and peak fitting under overloaded conditions to determine saturation capacity. The model proved predictive for yield and elution product concentration for a continuous multi-column process. The steric mass action (SMA) model was evaluated for either a set of gradient and frontal experiments or an inverse method for parameter estimation [410]. In this instance, the inverse method was found to be more predictive and to provide the best fit of model response and data. Modeling has also been used to determine best pooling strategies where the separation of monomeric antibody from high molecular weight (HMW) species was modeled with a general rate model and temperature dependent diffusion coupled to a SMA model [411]. This was found to be predictive at low loadings, but not high loadings and pH. However, it served sufficiently well to determine a robust pooling strategy to enrich for monomer. An AEX model determined the impact of electrostatic surface charge for deamidated product variants [412]. Metropolis Monte Carlo (MC) simulations provided a methodological framework for interaction analysis where electrostatic surface properties alone did not suffice. It was found that small distribution shifts had a big impact on retention times for different charge variants. The impact of salt and pH for IEX was explored in a mechanistic model [413]. Here the characteristic protein charge as a response variable of pH and the equilibrium constant was applied to the SMA model. The separation of a monoclonal antibody from product related HMW impurities was determined through a set of pH gradient experiments. The lumped rate model predicted separation of loadings up to 48 g/L. At preparative scale and with modern, high-capacity CEX resins, loadings of up to 120–150 g/L are feasible, indicating limitations to this approach. A deterministic model proved to be more predictive for higher loadings [286]. This model was capable of describing protein binding over broad ranges of protein and salt concentrations, which offers a suitable compromise until current, approximate mechanistic models are precise enough to predict industrial (high loading) scenarios reliably (Fig. 6).

One of the distinct advantages of this model was the ease of data generation. Batch isotherm data in combination with a systematic empirical interpolation (EI) scheme, a lumped kinetic model with rate parameters determined from HETP measurements for non-binding conditions, could be used to predict column behavior numerically. Two different modes of chromatography were assessed and successfully predicted. Membrane exchangers have also been modeled and a recent publication determined optimal parameters for purification of virus-like particles

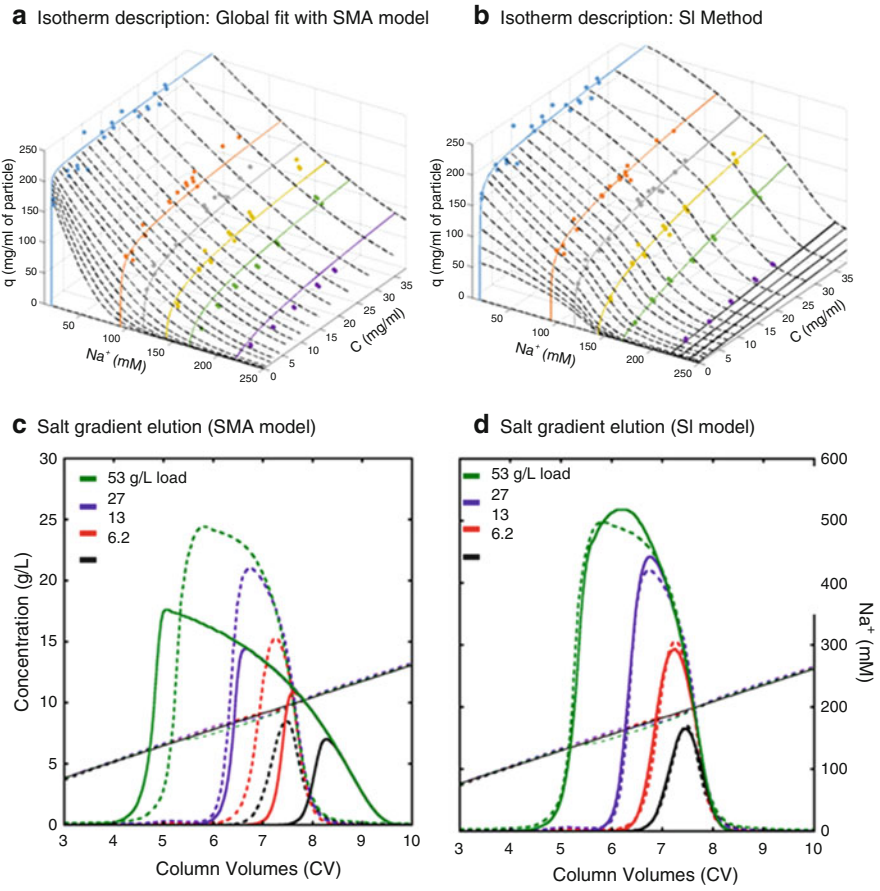


Fig. 6 (**a**) Isotherm prediction based on the steric mass action (SMA) model. (**b**) Isotherm prediction based on a systematic interpolation (SI) model. (**c**) Observed (*dotted lines*) and predicted (*solid lines*) elution behavior in a linear gradient (SMA model). (**d**) Observed (*dotted lines*) and predicted (*solid lines*) elution behavior in a linear gradient (SI model)

(VLPs) from Sf9 insect cells [306]. A radial lumped model in combination with a stoichiometric displacement model (SDM) isotherm was employed. SDM, which adds a solvent release term, and radial lumped models are better suited for modeling of membrane chromatographers and large molecules such as VLPs. A combinatorial approach of HTS and in-silico process modeling, simulation, and optimization allowed one to minimize the consumption of feed material. Although acceptable impurity levels were reached, recoveries were not as high as desired. This publication suggested that a better understanding of mass transport phenomena in membranes would be needed. Continuous purification processes have also been modeled [414, 415]. In one approach [223], a model was built of breakthrough experiments and then validated on a lab-scale multi-column system. A distinct advantage of this

approach was that the model lacked mathematical complexity. So its wider adoption may be more straightforward. Protein A performance has also been modeled targeting wash and elution pH specifically to minimize HMW formation [416]. An effective model required the foundational understanding of the DBC design space, which could be obtained either empirically or through modeling within process-relevant pressure flow constraints. Use of HTS was proposed to minimize column work and maximize data density. A lumped desorption-kinetic limiting model was able to simulate and predict packed column experimental outcomes and identify robust operating conditions. A pilot-scale confirmatory experiment served to ensure that larger-scale results matched predicted and small-scale results. Unfortunately, it was found that the design space for one antibody was not robust as it failed target impurity and recovery values at pH and conductivity control ranges that are impractical at preparative scale (± 0.1 units in pH and ± 1 S/cm).

Ideally, process development would shorten because of predicting chromatographic behavior based on physicochemical properties of a protein. First attempts at predicting behaviors based on properties have been published, although approaches taken vary. Quantitative structure–property relationships (QSPR) and quantitative structure-activity relationship (QSAR) models have been proposed early on [417]. QSPR and QSAR models of protein SMA parameters and free energy changes in IEC were modeled through a support vector machine (SVM), a regression technique for supervised learning models. Model proteins served to build molecular descriptors around physicochemical protein properties to describe stoichiometry, equilibrium, steric effects, and binding affinity in IEC. As a proof of concept, multiscale modeling was used to predict the chromatographic separation through use of isotherm parameters obtained from the QSPR models. Although this approach focused on single gradient separation, it could serve in theory to predict any preparative chromatographic separation. As an early attempt to simulate chromatographic behavior of proteins directly from their crystal structures, this was a first step toward going from primary sequence to purification with minimal process development investment. Different approaches, employing different models, were also described. One publication aimed at predicting IEX retention for different IEX resins [418]. A simplified colloidal model was employed, and insights on the advantages and disadvantages of this approach are provided elsewhere [419]. The distinct advantage of this approach was that protein sequence and stationary phase structural properties suffice to predict mAb retention factors as a function of pH and salt concentration. The effect of high mass loading was not studied here and it is not expected that this model would work well for high loading IEX steps because of limitations of this model at high concentrations [419]. With the advent of HTS, however, the same information can be obtained empirically within days and without the limitations of pH and concentration boundaries. Another publication employed molecular dynamics (MD) to model the retention of proteins in IEX [420]. The developed MD tool simulated interactions of proteins and adsorbers for a spectrum of protein and confirmed the validity of the model empirical with LGE experiments. Good overlap of model and experimental results was found. In addition, the main contributors to CEX binding consisted of arginine

and for AEX binding of aspartic acid residues. In another publication, protein sequence, structure, and dynamics were described by MD, not only to predict chromatographic separation but also with the goal of maximizing recovery of biological activity [390]. As seen before, sufficient granularity or resolution in the model was critical for predictive modeling of protein–ligand interactions. Here, modeling was simplified by studying insulin variants. As insulin is very small at 5.8 kDa, less computing power has to be invested to model the peptides surface properties. Affinity chromatography for mAbs was also modeled based on the structure and the free energy of interaction of the antibody and affinity ligand [421]. However, the effect of the environment (buffer solution, spacer, support matrix) has not yet been taken into consideration, although a procedure to do so has been outlined.

Interesting empirical approaches to gain a more fundamental understanding of protein adsorption were also recently described [422, 423]. Single molecules could be tracked to determine the role of pH for protein separations. This allows in situ study of molecular dynamics at the liquid–solid interface that determines the ensemble chromatographic elution performance. Translational surface diffusion was observed and it was recommended that good models would also consider translational surface diffusion for silica-based adsorption. Further studies elucidating differences in protein interactions driven by secondary and tertiary structure were also proposed.

Holistic process modeling in the spirit of quality by design (QbD) has also been published. One publication analyzed the impact of an IEX and HIC step and feed adjustments between the two steps [424] A CHO-expressed IgG1 product was analyzed in terms of pH and salt environment impact on product and impurities. A mathematical model encompassing kinetic and thermodynamic parameters was used to optimize operating conditions for column loading and chromatographic elution in the integrated process. Baumann et al. [425] integrated cell culture and downstream processing through a set of micro-scale cultivation experiments and chromatography modeling. This was served to optimize the entire process as a whole, which ensured that overall process output rather than separate optimization of titer and process was considered. It was found that the highest titer did not correspond to the most favorable overall outcome, stressing the importance of an integrated optimization approach. Although a model product expressed in *Escherichia coli* was employed, its approach is universal enough to foresee its implementation in industrial processes. A second paper considered integration of IEX downstream process optimization [426]. A flow sheet approach was taken based on salt gradient elution profiles and peak cutting for two IEX steps. The concerted approach performed better with the same time and effort investment, although a drawback seen was the inability to compute for the two columns in parallel. Feed adjustment between steps was not taken into consideration, which caused difficulties, and it was recommended to integrate ultra/diafiltration, dilution, and mixing unit operations through, for example, a black box model. Issues of suboptimal feed adjustments could however be modeled. An in-house software toolbox was developed to accomplish this. Modeling of the entire DSP is feasible in

theory, as the software can adapt to larger numbers of proteins and scale-up, so that it offers general utility in process modeling.

Modelling of plant utilization and its optimization has found applications as well. One group has published different approaches [53, 145] offering several different approaches to maximize facility usage. De-bottlenecking and cost considerations are stressed as well as visual aids to understand process robustness better. A decision-making tool also indicated that both affinity capture and buffer hold capacity would become bottlenecks first in legacy facilities. One of the main drawbacks of most commercially available as well as in-house facility modeling tools are accurate figures of cost. It can be quite difficult to obtain accurate figures which, in turn, lessens the accuracy of modeling where process economy is concerned. Computational fluid dynamics (CFD) is another useful tool in the decision-making process for biological processes. In downstream processing, temperature profiles, column and pump hardware impact, packed resin permeability impact, shear stress and channeling and vortexing could be esteemed and predicted by CFD [427]. Lastly, modeling has been utilized to gain a better understanding of in-process stability. Empirical models have been proposed to predict in-process hold times better [428]. Molecular simulations have also been employed [429]. The free energy of pairwise mABs association was studied with a conformational sampling algorithm and a scoring function. Weak interactions were explored with a computational method. The free energy landscape was explored with a steric interaction component, electrostatic interactions, and a non-polar component. Two model antibodies with the similar IgG1 backbones but small sequence differences were evaluated for differences in viscosity and propensity to aggregate. The simulations were found to be in good agreement with empirical observations and this model mAb can provide insights on the self-association responsible for bulk solution behavior and aggregation, important concerns in the manufacture of mAbs.

5 Concluding Remarks and Future Direction

Revisiting the theme of three key questions in understanding the intent of this review, the reader is cautioned that viability of a technology is not only dependent on the actual utility of a technology, but, as highlighted previously, a certain critical mass of early adopters or innovators is also needed to move a technology past the initial chasm. Once moved past its critical mass, the merits of a technology and the advent of competing, emerging technologies are the main drivers for success or failure of disruptive technologies. With that said, demand for high quality biologics should continue to increase in the coming decades and disruptive technologies are needed to help overcome some of the challenges. The boundary conditions, however, can change substantially. Amounts to be produced and the number of products will increase. Additionally, there will be fewer or even no blockbuster biologics because of a shift toward stratified medicine. Many of the current blockbusters will be “running out of patent” during the next few years. The trend toward stratified

therapeutics will support a change in plant design, aiming for highly flexible multipurpose facilities for small production volumes. Such a development will push innovation not only in the development of single-use technologies but also in the development of non-chromatographic, continuous, and flexible downstream operations. The implementation of new separation technologies on an industrial scale require significant investments in development, scale-up, and validation, including associated risks. To bridge the gap, innovations need to announce themselves by distinct increases in efficiency and cost reduction. Careful evaluation to avoid design-in of a non-sustainable technology continues to be critical. A successful, balanced evaluation requires a multidisciplinary team and an inclusive approach. To reduce production volumes, the need for facility flexibility and faster turnarounds increases. This can lead toward implementation of disposables in manufacturing, continuous processing, and dedicated but decentralized manufacturing concepts in containers.

Consequently, process development needs to adapt and truly integrate USP and DSP development. Cost increases of this approach toward integrated process optimization are offset by gains in overall process improvements. Integration of downstream unit operations reduces feedstream and processing adjustments. An integration of ion exchange and hydrophobic interaction chromatography would, for example, permit the use of only one buffer system for both operations [430]. Further trends in purification technology include other non-chromatographic processes such as membrane adsorption, crystallization, precipitation, or aqueous two-phase separation. Utilization of these previously difficult-to-optimize technologies is bolstered through high-throughput screening, modeling and in-depth understanding of the design space. Predictive biopharmaceutical process design will gain importance in process development as well through improved commercial viability. Furthermore, a strategic shift in biomanufacturing toward continuous processing along with SUS is gaining popularity, enabling companies to produce lower cost drugs with increased flexibility and to enable on-demand manufacturing of multiple products. This has created increased interest in the use of membrane adsorbers but these systems currently have much lower binding capacities than traditional resins and need significant improvement before commercialization. Advances in SUS can lead to its increased implementation in biopharmaceutical production. At present, single-use product lines already encompass a complete facility from storage bags to chromatographic columns, enabling a holistic, single-use, downstream process. Current and future research continues to address limits in scale, standardization, validation, and interactions of disposables and process, a major bottleneck in the implementation of a fully single-use process up to now [298]. Establishment of single-use membrane adsorbers, moving bed chromatography, countercurrent chromatography, two-phase systems, and novel chromatography resins is already on the horizon. Another new approach that will play a major role in DSP is quantitative structure–activity relationship (QSAR) modeling. Data analysis methods and statistics are applied to develop models that can accurately predict biological activities or predict the chromatographic separation of proteins based on their structures [431, 432].

Economic pressures coupled with the desire to gain access to the emerging markets make process innovation imperative [298]. The convergence of a growing interest in academia and industry, maturing of enabling commercially available technologies, and encouragement from regulatory bodies continue to drive bioprocessing toward process innovation and conversion of bioproduction from batch to continuous processing. An end-to-end bioprocessing platform offers a novel enabling solution to address the inefficiencies of the current non-standardized, large, inflexible, and low throughput facilities. Inherently, continuous flow processes are more conducive to process optimization and control, particularly with respect to steady-state product quality. Further, the end-to-end bioprocessing platform is lean, flexible, and portable, facilitating implementation in the emerging markets throughout the world.

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Fully Disposable Manufacturing Concepts for Clinical and Commercial Manufacturing and Ballroom Concepts

Berthold Boedeker, Adam Goldstein, and Ekta Mahajan

Abstract The availability and use of pre-sterilized disposables has greatly changed the methods used in biopharmaceuticals development and production, particularly from mammalian cell culture. Nowadays, almost all process steps from cell expansion, fermentation, cell removal, and purification to formulation and storage of drug substances can be carried out in disposables, although there are still limitations with single-use technologies, particularly in the areas of pretesting and quality control of disposables, bag and connections standardization and qualification, extractables and leachables (E/L) validation, and dependency on individual vendors. The current status of single-use technologies is summarized for all process unit operations using a standard mAb process as an example. In addition, current pros and cons of using disposables are addressed in a comparative way, including quality control and E/L validation.

The continuing progress in developing single-use technologies has an important impact on manufacturing facilities, resulting in much faster, less expensive and simpler plant design, start-up, and operation, because cell culture process steps are no longer performed in hard-piped unit operations. This leads to simpler operations in a lab-like environment. Overall it enriches the current landscape of available facilities from standard hard-piped to hard-piped/disposables hybrid to completely single-use-based production plants using the current segregation and containment concept. At the top, disposables in combination with completely and functionally closed systems facilitate a new, revolutionary design of ballroom facilities without

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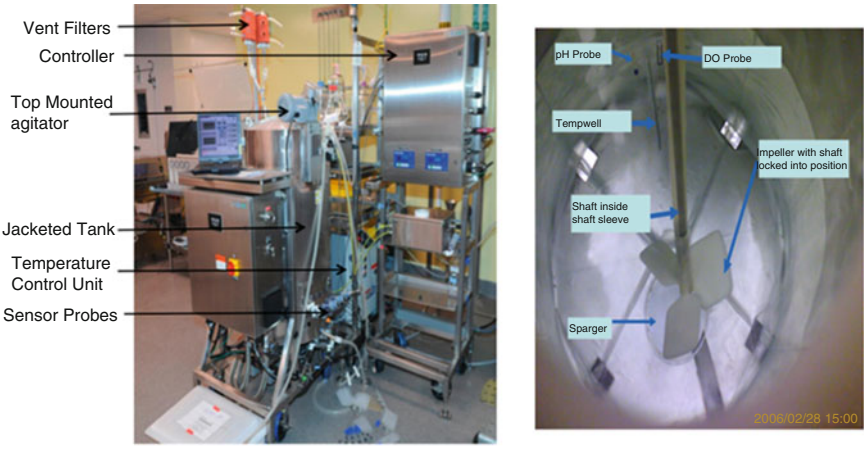
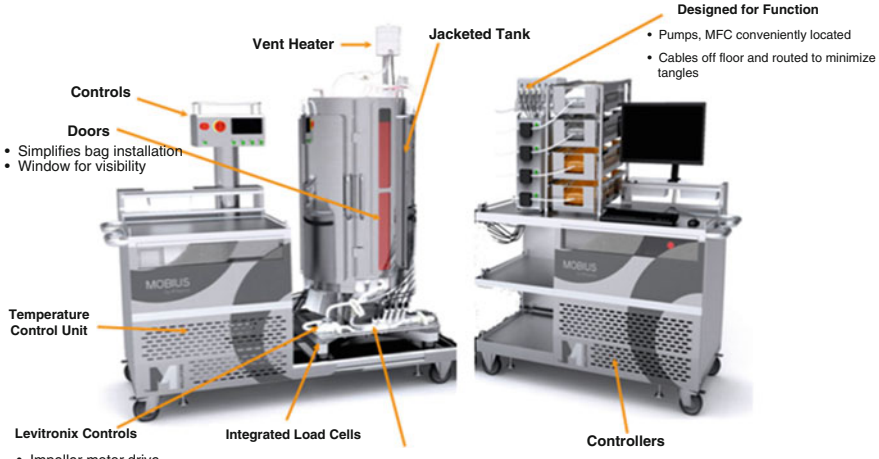
or with much less segregation, which enables us to perform good manufacturing practice manufacturing of different products simultaneously in unclassified but controlled areas.

Finally, single-use processing in lab-like shell facilities is a big enabler of transferring and establishing production in emergent countries, and this is described in more detail in 7.

Graphical Abstract



Courtesy: Thermo Scientific



Courtesy: Merck Millipore

Keywords Ballroom facilities, Disposables, Disposables-based manufacturing, E/L, Facility of the future, Monoclonal antibodies (mAbs), Single-use bioreactors (SUBs), Single-use equipment, Single-use systems

Contents

1 Introduction

The demand scenario is changing for many individual biopharmaceutical drugs such as monoclonal antibodies (mAbs). Market volume per drug decreases with the implementation of personalized medicine resulting in drugs for specific, high-responder subsets of patients,. In addition, increasing fermentation titers of up to 10 g/L for mAbs are leading to smaller fermentation volumes being necessary to accommodate individual biologics market demands. As a result, drug development, including production of clinical supply (up to 2,000 L) and lower volume commercial supply, now involves replacing current typical 10,000- to 25,000-L hard-piped, standard steel-based production plants. In this chapter, the status of single-use unit

operations, including benefits and risks of single-use technologies compared with highly automated, hard-piped processing, are addressed. This chapter also compares and discusses different state-of-the-art facility designs.

2 Status of Single-Use Manufacturing Unit Operations

Nowadays almost all processing steps for biologics production can be done in disposables up to a bag volume of around 2,000–3,000 L, except centrifugation, which still needs classical hard-piping. Such disposable-based unit operations include mixing/holding/distributing culture media and buffers, cell seed expansion and production fermentation, cell removal by depth filtration, disposable chromatography system and columns, and ultrafiltration (UF)/diafiltration (DF)/virus filtration. Many single-use units have already been an integral part of bio-manufacturing for a long time through integration into hard-piped setups (filters, etc.). However, the real innovation toward completely disposable processing came with the development of single-use bioreactors (SUBs), where now several systems up to a fermenter volume of maximally 3,000 L are commercially available, with 2,000 L being more commonly deployed. The following describes a snapshot of the currently available disposable-based unit operations using a standard mAb production process as base, including disposables-based options for bulk freeze and storage.

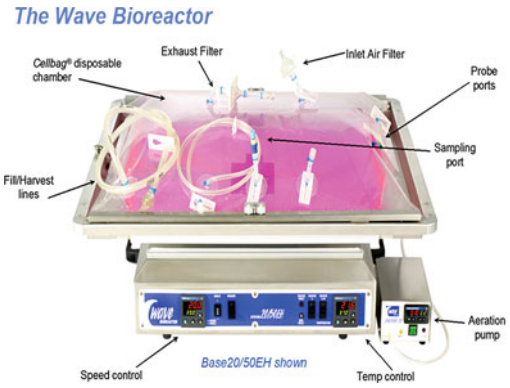
2.1 SUBs

SUBs are being used mainly to produce mammalian cells of sizes up to 2,000–3,000 L, with 2,000 L being more commonly deployed in industry. The bioreactors are available with a rocking motion and as stirred-tank bioreactors (STBs). STBs are available in sizes ranging from 10 L to 3,000 L for good manufacturing practice production and the rocker systems can scale down to 300 mL. General considerations for rocker-style or STBs include mixing capability and mass transfer (k_La), maximum/minimum working volume, impact of leachables on cell culture growth (testing with sensitive cell lines should be performed before film qualification), and robustness of the film under various stress conditions. It is also critical to ensure that the bag can handle the airflows required without breaking. It is critical to have a pressure sensor hardwired to the airflow in order to allow reduction in airflow if the pressure exceeds the bag pressure rating.

2.1.1 Rocker Bioreactor

The rocker-style bioreactors are simple and easy to use and work well for transient transfections, seed train operations, and initial inoculation. They consist of a jacketed platform/tray that holds the bag and sits on a motor. The agitation is non-invasive and is achieved by back-and-forth movement of the platform based on rocking angle and speed of the motor, which can be varied based on mass transfer requirements of the process. Most rocker bags come integrated with optical pH and dissolved oxygen (DO) sensors and can be controlled via DeltaV, UNICORN, and so on. Because pH and DO are critical to cell culture, it is important to test the performance of these sensors and to ensure the rocking motion does not induce noise and affect performance of the sensors. In some cases, the probes are inserted through a sleeve. It is critical in those situations to inflate the bag before inserting the probe through the sleeve to avoid breaking or crimping the sensor or probe. The temperature is controlled via a jacketed blanket; the temperature sensor is non-invasive as part of the platform tray. In certain systems, bags are placed directly on top of the heating blanket. However, it is not a recommended method because loss of temperature control can result in bag heating, which can cause bag leaks. The rocker-style bioreactors have the option to be integrated with load cells as well to record weight, which would be critical for some operations, such as perfusion.

The rocker bags can be used to perform multiple cell culture operations using the same bag because of low working volume capability. The system can be used to inoculate at low volume for low-density steps and then to add media for subsequent steps. The rocker system works well up to 50 L, and cell culture performance is similar to that of STBs. However, rocker systems are not recommended beyond 50 L because of mechanical constraints and k_La limitations at larger volumes (Fig. 1).



Courtesy: GE Healthcare

Courtesy: Sartorius-Stedim

Fig. 1 Rocker bioreactors

2.1.2 STBs

STBs are used to produce cell culture and are similar to their stainless steel counterparts, with the exception that the bioreactor comes in a bag. The bags come integrated with filters, sensors (pH, DO, temperature), an and agitator motor, which is housed in a jacketed (for temperature control) stainless steel vessel for support. The system is equipped with mass flow controllers for oxygen and pH control, and can be controlled via DeltaV, Rockwell, or other automation control systems. The aspect ratio for commercially available SUB is similar to stainless steel vessels (1:2).

Important considerations for SUBs include type of agitator, location of agitator, air flows, and agitation speed, among others. The bioreactors are available with either top-mounted or bottom-mounted agitator systems. One of the benefits of using SUBs with a bottom-mounted agitator is lower working volume, which allows inoculation and production in the same vessel, reducing the number of vessels, labor, and operating time. However, the design of the bottom-mounted agitator varies and can affect cell shear differently. Hence, it is critical to test the system for the intended application.

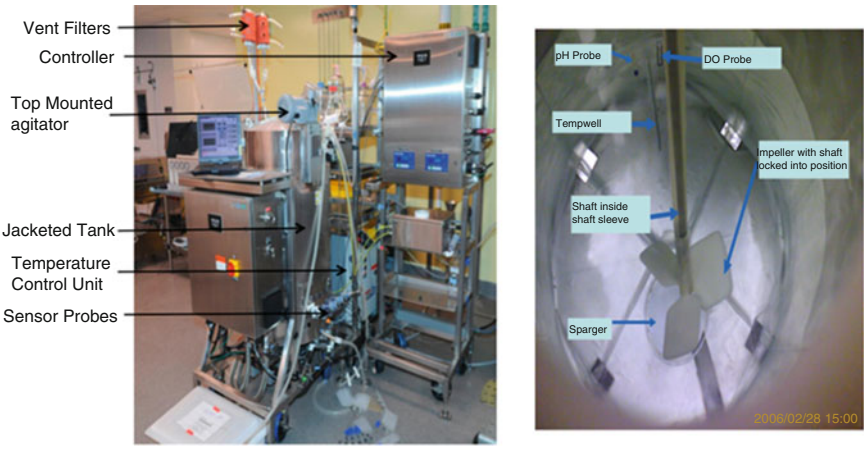
Mixing capabilities of each bioreactor should be evaluated by examining the interfacial mass transfer coefficient (k_La). Mixing can be optimized by varying agitation speed and oxygen/air flow through the system. It is critical to test k_La and CO_2 stripping to ensure the system meets the process needs. If replacing a traditional stainless steel vessel with an SUB, it would be beneficial to compare k_La and CO_2 stripping along with cell culture performance between the two systems.

Other considerations for an SUB include minimum and maximum liquid working volume, chamber diameter, overall reactor and fluid geometry, baffles, types of orifice such as open pipe, position of the impeller, blade count, blade pitch, impeller diameter, and agitation rate. The design of the bag is another critical aspect. The outlet tubing for harvesting should be at the bottom of the bag to ensure the entire cell culture is harvested. It is important that there are no sharp corners or folds in the bag, which can entrap cell culture or result in poor mixing.

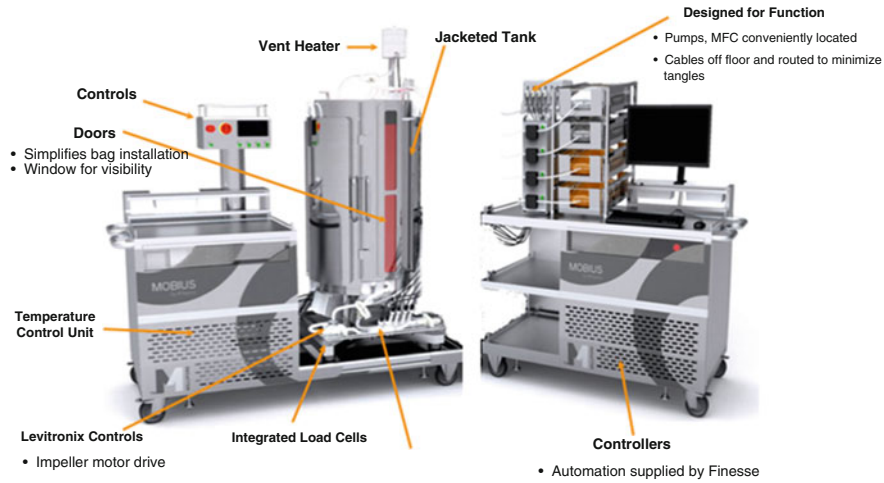
One of the drawbacks of SUBs and mixers is single sourcing as the hardware is tied to the consumables. Some work has been done to eliminate single sourcing issue [1]. The work shows similar performance in Unican compared to performance in dedicated vessels (Fig. 2).

2.2 Single-Use Harvesting

Centrifugation is used to separate cell debris from the cell culture fluid (CCF). The supernatant for mammalian cultures is further processed through depth filters, and the solid cells and cell debris are discarded. The filtered pool from depth filtration is further processed through sterile filtration to obtain harvested CCF.



Courtesy: Thermo Scientific



Courtesy: Merck Millipore

Fig. 2 Single-use bioreactors

For single use, there are currently two equipment options: single-use centrifuge followed by single-use depth filters or harvesting directly through single-use depth filters. The latter is more prevalent because of the lack of commercially available large-scale single-use centrifuges.

2.2.1 Single-Use Centrifuge

The disposable centrifuges are automated and contain disposable conical shaped chambers or single-use separation module and tubing sets to clarify CCF in a fully-

closed disposable format. Some centrifuges rotate about the horizontal axis instead of the vertical as do traditional centrifuges. The unique rotation allows for a flat bed of particles to form at the tip of each cone-shaped chamber rather than a slanted bed of particles that forms in traditional centrifuges. In addition, the fluid flow force and centrifugal force oppose each other, resulting in a bed of particles that remains in suspension through the centrifugation process. The concept is that this reduces cell shearing during centrifugation. Once the chamber is filled with particles, there is an option to wash the chamber to remove any product that exists in the interstitial space. After the wash cycle, the flow is reversed and the chamber is emptied prior to beginning the next cycle. The step would be equivalent to a discharge cycle on traditional disk-stacked centrifuges.

In another type of system, feed is pumped to the bottom of the separation module at its axis of rotation. The single-use module is mechanically sealed within an aluminum bowl and rotated at high speeds to generate large g-forces (up to 3,000 g at 6,250 rpm). Solids in the feed are separated via this force and collected at the perimeter of a flexible chamber and clarified liquid is pumped out at the top of the chamber.

Peristaltic pumps are typically used for feed inlet and the fluid path is controlled by pinch valves. The systems can be run either manually or in a fully automatic mode where volumes, flow rates, and bowl speed must be specified. The tubing manifolds may be sterilely welded to upstream and downstream unit operations or can come supplied with any number of sterile connectors.

Some of the key considerations to assess single-use centrifuges include processing time, particle size, turbidity, depth filter, and sterile filter area required post-centrifuging (Fig. 3).

2.2.2 Depth Filters

Depth filters in general are coarse, high capacity filters that retain contaminants primarily within tortuous passages that run along the vertical axis. Typical depth filters are composed of layers of cellulosic fibers and diatomaceous earth held together with a resin binder. The individual layers filter out contaminants predominantly by size-based capture, retaining components that are the same size or larger than the nominal pore size of the filter. The resin binder typically imparts a positive charge to the media, helping the filter retain smaller negatively charged molecular components such as DNA and RNA. Antibodies produced in the cell culture are not retained because of their small size and weak charge.

The depth filters typically require flushing with water or other liquid buffers to wash away as much of the inherent, loose organic material as possible from the filters prior to pumping CCF across the filters to collect the effluent (filtrate). The filters can be blown with pressurized air to push liquid out of the holdup volume of the filter capsules. Blowing down the filters serves to increase the yield of the filtration and reduce the volume of liquid that may spill from the filters during clean

Courtesy: Unifuge *Courtesy: kSep*

Fig. 3 Single-use centrifuge

up. The filters must then be removed from their holder and disposed of in the appropriate manner.

Depth filter performance should be tested separately if they are used in combination with a centrifuge vs direct filtration of CCF. A few considerations include lysis across the disposable harvest, product quality within the required range, processing time, and pressure drop across the filters. Excessive filtration time can negatively impact the product quality.

The disposable depth filters are typically available in different media grades within self-contained modules to meet application needs. In spite of modular units, a holder is still required which forces each capsule together and gaskets around each opening ensure a solid, watertight seal.

The depth filters can be used to harvest directly from an SUB up to 2,000 L in size. However, the number of modules required at 2,000 L is high and analysis should be performed with respect to finance, waste, footprint, etc. In some cases it might still be cheaper to use depth filters for a fully disposable facility instead of dealing with building a CIP/SIP (cleaning in place/steaming in place) facility just for centrifuging. Other technologies, such as precipitation/flocculation, can be considered to reduce depth filter area required.

The harvest is currently not closed but can be modified to a closed system if required by using aseptic connectors on tubing manifolds and depth filter capsules (Fig. 4).

Courtesy: PALL

Courtesy: Merck Millipore

Fig. 4 Depth filters

2.3 Single-Use Chromatography

Disposable chromatography systems can be used to purify protein to support up to 2,000 L of bioreactor harvest depending on the titers, column loading capacity, and flow rates. The disposable chromatography system consists of flexible flow kits supported by a network of pinch valves, single-use pump heads or peristaltic pumps, and single-use sensors, and can be used with traditional columns or single-use pre-packed columns. The system is supported by operating software such as Unicorn, DeltaV, and Rockwell, allowing the operator to monitor and control processes, as well as store and analyze generated data.

The flow kits are comprised of single-use tubing, connectors, and single-use sensors (pressure sensors, UV, pH, conductivity, temperature, and flow). The single-use flow kits come pre-gamma sterilized, pre-calibrated, and are typically available in high- and low-flow configurations to allow a range of purification of mass without compromising accuracy. It is important to note that the hardware is used to hold and operate the single-use components and is not itself single-use. Only process contact materials are designed to be single-use.

The system can be operated in a closed operation by modifying the flow kits with aseptic connectors. The buffer bags on the inlet can be connected aseptically to the flow kit. Similarly, on the outlet, the tubing manifold can be connected to product and waste lines using aseptic connectors. Closed processing can be critical if it is desired to operate in a lower classification room or operate upstream and downstream operations in one room. Closed processing can also alleviate the probability

of bioburden and endotoxin, resulting in reduced sampling and better controls. The columns can also be connected to the manifold using aseptic connectors. However, it is only possible if the same column is going to be used for multiple cycles. If the flow kit or column has to be changed, one might require to have manifolds with aseptic connectors or use connectors that can be used multiple times.

The flow kit can be designed to have few connecting points to reduce operator error as well as to reduce set-up time. It is critical to have pressure sensors before and after column to monitor any backpressure issues and to ensure smooth operation similar to traditional systems. It is critical to test the pressure rating of all single-use components that are part of the flow kit such as tubing, pressure sensors, and connectors. The component with lowest pressure rating should have its rating assigned as the pressure rating for the entire manifold.

The commercially available disposable flow and pH sensors are limited in accuracy and range. As flow sensor error can be as high as 10% at extreme ranges, the systems can be operated using pump calibration if the system is to be operated at the extreme end of the flow kit range to prevent compromising accuracy. A traditional pH sensor can be used as a single-use sensor to avoid cleaning validation because commercially available disposable pH sensors are limited by pH range.

Because of the limitations of commercially available single-use components and column sizes, an operating system with multiple cycles can be a viable option if it can be achieved within pool stability hold times (Fig. 5).

Fig. 5 Disposable chromatography columns

2.4 Single-Use Tangential Flow Filtration

Tangential flow filtration is a rapid and efficient method for separation and purification of antibodies. This method is widely used to concentrate antibodies (UF) and also for buffer exchange (DF). Separation can be achieved by means of a porous UF membrane that is classified by molecular weight cut off and usually ranges from 10 to 50 kD.

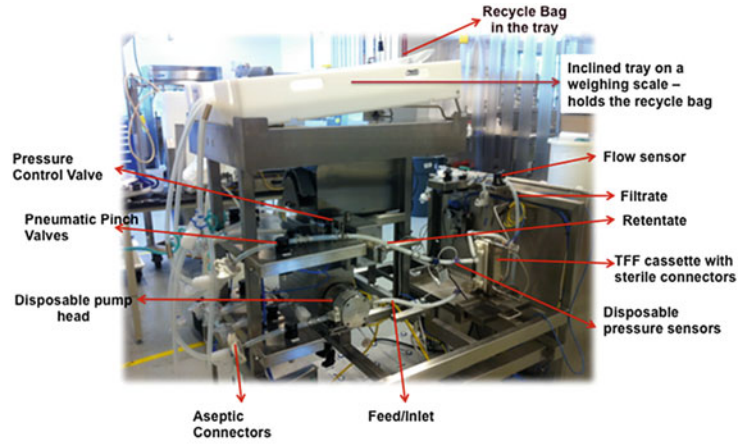
Single-use TFF is available for purifying protein from 20 g to 1 kg. The system hardware is comprised of pinch valves, recycle tank, pumps (feed and buffer), sensor transmitters, and tubing manifolds (feed, retentate, and filtrate). Similar to other single-use systems, hardware is used to support various consumables, which are single-use. The manifolds are integrated with single-use pressure sensors, flow sensors, conductivity, temperature sensors, and temperature control valves on the retentate line. The manifolds come pre-gamma sterilized and sensors are pre-calibrated similar to other single-use systems. The system can be used with different size tubing and bags to allow operation at varying flow rate/volumes without compromising accuracy at extreme ranges. This provides the flexibility to use the same hardware for multiple volume ranges.

The TFF cassette can be used in single-use or reusable format. The manifolds are connected to the cassette and recycle bag using aseptic connectors to ensure a closed process. The closed processing is important when dealing with potent molecules to prevent technician contact with any potent molecule. Additionally, closed processing can alleviate any bioburden issues and allow processing in a lower grade room.

Few critical elements include ensuring there is sufficient spacing/tubing length between different single-use components to avoid stress, which could result in leaks. It is critical to select the correct size tubing, connectors for the flow rates, and pressure required for the process. The single-use components should be tested for pressure rating and the component with the lowest rating should have its rating assigned to the manifold. The system should be tested post-gamma radiation, even for R&D evaluation, as few components can function differently pre- and post-gamma radiation.

The single-use components are typically not designed to handle pressure greater than 40 psi and should not be used to test TFF cassettes using the skid. The cassette should be tested separately if required or the system should be not be exposed to more than the lowest pressure rated component.

Another critical consideration is bag design for the recycle tank. It is important that the feed and retentate lines are not too close as this can result in short circuiting. When using a single-use mixer, the return should be either from the bottom or a dip tube should be used if the return port is at the top. The top port without a dip tube results in foaming and protein shear (Fig. 6).



Courtesy: PALL Lifesciences

Courtesy: Merck Millipore

Fig. 6 Single-use tangential flow units

2.5 Single-Use Mixers

Single-use mixer sizes range from 10 L to 2,000 L. They can be used for both upstream and downstream applications for buffer make-up, media make-up, pool holds, pool adjustments, etc. The mixers can have either top-mounted or bottom-mounted agitator systems. In both cases, the bags come with agitator built in. The agitator speed can be adjusted using the control box on the mixer. The mixers can be used with or without temperature control, the mixer being jacketed for temperature control.

It is critical to understand the impact of agitator style and agitator speed on protein shear and particle formation. Most of the bottom-mounted agitator systems

Fig. 7 Single-use mixers

are magnetically coupled impellers [2]. The bearing design is a critical aspect for designing/evaluating single-use mixers as a narrow clearance between the bearings may shear or grind the protein and generate particles [2]. The distance between the impeller and the drive unit is important in determining its impact on protein shear and particle formation.

When testing mixers it is critical to test not only liquid in liquid but also solid in liquid. It is important to challenge the system to the extremes of the operating range. The mixing time is dependent on agitator speed, operating volume, and viscosity. The mixing studies should be performed at both minimum and maximum operating volumes. Additionally, there should be a bottom port to pump out liquid after mixing.

Rocking systems can also be used as mixing vessels for small volumes. Rockers are equipped with temperature control and the rocking motion provides sufficient gentle mixing. However, it might not be sufficient for high-viscosity liquids (Fig. 7).

2.6 Single-Use Bulk Freeze Systems

Cell culture and many protein purification unit operations have now become fairly well-integrated into many traditional processes. One area of increasing focus that is becoming better established is the use of single-use bags for bulk freeze storage and shipping applications.

The increasing focus from both end users and vendors is now on bulk freeze systems, the next area of development and innovation for single-use manufacturing

lines. The bulk drug substance (BDS) containers for the transportation and storage of active pharmaceutical ingredients have been well-integrated into clinical operations. However, there are increasingly strong businesses as well as quality drivers to introduce single-use BDS containers into commercial operations. Some of the key drivers for the use of single-use bulk freeze systems vs steel components are the following:

- Reduce the risk of contaminating residuals between uses
- Reduce costly and time-consuming cleaning, steaming, autoclaving, and validation efforts
- Streamline logistics for receipt of BDS at fill sites (ease of storage and receipt of common containers)
- Reduce infrastructure (eliminate the management of stainless steel assets globally)
- Reduce resources and staff (eliminate the management and maintenance of large fleet of stainless steel tanks)

Although single-use applications may seem well-suited for most downstream applications, the use and fit of the technology has to be carefully considered to ensure that the material of construction (MOC) (of the BDS bag) is compliant with the following:

- Can the bags and/or shippers holding the bags meet domestic and international shipping standards?
- The need to control temperature conditions around the bag or in the shipper system
- A critical review to determine the compatibility of the film with operation at the low temperatures at which the BDS is shipped
- Chain of custody – isyour company in control of the shipper and its condition (temperature/time) from the plant to the fill destination?

Drivers for change- shipping and supply chain aspects.

The bulk freeze-thaw process entails complex operations that are time-sensitive and can be logistically challenging. However, the use of BioProcess containers (BPCs) provides flexibility that is increasingly important in today's versatile and fast-changing operational arenas. Incorporating a freeze-thaw process with multiple locations is an undertaking that requires experienced resources, capital, and a well-executed plan to manage the technologies. The routing and management of reusable stainless steel vessels in the traditional freeze-thaw process requires a large synchronized operation that is cumbersome and costly. A deviation in one part of the cycle can propagate delays throughout the entire operation. This can consume multiple resources and create long delays. Incorporating single-use disposable equipment helps break the chain of delays and reliance on specific tank manufacturers [3]. Having a source of ready-to-use single-use freeze-thaw vessels minimizes the risk of delays and contamination of the bulk freeze process. The use of single-use technology in the bulk freeze supply chain can lead to smooth and

efficient operations, at the same time minimizing the costs associated with reusable stainless steel systems.

Bulk freezing, transfer, and storage are important steps that ensure the final product is safely handled, stored, and promptly delivered to fill–finish sites and eventually to patients [4]. The vast majority of manufacturing for bulk freeze applications are still mainly comprised of steel tank systems. There are, however, several large disadvantages for stainless steel bulk freeze-thaw systems.

Stainless steel systems must be maintained in a clean state both pre-use and post-use. This requires costly and labor intensive cleaning and inspection. Part of this process also involves re-passivation of the steel interior surface to maintain a non-reactive contact layer for the BDS. The average large biotech company employees labor for many hours to maintain a steel tank's integrity via testing and costly upkeep of gaskets and seals. Looking after the multiple different sized tanks and the associated configurations that fit a particular plant or unit operation is challenging, not to mention the shipping validation required to ensure that the steel tanks are in a state of microbial control at all times. Because of the extensive support needed for bulk freeze tanks, a large dedicated labor force to support and sustain the operations are commonplace in the industry. It is because of these challenges that the growing investigation and acceptance of disposable bulk freeze-thaw systems have started to make inroads into both new and existing plants. For all of the reasons discussed earlier, disposable bulk freeze-thaw systems are following the overall trend of single-use products and are moving into mainstream manufacturing.

2.7 Cold Temperature Compliance Challenges Using SUT (System Under Test) Systems

Exposure to cold temperatures is still the primary challenge in this emergent technology. Even though the currently marketed bulk freeze products are being sold for cold temperatures, the MOCs from which they are made may not currently be a good match for temperatures below −55°C. Current offerings include polyethylene (PE) bags and ethylene vinyl acetate (EVA) bag materials. None of the commercial bags currently offered are a pure form of PE or EVA but a mixture of extruded films that contain multiple layers, and in some cases contain vapor barriers such as ethylene vinyl alcohol (EVOH). The EVOH barriers in bags tend to make things difficult when operating at low temperatures because of their high Tg (glass transition state), which makes them brittle and thus prone to crack at lower temperatures. Understanding the layers of the membrane/film is key to a successful implementation of a bulk freeze system because of the fragility at colder temperatures. The manufacturer of the BPC for bulk freeze offers you technical specifications on the films' brittleness, cold crack temperature, glass transition

temperature, and many more physical tests that describe the films' attributes at various test conditions and temperatures. For a full discussion of this topic see [5].

Typical single-use systems are validated for operation at the normal biopharmaceutical operating temperatures of 4–70°C. Because many freeze processes need the product to be at −70°C, it is important to select components that are compliant and tested at this lower temperature. The polymers that make up the film and other single-use components are recommended to have cold crack temperatures that reach at least −80°C. BPCs made from films that can handle these temperatures are starting to become available for commercial use in these applications. These criteria can be confirmed in handling tests where the assemblies go through several 48-h cyclic processes at temperature cycles from −80°C to +4°C.

2.7.1 Decoupling Bulk Freeze Operations

Decoupling the production of biologics BDS from the final drug product can provide flexibility and cost savings in the manufacturing. Many protein purification steps require the production of biologics in campaigns which produce large amounts of BDS that may need to be stored for lengthy periods until the fill slots are scheduled within the drug product fill facilities. The storage of BDS in the liquid state at typical temperatures of 2–8°C for long periods can provide challenges for maintaining product quality attributes. Most mAb processes store the drug substance in a frozen state because the solid phase of the frozen drug substance is a more stable environment for the protein.

Even though stainless steel systems have a proven and established track record of capabilities at lower storage temperatures, stainless tanks are resource intensive.

A number of challenges for stainless tanks are as listed below:

(1) Complex vessel tracking in a larger company matrix
(2) Storage areas to maintain, clean, and store empty vessels
(3) Extensive labor and expenses to support (CIP, SIP, maintenance, etc.)
(4) Monitoring program for bioburden contamination because of the long-term storage of empty vessels
    (a) High operating costs for the annual cleaning validation
(5) High capital expenditure for stainless steel vessels
    (b) Long lead times for stainless steel vessel fabrication
(6) Inconsistent validation potential for the older/different configured vessels
(7) Safety concerns because of ergonomic vessel handling (size and weight)

2.7.2 Simplification in Cold Chain Logistics

The complex routing inherent with the stainless steel tank freeze-thaw process can significantly impact supply chains that have a focus on "just-in-time" operations. A

small delay in one section of the complex route can propagate and have significant impact throughout the entire operation, and of course the delays occur at the most inopportune times. These unpredictable conditions with the potential to have a wide impact raise a significant concern for the lean operation that relies on "just-in-time" manufacturing concepts. These concerns have prompted investigations into the use of single-use disposable products. There are prior positive experiences with single-use technologies that have resulted in the implementation of disposable freeze-thaw processes at small volumes. Eliminating the cleaning and preventative maintenance loop of stainless steel tanks allows the operation to change significantly when the single-use disposable method is adopted.

Utilizing the single-use disposable approach to bulk freezing can simplify the process with freeze-thaw steps in a number of ways. The typical single-use disposable process features:

1. Simple vessel tracking system by incorporating one-way logistics
2. Minimal space required to maintain secondary containers
3. No vessel storage needed – BPC are closed systems and gamma sterilized by the manufacturer
4. Bulk freeze BPCs can be ordered quickly with short lead times relative to stainless steel
5. Relatively lightweight BPC components

Cold chain logistics clearly benefit the downstream process by providing a hold step that decouples the downstream process from longer-term fill schedules. Some of the more common benefits of single-use bulk freeze-thaw include:

1. Ease of transport between drug substances and drug product contract manufacturing organizations (CMOs) for further processing steps
2. Removal of CIP/SIP steps
3. No need for change over of equipment between products and thus quicker product change over
4. Quicker return to service because of reduced preventative maintenance and calibration activities
5. Simplification of cold chain logistics
6. Reduced risk of contamination because of prior irradiation – no need to open system
7. Reduced labor because of the elimination of washing and autoclaving of tanks
8. Reduced gowning requirements
9. Quicker return to service because of reduced preventative maintenance and calibration activities

Considerable care must be taken to understand both the temperature required for the BDS's stability over time and the temperatures that your shipper containing the BDS bag encounters during cold chain shipping. Being aware of the coldest temperature your bag system, the shipper, and the BDS can handle is an often overlooked technicality that can spell failure for the integrity of your shipments.

2.8 Sterility – Inventory of SUT Bags

Because gamma irradiated assemblies/BPCs have a specific shelf life for validated sterility from the vendor (often covered by gamma irradiation load patterns developed by the irradiator), careful supply chain planning needs to be made, thereby reducing the risk of needlessly having to dispose of unused expired bags.

Normally the shelf life for the gamma irradiated bag is several times longer than the delivery time for the prepackaged assembly. The inventory kept in hand in today's just-in-time manufacturing environment is usually well within the shelf life limit. Having an established delivery time from your vendor by agreement, experience, or both is the best way to make sure you have enough single-use products for your operation's needs without generating scrap.

Single-use BPCs are often manufactured as part of assemblies connected by tubing and filters that are closed systems. Once sterilized, the closed system remains sterile unless it is opened. As the assemblies typically need to be connected to other process equipment, the assemblies include sterile connectors that allow one to make these connections and retain the sterility via these sterile connections and therefore for the entire assembly. Because filters or other portions of the pre-gamma irradiated assemblies may need to be flushed, designing appropriate flush bags via Ts or other sterile connectors can be a challenge to sterile boundaries and require specific operator training, as the majority of the fluid management is manual pinch valves rather than automated valving.

2.9 Validation and Shipping

Validation of the bag chamber and all the associated tubing, connectors, and ancillary single-use components is an important factor. A well-planned implementation program should address the validation issues for material compatibility and applicability of single-use components at the extreme conditions of the freeze-thaw operation.

Part of a successful tech transfer for a bulk freeze technology includes detailed standardized testing. This level of shipping testing challenges both the BDS bag and shipper to a set of standardized shipping tests, including drop testing, side impact loads, vibrational testing, and simulated altitude chamber change testing. Additional areas of testing for BDS containers include the study of any effects of light, or pH changes occurring because of interactions with the plastic during storage and shipping in the single-use containers.

3 Pros and Cons of Using Disposables

As already outlined, there are many advantages of using disposables compared to standard piping and tank-based processing [6, 7].

One major advantage is that pre-sterilized single-use systems can be used in a lab-like environment. This is well-suited to small-scale research and development activities, as no supporting engineering infrastructure regarding, for example, SIP, CIP, utilities, hard-piping, and automation is needed to operate such processing units. This even enables universities to do bioprocessing on a reasonable scale in their labs.

Another advantage is the time and cost savings in plant construction and operation. The main contributors here are capital costs, reduced consumption of utilities such as gas, electricity, and water (purified, WFI), as well as reduced staffing. One out of several evaluations is shown in [8] as a case study by Roebers et al., presented at the *IBC Life Sciences Bioprocessing Meeting, October 2009* (Table 1).

The achievable time savings vary depending on the extent of disposables usage. Most facilities still involve non-disposable unit operations. In such a case of hybrid design, time savings in the early engineering project up to mechanical completion are sometimes marginal, but time savings during start-up, including qualification and validation, can be very pronounced (up to 70%), as equipment qualification using disposables is very limited, and no SIP and CIP processes are needed. The sometimes very lengthy cleaning validation of vessels and pipes with product contact is not needed for single-use because the bags are discarded after each run. The case study by Roebers indicates that almost all aspects of plant design and operation, except for materials/consumables, may benefit from the use of disposables instead of hard-piped systems.

In summary, all this leads to overall much reduced cost of goods, although the material costs in the form of single-use units are increased. However, this is more than compensated for by the lower capital depreciation alone, as the capital costs using disposables are in the range of 20–40% compared to hard-piped arrangements.

Table 1 Cost and operations comparison of a hard-piped (2 × 15,000-L fermenters) vs single-use (10 × 2,000-L fermenters) upstream facility. (Study of Elan Corp for MoAb production; Roebers, IBC Life Sciences Bioprocessing Meeting, Oct 2009)

Parameter	Hard-piped	Single-use
Titer (g/L)	3	3
Mfg capacity (tons/year)	1	1.2
Capital cost (millions of €)	350	145
Capital cost (€/kg)	100	35
Gas supply (%)	100	12
Electricity supply (%)	100	37
Water supply (%)	100	8
Manufascturing area (%)	100	17
Staff (%)	100	41

Facility assumptions: hard-piped with 2 × 15,000-L fermenters, single-use with 10 × 2,000-L fermenters

Another advantage of disposables-based plants is the option to perform short product campaigns efficiently in multi-purpose facilities, including fast product turnover by simply replacing used by new bags. In addition, it is comparably easy to transfer a disposable-based manufacturing process to a second site, which is of increasing interest for producing a drug locally for regional markets. As the same disposables-based units combined with low qualification and validation efforts can be used at all envisioned sites, the risks for process transfer and achieving biochemical comparability, identical product quality, and finally regulatory approval should be strongly reduced.

However, there are also several limitations and risks in using disposables, which need appropriate risk mitigation strategies as outlined in [6, 7].

An overall scale-related limit is always the maximal volume for handling and operating disposables. For fermenters and larger hold bags it is expected to be in the 3,000 L range, for portable systems about 1,000 L.

Compared to automated, hard-piped systems, working with single-use systems is labor intensive, because assembling of individual pieces to operational units is done manually by tube welding or pre-sterilized connectors integrated in the disposable unit operations to be connected before use. A very well-trained workforce is needed to minimize operator errors. Finally, as mentioned before, using disposables increases the material costs and leads to more waste, which has to be addressed.

A big issue is the dependency on vendors. There are several integrated systems being developed by individual suppliers, which are not compatible, that is, it is not possible to interconnect systems from different suppliers to a large functionally closed processing unit. In order to comply with the desired second supplier concept in bio-manufacturing for SUBs, one has to show biochemical comparability and product quality in two bioreactor types before they can be used for commercial production. This is a big additional development and validation effort, until two adequate systems are licensed. In addition, it is necessary to get improved quality control by the suppliers. For example, bags should be pressure tested before delivery to reduce failure rates. It would also be advantageous to get full supporting validation packages, including E/L data and regulatory support files from the suppliers to make regulatory filing simpler. Currently, vendors and users are working together to come to standardized inter-connectable single-use systems as well as acceptable validation guides for E/L in several working groups, such as the Biophorum Operations Group (BPOG). As this is still in development and individual companies have their own solutions, the latest progress can be taken from the websites shown. The goal for E/L validation is a clear standardization of activities needed per processing phase, which is also accepted by regulatory agencies. A recommendation of how to approach E/L studies is summarized next.

3.1 Key Recommendations for E/L Studies

When new MOCs are introduced into a process application, E/L data should be generated to assess the impact on process and patient safety. Extractables study is

performed using worst case conditions and can be useful for assessing the risk of the component in any application. Leachables information reflects the interaction of the process with the equipment and, therefore, related studies use the actual product and process conditions to determine which chemical species migrates from the component.

3.2 Extraction Conditions and Procedures

The requirements for conducting the extractable study include:

- Solvents used should represent extremes in pH, ionic strength, polarity, and surfactant concentration
- Solvents and conditions must be as extreme as or more extreme than those that would be encountered in actual manufacturing applications
- Multiple time points should be included in the study as the extractable profile changes over time (e.g., volatiles would reduce over time whereas non-volatiles would increase over time)
- Higher temperature can be used to simulate longer hold at lower temperature
- Higher surface area/volume ratio should be targeted for worst case conditions
- Negative controls to establish background levels are required
- When recirculation methods are used in extractables testing, inert materials such as PTFE should be used for the remainder of the system
- Starting and ending volumes of test solvent should be recorded to compensate for evaporation in final calculations

Extraction conditions are critical in determining the chemical profile and levels of individual extractables. The extraction conditions must be appropriately selected and justified, well-described, and reported, including temperature, pH, and time. If a suggested model solvent is not chemically compatible with the MOC (e.g., C-flex® tubing compatibility with ethanol is poor), a similar model solvent within the compatible range should be used, or a rationale should be provided for why a similar solvent is not used.

The common extraction model solvents included comprise a broad range of representative solutions chosen to bracket the typical process conditions used in biological manufacturing [9] (Table 2).

Table 2 Extractable conditions for single-use components

Chemical conditions	Model solvent
Low pH aqueous solution	$\geq$0.1 M H_3PO_4, target pH $\leq$1
High pH aqueous solution	$\geq$0.5 M NaOH, target pH $\geq$13
Low ionic strength, polar aqueous solution	High purity water, conductivity <0.2 mmho/cm
High ionic strength aqueous solution	5 M NaCl, conductivity >220 mmho/cm
Polysorbate-containing solution	$\geq$1% polysorbate 20 or polysorbate 80
Low polarity solution	50% ethanol

4 Impact of Single-Use Systems on Plant Design and Operation

A key challenge to using disposable systems is their impact on plant design, costs, and operation. However, because steel plants are currently state-of-the-art and as not all unit operations can reliably be performed as single-use, there are several plant designs currently realized [8, 10], each having its own special characteristics.

4.1 Stainless Steel-Based Standard Facility

This represents the established current state-of-the-art for biomanufacturing using, for example, a fermentation six pack of 10–15,000-L fermenters with, in many cases, two purification suites. These facilities are very expensive, fully hard-piped, and highly automated. They have the usual operational and area segregation and containment concept, minimizing cross-contamination of any kind. Start-up and validation are lengthy, including automated SIP and CIP processes, but lead to very reliable, operator error-independent processing. This is and remains the choice for large volume products.

4.2 Stainless Steel/Single-Use Hybrid Standard Facility

The first integration of disposables into bio-production came by introducing disposable units into hard-piped facilities simply to make operations more efficient. A good example for this is harvest clarification replacing cross-flow microfiltration, which is difficult to maintain and clean, as well as centrifugation for cell removal by large depth filters. In the meantime, with more disposable systems available, true hybrid facilities are being built with hard-piped parts as well as flexible stand-alone units based on disposables, but still using the standard segregation and containment concept.

A recent hybrid pilot facility at Bayer was constructed with the goal of directly comparing a 2,500-L hard-piped steel fermenter with single-use production fermenters. It is designed to run two production fermenters in parallel, the steel fermenter and an SUB of 1,000 L or 2,000 L. This represents an excellent development tool for establishing biochemical comparability and product quality in both systems. This is essential if clinical production is done in disposables, but future manufacturing needs larger hard-piped fermentation systems because of very high product demand. However, in all these hybrid plants the potential time and cost savings from using disposables are limited because the hard-piped part determines time and costs.

4.3 Single-Use-Based Standard Facility

To utilize fully the advantages of single-use systems, the first mainly disposable-based plants are being built, implementing the advantages of a smaller footprint, lab-like environment, reduced costs and time schedules, but still using the established segregation and containment concept. They fully realize all the advantages of using disposables and rely mostly on manual operations. However, there are still some non-disposable unit operations integrated such as large UF/DF or chromatography skids, which are individually cleaned and maintained using portable clean out of place units, thus avoiding the complex hard-piped CIP infrastructure.

5 Facility of the Future: The Ballroom Concept

The ballroom concept represents an innovative concept to enable parallel processing of different products in a shared low classification containment without or with only limited segregation, which in the extreme would mean operating all closed units in one area using air control but no air classification (Fig. 8). The concept was first described in a publication by Chalk et al. [11]. It is based on the key assumption that technological advances, including single-use systems, have continuously reduced the risk of environmental impact on processing, the main reason for the high segregation in standard plants. Nowadays most process steps can be securely performed closed or functionally closed. The few remaining open processing steps are being addressed separately, that is, using small areas of classical containment or isolator technology.

The basic concept in ballroom set-ups is that, in a closed or functionally closed system, the process stream is isolated from the environment so that environmental control is of less importance. Basically, a potential breach of the closed system is the major risk to be addressed by intense microbial monitoring, as well as proving that no contaminations or cross-contaminations have occurred. Maintaining the closed system status is very important and has to be addressed by a risk-based approach with appropriate risk mitigation strategies considering each process step or operation. In the case study [11], the following risks were addressed and mitigation strategies provided using detailed failure mode and effects analysis tools: temporary breakable connections, open manipulation in the process stream, charging raw materials during media and buffer preparation, equipment preparation, cleaning or maintenance, in-process sampling, and an unexpected breach of a closed system element.

Compared to the standard clean room class segregation in current plants separating upstream and downstream, as well as between the different virus removal/inactivation steps, this revolutionary ballroom concept has still many hurdles to pass before it is a viable alternative for manufacturing clinical or commercial

Fig. 8 Schematic layout of the ballroom facility

material. Regulatory acceptance in different regions is a major challenge, particularly avoiding the pre- and post-virus depletion steps segregation, as viruses can potentially pass sterile filters, which represent an important boundary to maintain and claim closed processing in single-use set-ups. More realistic is to implement only parts of the ballroom concept, that is, maintain separation of upstream and downstream segregation but run several products in parallel in one of these areas.

Single-use-based ballroom plants are by far the most beneficial economical versions, both for plant design, costs, start-up as well as for processing, resulting in the lowest among the described options. Unit operations, based on disposables, aseptically connected or even sterile assembled by tube welding or disposable sterile connectors, represent a good basis to fulfill the closed processing requirements of the ballroom concept. However, in practice it is considered risky at this time to neglect completely segregation and area containment, as it is not yet fully understood, even by the regulatory agencies, that the closed state can be maintained without breach during operation. Therefore many users are choosing a less risky route. What is being considered is a ballroom approach for upstream operation excluding the seed expansion, which has to be done classically in a small separate class C cleanroom. However, because of the easy set-up and exchange of equipment using disposables, it should be low risk to operate several products in parallel in an upstream suite, which may still have class C environment. Also, in the downstream area, it seems possible to run several trains in parallel without cross contamination risk. However, for final processing of the drug substance, a switch after the final virus nano-filtration into a separated standard class C environment for bulking is considered an acceptable risk.

In addition, it is easier to implement elements of the ballroom concept for clinical production vs commercial manufacturing, simply because regulations are less strict. An example of this has been proven at pilot scale performing fully closed unit operations in one manufacturing area, resulting in acceptable KPIs and microbiological acceptance criteria [12]. In the end, each manufacturer has to find the right balance between innovation and risk management.

6 Status of Disposables-Based Manufacturing

Hybrid facilities using hard-piped and disposable elements are widely used in the industry for both clinical and commercial production. If one looks at the CMO scene, clinical production based on the 1,000- or 2,000-L scale SUBs is expanding rapidly and is difficult to get access to as a potential customer. However, it remains to be seen whether and how comparability to large scale steel fermenters can be established, when commercial demand needs another large scale-up.

Pure disposable facilities are now getting established. BI in China is a published example (Sartorius website under Integrated solutions, client case studies, Boehringer Ingelheim). However, they are limited in scale because, in the case of high titer 2,000-L SUB fermentation, the corresponding downstream capacity is

quite volume–demanding, making completely disposable solutions unrealistic, except when the required chromatography steps are frequently cycled or continuous downstream processing is used.

Disposable manufacturing in pure play ballroom plants is currently still very risky as outlined above, and therefore not yet realized, including world-wide licensure.

7 Concept for Emergent Markets

7.1 Emerging Markets

The incidence of several cancers is rising rapidly in many countries, especially in urban areas within emerging markets. The prevalence of Western diets, sedentary lifestyles, cigarette use, and environmental pollution has contributed to the increase in cancer rates, but access to proper treatments is limited in emerging markets. In addition, the healthcare industry is faced with an aging population, increased unemployment, and other economic pressures that further limit patient access to cancer treatments.

There are also significant challenges in reaching emerging markets, including lack of infrastructure, trained personnel, and major differences between local private and public healthcare. Despite the many challenges, emerging markets constitute one-third of the global pharmaceutical market. To serve these markets, some pharmaceutical companies have begun assessing new manufacturing technologies to allow for flexible, scalable, and cost-efficient production. Currently available single-use technologies have been found to provide economic advantages over stainless steel components, and have been adopted as one solution to reduce production costs and enable flexible facility designs. The lack of infrastructure and supply sources has also driven companies to investigate the deployment of modular facilities which can be built off-site, so that the prefabricated building blocks can be shipped and assembled in proximity to the end market. The emergence of these new challenges continue to prompt companies to adapt their innovation strategies and their operating models to fit the needs of a changing global healthcare market.

7.2 Expectations and Applicability

The purpose of E/L studies is to determine which chemical species might migrate from process-contacting components. Extractables information can be useful for assessing the risk of components in a wide variety of applications. On the other

hand, leachables information reflects the interaction of components with actual process and product conditions.

E/L assessments present several challenges in pharmaceutical development as studies must account for a wide range of solvent conditions, exposure times, exposure temperatures, exposure areas, and other factors. For example, the same MOCs for a bag may be found to generate different E/L profiles depending on their application (e.g., a mixing bag in short but dynamic contact, as opposed to a storage bag in long but static contact). Special attention must be taken to ensure that the appropriate conditions are simulated to be representative of the processing conditions in question.

Upon obtaining E/L information, it is expected that a risk-based approach is taken to determine whether there is a potential risk to patient safety. Regulatory agencies have already outlined general requirements for E/L assessments, and different tolerances for risk have been determined depending on factors such as a drug's route of administration. The E/L information should be analyzed and compared to regulatory guidelines such as the International Conference on Harmonization (ICH) Guidelines and European Pharmacopeia (EP) Monograms.

7.3 Quality Risk Management in E/L Studies

Guidelines for quality risk management in the context of E/L information have been described by ICH Q9. Several tools are available to assess risks associated with patient safety, product quality, and overall process performance. For example, if an automated process is implemented for component manufacturing, fewer samples may be needed when compared to a manufacturing process that relies on manual labor that may be prone to process variability, therefore requiring more samples.

7.4 Risk to Patient Safety

A health-based safety assessment is conducted to evaluate the overall risk to patient safety. More specifically, a toxicological assessment is conducted for the worst-case impact of impurities to patient safety. The toxicological assessment must be conducted in a way that is compliant with regulatory guidelines. It is important to note that regulatory authorities focus primarily on patient safety by considering the end users who implement single-use components in drug manufacturing, and also by considering the vendors who supply the single-use components.

How to determine whether you should build a new traditional steel-based facility or a single-use technology facility, or modify an existing facility in your network, can be a complex decision requiring the assessment of many variables [13]. Areas to explore when asking this question include the following:

- Do the molecules you are planning to make fit nicely in your existing network?
- Can you leverage recent advances in single-use technologies and flexible manufacturing to scale your production to the demand required?
- Why is this so difficult to figure out?

When getting involved with initiatives such as this, it is important to consider several aspects, especially for emergent markets. An assessment by a competent team of biotech experts within your organization to focus on strategically analyzing the comparisons of building vs CMO utilization is key. Additionally, exploring the financial return and comparison between a completely new greenfield site using disposables vs modification to existing manufacturing spaces to allow for new product introductions should be considered.

Demand drives to a large extent the decision on what type of plant to build. In scenarios where —one or two drugs are manufactured over a year with limited product changeovers and minimized down time, traditional steel plants are still justified when dedicating capital equipment to a limited number of changeovers. The time, resources, and testing required to support product changeovers, including cleaning validation, are key financial drivers when determining the facility design and make-up.

Single-use facilities or hybrid facilities (those facilities containing a mix of single-use technologies and steel unit operations) allow for a much quicker changeover of equipment, simultaneously negating the need for cleaning validation.

When performing an analysis for which the site fits your needs, consider what the peak demand for the products being manufactured at the new site would be. Many factors need to be explored:

- What are the ranges of titers, yields, and total weights required for the plant annually?
- How long do you want to allow for changeover?
- How many molecules and or new transfers do you expect to move into the facility in a year?
- Are you manufacturing hard-to-clean (to show removal) molecules (conjugates, highly potent proteins that require harsh cleanings and copious cleanings to show removal?)
- Are you manufacturing both clinical and commercial phase materials in the same areas?

These are all key inputs to help evaluate the type of facility that meets your company's needs.

Other areas that must be evaluated in scenario planning when comparing traditional steel facilities against SUT facilities are the following:

- Capital required to build the facility
- Technology transfer cost for each product (sending and receiving site)
- Maintenance of facility (running cost/idle cost)
- Operation of facility and associated staff when producing and between changeovers
- Raw materials associated with each scenario

- Consumables (naturally higher cost on disposables for SUT facility)
- Timeline for each product introduced (when are the products introduced in the life cycle of the plant)
- Demand curve of each product (several long campaigns vs multiple changeovers)

7.5 Qualification of SUTs

Front loaded costs (tech transfer through qualification runs) dominate the overall cost with small demand products because of the run rate of the new molecules and the high cost of qualification, sampling, and validation to support each transfer.

7.6 Other Important Strategic Drivers Not Included in Financial Analysis

Regarding agility and focus, single-use unit operations allow for modular options.

In the rapidly changing and competitive environment of tech transfers, having the ability to add capacity quickly to an existing plant when market needs change rapidly can positively affect standard ROI calculations. The ability to have manufacturing capabilities that can more easily integrate new technologies (such as new unit operations without significant capital cost) that are mobile and can be reconfigured rapidly rather than modifying a hard-piped facilities is a game changer and is not easily quantified in terms of payback.

7.7 Areas of Concern for SUTs

Transferring processes from a flexible facility to a stainless steel facility in some instances may require time for redevelopment and new regulatory filings. Regulatory heath agencies may view processes in stainless steel and disposables as inherently different if there are not adequate comparability data. Additional concerns are that many unit operations are currently specific to the vendor who has developed or launched the unit operation (e.g., an SUB bag with sensors is not easily utilized in a competitors external holder/capital procured) leading to costs and risks associate with single sourcing of the products in a flexible facility. Other areas of concern are the development of the process step that may have been designed for a flexible facility; it may have been scaled with respect to flows, pressures, and cycle to fit a SUT application vs larger throughput applications

which require less time (centrifuge vs depth filtration, 1.4 M columns vs multiple cycles on 60-cm pre-packed columns).

Although flexible facilities utilize single-use materials which greatly reduce the risks related to cleaning steel as well as validation failures related to bioburden excursions, there are other areas of which one needs to be aware.

When considering flexible facilities, there is shift in risk away from your internal maintenance/cleaning of stainless equipment to external supplier quality which must be proactively managed. Along with this management of the single-use technology items comes the need to manage aggressively vendor initiated changes and the assessments for potential product/patient impact.

7.8 Outlook/Future Trends

Single-use technologies based on pre-sterilized disposables is a maturing field allowing most cell culture process steps to be performed in disposable instead of hard-piped unit operations. This leads to simpler operation in a lab-like environment. However, because the disposable industry is still comparatively new, there are many open issues such as validation, secure supply at high standard and standardization, quality control, etc., which have to be addressed or improved, respectively.

Disposable-based flexible facilities with functionally closed operation units are developing into a viable alternative to the standard hard-piped facilities, particularly for lower volume products. Such facilities are faster to build, have smaller footprints, and involve less complex engineering. In addition, they are easier to qualify and validate, lower in costs, and easier to operate, especially in a multipurpose environment with frequent product changes. They still have the same or a similar segregation concept compared to classical plants.

Finally, the ballroom plant design may be the concept of the future with less or no segregation and containment. It is nowadays at least technically possible, because of improvements in functionally closed systems operation and monitoring, if corresponding risk mitigation strategies are applied. This may result in less or unsegregated plants with low containment requirements, which may even enable parallel operation of several products at a time. However, issues such as how to handle steps, where functionally closed operation is not possible or available, as well as regulatory acceptance, still need to be addressed.

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Trends in Process Analytical Technology: Present State in Bioprocessing

Marco Jenzsch, Christian Bell, Stefan Buziol, Felix Kepert, Harald Wegele, and Christian Hakemeyer

Abstract Process analytical technology (PAT), the regulatory initiative for incorporating quality in pharmaceutical manufacturing, is an area of intense research and interest. If PAT is effectively applied to bioprocesses, this can increase process understanding and control, and mitigate the risk from substandard drug products to both manufacturer and patient. To optimize the benefits of PAT, the entire PAT framework must be considered and each elements of PAT must be carefully selected, including sensor and analytical technology, data analysis techniques, control strategies and algorithms, and process optimization routines. This chapter

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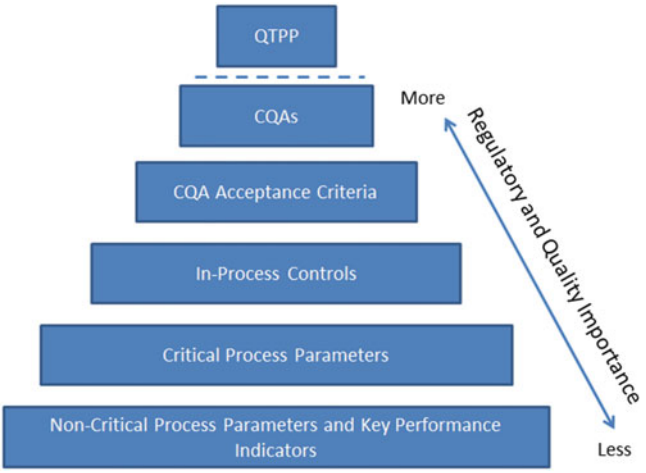
discusses the current state of PAT in the biopharmaceutical industry, including several case studies demonstrating the degree of maturity of various PAT tools.



Graphical Abstract Hierarchy of QbD components

Keywords Bioprocess monitoring and control, PAT, QbD

Contents

Symbols and Abbreviations

ANN	Artificial neural network
API	Active pharmaceutical ingredient
CIP	Cleaning in place
CPP	Critical process parameter
CQA	Critical quality attribute
EMA	European Medicine Agency
FDA	U.S. Food and Drug Administration
HPLC	High-performance liquid chromatography
ICH	International Council for Harmonisation
mAb	Monoclonal antibody
MIR	Mid-infrared
MOC	Material of construction
MPC	Model predictive control
MSPC	Multivariate statistical process control
MVDA	Multivariate data analysis
NIR	Near infrared
OUR	Oxygen uptake rate
PCV	Packed cell volume
PHC	Personalized healthcare
QbD	Quality by Design
QC	Quality control
ROI	Return on investment
RQ	Respiratory quotient
RVR	Relevance vector regression
SVR	Support vector regression
VCD	Viable cell density

1 Content and Introduction

The development and manufacturing of biological therapeutic products should be guided by two fundamental principles:

- Quality cannot be tested into a product; it must be built into it.
- Product and process variability should be understood and controlled.

This means that manufacturers of biologics must develop mechanisms and tools to measure the quality of their raw materials, intermediates, and products during processing, not just at the final stage of production of the drug substance or drug product. They must measure, characterize, and attempt to reduce variability in processes and materials (raw materials, intermediates, and final products).

Over the past decade, these two principles have led to the development of a series of Harmonized Tripartite Guidelines by the International Conference on Harmonization (ICH) of Technical Requirements for Registration of

Pharmaceuticals for Human Use, on drug product development (ICH Q8), quality risk management (ICH Q9), quality systems (ICH Q10), and drug substance (active pharmaceutical ingredient (API)) development (ICH Q11).

These guidelines were also the foundation stone of the Quality by Design (QbD) initiative, which has significantly affected the development and manufacturing of biopharmaceuticals in the past decade. QbD is a system that encompasses new drug development and manufacture to define better the manufacturing processes, their control, and the analytical methodologies used to evaluate their performance.

As shown in Fig. 1, QbD is based on a hierarchy of components. The quality target product profile is the description of the quality characteristics of the product. It serves as the "goal" (i.e., begun with the end in mind). The critical quality attributes (CQAs) are the quality characteristics of the drug substance and drug product that affect the safety, efficacy, immunogenicity, and pharmacokinetic characteristics of the final drug. Specifications (including raw materials, drug substance, drug product, intermediates, and in-process controls) define analytical methodologies and their associated limits, as these ensure that the CQAs are attained by the product. Critical process parameters (CPPs) are those process parameters that have an impact on product quality, which must be controlled within an acceptable range in order to ensure that product quality is met.

These QbD components define the control strategy, which is the "control system" for manufacturing.

The corresponding guide, entitled "Process Analytical Technology (PAT)" was published by the U.S. Food and Drug Administration (FDA) in 2004 [1]. PAT is a methodology that is predominantly used for the in-line, on-line, and at-line measurement of process adherence and/or compliance with the process parameters and attributes as defined in the QbD. PAT can be seen as an enabling technology for

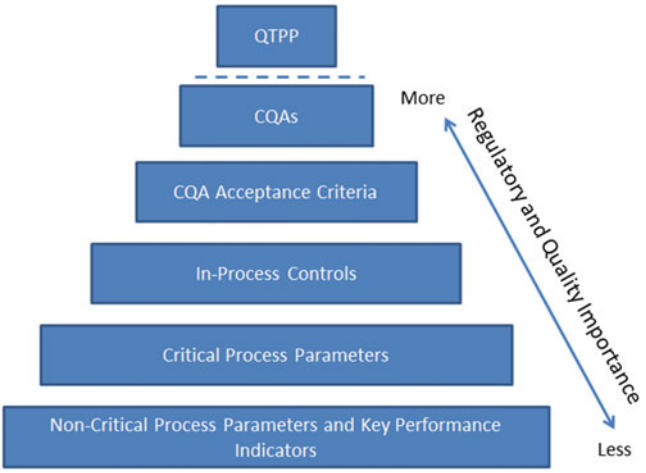


Fig. 1 Hierarchy of QbD components

QbD that enhances the probability that the manufacturing process of a drug substance and/or drug product produces material that predictably meets the quality requirements.

PAT can be defined as a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality (ICH Q8). It also encompasses any measurement of a process variable or CQA that is performed often enough and reliably enough to permit the process to be adjusted to ensure that there is a high probability that the output of the unit operation meets its CQAs.

The two guiding principles for bioprocessing (quality must be built in, and variability must be controlled) affect both development and manufacturing. Development must "design" and demonstrate these principles and manufacturing must implement them. PAT facilitates the implementation of these principles by manufacturing, providing "real-time" measurement of process and product parameters and attributes. The lack of PAT or the lack of the use of rudimentary concurrent analytical tools (such as pH, flow rate (gas and/or liquid), volume, and pressure control) usually means that control is ex post facto, relying on quality control (QC) results that are often only available days after the events have occurred.

The "real-time" analysis of the process and its performance facilitates control of the process by implementing feedback or feedforward algorithms or manual adjustments, for example, adding an acid or base, or modifying gas flow rate and/or constituent gases, to control pH. It facilitates quality by controlling process or product attributes (e.g., purity or impurities can be controlled by manipulating the process stream in downstream operations, or the concentration of raw materials, such as the carbon or nitrogen source, in upstream operations) in order to select or deselect specific attributes. Moreover, process/product variability can be measured and/or controlled, for example, variations in temperature, purity, impurities, and pH can be minimized by feedback/feedforward control systems, as previously indicated.

In addition to achieving QbD goals, such as consistently meeting CQAs, biotechnology/biologics processes should maximize operating efficiency (e.g., yield, throughput, cycle time reduction). Therefore, applications focusing on improving operating efficiency (e.g., yield) can also be included within the definition of PAT. In general, it is just these that generate a measurable return on investment (ROI), and this can justify a company's investment in PAT.

PAT has the potential to be a "disruptive" technology in biopharmaceutical manufacturing, (i.e., a technology that changes manufacturing, effectively making current technology obsolete).

Finally, a current trend for biopharmaceutical processes is the more frequent use of continuous and/or completely automated operation [2, 3]. This is unattainable without appropriate PAT, as process decisions must be made in "real time" and PAT provides the sensors required to afford the "real-time" control required.

2 Current PAT Applications, Standards, and Strategies

For biologics processes, numerous examples of the application of PAT tools have been reported in the literature (e.g., [4]). Examples include:

- Monitoring and controlling the testing of raw materials by spectroscopic methods such as near-infrared (NIR) and two-dimensional (2D) fluorescence spectroscopy, including multivariable data analysis
- More rapid at-line or even in-line monitoring of fermentation processes:
  - Specific parameter monitoring (e.g., cell density measurement by dielectric spectroscopy)
  - Process fingerprinting (e.g., mid-infrared (MIR), NIR, 2D fluorescence spectroscopy, Raman and other techniques, such as localized or enhanced surface response instruments)
  - Soft sensors
  - Monitoring media components such as amino acids
  - Status of off-gas analysis: techniques and applications
- Closed-loop control of fermentation processes with in-line monitoring signals
- Process supervision using process models (e.g., by multivariate statistical process control (MSPC), later integration of raw material, starting conditions, early process deviation detection (predictive/preventive mode))
- Tools for data management and data analysis needed to deal with high data volumes and supported new data formats (e.g., spectra); ability to correlate large volumes of data from different sources in order to detect new patterns and solve problems faster
- Monitoring cleaning-in-place (CIP) processes

Although all these examples demonstrate the applicability and value that PAT can have for biologics manufacturing, there are few examples of the implementation of PAT tools in testing for release that have developed into real-time release strategies. Likewise, no examples are known of a totally integrated "PAT" strategy or approach encompassing the entire development and manufacturing process of a biologic.

2.1 Raw Materials Screening

Raw materials used as media components in bacterial fermentations or in mammalian cell cultures have typically been a major source of process variability, and have even been reported where purely chemically defined media have been used [5]. NIR and Raman spectroscopy have been successfully implemented by many pharmaceutical companies to identify raw materials upon shipment, and handheld sensors are now used, as seen in Fig. 2.

Fig. 2 Handheld NIR material analyzer (Thermo Fisher microPHAZIR RX, http://www.thermoscientific.com)

In principle, screening incoming raw materials using spectroscopic methods makes it possible to characterize raw materials quickly as "suitable for use" before they are used to prepare media. As the measurement is performed before the raw material enters the process, there is no risk that the sensor may breach sterility. In addition, because many of the raw materials are dry powders, NIR spectra can be obtained without the presence of a large water signal. Examples of the use of NIR spectroscopy for this purpose have been given by Kirdar et al. [6], Jose et al. [5], Lee et al. [7], Hakemeyer et al. [8], and Prajapati et al. [9]. A comparison between NIR, Raman, and other spectroscopy methods for a case study of raw material characterization can be found in Trunfio et al. [10].

2.2 PAT Tools in Bioprocesses

2.2.1 In-line Measurements

Electrochemical sensors, such as those used for pH and dissolved oxygen, have been commonly used for decades in bioprocesses. These are obviously also examples of in-line sensors and can be considered as PAT tools. As they are generally accepted and have been used for a long time, they are not discussed here.

In-line measurements in bioreactors can be especially challenging because bioreactors are unique in that they can be thought of as a conglomeration of tiny bioreactors. This means that the actual pharmaceutical product is produced within the individual cells that are suspended within the culture broth and either maintained within the cell walls until additional downstream processing takes place (cell lysis) or secreted into the media. This is obviously a different and considerably more complex scenario from that observed in small-molecule manufacturing, where the product is generally produced within a homogenous solution in the chemical reactor.

In-line sensors for bioreactor monitoring may use NIR, MIR, Raman, 2D fluorescence, or dielectric spectroscopy.

2.2.2 NIR Measurements in Bioreactors

In-line optical sensors typically allow short analysis times, ranging from seconds to minutes per analysis, depending on the number of spectra and amount of data processing required. Although an analysis every 2 min is typically unnecessary for a single bioreactor, this short analytical time does make it possible to interface a single spectrometer to multiple bioreactors. In-line sensors offer the significant advantage of not having to draw a sample from the bioreactor in order to perform the analysis. This is a major advantage of these technologies as it reduces the risk of contamination and potential loss of sterility that is considered to accompany on-line measurements and their associated on-line sampling devices. The disadvantage of these optical sensors is that culture media are generally a challenging matrix for NIR, as water absorbs NIR strongly. This necessitates the use of short path lengths for NIR, which then raises the risk that suspended solids may block the bioreactor. Furthermore, these probes are susceptible to the formation of superficial biofilms, which can also negatively impact the analysis.

The complex fermentation matrix gives rise to multiple overlapping absorptions in the NIR spectra and this requires the use of chemometrics to build correlations between the reference method for analyte concentration and absorptions in the NIR spectrum. Developing these calibration models is not a trivial task – as noted by Calvalhal and Saucedo [11]. The accuracy and robustness of these models is highly dependent on spectral variation in anything present in the media, including the cell line. Robust quantitative models must include these spectral variations (e.g., changes in raw materials, cell lines, etc.) in the model to compensate for the variation and to maintain accurate analyte predictions. However, even being aware of when this spectral variation is impacting the accuracy of the calibration model can be a challenge without running samples by a reference method. It is likely that this complexity and the amount of effort to construct and maintain robust quantitative models is one of the major reasons that NIR has not seen broad and routine use across the biotech industry. There have been numerous articles from academic institutions describing the successful application of NIR for monitoring various analytes in fermenters [12, 13]. In spite of this, NIR does not seem to have

been widely and routinely used by major biopharmaceutical companies in production bioreactors. The low limit of detection and the high level skills required to calibrate and maintain might be reasons why NIR is not widely used in industry.

One possible reason for this apparent success of NIR in academia, but relatively poor acceptance in biopharma manufacturing, may be the lack of raw material variation seen in the limited number of fermentation runs performed by the academic institutions. This gives the impression that quantitative NIR measurement models in a fermenter are more robust and stable than is observed when these techniques are applied at an actual production facility, where variation in raw materials over an extended period of time includes spectral variability that may have not been present in the original calibration set. This requires that calibration models should be constantly evaluated and frequently updated to include this new spectral variability. Saucedo et al. [14] proposed a "hybrid" technique, whereby analytes are spiked into media not included in the original calibration set, so that an existing calibration can be extended to applications not included in the calibration set. In spite of the challenges of using NIR for bioreactor monitoring, there appear to be have been several successes, including ammonia, glutamine, and glucose [15–17].

2.2.3 Raman

Although not as widespread as NIR, Raman probes for bioprocess monitoring are commercially available and reports of their application have been published recently [18–22], for example, for measuring product concentration in-line.

Berry et al. [21] describe the use of Raman for in-line glucose measurement and control, in order to keep the glucose concentration below a specific threshold and thus to reduce glycation of target protein. This is an example of a true PAT application used to control the CQAs directly with a closed loop system.

The challenge of Raman spectroscopy is similar to that of NIR probes. It is difficult to extract the required information consistently and reliably, and to deploy the technique in an on-line or at-line setting.

2.2.4 Dielectric Spectroscopy

Knowledge of the growth rate of viable cells in the bioreactor is critical in understanding how a bioreactor is proceeding. Information about the cell density or cell growth rate is of quite fundamental importance, as it indicates whether or not the bioreactor is proceeding normally. With a dependable viable cell count sensor, it should be possible to ensure feedback control of nutrient feeds such as glucose and/or amino acids by using metabolic models developed off-line and real-time data from the in-line viable cell count sensor to determine the rate with which metabolism is proceeding. Numerous technologies to sense cell density have been

tried in recent decades, including NIR and fluorescence spectroscopy. These are described in later sections.

However, the most successful sensor to date for viable cell growth rate seems to be the dielectric spectroscopy or capacitance sensor. These sensors can differentiate viable from non-viable cells by exploiting the fact that viable cells have an intact, non-conductive cellular membrane surrounding the conductive cytoplasm. When an alternating current is applied, the viable cells act as tiny capacitors. The data obtained can be correlated with an off-line cell counting method, such as trypan Blue staining and manual or semi-automated counting. A common issue with this sensor is that it correlates well with the off-line measurement in the early stages of cell growth, but, with some cell lines the correlation begins to deteriorate considerably in the late growth phase [23].

Publications by Bend Research [24] and Aber Instruments [23] describe a new approach to processing the data by using the concept of an area ratio algorithm. They claim that this predicts viable cell volume fraction better than current methods.

Several applications of dielectric spectroscopy have been described [25, 26] that include the use of the biomass probe to determine the transfer time point from inoculum and seed bioreactors to the next reactor stage. This made it possible to perform the transfer at a much more precise cell concentration than is possible with off-line analysis. Other examples included using the probe to monitor retention filters in perfusion bioreactors [27], with the signal from the dielectric sensor being used to detect filter breakthrough.

2.2.5 In-line Fluorescence Measurement

Many substances occurring in biochemical reactions are fluorescent species, such as NAD/NADH and flavonoids. These compounds produce strong fluorescence signals that can be used to monitor the progress of these metabolic reactions. Since the 1980s, in-line fluorescence probes monitoring NAD/NADH fluorescence for bioreactors have been used to determine rates of viable cell growth. More advanced rapid scanning 2D fluorescence instruments – coupled with chemometric data processing – have now opened the possibility of examining other biochemical reactions involving other fluorescent products such as $FADH_2$ and $FMNH_2$. There are hundreds of biochemical reactions with fluorescent substrates or products that can be used to monitor the progress of cellular metabolism. As such, 2D fluorescence spectroscopy would seem to be a useful tool to understand intracellular reactions that may impact CQAs. Hantelmann et al. [28] proposed 2D fluorescence as an alternative to mid-IR or NIR as a tool for controlling glucose feed. The fluorescent method could predict the change from oxidative to oxido-reductive metabolism. The data from the sensor was used as an input to a feedback control loop to control the feed rate of glucose in a fed-batch reactor to below a value where ethanol was produced. Although this is not specifically of interest in bioreactor

monitoring, Schwab and Hesse [29] described the use of 2D fluorescence for the real-time measurement of product aggregation. This could be of importance in antibody and other biotech products if the processes are operated at high concentrations where product aggregation is favorable.

2.2.6 Model-Based Sensors

Soft sensors, model-based sensors, or what are sometimes referred to as "neural networks" are sensors that use multiple existing inputs to predict a process variable [30–35]. These systems are a sort of hybrid system in that they may use information from in-line, on-line, at-line, or off-line measurements to generate a value. These systems frequently use less than optimally calibrated sensors to generate some fairly accurate information about the state of a bioreactor. There are several good examples of when data from single sensors correlate poorly with the predicted value but excellently when combined with other sensor inputs [36]. This article models data from many of the sensors described previously, including NIR, dielectric spectroscopy, 2D fluorescence spectroscopy, and proton transfer reaction mass spectroscopy.

2.2.7 Trajectory Analysis

Most of the methods described above have the potential to allow fingerprinting of the culture by "trajectory analysis." For example, the use of NIR for trajectory analysis has been proposed by various authors as a means of rapidly assessing whether a process is proceeding "normally," for example, Hakemeyer et al. [16]. Sandor et al. [37] discuss the potential applications of NIR to bioreactor monitoring and include the use of NIR trajectory analysis. Trajectory analysis offers the advantage of not requiring a calibration model to be built which may fail or require frequent updating as raw materials vary. Information obtained this way could potentially be used to determine rapidly whether a fermentation is not proceeding "normally." If this is not the case and the trajectory leaves a predefined range, there are two options. It is possible either to drill down to the actual problem and try to solve it or to confirm that the process is too far from the limits, cannot be saved, and should therefore be terminated. Such drill-down diagnoses rely, for example, on Q-residual contribution to identify the root cause of the problem. However, there are still lots of questions about this approach (e.g., confirmation of causality, signal resolution).

A long-term objective could be that the process should be thoroughly understood and designed in such a way that all sources of variability are identified and well-controlled. This could eventually be an acceptable method of rapidly releasing product for forward processing (real-time release).

2.2.8 On-line Measurements

On-line sensors are those analysis technologies which lie outside the fermenter. They differ from at-line or off-line analyzers in that they are capable of automatically sampling and analyzing the bioreactor contents without manual intervention by the production personnel. The drawback to this type of system is that they require a sampling interface to the fermenter that enables a sample to be drawn and delivered to the analyzer, while ensuring that the fermenter remains sterile.

2.2.9 Sampling Devices

Developing a suitable sterile sampling device has turned out to be a challenging task. Historically, the approach has been to utilize sample probe material with a pore size small enough to provide a sterile barrier. This approach was first described 30 years ago in a patent from Eli Lilly and Company for a sintered stainless steel sampling probe [38]. Several commercial systems are now available that utilize this approach of using filter material pore size to create a sterile barrier. Besides the obvious concerns about potential sterility breaches caused by these sampling systems, the use of small pore-size filters to provide a sterile barrier excludes the cells from the extracted sample. Therefore, it is normally possible to measure the biomass or cell concentration with cell counters such as the Cedex HiRes® analyzer. Alternatively, a steam-in-place-type sampling device can be used that enables the entire contents to be extracted from the bioreactor for analysis. Withdrawing an intact sample enables off-line viable cell counting from the sample [39]. BaychroMAT® seems to be one of the best automated on-line sampling and analysis systems, although automated on-line sampling devices, in general, have not been widely accepted by the biopharmaceutical community.

2.2.10 On-line Liquid Chromatography

Despite the challenges described above, on-line sampling devices offer the advantage that they can be combined with technologies with superior sensitivity, resolution, and robustness compared to spectroscopic measurements, such as chromatography-based methods.

As the chromatographic column generally eliminates any matrix effect, simple, direct linear calibrations can be used that avoid the need for chemometric models to correlate an analyzer to a reference method. For example, interfacing on-line chromatography with electrochemical detection can provide a complete amino acid analysis, as well as carbohydrate analysis. Organic acids and even trace metals can also be analyzed by on-line chromatography. Furthermore, on-line HPLC analysis even has the potential to measure CQAs during production if the product is released into the media.

If the process is adequately understood, it may be possible to perform feedback control of various CPPs, such as controlled feeding of a particular amino acid or sugar to ensure that CQAs are achieved. The use of online chromatography in a pilot plant to study the uptake rate of key amino acids, coupled to feedback control of the amino acid feed rate in the bioreactors, was shown by Rapoport et al. [40]. Online chromatography or high-performance liquid chromatography (HPLC) are typically not thought of as "real time," but solid phases with smaller particle sizes are now enabling chromatographic analyses to be performed much more rapidly. For example, amino acid profiling via pre-column derivatization and HPLC analysis can be performed in as little as 10 min in some cases, with the longest time being approximately 30 min per profile, with direct injection into an HPLC with electrochemical detection. Although 30 min may not resemble "real time," it is adequate for the time scale of a typical bioreactor and can even be fast enough for a single analyzer to be multiplexed to more than one bioreactor. A recent article described the use of a 2-dimensional HPLC assay to monitor charge variants in an monoclonal antibody (mAb) product [41]. Although the title describes the analysis system as at-line, it is actually an on-line system with automated sampling performed by a Groton Biosystems sampler. This is an excellent example of the capabilities of HPLC to monitor a CQA that can be adapted to on-line monitoring of the bioreactor as it produces the product.

2.2.11 Mass Spectroscopy

Mass spectroscopy has been utilized for several decades for off-gas analysis in fermentation [42]. Although this is technically an on-line measurement, as a sample (headspace vapor) is extracted from the bioreactor, there is no direct contact with the bioreactor media. This obviates the concerns about compatibility of materials of construction (MOC) often associated with in-line sensors, as well as issues about breaching sterile containment, as the sensor is placed outside the sterile environment. Historically, low resolution mass analyzers have been used to measure O_2 and CO_2 in the off-gas to calculate the oxygen uptake rate (OUR) and respiratory quotient (RQ). Within biopharmaceutical manufacturing, off-gas analysis is very well-established in microbial (*Escherichia coli*) fermentations, but is rarely used in mammalian cell culture. With high resolution mass spectrometers or proton transfer reaction mass spectrometry [43] it is also possible to measure other volatile metabolism products, such as organic acids, which may provide interesting information about the state of cellular metabolism. Although mass spectrometers are fairly expensive, their very short analysis time (typically of the order of tens of seconds) makes it very easy to use one multiplexed instrument to monitor 10–20 fermenters or more. Multi-port sample multiplexing valves or sample multiplexing panels of assembled components can be constructed that fit into a compact space.

2.3 Closed Loop Control of Fermentation Processes

Biopharmaceutical production processes that rely on living organisms as "producers" of an intended molecule are often chaotic in nature. Initial conditions cannot be exactly reproduced, and minor differences in starting conditions, such as make-up and/or inoculum strain history, can have a significant impact on the performance of the culture. Furthermore, once a process has been "set," manufacturing is effectively "along for the ride," with minimal ability to control or influence performance of the culture. There are many reasons for these "traits" (e.g., chaotic determinants may be working cell bank (WCB) vials and their storage conditions, batches of media components, etc., and minimal control capability, e.g., dO_2, pH, temperature, pCO_2, agitation rate, and nutrients by bolus or continuous feed). Some countermeasures have been developed (e.g., randomization of WCB vials in storage and utilization in cultures, large batches of media to feed several cultures, etc.). However, the development of the culture process and conditions, particularly utilizing QbD methodologies, should provide the knowledge to control these processes more effectively.

If appropriate, PAT is implemented in the culture processes on the basis of optimized knowledge of the development, which should provide the opportunity to address these issues by creating closed control loops that effectively stabilize the processes.

Development activities, particularly those utilizing QbD principles, should provide a thorough list of CQAs for the product. The CQAs for N- or C-terminal modifications of structure, amino acid sequence, and product-related impurities – including different post-translational modifications – are often directly impacted by culture conditions. Once these CQAs have been identified, development studies should determine the culture conditions that impact each of these CQAs. This knowledge provides information about the PAT tools that need to be identified and used to control the culture process.

Studies conducted with *E. coli* fermentations more than 20 years ago led to the development of fed-batch cultures, where cell number, RQ, and glucose concentrations (in the fermenter) were used to optimize the fermentation for titer [44]. This system, although rudimentary today, was a break-through for fermentation at the time.

Another recent example from Zupke et al. [45] describes the use of predictive modeling control of the glycosylation pattern, based on the analysis of cell culture media components. The level of high mannose species in a therapeutic antibody could be influenced by raising or lowering the level of mannose in the cell culture media. Moreover, the authors generated a model of predictive control, which allowed the running process to be adapted to meet the desired product quality.

Various published case studies [46–53] demonstrate that PAT can be used for the real-time control of culture processes with closed loop controllers. Nevertheless, other factors seem to have inhibited the implementation of PAT in bioprocesses. These apparently include concern about the complexity of PAT in the

manufacturing environment, concerns about development resources to appropriately define processes and analyses to support these processes, and a fundamental lack of understanding of the potential power in PAT systems and real-time process management.

2.4 PAT Applications in Purification Processes

PAT has been used, as previously indicated, in downstream processes to control purity, maximize yield, and reduce cycle time with significant unit cost benefits. Compared to applications in cell culture, implementation of PAT for use in downstream processes, particularly chromatography, has turned out to be challenging. This is largely because of the rather long time to result of applied analytical methods compared to the short time available for decisions impacting the process. In many cases, the time needed for analysis exceeds the time window for process decisions, so that it is difficult to implement PAT tools.

It is worth highlighting the fact that in-line UV and conductivity sensors, that is, true PAT tools, are routinely used in all chromatography and ultrafiltration/diafiltration operations today. They allow process monitoring, for example, control of chromatography gradients, endpoint analysis of concentration steps, and, most importantly, setting specific pooling criteria for defined product quality.

For over two decades, HPLC has been used as additional application to assess process performance continuously. Particularly in cases where a process decision based on UV is not optimal, for example, pooling during a high-resolution separation, HPLC makes it possible to differentiate between the product of interest and other species. On-line monitoring of these product- and process-related impurities also accounts for differences in load material and thus can ensure consistent product pool quality.

On-line reversed-phase HPLC was used to measure aggregates in the purification of recombinant human insulin-like growth factor-I (IGF). The assay allowed real-time control of purified IGF collection [54]. In a similar set-up, antibody loading onto protein A affinity chromatography was reliably controlled through an on-line chromatographic assay. This allowed real-time measurement of antibody breakthrough during the load phase, thus ensuring constant yield despite differences in titer and protein A capacity [55].

Rathore et al. [56] developed an on-line size exclusion chromatography technique that allowed real-time decision-making on chromatographic pooling. The method allowed consistent product pool purity, despite large variations in the initial feed purity. Although initially performed only on a small scale, a similar setup has successfully been implemented on a large-scale pilot plant. Pooling criteria were defined by on-line HPLC in this large-scale setting. Pool purity levels achieved with the PAT setup were comparable to product quality achieved with pooling based on fractionation followed by off-line analysis. These results show that on-line HPLC

analytics and implementation in a large-scale purification process is both feasible and beneficial in order to achieve consistent product quality.

Antibodies are the most common biological therapeutics. Non-antibody biologicals, for example, enzymes, often pose greater challenges to downstream processes, particularly when preparing these molecules for further analytical characterization. Brower et al. [57] recently proposed an on-line HPLC method based on single-step immunoaffinity purification that could respond to these problems in sample preparation and be used as a PAT tool in these processes.

A combination of HPLC, differential refractometry, and multi-angle laser light scattering (HISEC-RI-MALLS) was used to characterize a therapeutic human immunodeficiency virus envelope protein [58]. The combination of these methods allowed the investigators to obtain the molar mass of macromolecules, independent of their shape or hydrodynamic radius, and is thus an ideal tool to define oligomerization states of these molecules. The method proved to be comparable to orthogonal methods, such as native polyacrylamide gel electrophoresis and ultracentrifugation analysis. Although the latter are time-consuming and thus inadequate for on-line monitoring, the HISEC-RI-MALLS analysis is a suitable method for manufacturing control.

In addition to monitoring chromatography unit operations, HPLC can also be used to provide guidance for the development of filtration steps such as microfiltration. Microfiltration processes have more recently been employed as downstream purification steps in tandem with selective precipitation of either product or impurities. Large-scale implementation of these microfiltration purification processes is particularly challenging because of the deposition of insoluble material on the membrane surface or inside membrane pores. To circumvent the scale-up problems, Watson et al. [59] implemented an ultra performance liquid chromatography-based method to monitor impurities and combined these at-line results with a mass balance-based model of ideal filtration performance. The results were used to define large-scale operational changes and increased step yield by $>20\%$. This example also shows how PAT tools can be used successfully during process development to guide transfer into large-scale manufacturing processes.

In addition to these on-line and at-line experimental tools, multivariate data analysis (MVDA) has been applied to analyze downstream processes, particularly to ensure consistent column packing. Principle component analysis (PCA) has been shown to be a suitable tool to assess process performance across a column lifecycle [60]. Signs of column underperformance, including backpressure buildup and inefficient deoxyribonucleic acid clearance, could be predicted using chemometric analysis.

Another approach to analyze column integrity is transition analysis of chromatographic profiles. In general, these studies use signals from step transitions between buffers of different conductivities to describe the same dispersion parameters as the traditional pulse injection method. Bork et al. [61] showed that it is feasible to monitor large-scale manufacturing processes by this method and to establish control limits based on historical data to prevent issues during manufacturing (see Sect. 3.3).

2.5 Monitoring CIP Processes

CIP processes may only be secondary processes for the biologics manufacturing industry, but they are especially suited to implement PAT tools.

There is a lower risk from a QA/QC perspective than for other potential PAT applications, as the biopharmaceutical product is not directly involved. In addition, the point at which the CIP solutions are monitored is downstream of the actual production tank, so there is no risk of contamination if a sensor or sampling device is in contact with product during a production run, as is the case with the other examples listed above.

CIP monitoring following a PAT approach involves using on-line or in-line analytical technology to determine when the end point of the CIP cycle has been reached by actual measurement of the removal of a potential process contaminant rather than by using a fixed time or volume, on the assumption that this represents removal of the process contaminant. The on-line or in-line technology could be used to monitor the rinse water for the presence of contaminants, as well as residual cleaning agents. When these contaminants are measured below the target level, the CIP cycle is stopped automatically and the tank can be returned to service. The key quantifiable benefits of these approaches are:

- Reduction in the use of rinse water and the costs to generate that water
- Reduction in overall CIP cycle time, allowing the tank to be returned to service sooner and thus increasing production throughput

CIP monitoring is a PAT project that has excited fairly widespread interest in the biopharmaceutical industry for the reasons mentioned above. The gold standard for determining that a piece of equipment has been thoroughly cleaned is the swab test, followed by off-line HPLC analysis. Although this is a very sensitive method to determine cleanliness, it is not suitable for rapidly and/or automatically determining the CIP cycle endpoint because of the delay in the off-line HPLC analysis. To automatically determine the CIP endpoint from contaminant analysis, one needs to use a measurement technology that can be applied on-line or in-line. On-line/in-line analysis of the CIP solution involves installation of an on-line or in-line sensor downstream of the process equipment that is being cleaned. Depending on whether or not the equipment is cleaned by a fixed or portable CIP system, the sensor can be installed in the drain line of the process equipment or in the drain line of the portable CIP system. Technologies that have been successfully applied in this way include:

- On-line total organic carbon (TOC) analysis [62]
- In-line conductivity [62]
- Light-induced fluorescence [63]
- In-line UV spectroscopy [64]

2.6 Data Management: Availability for Monitoring, Control, and Analysis

Data management, that is, data acquisition, data analysis, data storage, data retrieval, and data correlation, are significant issues, particularly in the highly regulated pharmaceutical industry, but also in process development.

Monitoring and controlling processes and the analysis of historical data require the provision of meaningful data from bioprocesses, in appropriate formats and with all necessary metadata. Data structures from fermentation processes are complex and often need harmonization prior to use. Non-contextualized and non-aligned numerical values are worthless. To this end, it is important to provide all metadata necessary to align the different data types (e.g., in-line and at-line data) and formats. It may be necessary to prioritize data if more than one analysis of the same type is available per sample. The interpolation of data to align in-line data with at-line or off-line data can employ various algorithms.

Another challenge is the integration of additional process information and event logs, recorded within different components (e.g., in an electronic lab notebook by the supervisor, in the historian, sample management by an operator, or by the system itself) along the course of the process. This information is often needed to interpret data correctly.

The integration of data formats such as, for example, spectra or information derived from this with classical in-line data (e.g., pH, dO) and at-/off-line data (e.g., glucose concentration in a sample from the biosuspension) is still a challenge, especially in a regulated setting where all components have to be fully validated. This is particularly true if you wish to automate your data in such a way that they can also be used for automatic control of the bioprocess.

The performance and availability of the systems for data management have to be high, which in turn increases the operating cost.

Many commercial systems are available for data management, (e.g., PI, Discoverant, etc.), but the industry seems to suffer from the DRIP issue ("data rich – information poor"). The sheer volume of data that can be collected can be daunting. One example that illustrates this is the on-line pH data from a bioreactor: With a sampling rate of fractions of seconds, and a culture period of weeks, this can result in millions of data points per batch. However, in many cases the vast majority of the data collected is not particularly useful; for example, in the pH example, approximately >200 points would provide useful information when the pH was shifted or the process went out of control because of equipment failure.

Application of the above-mentioned PAT tools generates huge amounts of data. Data management and correlation require the services/expertise of trained statisticians and/or chemometricians. In the final analysis, the quality of process supervision depends on the quality of the data management.

Data management and analysis tools commonly used in the biotechnology industry include, but are not limited to:

- Filtration
- Compression
- Statistical process control
- Multivariate statistical process control
- Time-sequence analysis
- PCA

Data compression consolidates data based on a predetermined algorithm. Statistical process control (SPC) is a univariate analysis of process parameters and attributes. Process parameters and specifications should typically be monitored using SPC (this is becoming a regulatory expectation and industry standard practice). MSPC consolidates a number of parameters and/or attributes into a single control chart. The calculation of the T^2 for means and T^2 for dispersions should be established by a statistician/chemometrician, in conjunction with process experts. PCA can be thought of as revealing the internal structure of the data in a way that best explains the variance in the data. A statistician/chemometrician should be consulted for establishing PCA data monitoring.

If mathematical models are employed in the scope of process control, operators with an appropriate qualification are required. The use of models within a regulated bioprocessing environment is still not at all common and one can expect much discussion, at least with QA, until approval of such a strategy.

3 Case Studies

3.1 On-line Estimation of Biomass Concentration

For state estimation, one usually employs static estimators [65]. Current classical engineering correlations are being replaced by advanced estimation techniques. Artificial neural networks (ANN) have primarily been proposed for this purpose [34]. When they are trained on the cumulative signals of the OUR and the carbon dioxide production rate (CPR) as well as on the total base consumption, they lead to excellent estimates of biomass. The reason for cumulating the original signals is to improve the signal-to-noise ratio and thus increase the information content about the process. In addition, as the biomass and its metabolic products accumulate during the cultivation, these masses are better correlated with the cumulative signals of OUR and CPR.

Examples from *E. coli* cultures showed that the data from all cultures in a given process can be described by a single ANN [32] with rather small estimation errors in a biomass concentration of about 0.5 (g/kg) or only 1%. An important advantage of these static estimators is that they can be set up immediately after a distortion in the system if their input signals are still available. This property is not guaranteed with the dynamic estimators discussed later.

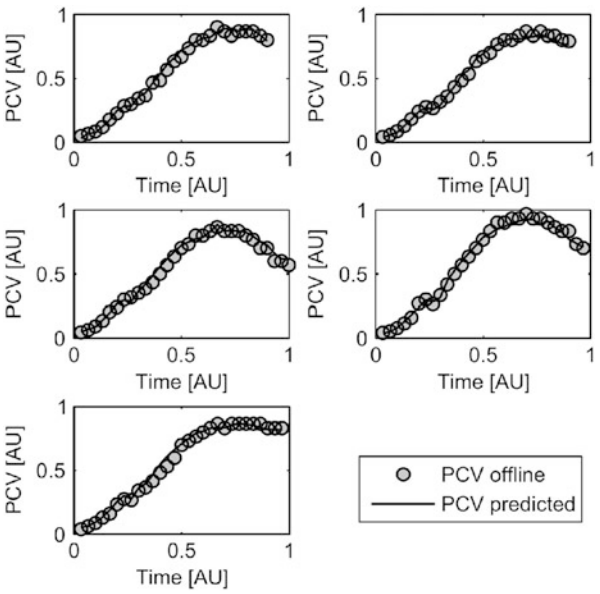


Fig. 3 ANN-based estimation of PCV signals in five independent batches of a recombinant mAb-cell culture production process based on on-line signals of the aeration rate of oxygen, total gas supply, base consumption, and culture volume [66]

The application of ANNs for biomass estimation is not restricted to microbial systems. As shown in Fig. 3, they deliver reliable values in large-scale industrial cell cultures for a recombinant therapeutic antibody. Thus, such ANNs are not restricted to laboratory-scale experiments. Although the data depicted in Fig. 3 had to be normalized in time and packed cell volume (PCV) for proprietary reasons, it is becoming clear that performance can be estimated well with this technique.

Recent developments, particularly in the machine learning community have provided some further estimator developments that compete with ANNs. The key expression is "kernel techniques." These allow us to estimate biomass, for instance, by means of a linear combination of nonlinear functions, referred to as kernel functions [67]. An important example of such a kernel function is the radial basis function, that is, a Gaussian bell. These kernels are data centered, that is, centered at the available measurement points. A key advantage of these representations is their sparseness, meaning that well-performing mappings can be obtained with a few basic functions only. In this way, irrelevant terms of the linear combination are discarded. Two important variants of the kernel techniques are the "support vector regression" (SVR) [68] and the "relevance vector regression" (RVR) [69]. SVR determines the regression parameters in a way similar to conventional least square techniques but with different objective functions, whereas RVR uses Bayesian statistical approaches for that purpose [70]. The latter has the advantage that it automatically performs some model complexity control by automatically removing all irrelevant terms in the sense of Ockham's razor and thus avoids over-fitting, a problem appearing with most other estimation techniques [71]. The most significant benefit of both techniques is that they result in a globally optimal solution [72].

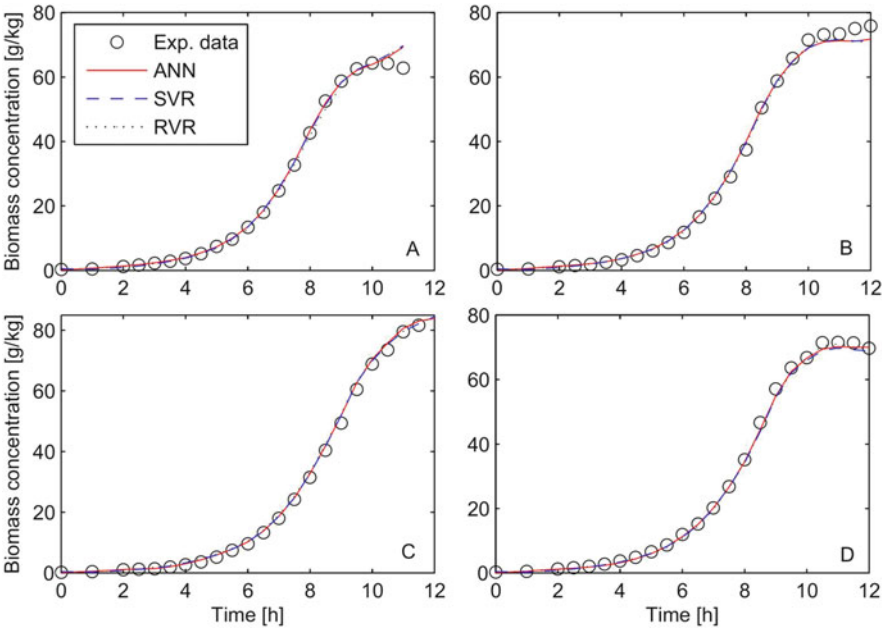


Fig. 4 Comparison of results obtained with ANN, SVR, and RVR. All were trained on the same data set [73]. Estimates made using these three techniques are shown for four different experiments. The data shown are validation data, not used for training the estimators [66]

Figure 4 shows a comparison of the estimates derived from the three static estimators mentioned after training on the same data set. All three techniques give essentially the same estimates across the entire fermentation. This is what we expect, but the important point to note is that the kernel methods need a significantly smaller number of coefficients and, as is important to validation procedures, the same set of coefficients is yielded when the calculation is repeated. It should also be noted that all three estimators depict the same deviations from the data in the upper left corner for culture times above 9 h. This suggests systematic errors in the measurement data or in process behavior and not in the estimation techniques. These alternatives to ANNs should appear more often in biotechnology literature in the near future, as they significantly simplify model complexity control (or over-fitting) and model design issues [74].

3.2 Dielectric Spectroscopy as Basis for Feed Rate Control in Mammalian Cell Culture Processes

Viable cell density (VCD) is an important variable in mammalian cell fermentation that is routinely used for monitoring and manual control. Dielectric spectroscopy

can be employed to predict VCD in mammalian cell fermentation. This can be implemented by two different strategies:

- Permittivity difference at two distinct frequencies to be correlated with VCD
- Integration of all spectral information (permittivity values over the entire frequency band) and prediction of VCD by using a multivariate calibration model (to be developed in advance)

Better results in terms of noise and prediction power in later process phases can be obtained by employing the second strategy. Processes that need tight monitoring and control of process variables (e.g., nutrient concentration) can benefit from dielectric spectroscopy in two ways:

- Much higher resolution (down to 0.5/min)
- Diverse options for process control based on the predicted VCD (can be integrated into classical control approaches)

One example of an application is the prediction of VCD by dielectric spectroscopy according to the second strategy used as input for an algorithm that calculates the feeding rate, in order to control the nutrient concentration level. This algorithm also integrates other in-line variables and the resulting signal is transferred to a controller driving a conventional bioreactor feed pump. This control concept assures that:

- The nutrient concentration (in biosuspension) is very low so as to provide high product quality
- The feeding rate is high enough to support optimal cell growth and productivity

The benefits of such a control strategy are:

- Lower process variability
- Less sampling of biosuspension and less at-line analytics necessary (because no manual control is based on this)
- Relieving the operators' workload and thus increasing throughput (more processes per time interval)

In Fig. 5, the VCD during the process of mammalian cell fermentation for the production of a mAb in a 1-L bioreactor is compared to VCD analyzed by Cedex (Custom Biotech, Roche Diagnostics GmbH, Germany). The multivariate prediction model uses the entire spectral information of the dielectric spectroscope. The predictive power of this method is obvious. Even in later process phases, where prediction is sometimes not of high quality when based on only two frequencies, there is a good correlation with the off-line analyzer. However, an offset is observable within this process phase. After taking into account the measuring tolerances of an at-line method (and the manual steps within), the quality of the predicted VCD is still good enough to be used for process control.

Figure 6 shows the effect of this type of process control on the nutrient concentration to be controlled in comparison to a manual control setting, where the VCD of the at-line analytical device is used as input for the algorithm calculating the feed

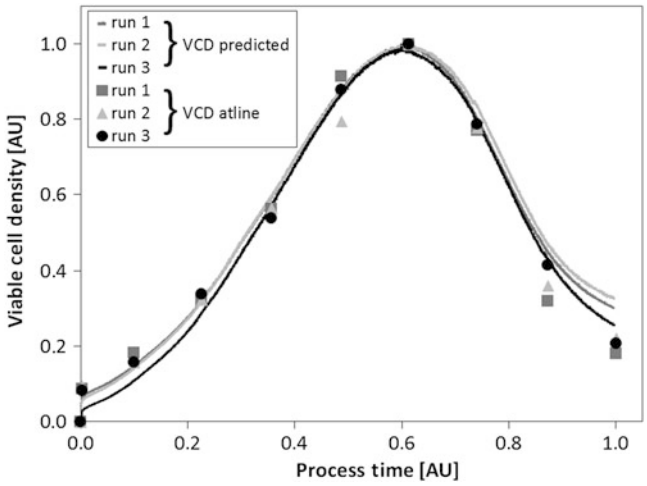


Fig. 5 VCD during the process of mammalian cell fermentation for the production of a mAb in a 1-L bioreactor. *Symbols* (VCD at-line): VCD analyzed by Cedex (Custom Biotech, Roche Diagnostics GmbH, Germany). *Lines* (VCD predicted): prediction of VCD based on dielectric spectroscopy (evo456XL, Hamilton, Swiss)

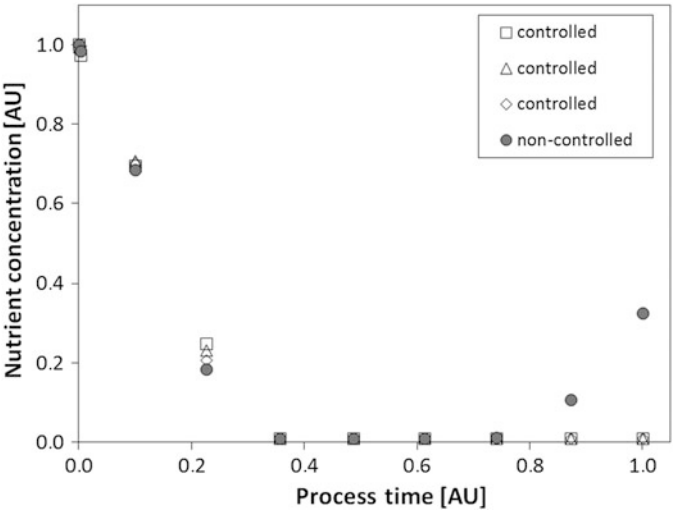


Fig. 6 Nutrient concentration during the process of mammalian cell fermentation for the production of a mAb in a 1-L bioreactor. *Open symbols* (controlled): nutrient concentration employing feed rate control based on dielectric spectroscopy. *Filled circles* (non-controlled): nutrient concentration with manual control of feed rate based on at-line determination of VCD by Cedex (Custom Biotech, Roche Diagnostics GmbH, Germany)

rate. It can clearly be seen that the automated control keeps the glucose concentration at a low level. It is important to mention that product quality was within the acceptance range for the mAb produced, in comparison to the manually controlled fermentation runs.

Effectively, no operator activity is necessary for the controlled runs based on in-line dielectric spectroscopy. The samples have only been drawn and analyzed to show that the approach works as expected.

This is only one example of how to apply dielectric spectroscopy as input for fermentation control. As this technique can be integrated in several control concepts, there are more options for its use.

3.3 On-line Integrity Monitoring of Chromatography Columns

One factor that can have a significant effect on chromatographic performance is the quality of the column packing [75]. The current standard procedure for testing the quality of a packed bed liquid chromatography column is to use a non-absorbed tracer to perform a pulse-injection experiment. The injected tracer solution is assumed to be a Dirac pulse. The pulse exits the column as a peak because of axial dispersion. Plate number, N, describes the degree of the dispersion, which is influenced by the packing quality of the column bed.

A related term, "height equivalent to a theoretical plate (HETP)," provides a measure of peak broadening in relation to the distance the tracer has traveled in the chromatography column. The mathematical definitions of N and HETP are given by the following equations:

$$N = {V_r}^2/\sigma^2 \tag{1}$$

$$\mathrm{HETP} = L/N \tag{2}$$

where V_r is the retention volume, which is defined as the volume that has passed through the column, from the time when half the tracer is applied to the time when half the tracer has exited the column. In other words, V_r is the mean exit volume of the injected tracer, σ^2 the variance of the exit volume distribution, and L the column length. On the basis of the normal density function, the width of a curve at half peak height, W_h, is equal to $2\sigma(2\ln 2)^{1/2}$. Because the peak generated by the tracer as it exits the column is assumed to follow a Gaussian distribution, N is usually calculated with the simplified formula shown in Fig. 7.

In recent years, efforts have been made to use process chromatography data directly to determine column efficiency in order to achieve real-time monitoring [61, 76, 77]. The common approach taken in these studies is to utilize information from step transitions between buffers of different conductivities to describe the same dispersion parameters as the traditional pulse injection method. One method is

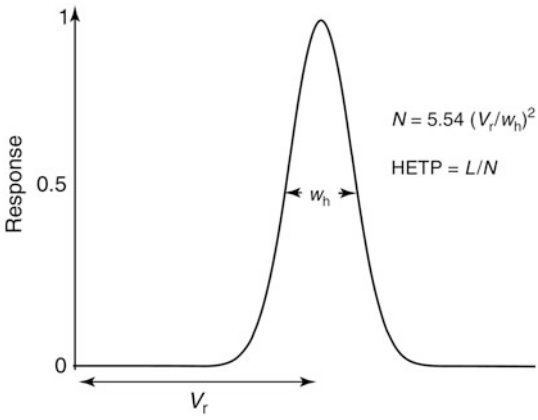


Fig. 7 Plate calculation via the traditional pulse injection method [61]

to transform a breakthrough curve or a washout curve into a peak by taking the first derivative [77]. The dispersion parameters are then derived from peak position and shape. To avoid the inaccuracy in the calculation caused by assuming a normal distribution, algebraic functions other than the normal probability density function were evaluated, and a function that can describe a large number of step transitions has been identified. However, arbitrarily assigning a predetermined function to represent unknown distributions has disadvantages.

Depending on the column packing quality and the running conditions of the chromatography, there are transitions that differ significantly from the chosen function and cannot be adequately represented by it. In these cases, the forced fitting of transitional data to the function would cause loss of information. Another method is to treat the exit volume of the solution that is replacing the original solution in the column as a discrete random variable [76]. The incremental change in a response signal, such as conductivity, serves as the frequency of each exit volume. The starting point for the transition occurs when zero L of the displacing buffer has run onto the column. The conductivity recorded at this point corresponds to that of the buffer on the column at that time ($C_{\min}$). After a sufficient amount of the displacing buffer has flowed through the column, the conductivity reaches a new equilibrium ($C_{\max}$). $C_{\max}$ is equal to the sum of $C_{\min}$ and the definite integral of dC, which is integrated from $C = C_{\min}$ to $C = C_{\max}$. To simplify the calculations, C may first be normalized using the following equation:

$$C_{\text{normal}} = (C_i - C_{\min})/(C_{\max} - C_{\min}) \tag{3}$$

As shown in Fig. 8, V_r and σ^2 can be calculated from the transition curve using the rectangular approximations of their integral forms.

Monitoring the integrity of large-scale packed bed liquid chromatography columns using transition analysis can provide useful information about the process. Figure 9 provides an example of how the HETP value derived from the transition analysis of a protein A chromatography column changed over multiple cycles of processing. The values increased with time after initial column packing (A).

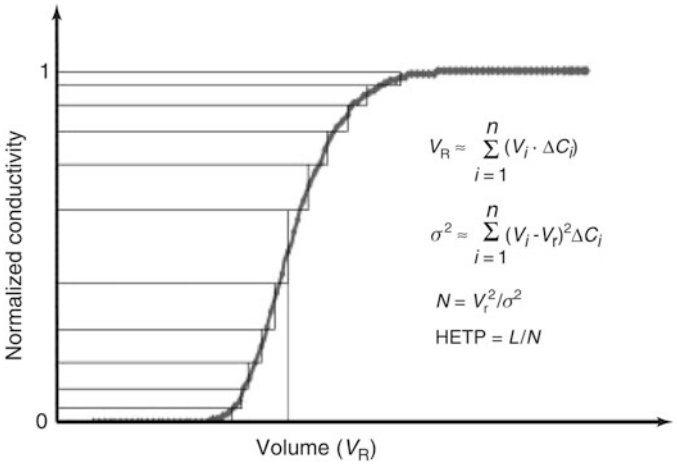


Fig. 8 Plate calculation via the transition analysis method

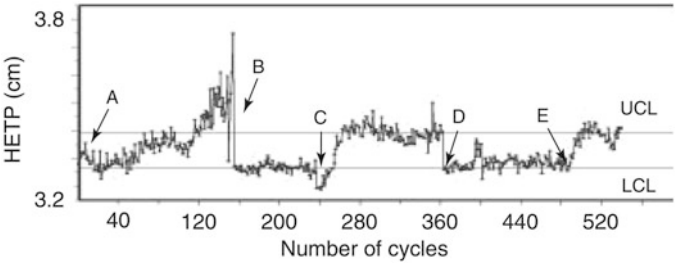


Fig. 9 Control chart of protein A column HETP derived from transition analysis [75]. *UCL* upper control limit, *LCL* lower control limit

Increased measurement variability was also observed as integrity decreased. It was subsequently shown that this was caused by the formation of a headspace on the column. When the top flow adapter was lowered to eliminate the headspace, the HETP values were restored to their original values (B). However, subsequent repacking of the column once again resulted in rapid degradation of the column integrity because of insufficient consolidation of the resin during the packing procedure (C and E). The flow adapter was lowered again after the second packing (D) to improve column performance.

3.4 Alternative and Rapid Microbiological Methods

Bioburden testing plays a crucial role in ensuring product quality during pharmaceutical manufacturing. It spans the complete process, starting with testing raw

materials and ending with the final drug product. Despite its role, the vast majority of tests are still performed using traditional methods originally developed well over 100 years ago [78, 79].

The major disadvantages of these methods are the long time-to-result and the lack of automation. In routine pharmaceutical bioprocessing, results from microbial testing, for example, pre-filtered cell-culture media or in-process controls for purification, are often obtained after use of the respective material. Thus it is not possible to react to a positive result in a timely fashion, which may result in the loss of an otherwise acceptable manufacturing batch. In addition, the large number of samples – particularly for water and environmental testing – necessitates automated routines that cannot be implemented with classical methods.

Several commercially available alternative and rapid microbial methods (ARMM) have addressed this issue (for a comprehensive overview see http://rapidmicromethods.com/files/matrix.php). Growth-based methods rely on traditional culture of samples but utilize sophisticated, highly sensitive methods for detection [80]. Non-growth-based methods, including, for example, spectroscopic methods or fluorescent labeling, allow even faster detection of microorganisms, even in real time [81, 82].

Parveen et al. [80] evaluated ARMM based on the detection of growth by adenosine triphosphate (ATP) bioluminescence (Rapid Milliflex® Detection System) and CO_2 monitoring (BacT/Alert and the BACTEC systems). The former was shown to be acceptable as an alternative sterility method and took 5 days rather than 14 days in the compendium method and even proved to be superior to compendium methods with respect to detection time and sensitivity. Technologies based on CO_2 monitoring were not as effective as the bioluminescence methods, but provided a potential inoculation sterility method for products that do not contain preservatives or antimicrobial agents.

Additional studies with bioluminescence-based ARMM showed that this technology offers a rapid sterility method and yields results that are equivalent to or better than those obtained with the traditional method. With an incubation (enrichment) phase, the results were easier to interpret and to compare with the methods in the compendium, as both rely on the growth of the microorganisms under conditions similar to those described in the pharmacopoeia [81].

Solid-phase cytometry as used in the ScanRDI® system is based on fluorescent labeling of viable microorganisms collected on a filter membrane and their subsequent automated detection and enumeration by a laser-scanning instrument. The ScanRDI® system appeared to as sensitive as the standard method.

Despite a number of regulatory guidance documents on the validation of ARMM [83, 84], these have been rarely implemented within the pharmaceutical industry.

Novartis and Sandoz have implemented rapid sterility testing based on bioluminescence in a routine setting [85, 86]. In addition to quantification, Grey et al. showed that it is possible to identify non-sterile findings even after using

bioluminescence reagents that disrupt cell walls. Regrowth of the treated microbial cells and subsequent genotypic identification reproduced feasible and robust results and showed sufficient recovery.

As with other PAT tools, regulatory acceptance and return-on-investment remain the greatest hurdles. As vendors, regulators, and pharmaceutical companies continue to build and share experience in the use and implementation of ARMM, it should become increasingly easy to gain rapid approval and thus to support these procedures as routine PAT tools.

3.5 Residual Moisture by NIR

In addition to the applications in bioprocessing described above, NIR spectroscopy has been widely used to determine water content in both small molecules and lyophilized biological products [87]. Residual moisture is a CQA in these formulations because it can dramatically influence stability and thus the shelf-life of the product. As water shows two prominent and distinctive bands in the NIR spectra, at 5,155 and 6,895/cm, NIRS is particularly well-suited for this application. Examples include determination of residual moisture in powders or granulates [88–90], tablets or capsules [91, 92], and in lyophilized vials or in solutions [93].

The advantage of this technique when compared to the traditional methods, such as the Karl Fischer titration or thermogravimetry, is that it offers rapid, non-invasive, and non-destructive measurements, especially when applied as an on-line tool, for example, in fill/finish manufacturing lines [94]. The improvement in time-to-result allows us to measure entire manufacturing batches for their residual moisture, unlike traditional procedures, which focus on representative samples only. Analysis is performed non-invasively, avoiding contamination through atmospheric moisture. In addition, hazardous or highly active compounds, for example, antibody–drug conjugate, can be handled with a much lower safety risk. Finally, the non-destructive nature of the application allows additional, orthogonal methods to be performed, for example, to confirm out-of-specification results or for use in stability testing.

For absolute quantification of residual moisture by NIRS, a chemometric model has to be developed that uses classical methods as reference, such as the Karl Fischer titration. Although small physical or chemical variations (e.g., surfactant or buffer content) can be accommodated by a standard NIR calibration, significant formulation changes may cause alterations in spectral features and thus invalidate the multivariate model [95]. Such calibration is specific for a fixed formulation and product configuration, and thus makes the method highly suitable for use in routine manufacturing, but not for development applications with a high product turnover.

4 Conclusion and Outlook

4.1 Status of PAT Implementation in the Biopharmaceutical Industry

In general, it is difficult to come to an accurate assessment of the implementation of PAT in the biopharmaceutical industry. There have been many publications and/or presentations describing the use of PAT in the biopharmaceutical industry following the release of the FDA's PAT Guidance Document, but it is difficult to assess from these presentations/publications how broad the use of the described applications actually is. Many of the applications described in these presentations/publications imply that the techniques are in routine use, but internal discussion often suggests something entirely different. It would appear that, in many cases, the literature is describing applications that have only been explored for possible use or have only been utilized in a few isolated cases, but not implemented on a broad, routine basis. This is also true for research originating from academic institutions where PAT research is stated to be performed and/or supported through collaboration with a biopharmaceutical company, but the research appears to be only work supporting a student's thesis and never routinely applied at the sponsoring company.

In spite of the support and pressure from regulatory agencies over the past 10 years to implement new approaches to the QC of biopharmaceutical manufacturing (ever since the FDA published the PAT guideline in 2004), the consensus opinion across the industry seems to be that progress on this initiative has been patchy at best. PAT has seen increasing use in the production of small molecules [96, 97], but little change has been observed for biologics manufacturing, especially in fermentation and cell culture operations. There have been developments in the use of various technologies for monitoring and controlling these operations (e.g., NIR, Raman, fluorescence, on-line HPLC, model predictive control, etc.) at academic research centers and some exploration of these technologies within biopharmaceutical companies, but these technologies seem not to have found broad, routine use across the industry.

The sensing technologies that are used routinely continue to be those that have been used for decades to monitor and control the environment in which the cells are grown (e.g., pH, dO, temperature, etc.). The major reasons why the industry seems to be so reluctant to implement new technologies seem to be:

- The complex regulatory environment
- The level of process understanding within the fermentation/cell culture field
- The difficulties in implementing the available technology
- A lack of understanding of the ROI that could be generated

The culture of the biopharmaceutical industry has historically been very conservative. From a regulatory perspective, it is a risk-averse culture. New, previously unapproved technologies are typically only used in manufacturing when they offer

the only way to produce a product. Companies often intentionally avoid being at the leading edge of manufacturing technology because of fear that it may raise concerns from the regulatory agencies reviewing their license application and, as a result, delay approval of their product. This ultimately delays the product reaching the marketplace and generating a stream of revenue.

This "regulatory paranoia" has severely slowed the implementation of PAT in spite of the fact that documented use of process analytics by the chemical industries dates back as far as the 1940s. The common retort to this observation is that the pharmaceutical industry is "different" and the chemical industries are not regulated. Although this observation is accurate to some degree, the nutrition industry has been using process analytics for well over a decade in processes that are indeed regulated by the FDA. A comment was once made by Mark McClellan (at that time FDA Commissioner) to the Wall Street Journal at around the time that the FDA released the PAT Guidance Document that the science of drug manufacturing was behind "that of potato chip and soap making" [98]. Similar observations have been made when comparing the pharmaceutical industry to other industries. For example, specifications for the uniformity of suspensions in paint are more than an order of magnitude tighter than USP quality specifications for pharmaceuticals, and processes in the semiconductor industry approach precision of 5 sigma, whereas the average pharmaceutical process is only able to achieve 2.5 sigma.

In the USA, the FDA has recognized the industry's reluctance to implement new technologies. In response to the industry's concerns, the agency initially openly invited any company considering the use of QbD, and potentially PAT elements, to join the FDA pilot program on QbD and to schedule discussions with them early in the process planning stage to ensure there are no surprises that delay approval. However, the reports have been mixed on how successful this approach has been. This initial approach of using the collaborative approach between regulator and manufacturer as a "carrot" to encourage the use of PAT by the manufacturers is felt to be moving more toward a "stick": The impression is that if the regulatory agencies do not feel convinced that the manufacturer truly understands how their process impacts product quality they risk delays in obtaining approval. Even if single health authorities such as the FDA are more open to new approaches, it has to be acknowledged that biopharmaceutical companies often operate globally, and that they need not only the FDA's consent on the manufacturing strategy but also that from more than 100 other health authorities which might be much more conservative in their thinking.

The topic of demonstrating process understanding leads to another challenge which hinders the implementation of PAT in bioprocessing in general, and especially in fermentation and cell culture. Unlike in small molecule API production, where the kinetics and organic chemistry mechanisms that impact production of the molecule of interest and generation of impurities are well-understood and controlled, this same level of understanding and control has not been achieved in biologic processes. The higher level of process understanding and control for small molecule API manufacturing enables the identification of process parameters that maximize production of product and minimize impurities, and then controls

those factors to meet CQAs specifications consistently. For biologic processes this level of understanding and control is much more difficult to achieve. As was stated earlier, the bioreactor is basically a vessel for producing cells, where the cells are the reactors that produce the product. Fermentation science has a fairly good understanding of what impacts the production of cells and has measured and controlled those factors (e.g., pH, temperature, dO, nutrient sources, etc.) to maximize cell density for decades.

This same level of understanding and control has not been achieved for the biochemical reactions taking place within the individual cells. It is these reactions that actually impact the CQAs of large molecule APIs, such as glycosylation, sialylation, etc. Achieving this level of understanding is no trivial task because of the complexity and adaptability within the cell's biochemical pathways. Although there has been significant progress in academic institutions toward mapping and modeling these pathways in some specialized cases [99], the industry is far from achieving this goal for every large molecule product, especially not for large glycosylated proteins produced in mammalian cells. Given the numerous cellular pathways, the number of analytes involved in the pathways, and the complexity of the analytical instrumentation and methods required to make these measurements, it is likely that the best that can be achieved with respect to cellular PAT is the development of models describing these pathways and on-line measurement and control of the extra-cellular analytes that impact these pathways. These extracellular measurements can then be used as the inputs to update continuously the on-line, metabolic models used in a model predictive control loop.

The technology issues limiting the implementation of PAT in biopharmaceutical production were described earlier: In-line analytical measurements are primarily spectroscopic measurements such as mid-IR, near-IR, Raman, and fluorescence. It is probable that these measurements are always challenged when it is attempted to predict single analyte concentrations in bioreactors, because of the chemical complexity of the cell culture broth and the limited resolution and sensitivity of spectroscopy-based techniques. The lack of resolution requires the use of multivariate calibration techniques, with the result that their long-term application in bioreactors are likely to be difficult without constant "re-validation" of the model. This is largely impacted by the variability observed in raw materials used in fermentation-based reactions. As more and more cultures are switched to defined media, this may become less of an issue. That said, the importance of trace molecules that are below the detection limit of spectroscopic techniques and the co-linearity that is very probably occurring within the multivariate system make it very difficult for spectroscopy to be used to predict the concentration of individual analytes other than those present in high concentrations (e.g., glucose). The most promising application of spectroscopy is to move away from attempting to use it as a substitute for a lab-based test of an individual analyte to using it as part of a MVDA scheme, where multiple principle components from a spectrum are correlated to an event or events in the bioreactor.

Measurement of specific analytes (particularly those that are present in low concentrations) can currently only be achieved with at-line technologies. In order to use

these analytical methods on line, an automated sampling system must be used to interface the analyzer to the bioreactor. As described earlier, on-line sampling methods must provide a sterile barrier between the bioreactor and the analyzer. This has historically been done either by sterilizing the sampling device prior to opening the sampling device to the bioreactor or by providing a filtration media between the bioreactor and the sampling device that provides a sterile filter. The issue that is impeding the widespread use of on-line sampling interfaces is primarily related to the perceived increased risk of bioreactor contamination that can occur with these devices, as well as the added effort and complexity required to set up the sampling devices.

The last possible reason for the slow advance of PAT in biopharmaceutical manufacturing is that the industry fails to understand the potential financial gains this could bring. This may be because some promises of PAT seem to be very "abstract."

From a process development perspective, utilizing PAT can:

- *Increase process understanding*. Utilizing multivariate experimental designs allows the identification of the factors that impact the CQAs.
- *Accelerate time to market*. Utilizing on-line/in-line measurements and automated experimental control allows unattended 24/7 experimentation to develop process understanding and define the design space more quickly and efficiently. Automated operations can obviously have a huge ROI from labor savings and increased utilization of R&D infrastructure.

A challenge to this, though, is that it really takes a leap of faith to believe that these advantages can in fact be realized by implementing PAT and, if they are achieved, how much of a measurable advantage is realized compared to the conventional approaches to process development that have been used historically. This is a question that appears still not to have been answered.

On the other hand, common sense indicates that generating more data and utilizing MVDA techniques to mine that data to generate valuable process knowledge should speed up process development and avoid future problems in manufacturing. There has as yet been no published study that quantitatively compares the advantages of the PAT approach to the conventional approach of process development.

From a manufacturing perspective, utilizing PAT can:

- *Reduce process variability*. The increased understanding of what and how CPPs impact CQAs enables the process to be operated in a flexible manner within the design space, leading to reduced variability in the process output. This can results in two things: reduced risk of failure and more consistent process yield and quality. By reducing process variability, the risk of product failure is also reduced. This can lead to a reduction in inventories of in-process material and final product necessary to protect against in-process failures interrupting production flow. It also enables better production planning.

- *Increase operating efficiency*. By utilizing process analytics, off-line QC assays that create delays in forwarding processing in-process materials can be eliminated. This should decrease overall cycle time and increase production throughput. This may be achieved by moving in-line assays on-line, where they can provide continuous product monitoring, or possibly just a more robust/capable process that eliminates the need to perform off-line verification of product quality.

Once again, these gains are "abstract" and hard to quantify, so it is hard to compare them against the perceived risks.

Although there are now mature technologies that can be readily adapted to biopharmaceutical manufacturing as on-line sensors for the measurement and control of CQAs, the key regulatory message is to ensure that multivariate understanding is obtained from the use of these technologies supporting the whole QbD paradigm.

4.2 Influence of New Formats and Continuous Manufacturing on PAT

There has been much discussion in recent years on the move toward personalized healthcare (PHC) or personalized medicine, where an API is modified in such a manner as to target a particular patient population (e.g., metabolism). Does this change in the industry have an impact on PAT implementation? The authors believe that the answer to this is yes; PAT should have a much greater impact on this than it has had on conventional pharmaceutical manufacturing. Why is this?

If we look at conventional pharmaceutical manufacturing, we find that it is performed as a batch operation with, in most cases, the batch size being very large relative to the market's short-term need. The excessively large lot size produces an in-process intermediate or final product that is typically not required for the market at the time of manufacture and is stored in a warehouse until it is required to fulfill market demand. At this time, it is pulled from stock and either further processed or shipped. This approach of manufacturing large lot sizes leads to production operations being "campaigned", that is, a particular product may be manufactured in a plant ahead of time until sufficient inventory is generated, then operations are stopped and changed over to produce a separate product. The driver for operating in this manner is that large lot sizes minimize the per kilogram cost of ancillary operations, such as QC, regulatory, etc. That is true given the way these operations have been performed historically, for example, run a lot, sample the lot, run QC assays on the sample, confirm the lot meets CQAs, and release the lot. If the lot fails to meet CQAs, it is re-processed if there is an approved re-work process or disposed of if not and written off as a loss. Although this is an effective way to minimize costs per kilogram, it can create a large amount of waste in manufacturing because of start-up failures, down-time to clean and changeover equipment, etc.

Start-up failure is a nearly unavoidable issue that occurs any time a unit operation is started after being down for a period of time. It is a result of the learning curve. The more frequently someone performs an operation, the quicker they get up to speed on that operation. By running large size lots infrequently, we are creating numerous periods of time where it may take days, weeks, or even months to get past the initial learning phase and into routine, error free operation. On the other hand, if we run smaller lot sizes more frequently or, better still, run manufacturing in a continuous mode, the start-up failure phase would be much shorter and, in the case of a continuous process, would only occur during the initial start-up of the continuous process. The question becomes "How do we minimize the impact of QC and regulatory affairs costs per kilogram if we create smaller lot sizes?" The answer is either to develop processes that are so robust that the need for QC is either minimized or eliminated or to move the QC on-line through the use of process analytics.

If the industry moves to continuous processing, the critical operating parameters could be monitored and controlled continuously using validated sensors, so that variability in the output of the process would be minimal, the process capability would be increased, and the need for QC release testing would be minimized or eliminated, thus enabling real-time release. If there were still a need for the "belt and suspenders" approach, it would be possible to install process analytics on the process to provide continuous verification of the product's CQAs. An additional advantage to continuous production is the likelihood that continuous manufacturing equipment is much smaller in scale and potentially resemble pilot plant or even laboratory-scale equipment. If this occurs, it might be possible to build a pharmaceutical plant in a modular container that could be constructed and validated off-site, then shipped to the location where it is needed or even moved from location to location as needed, for example, production of a vaccine during times of need for inoculation.

Manufacturers are being increasingly required to produce in those regions and countries in which they sell their products. Furthermore, as the industry moves toward PHC, production is probably pressured to move to smaller lot sizes. This can lead to an increased push for continuous processing methods. If this occurs, the need for PAT should increase as well.

4.3 Regulatory Oversight

The regulatory perspective on PAT has evolved out of several emerging scientific disciplines that have or could have the greatest impact on PAT in the biopharmaceutical industry in the future. In the US, the Critical Path Initiative has several manufacturing requirements that favor companies with flexible and scalable manufacturing capabilities. The remaining areas of emerging sciences that have a global impact and are the current subjects of both the FDA and EMA to be

discussed are personalized medicines, systems biology, stem cell research, and translational medicine.

Regulatory oversight generally can be correlated to traditional means of enforcement through guidance, inspections, and annual reporting. However, although it has generally been stated that the drivers for biopharmaceutical manufacturers buying into PAT strategies primarily come from manufacturing needs and not regulatory pressures, the reality is that regulators have the ability to drive PAT in the future. The evidence for this comes from the current regulatory climate, in which two related sets of activities have spurred on the increasing inclusion of QbD in BLAs and MAAs [100]. However, there are several examples where manufacturing needs can clearly drive the use of PAT tools. For instance, it has been observed that 50% of the biopharmaceutical formulations produced are freeze dried. This necessitates measuring the amount of moisture in the preparation. Both Raman and NIR spectroscopy are suitable tools (see Sect. 3.5). That is, they both have regulatory (compendial) standing. Coupled together for the processing of freeze-dried products, they can be used to measure simultaneously the spectral stability of the product itself (the crystalline material) and the amount of residual moisture during the freeze-drying process. It would make good business sense then to adopt these modes of controlling the product quality, as these procedures can be totally automated (less reliance on analysis by highly skilled and costly laboratory personnel who are prone to human error) and are highly accurate and precise, thus providing regulators with a high degree of assurance that lot to lot variability can be controlled and minimized. On the basis of the regulation and compendium, the single most useful technology that has the greatest potential for providing real-time quality assurance is MVDA and especially MSPC. Similarly, single use technology, perhaps in combination with PAT, and single use biosensors, are either starting to become or should become a mainstay in the industry. These technologies can have a tremendous impact on speed to market while preserving and even enhancing drug substance and drug product quality.

Chemometric methods for spectroscopy are the most common MVDA method seen in pharmaceutical regulatory filings [101]. FDA supports implementation of MVDA, using a science- and risk-based approach. The FDA recommends initiating discussions with the Agency before implementation of novel approaches. Future growth opportunities for MVDA are:

- Identity of complex molecules
- Routine use of MSPC as part of continual process verification
- Monitoring in support of real-time release testing (RTRT)
- Integration of multiple sensors for control of entire process
- Implementation of feedback or feedforward control (e.g., MPC)
- Control to a process signature
- Control of continuous manufacturing
- Further development of data management tools

Submitted models are considered on the basis of the level of detail in the submission and depend on the importance of the models to the overall control strategy:

- Low Impact Model (e.g., models for development)
  - General discussion of how the model was used to make decisions during process development
- Medium Impact Model (e.g., design space models)
  - More detailed information about model building, summary of results and statistical analysis
  - Discussion of how the model fits into the control strategy
- High Impact Model (e.g., RTRT models)
  - Full description of data collection, pretreatment and analysis
  - Justification of model building approach
  - Statistical summary of results
  - Verification using data external to calibration set
  - Discussion of approaches for model maintenance and update

4.4 Outlook

In the coming years, little or no further change in PAT-based manufacturing can be anticipated from the regulators, as the required guidance for industry to adapt newer manufacturing strategies are mostly in place (some now for at least 10 years), they have been relatively harmonized in the US and EU, they have or should begin to be internalized within their respective regulatory structures, and, as has been stated time and time again, the current system is already sufficient to support the required quality of the marketed product. Nevertheless, from the regulator's perspective, these newer tools represent the future.

Regulatory expectations, with respect to process knowledge and process control, are significantly increasing. Recent FDA Form 483s received by several companies demonstrate this clearly by citing: "inadequate investigations," "processes lack control," "investigation did not link process control and the "deviation" with changes in impurities levels reported in the Annual Product Review", etc. In addition, as indicated previously, expectations are changing with respect to "the product met specifications." Product meeting specifications is necessary, but may not be sufficient, that is, the product must conform and/or must be comparable from batch to batch. Changes in impurity levels, over time, are starting to be viewed as the result of a process that may not be "in control." *Quality cannot be tested in, it must be built in*. PAT is a "disruptive technology," that is, it changes the way companies view control of their processes, and it enhances their ability to control their processes. PAT enables major "leaps" in process understanding, through real-time data acquisition for CPPs and CQAs, which cannot be achieved by off-line analysis as effectively, particularly from a cost perspective. The real-time component of PAT allows or facilitates process control systems that ensure product quality, with respect to CQAs, from batch-to-batch. *The process is the product!*

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Next Generation Biopharmaceuticals: Product Development

Roman Mathaes and Hanns-Christian Mahler

Abstract Therapeutic proteins show a rapid market growth. The relatively young biotech industry already represents 20 % of the total global pharma market. The biotech industry environment has traditionally been fast-pasted and intellectually stimulated. Nowadays the top ten best selling drugs are dominated by monoclonal antibodies (mABs).

Despite mABs being the biggest medical breakthrough in the last 25 years, technical innovation does not stand still.

The goal remains to preserve the benefits of a conventional mAB (serum half-life and specificity) whilst further improving efficacy and safety and to open new and better avenues for treating patients, e.g., improving the potency of molecules, target binding, tissue penetration, tailored pharmacokinetics, and reduced adverse effects or immunogenicity.

The next generation of biopharmaceuticals can pose specific chemistry, manufacturing, and control (CMC) challenges. In contrast to conventional proteins, next-generation biopharmaceuticals often require lyophilization of the final drug product to ensure storage stability over shelf-life time. In addition, next-generation biopharmaceuticals require analytical methods that cover different ways of possible degradation patterns and pathways, and product development is a long way from being straight forward. The element of "prior knowledge" does not exist equally for most novel formats compared to antibodies, and thus the assessment of critical quality attributes (CQAs) and the definition of CQA assessment criteria and specifications is difficult, especially in early-stage development.

Keywords Antibodies, Chemistry, manufacturing, and control (CMC), Drug product development, Next-generation antibody formats

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Contents

1 Introduction

In 1982, human insulin was the first approved drug produced by recombinant DNA technology. Since then, several recombinant proteins and numerous monoclonal antibodies (mABs) have been commercialized. Therapeutic proteins have become a standard therapeutic modality for severe diseases [1].

Therapeutic proteins show a rapid market growth. The relatively young biotech industry already represents 20% of the total global pharma market [2]. The biotech industry environment has traditionally been fast-paced and intellectually stimulated. Although the first therapeutic proteins produced were “non-antibodies” (e.g., rhInsulin, Interferon a2a, or epoetin a), nowadays the top ten of the best selling drugs is dominated by mABs [2].

The breakthrough of mABs can be explained by several biotechnological milestones: discovery of hybridoma technology by Koehler and Milstein [3], phage display by Smith [4], humanization by Winter [5], and several technical improvements in fermentation and purification [2]. mABs offer several benefits over other recombinant proteins, such as long serum half-lives, mostly mediated by FcRn recycling [6], and target specificity. The possibility of tailoring the mAB binding to any biological target has led to the development of marketed products across a variety of different disease areas, with a focus on oncology and autoimmune disorders [7].

2 Next Generation Biopharmaceuticals

Despite mABs being the biggest medical breakthrough in the last 25 years, technical innovation does not stand still. The driver for next generation biopharmaceuticals is to develop even better drugs. The goal remains to preserve the benefits of a conventional mAB (serum half-life and specificity) whilst further improving efficacy and safety and to open new and better avenues for treating patients, for example, improving the molecules' potency, target binding, tissue penetration, tailored pharmacokinetics, reduced adverse effects, or immunogenicity. For example, several protein drugs were reported to promote immunogenicity, protein pharmaceuticals are currently limited to the cell surface or extracellular targets, and they have to be administered via the parenteral route. However, the delivery to tumors, which would be desirable, remains very inefficient [8].

Another driver for next generation formats is the competitive biologic market in certain diseases. Different companies strive to develop drugs in overlapping therapeutic areas. For example, in rheumatoid arthritis several therapeutic modalities exist. Anakinra® binds and blocks the IL1 receptor and Rilonacept® is an IL1 receptor Fc-fusion protein which directly binds IL1. A variety of molecules bind to TNFα: Ethanercept® is a TNFα-receptor Fc-fusion protein, Cimzia® is a Fab-PEG conjugate, and Adalimumab® is conventional mAB [9, 10]. In addition, the rise of biosimilars challenges the innovator drug maker to improve existing therapies and develop next generation biopharmaceuticals which outperform first generation mABs [11].

To improve existing treatments, several antibody drug conjugates (ADCs) and fusion proteins have been successfully marketed in the past (Table 1).

2.1 Antibody Drug Conjugates

Despite the remarkable pharmacological performance of mABs, some challenges remain. MABs may show weak tissue penetration or low cytotoxicity. In contrast, small molecule cytotoxics feature no target specificity and high non-target toxicity. ADCs contain cytotoxics chemically linked to a mAB and aim to combine the desirable properties of the two therapeutic modalities [12].

The three building blocks of the ADC – the mAB, the linker, and the cytotoxic agent – should be individually chosen to tailor the performance to the desired pharmacological profile. All three marked ADCs (Table 1) featured a conventional mAB, although other mAB scaffold formats are possible [13]. For example, a linked Fv (or scFvs) (Fig. 1) or fragment antigen-binding (Fab) with short in-vivo half-lives can be of advantage when using hydrolysis-sensitive linkers to reduce free plasma cytotoxics or to increase tissue penetration and accumulation [8].

The cytotoxic payload kills the target cell. Current payloads have a picomolar or better potency and exceed traditional cytotoxics such as doxorubicin or paclitaxel.

Table 1 Examples of marketed next generation biopharmaceuticals

Marketed product	Manufacturer	Disease area	Technology	FDA approved
Mylotarg®	Pfizer	Oncology	ADC	2001
Adcetris®	Seattle Genetics	Oncology	ADC	2012
Kadcyla®	Roche	Oncology	ADC	2013
Eloctate®	Biogen Idec	Blood	(Factor VIII) FC-Fusion	2014
Alprolix®	Biogen Idec	Blood	(Factor IX) FC-Fusion	2014
Amevive® (withdrawn)	Atellas and Biogen Idec	Autoimmune disorder	(CD2 binding) FC-Fusion	2003
Enbrel®	Amgen and Immunex	Autoimmune disorder	(TNF receptor) FC-Fusion	1998
Orencia®	Bristol-Meyers-Squibb	Autoimmune disorder	(CD80/CD86 binding) FC-Fusion	2005
Eylea®/ Zaltrap®	Regeneron and Sanofi-Aventis	Macular degeneration/oncology	(VEGF-A receptor/VEGF-B receptor) FC-Fusion	2011/ 2012
Arcalyst®	Regeneron and Sanofi-Aventis	Autoimmune disorder	(IL1 receptor) FC-Fusion	2008
NPplate®	Amgen	Blood	(Thrombopoetin-binding peptide) FC-Fusion	2008
Eperzan®/ Tanzeum®	GlaxoSmith Kline	Metabolism	(Glucagon-like peptide1) Albumin-Fusion	2014
ReoPro®	Eli Lilly	Blood	Fab	1997
Cimzia®	UCB	Autoimmune disorder	Fab-PEG	2007
Lucentis®	Genentech/ Novartis	Macular degeneration	Fab	2006

They are all naturally derived compounds and either bind DNA (calicheamicins), inhibit the tubulin (auristatins or maytansinoids), or promote alkylation (duocarmycins). The drug antibody ratio (DAR) defines the number of cytotoxic molecules per carry protein and is an important characteristic for ADC potency or ADC physiochemical stability [14].

Several biochemical conjugation strategies were developed to link the cytotoxic to the carrier protein [15]. Conjugation strategies can be divided into non-specific and genetically engineered site-specific conjugation. Non-specific conjugation utilizes random lysine on the mAB to couple amine-reactive payloads or uses cysteine in the hinge region to couple a thiol-reactive payload [15]. Both pathways result in highly heterogenic DARs and significant variability in conjugation sites. For example, the DAR of an ADC coupled by cysteine conjugation can be 0, 2, 4, 6, or 8 [16]. These product inconsistencies possess several challenges during manufacturing/administration and can influence the pharmacodynamics/pharmacokinetics (PD/PK) profile of the product [17]. To address these challenges, site-specific conjugation strategies were developed. Genetically engineered site-specific cysteine conjugation was achieved by eliminating cysteine conjugation sites through mutation of 1-4 cysteine amino acids to serine [18]. Junutula et al. replaced

serine, valine, or alanine by cysteine [19]. Theses genetically engineered unpaired cysteines are used for site-specific conjugation of the payload without disrupting structure and functionality of the mAB (THIOMAB). THIOMABs showed homogeneous DARs with an increased safety profile [20]. However, they possess the risk of cysteine or glutathione adducts derived in the fermentation process. Therefore, THIOMABs usually require a reduction and re-oxidation step. Several other site-specific conjugation strategies were developed. For example, enzymatic conjugation, which utilizes a glutamine tag, unnatural amino acids being introduced to the carrier mAB, or the mAB glycans being used [18, 21].

The linker between the carrier mAB and the cytotoxic determines possible bioconjugation strategies and influences PD/PK and the toxicological profile of the drug. Linkers are usually bifunctional, one functional group of the linker reacting with the cytotoxic and the second functional group with a specific amino acid on the carrier mAB. The linker should be stable during storage and during in-vivo circulation in the blood serum to prevent unwanted side effects of free cytotoxics [15]. However, the ADC should release the payload at the target site to ensure a desirable potency profile. This can be achieved by cleavable linkers, which are unstable at the lysosome pH, sensitive to reduction by endogenous thiols, or sensitive to cleavage by proteases. Non-cleavable linkers rely on the proteolytic cleavage of the carrier mAB itself. ADCs featuring cleavable and non-cleavable linkers were both successfully commercialized in the past. For example, Adcetris® uses a cathepsin (protease) cleavable linker with *p*-aminobenzamidine as a spacer to conjugate monomethyl auristatine E to Brentuximab [22, 23]. Mylotarg® uses a hydrazine linker including a stable S–S bond to attach calicheamicin to Gentuzumab [24]. Kadcyla® features the non-cleavable linker SMCC to conjugate Mertansine to Trastuzumab [25, 26]. A variety of next generation linkers were introduced which utilize non-native amino acids (ReCode®) [27], azido- or alkynyl-"click-chemistry" (Biociphering®) [18], or aldehyde placement on specific amino-acids (SMARTtag®) [28].

2.2 *Fusion Proteins*

Fusion proteins are recombinant combinations of two or more therapeutic proteins. Specifically, Fc-fusion protein are the most advanced and promising strategy to leverage desirable properties of two molecules [29]. Since the launch of the TNF-α-receptor-FC fusion protein Enbrel® in 1998, several other Fc fusion proteins have been successfully commercialized (Table 1). The fusion of an Fc part of a mAB to a conventional protein significantly enhances the serum half-life. Fc-fusion half-life extension is achieved by two mechanisms, the first being FcRn recycling, which prevents proteolysis in the lysosome or in the case of small proteins/peptides by increasing the molecular weight of the final fusion protein over the glomerular filtration cut-off (ca. 50 kDa) [30].

In addition, two or more fused therapeutic proteins can achieve specificity to more than one target. For example, the bispecific mAB Ang2-VEGF-A CrossMab® binds angiopoetin-2 and the vascular endothelial growth factor (VEGF) A and features the FC part of a conventional mAB [31]. This enables a synergistic therapeutic effect in preventing angiogenesis and enables long half-lives. Aflibercept is a peptide FC-fusion protein, which binds VEGF-A and VEGF-B [32].

Other fusion protein formats such as Albiglutide are peptide–albumin fusion proteins. Albiglutide also features a significantly increased serum half-life compared to the Glucagon-like petide1 alone [33].

2.3 Other Next Generation Biopharmaceutical Formats

ADCs and Fc-fusion proteins are the most prominent class of next generation biopharmaceuticals. However, several other formats have been developed to meet specific medical challenges (Fig. 1). Lucentis®, ReoPro®, and Cimzia® are all examples of successfully commercialized Fabs [34]. Decreasing the size of the conventional mAB is associated with increasing tissue penetration or influencing the pharmacokinetics. Yet, controlled clinical studies are not to be found. In mice, linked Fv domains (also called scFv), mini-bodies, or Fabs showed an increase tumor/blood concentration ratio compared to conventional mABs [35]. The high tumor tissue penetration was explained by better tissue penetration properties and decreased FcRn recycling.

3 Drug Development Challenges of Therapeutic Proteins

3.1 Degradation Pathways

Several comprehensive review articles were published in the past detailing the different protein degradation pathways [36–38]. Therefore, protein degradation is only briefly discussed in this chapter.

Proteins can exhibit chemical and physical instabilities, which may or may not compromise their therapeutic effect and/or safety. That is to say, product-related impurities may or not be a critical quality attribute (CQA). The most relevant and most common chemical instabilities comprise deamidation, oxidation, isomerization, hydrolysis, glycation, and disulfide formation/breakage. Asparagine deamidation is influenced by the formulation pH, temperature, and amino acids in direct proximity. The reaction kinetic is favored at elevated temperatures, for most proteins in neutral to basic conditions (pH 6–10) and by direct proximity of serine, threonine, and glycine to asparagine. Detection can be via analytical methods analyzing charge. Deamidation is often considered as a major and critical

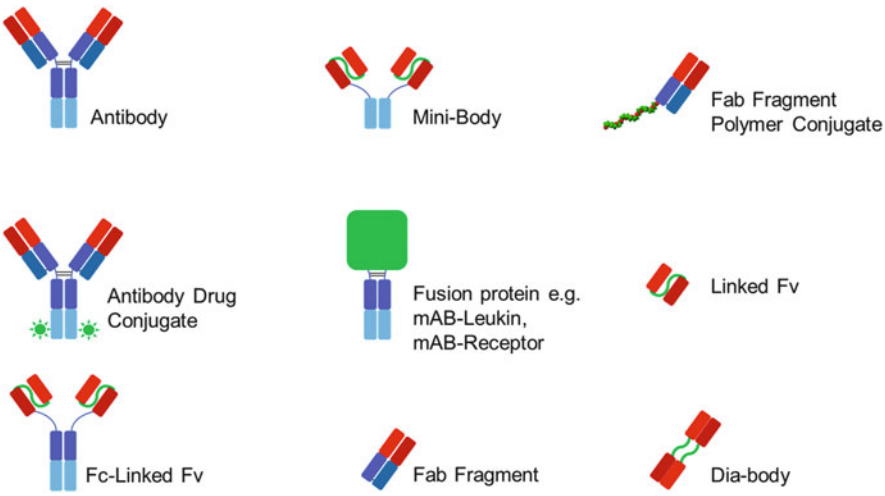

Fig. 1 Examples of formats of next generation biopharmaceuticals

degradation pathway. In various examples, deamidation in a binding region has been shown to be related to reduced or diminished binding activity and, thus, efficacy [39]. For antibodies, charge-based methods typically work well based on platform approaches and prior knowledge, and the CDR region would be specifically critical for possible deamidation sites. For next generation biologics, the binding region and mode of action may not be fully established, especially during early-stage development. In particular, charge-based analytical methods may be very difficult to establish for early-stage formulation development. This can make deamidation for next generation molecules a special challenge, especially in early development.

Oxidation is another major protein degradation pathway. The protein can, for example, exhibit oxidation at histidine, methionine, cysteine, tyrosine, and tryptophan residues. In molecules containing a single cysteine amino acid, covalent aggregation (disulfide formation between two monomers) is very likely to occur and, thus, the presence of cysteine residues that are not coupled usually raises a flat in formulation development. Lately, protein oxidation has received increasing attention across industry as it can influence conformational stability, which may impact target binding affinity and FcRn recycling (i.e., having a possible relevance for pharmacokinetics) as well as promoting aggregation [36, 40], and increased oxidation has been associated with immunogenicity when connected to excessive aggregation [41].

In addition to chemical instabilities, proteins show physical instabilities via denaturation, aggregation/precipitation, or surface adsorption [42]. Denaturation represents a change of the three-dimensional structure of a protein and can be promoted by elevated temperatures or other environmental factors such as the formulation composition. Industrially, denaturation is often an irrelevant endpoint

given that drug product storage and handling is typically considerate of the sensitivity of biologics. For example, biologic drug products are typically stored refrigerated and products would thus not be exposed to significant temperatures. Protein aggregates are formed by multimeric (oligomeric) species and can be described by several properties. For examples, the protein conformation (native or denatured), the type of bond (non-covalent, covalent, etc.), reversibility, solubility, size, or morphology are typical factors to classify protein aggregates [38]. Protein aggregation remains one of the major "hot topics" related to protein stability given the speculation about the connection between aggregation and undesired immunogenicity in patients [43]. However, predictivity of immunogenicity remains challenging [41] and current studies suggest that aggregates and particles at relevant levels would not be immunogenic, unless significantly chemically modified (oxidized) [41].

In general, the chemical and physical degradation pathways can be interdependent. For example, deamidation and oxidation may lead to conformational changes, which may promote aggregation, and aggregated and/or denatured protein may be more easily chemically modified, for example, oxidized.

3.2 Ensuring Product Stability

Protein pharmaceuticals are complex macromolecules per se, which require combined and interdisciplinary efforts from drug substance and drug product development to achieve an adequate product quality. The next generation biopharmaceuticals provide some additional challenges [44]. For examples, the development of an ADC includes not only monitoring mAB stability but also assessment of linker stability, DAR, and free cytotoxic drug [18].

Developability assessments can help to select a lead candidate that would possibly show better stability by evaluating – and replacing – critical/non-critical degradation hotspots (e.g., in the target binding region). The results of the developability assessment trigger an adequate formulation and process development strategy [45].

In general terms, the preferred dosage form for biopharmaceuticals is the liquid formulation. Liquid dosage forms enable seamless manufacturing and convenient administration; however, they are less stable than lyophilized products [46]. Conventional mABs in most cases do not require lyophilization of the final drug product. The majority of mABs commercialized between 2013 and 2015 are liquid drug products (Table 2). In contrast, next generation biopharmaceuticals have often been lyophilized (Table 3). There can be many reasons for this. For some ADC formats, where there is significant sensitivity of the linker region to water (hydrolysis for the conjugated), it is obvious that the presence of water in the final drug product should be minimized, and thus drying of the product is required. In other cases, next generation biologic formats can exhibit complex instability reactions, or the panel of analytical methods to make a thorough assessment of formulations is incomplete or insufficient. In these cases, lyophilization may be performed to

Table 2 Formulations of conventional mABs (2013–2015)

Product	Company	Date	Liquid/ Lyo	Formulation
Cosentyx	Novartis	2015	Liquid	Histidine buffer, Methionine, PS80, Trehalose
Unituxin	United Therapeutics Corporation	2015	Liquid	Histidine buffer, PS20, NaCl
Cyramza	Eli Lilly	2014	Liquid	Histidine buffer, NaCl, Glycine, PS80
Sylvant	Janssen	2014	Lyo	Histidine buffer, PS80, Sucrose
Entyvio	Takeda	2014	Lyo	Histidine, Arginine, Sucrose, PS80
Keytruda	Merck Sharp	2014	Lyo	Histidine buffer, Sucrose, PS80
Simponi Aria	Janssen	2013	Liquid	Histidine buffer, Sorbitol, PS80
Actemra	Roche	2013	Liquid	Phosphate buffer, Sucrose, PS80
Gazyva	Roche	2013	Liquid	Histidine buffer, Threalose, Poloxamer 188

reduce the risk of the development of the product. Yet, if the target product profile (TPP) of the product foresees and desires a liquid product for commercialization, the use of a lyophilizate in early-stage development has significant downsides. For example, when planning to change from a lyophilizate toward a liquid formulation of a given product, the quality and quantity of product-related impurities on stability is likely to be different (higher) with the liquid product, raising questions on whether efficacy and especially safety are unchanged in the liquid formulation compared to the lyophilizate, for which preclinical and clinical data have been obtained. Analytical data such as comparison of impurity profiles on stability are required in these cases, including an evaluation of potency (bioassay). The change evaluation, however, can in some cases lead to the conclusion that an additional safety evaluation, either preclinically or clinically, is warranted, challenging and extending the overall development timeline. In other cases, the shelf-life of the liquid formulation needs to be kept sufficiently short to ensure that impurity levels match the level of impurities found end-of-shelf-life in the lyophilized drug product.

Another consideration of the suitability of formulation parameters relates to considering the various components of conjugates, fusions, and the like. For example, suitable formulation parameters of the carrier mAB of an ADC might not necessarily be suitable for the linker/cytotoxic or suitable formulation parameters of one part of a fusion protein might not necessarily be suitable for the second part of the fusion protein [18].

Table 3 Formulations of next generation biopharmaceuticals

Marketed product	Manufacturer	Date	Technology	Liquid/ Lyo	Formulation
Mylotarg®	Pfizer	2001	ADC	Liquid	Histidine, NaCl, Aluminum phosphate, PS80
Adcetris®	Seattle Genetics	2012	ADC	Lyo	Citrate buffer, Trehalose, PS80
Kadcyla®	Roche	2013	ADC	Lyo	Histidine buffer, Sucrose, NaCl, Methionine, CaCl
Eloctate®	Biogen Idec	2014	(Factor VIII) FC-Fusion	Lyo	Histidine buffer, NaCl, CaCl, Sucrose, PS20
Alprolix®	Biogen Idec	2014	(Factor IX) FC-Fusion	Lyo	Histidine buffer, Mannitol, Sucrose, PS20
Amevive® (withdrawn)	Atellas and Biogen Idec	2003	(CD2 binding) FC-Fusion	Lyo	Citrate buffer, Glycine, Sucrose
Enbrel®	Amgen and Immunex	1998	(TNF receptor) FC-Fusion	Liquid/ Lyo	Phosphate buffer, Arginine, NaCl, Sucrose/ Sucrose, Mannitol, Thromethamine
Orencia®	Bristol-Meyers-Squibb	2005	(CD80/CD86 binding) FC-Fusion	Lyo	Phosphate buffer, Poloxamer188, Sucrose
Eylea®/ Zaltrap®	Regeneron and Sanofi-Aventis	2011/ 2012	(VEGF-A receptor/VEGF-B receptor) FC-Fusion	Liquid	Phosphate buffer, NaCl, PS20, Sucrose/Phosphate buffer, Citrate, NaCl, PS20, Sucrose
Arcalyst®	Regeneron and Sanofi-Aventis	2008	(IL1 receptor) FC-Fusion	Lyo	Histidine buffer, Arginine, PEG3350, Sucrose, Glycine
NPplate®	Amgen	2008	(Thrombopoetin-binding peptide) FC-Fusion	Lyo	Histidine buffer, Sucrose, Mannitol, PS20
Eperzan®/ Tanzeum®	GlaxoSmith Kline	2014	(Glucagon-like peptide1) Albumin-Fusion	Lyo	Phosphate buffer, Mannitol, Trehalose, PS80
ReoPro®	Eli Lilly	1997	Fab	Liquid	Phosphate buffer, NaCl, PS80
Cimzia®	UCB	2007	Fab-PEG	Lyo	Lactic acid, sucrose
Lucentis®	Genentech/ Novartis	2006	Fab	Liquid	Histidine buffer, Trehalose, PS20

3.3 Providing Stability in Liquid Formulations

Important parameters of a liquid protein formulation comprise the choice and quantity of buffer and pH, surfactant, target protein concentration, tonicity modifier, amino acids, sugars, and salts [37, 47].

The pH of the formulation is the most significant parameter impacting chemical and physical stability of a protein. For example, methionine hydrolysis and met oxidation are promoted in an acidic environment, whereas deamidation, β-elimination, and disulfide exchange are often favored at basic pH. The pH of a formulation also dictates protein net charge influencing physical stability by electrostatic interactions. Repulsive interaction between molecules contributes to physical stability. However, an increased number of charged groups can compromise conformational stability as the charge density is higher in the folded state. Proteins are usually formulated one or more pH units away from their isoelectric points [37, 47].

Several specific challenges regarding the choice of an adequate pH for next generation biopharmaceuticals exist. For example, fusion proteins feature more than one IEP. Therefore, the selection of the formulation pH is non-trivial and stability of various subunits may behave differently. In the case of ADCs, stability of the linker and the cytotoxic drug payload need to be considered. For example, hydrazone linkers are acid labile as they are designed to release the cytotoxic at low pH in the lysosome [48]. In contrast, ester linkers are sensitive to hydrolysis in a basic environment [49]. In addition to pH, other degradation pathways were postulated: Calich is susceptible to photolysis, T-DM1 to oxidation [18]. Physical stability can also be different in conjugates or fusion proteins. For example, ADCs often show a lower Tm compared to mAbs, and colloidal stability often decreases with increasing DARs [17]. In particular, cysteine conjugation was reported to affect physical stability more than lysine conjugation. With ADCs, the level of DARs can also change (increase) hydrophobicity. Although this may not be an immediate issue in the drug product formulation, product dilution in solutions for infusion such as 0.9% saline may lead to issues such as aggregate or particulate formation, which is interestingly dependent on DAR. One reason for this is that stabilizing excipients are diluted in the carrier solutions. Thus, these products may show instability in infusion bags, depending on DAR, and if DAR varies batch-to-batch, these issues can be very cumbersome to identify and tackle. Again, physical and chemical instabilities can be interconnected. Groups susceptible to chemical degradation, which are buried on the carrier mAB, might be exposed after the conjugation of a hydrophobic cytotoxic.

Nonionic surfactants are almost always included in a protein formulation to decrease interfacial-mediated protein degradation, especially physical degradation (aggregation and particle formation). They are amphiphilic, surface active molecules, which competitively replace proteins from hydrophobic/hydrophilic interfaces, such as ice/water or air/water interfaces [47]. Surfactants also prevent adsorption to surfaces, such as stainless steel tanks, plastic bags/tubings, or filters, and also the primary packaging containers and administration tools. As mentioned above, ADCs display an increased hydrophobicity and show an increased sensitivity to interfacial stress [50]. This is also reflected in the prescribing information of ADCs. For example, the label of Kadcyla does not allow shaking and freezing of the reconstituted or diluted drug product.

Tonicity modifying excipients are used to adjust the osmolality of a formulation to physiological acceptable concentrations. Common excipients are sugars such as sucrose, trehalose, amino acids such as glycine, methionine, or arginine, or sodium chloride [37, 47]. Each of these excipients also has its specific challenges and thorough assessment and knowhow is required for final product formulation design.

3.4 Stabilizing a Protein by Drying

Obviously, a liquid formulation is the most economic and easiest solution for drug product manufacturing and convenient administration. Lyophilized drug products require expensive and time-consuming processing and a reconstitution step prior to actual usage.

As mentioned above, next generation biopharmaceuticals can pose specific challenges regarding product development. In contrast to conventional proteins, next generation biopharmaceuticals often require lyophilization of the final drug product to ensure storage stability over shelf-life time (Table 3). Common exceptions to this rule are Fabs, which show sufficient stability in a liquid formulation (Table 3).

In general, lyophilization of next generation biopharmaceuticals follows the same rules as conventional proteins. However, in many cases, lower doses are required.

Critical process parameters of lyophilization formulation and process development are the collapse temperature (T_c) [51], the glass transition temperature in the frozen state (T_g') [51], the eutectic temperature of crystalized solutes (T_{eu}), and the glass transition temperature of the dried product (T_g) [52]. Above the T_c the lyophilized product collapses during freeze drying. Collapse means the loss of the macroscopic cake structure.

The amorphous phase collapses on the crystalline phase, if the temperature is between T_g' and T_{eu}, but no macroscopic collapse can be observed. It is current dogma that the drug product is freeze-dried below the T_c of the formulation and features a pharmaceutically elegant appearance.

3.4.1 Process Development: Lyophilization Cycle Design [53]

A typical lyophilization cycle consists of three stages: freezing, primary drying, and secondary drying.

3.4.2 Freezing

During the freezing phase most of the water is removed from the drug/excipients forming an interface between the two phases. The freezing process is responsible

for several protein instabilities. For example, freeze concentration of the protein increases protein/protein interaction or significantly increases the ionic strength in the freeze concentrated solute, leading to aggregation. Crystallization of one buffer component changes the pH of the formulation [54]. Finally, the formation of an ice/aqueous interface may cause surface-induced degradation [46, 55].

The cooling rate influences protein stability and primary drying time. The cooling rate has a direct impact on supercooling. Supercooling is the temperature difference between the temperature of thermodynamic ice formation and the actual temperature of ice formation [56]. The supercooling effect is influenced by several factors; however, it is usually −10 °C to −25 °C. Large supercooling effects cause small ice crystals with large ice surface area and high product resistance during primary drying (undesirable for process efficiency and protein stability), whereas small supercooling effects lead to large ice crystals with small ice surface area and low product resistance (desirable for process efficiency and protein stability). Slow cooling rates or pre-cooled shelves lead to low supercooling, but also prolongs the time of the protein in the freeze concentrated solute, which can cause protein instability [53]. In addition, slow cooling rates may cause protein instability in phase separation prone systems, for example, formulations containing polymers as a stabilizer. After the freezing phase the complete formulation should be in the solid state. The formulation should be below the glass transition temperature of the frozen state (T_g') if the protein is in the amorphous phase or below the eutectic temperature (T_{eu}) if the protein is in the crystalline state. Recently, controlling the ice nucleation during the freezing step has received significant attention as it offers several benefits: the process of controlled nucleation can lead to larger pore sizes with a reduced primary drying time. In addition, the temperature of ice nucleation can be selected for all vials, which increases product homogeneity. Several methods were described to induce ice nucleation: for example, the ice fog method, the depressurizing method, or mechanical agitation [57].

3.4.3 Annealing

Annealing is holding the drug product at a defined temperature (above the final freezing temperature) to allow crystallization of crystalline formulation components. Crystalline components are usually the bulking agents of a formulation such as mannitol or glycine. The ideal annealing temperature is a compromise between crystallization rate and crystallinity [53]. A sufficiently high annealing temperature is required to ensure a fast crystallization rate; however, a sufficiently low annealing temperature is required to ensure high crystallinity. The annealing temperature should usually be between the T_g' of the amorphous phase and T_{eu}. Preferably, annealing is not employed for industrial drug products and, thus, the use of mannitol or glycine is often discouraged.

3.4.4 Primary Drying

The primary drying phase requires the longest time fraction of the lyophilization cycle. Optimization of this step leads to an overall economic efficiency of the lyophilization process [58, 59]. The most important parameter, the product temperature (T_p), dictates primary drying time. An increase of the T_p by 1 °C decreases the primary drying time by ca. 13%. Therefore, the T_p should be as high as possible (as close as possible to T_c). However, optimizing T_p remains a challenge because T_p cannot be directly controlled and is a complex interplay of the shelf temperature, the chamber pressure, the formulation (product resistance), the used lyophilization equipment, and the primary packaging [60].

The shelf temperature is usually 5–40 °C above the T_p and provides the heat for the ice sublimation process. The heat transfer from the shelf to the frozen product is mainly influenced by the chamber pressure (gas conduction) and the heat transfer coefficient of the primary packaging (direct conduction). The primary drying end point can be detected by a decrease in water vapor pressure in the product chamber or an increase of T_p to the shelf temperature. In addition, the primary drying end point can be predicted by in silico calculations [53].The primary drying removes all frozen water from the product. However, amorphous products still contain 5–20% of adsorbed water.

3.4.5 Secondary Drying

The third phase of the lyophilization process is secondary drying, which reduces the residual water content to around 1% [61]. Sufficiently low residual water content is necessary for optimal product storage stability. Water is a plasticizer of the amorphous phase and an increase of the residual water content by a few percent usually lowers the glass transition temperature of the dried product below room temperature. During the secondary drying phase the shelf temperature is slowly increased to prevent collapse. The probability of collapse is higher at the beginning of secondary drying as the product still contains a reasonable amount of water. Crystalline products are at no risk of collapse. It is usually favorable for product stability to run a high shelf temperature for a short period, compared to a low shelf temperature for a longer time [62].

3.4.6 Formulation Development: Choice of Excipients

A lyophilization formulation usually comprises four excipient components: buffer system, stabilizer, surfactant, and/or bulking agent, which also function as tonicity modifier [63].

The buffer system controls the pH of the drug product and is critical to minimize protein degradation during processing, storage, and reconstitution. The choice of an

adequate buffer system is even more restricted for lyophilization formulations compared to liquid formulations. Several buffer systems have undesirable properties for lyophilization. For example, sodium phosphate buffer systems show a significant pH drop during freezing as the dibasic form Na_2HPO_4 selectively crystalizes during the freezing step [64]. Acetate buffer systems are volatile [65] and, thus, are not (easily) usable for formulations intended for lyophilization. The ideal buffer system provides pH control over a broad temperature range, is non-volatile, and has a high T_c and T_g [66]. Typical buffer systems for lyophilization are histidine buffers or tris buffers [67]. Tris buffers, however, show temperature-dependent pH and may be critical from a safety perspective for some patient populations, depending on total product volume to be dosed and route of administration. Citrate buffers show adequate properties toward protein stability; however, they have been connected to pain after administration at the injection site [68].

The stabilizer and the protein form the amorphous phase in the lyophilized solid drug product. Stabilizers thermodynamically protect the protein during freezing and minimize protein unfolding by replacing water molecules during the drying phase [69]. Stabilizers should be non-reducing (e.g., glucose shows the Maillard reaction [70]) and remain in the amorphous phase during lyophilization. Typical stabilizers are sucrose and trehalose. Both disaccharides have proven effective stabilization properties. Of note, depending on storage conditions, sucrose may invert to glucose. Trehalose has a higher T_g and is less susceptible to acidic hydrolysis, whereas sucrose was observed to be more effective in inhibiting protein unfolding during drying. The stabilizers are used in at least 1:1 stabilizer: protein (w/w) ratio and show best stabilization effects at a 5:1 ratio [69].

Surfactants can stabilize protein during processing (e.g., vial filling), during the freezing step by reducing ice/water interfacial stress, and during the rehydration step by reducing solid/water interfacial stress and by serving as a wetting agent [71].

Finally, a crystalline bulking agent forms a mechanical stable, pharmaceutically elegant cake. The bulking agent should be completely crystallized during the freezing step. Incomplete crystallization of the bulking agent can compromise protein stability by crystallizing from the solid during storage and can depress product T_g. Typical bulking agents are sucrose or trehalose. Mannitol and glycine can also be used but require a more complex lyophilization process and also typically do not add to protein stability [46, 63]. Mannitol was also observed to cause vial breakage in some cases.

3.5 Stress Conditions During Processing

During upstream and downstream processing, drug product formulation, and fill&finish operations, proteins encounter several critical process steps which may compromise product stability [40]. Compared to conventional mABs, next

generation biopharmaceuticals are often more sensitive to process stress and physical degradation. For example, ADCs are significantly more hydrophobic than their carrier mABs alone and the Tm of an ADC is lower with an increasing DAR compared to an mAb. Both aspects may promote and increased adsorption to process equipment and may lead to surface induced degradation. In addition, special cleaning and handling procedures are required for ADCs. Therefore, special emphasis is given to an adequate process design and development to ensure product quality.

3.5.1 Freezing and Shipping

The drug substance bulk is preferably and typically stored in the frozen state to ensure protein stability during production hold step and shipment and to minimize risk of microbiological growth which could also occur in the refrigerated state (considering that drug substance is not sterile but low bioburden). A storage temperature below the maximally freeze concentrated solution is suggested, which is usually not warranted by storage at or around −20 °C [72, 73]. In addition, cryoprotectants such as trehalose or mannitol may show excipient crystallization that may or may not lead to protein instability in the frozen state [74]. Thus, drug substance bulk is preferably stored below −40 °C. The manufacturing of an ADC usually comprises several freeze/thaw and shipping steps. The mAB and the cytotoxic agent of an ADC are often produced in different facilities. Transport of the mAB intermediate to the conjugation facility and transport of the final ADC to the fill&finish facility are required. The mAB intermediate should be stored in a formulation that provides sufficient stability yet does not significantly negatively impact the following conjugation process. For example, a surfactant is in most cases preferably avoided for mAB intermediate drug substances [18]. The final ADC should be stored in the final formulation buffer to avoid processing such as buffer exchange at the fill&finish facility.

3.5.2 Mixing, Filtration, and Filling

During product manufacturing the drug substance bulk solutions need to be thawed and subsequently homogenized. Different mixer designs are available. Magnetic button mounted mixers with no contact between the impeller and the driver are often preferred as they are considered to feature lower stresses and easier cleaning [75]. Bags are usually homogenized with horizontal shakers. Therapeutic proteins are manufactured aseptically in combination with sterile filtration. Filter materials need to be compatible with the protein and its excipients and fit the filtration process parameters [76]. The filling process represents another critical step during drug product manufacturing. The choice of the filling pump and process parameters is key as they need to be compatible with the drug product solution [77–79].

As mentioned above, next generation biopharmaceuticals are often less stable than conventional mABs. Fusion protein can be susceptible to protein aggregation, and the hydrophobic nature of ADCs can lead to adsorption to process equipment, unfolding, and interfacial induced aggregation during the filling process. Adsorption is especially of concern with low dosed ADCs as adsorbed material may significantly lower the protein concentration in the final drug product.

4 Specific Challenges with Formulating New Molecule Formats

Monoclonal antibodies can be considered a commodity nowadays, and formulation and product manufacturing can in most cases rely on prior knowledge. Sequence analysis and developability is, however, recommended to ensure that the molecule does not contain a specific "instability hotspot" or liability that would prohibit or complicate the use of such platform knowledge. For example, if the pI of an mAb is in the unusual range of 5–6, this would trigger further assessment. Another example is when instability hotspots reside in the CDR region of the mAb, and thus would likely be expected to impact on binding, potency, and thus possibly efficacy. This includes examples such as exposed methionine or tryptophan residues which can be easily oxidized, deamidation hotspots (similar to DG residues), exposed lysines (which could be glycated), or any type of unpaired thiol group (single cysteine amino acid), as the latter are expected to lead to covalent dimers, which are typically undesirable.

An mAb that does not show specific liabilities of concern would qualify for using platforms, including the use of platform formulations, which can yield liquid mAb formulations, including those for subcutaneous use and in the range of 150 mg/mL, predefined and already qualified standard container closure systems, and platform manufacturing processes. Such platform manufacturing processes typically involve using standard unit operations, standard technologies and types of equipment, and standard parameterization.

4.1 Sequence and Molecular Assessment of a Novel Format

The first evaluation of a novel format would also include an evaluation of the molecule structure and primary sequence. It is important to evaluate the expected regions relevant to binding and activity, as changes in amino acids in this region may change potency (and efficacy), and thus may need to be evaluated with even more scrutiny. The assessment of the molecule should also consider whether the novel protein contains various domains. These could be differently charged and have a different pI which may or may not complicate analytics as well as stability,

depending on how the chosen formulation pH would impact on respective subdomains.

4.2 Analytical Methods for Product Development

Without adequate analytical methods, covering all the different possible degradation patterns and pathways, product development is far from being straightforward. Specifically, analytical methods should be in place to be able to monitor product- or process-related degradants. Examples include methods to monitor (soluble) aggregates and protein particles (insoluble matter), including SE-HPLC, AF4-LS, light obscuration, low imaging, and visible particles. Most of these methods require, typically, only minor adaptions for novel formats, but, of course, this is case-by-case assessment.

Methods to monitor charge variants (and thus deamidation) include iCE280, IEC, and IEF. Depending on the molecule format and charge variants already present in material after processing, this assessment has been found to be challenging in many instances. Platform methods may not readily apply, analytical separation of charge variants may not be an issue, and quantification of variants certainly isn't, thus requiring the grouping of different variants.

Generally, given the low level of knowledge on identity of product-related variants in an analytical data set such as a chromatogram, it is unusually difficult to assign criticality. The element of "prior knowledge" does not exist equally for most novel formats compared to antibodies, and thus the assessment of critical quality attributes (CQAs) and the definition of CQA assessment criteria and specifications is difficult, especially in early-stage development. As an example, higher molecular weight species (soluble aggregates) in many monoclonal antibody products are in most cases specified not to exceed 5% (or in some cases 10%) (means, >90 resp >95 content of monomer in SE-HPLC). For non-antibodies, 5–10% aggregates may be well acceptable if efficacy and safety are not impacted (i.e., if aggregates would be non-CQA, or if CQA acceptance criteria are sufficiently broad).

This lack of prior knowledge may suggest that for early-stage analytical development, some further resources should be invested into trying to identify degradants or impurities more extensively than in mAbs, or at least to assess groups of impurities on their potency. As an example, acidic variants in a charge assessment can be purified from main peak and basic regions and evaluated for potency in a bioassay that is found relevant to the molecule's mode of action (MoA). The same assessment would include purified basic region and main peak. Relevant endpoints to consider include any structural element of the protein possibly relevant for drug safety or efficacy, including potency (MoA), changes in FcRn binding (if relevant for the specific molecule for, e.g., PK), and changes in Fc_{γ} binding (if relevant to the specific molecule's MoA).

4.3 Formulation and Dosage Form Strategy

Given that the assessment of safety and efficacy finally requires relevant preclinical species and clinical testing in humans, attention must also be paid to the related preclinical study material and clinical supply. Specifically, the quality and quantity of impurities in toxicological material (vs. later clinical testing material) should relate to allow sufficient safety factor evaluation when proceeding to first-into-human (FIH) studies. It is generally recommended to use drug products with the same formulation and comparable drug substances in a Tox and FIH study. Using a drug product would also facilitate the assessment of comparability between Tox and FIH study materials, given that the list of analytical tests would compare and preferably be identical. In cases where a drug substance would be used for GLP Tox testing, important tests, such as for particulates, would typically not be performed, and thus that information from the technical package would be missing. Also, the dosage form may play a role here. For example, an ADC with a water-labile linker would typically require a sufficiently dried formulation and drug product. If using a formulation or configuration where hydrolysis of the linker would not be sufficiently slowed down, the conjugated drug may be exposed in solution and preclinical test species much quicker, and may exert its pharmacological and toxicological effects.

The general choice of a parenteral dosage form is either liquid or dried. Lyophilization, that is, freeze-drying is the standard technology of choice although spray-drying may have its niche applications, for example when considering special routes of administration such as pulmonary dried powder formulations. Given that the impact of process- and product-related degrades is not deeply understood for novel protein molecules, it is relevant to test representative or "worst case" levels of these early on, in, for example, acute and chronic toxicological studies. It may be counter-intuitive, but considering a liquid formulation should, from these considerations, be the likely preferred dosage form when any commercial launch configuration would also require a liquid dosage form. Lyophilization is a means of stabilization but may prevent some of the degradation reactions and thus minimize the development scientists' ability to understand their impact on biological reactions and safety. Of course, lyophilization is and must be the preferred dosage form and way of stabilization for any water-sensitive molecules (such as ADCs with water-labile linkers). Lyophilizates may also be preferred in cases where the actual analytical methods available for formulation assessments are insufficient to provide enough insights and to allow a thorough and holistic formulation evaluation. The use of freeze-drying in these cases may thus also be a derisking measure. We assume that with increasing numbers of non-antibody novel formats in development, the number of lyophilizates in the commercial market is expected to increase.

4.4 Some Specific Formulation Challenges with Novel Formats

As briefly mentioned above, there are some typical challenges with novel formats, including:

– Aggregation, particle formation, and viscosity

These can relate to protein/protein interactions and charge and charge distributions are expected to be relevant to impact on these. As mentioned, charge variations of subunits of fusion proteins can lead to dipole moments, aggregation, viscosity or other findings.

– Oxidation

Amino acids that can be oxidized are commonly found in a proteins' primary sequence. This includes methionine and tryptophan. Having adequate analytical methods in place to monitor protein oxidation is often quite a challenge. Peptide mapping and mass spectrometry may be required to monitor and evaluate levels of oxidation in different formulations, and these may be quite cumbersome and require significant resources. Oxidation can be generated by a variety of factors. Metal ions are ubiquitous and may lead to reactive oxygen species (ROS) via Fenton or Haber Weiss reactions [80] and these ROS can subsequently oxidize proteins. Peroxides may also be introduced via raw materials. Polysorbates are specified in the USP and Ph. Eur. with "NMT 10 ppm" of peroxide. However, these levels of peroxides may be very critical for formulations of antibodies and other formats, including Fab fragments but also any other protein that is oxidizable. The oxidative degradation of polysorbate would further accumulate and generate peroxides [81]. Thus, even a well-monitored polysorbate at release with "minimal peroxide" bears the risk and likelihood to generate peroxides during storage of the drug product formulation. The concentration of polysorbate is recommended to be monitored for stability of liquid, but also lyophilized drug product. The peroxides generated during oxidative polysorbate degradation would further lead to degradation of the surfactant, but also oxidize protein at the same time. This is why it is also imperative to monitor protein formulations for oxidation when using surfactants that can degrade via oxidative pathways. Finding protein oxidation can also be a hint toward oxidative polysorbate degradation if the latter has not been thoroughly monitored. The complexity of possible impact of process-residuals or degradation of polysorbates can in some cases also suggest the use of poloxamer in formulation development. Although polysorbate 20 and 80 remain the most preferred surfactants in protein formulations, the evaluation of poloxamer early in formulation development may be helpful.

5 Summary

Monoclonal antibodies are a commodity nowadays and offer significant potential for applying prior knowledge and using platform processes. Novel protein formats, however, may provide specific challenges to drug product development. This may include the lack of knowledge about criticality of quality attributes, the complexity of process- or product-related degradation patterns, challenges related to analytical methods and setting specifications, but also, specific stability liabilities such as aggregation, deamidation, or oxidation. All of the above likely lead to an increased use of lyophilization for drug product stabilization.

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Continuous Manufacturing of Recombinant Therapeutic Proteins: Upstream and Downstream Technologies

Rohan Patil and Jason Walther

Abstract Continuous biomanufacturing of recombinant therapeutic proteins offers several potential advantages over conventional batch processing, including reduced cost of goods, more flexible and responsive manufacturing facilities, and improved and consistent product quality. Although continuous approaches to various upstream and downstream unit operations have been considered and studied for decades, in recent years interest and application have accelerated. Researchers have achieved increasingly higher levels of process intensification, and have also begun to integrate different continuous unit operations into larger, holistically continuous processes. This review first discusses approaches for continuous cell culture, with a focus on perfusion-enabling cell separation technologies including gravitational, centrifugal, and acoustic settling, as well as filtration-based techniques. We follow with a review of various continuous downstream unit operations, covering categories such as clarification, chromatography, formulation, and viral inactivation and filtration. The review ends by summarizing case studies of integrated and continuous processing as reported in the literature.

Keywords Biomanufacturing, Continuous, Continuous chromatography, Downstream, Integrated, Perfusion, Upstream

Contents

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1 Introduction

Although the vast majority of current biotherapeutics are manufactured via upstream and downstream batch operations [1–3], continuous manufacturing is receiving serious consideration in the biopharmaceutical industry [4–9]. Over the past years, continuous technologies for individual unit operations have been developed and applied in both industry and academia. As these technologies have matured, examples of integration of continuous operations are emerging with increasing frequency. Ultimately, this progression could lead to fully integrated and continuous end-to-end bioprocesses. Such processes potentially offer several advantages, as enumerated below.

First, from a cost-of-goods perspective, continuous manufacturing unlocks process intensification and integration opportunities, increasing productivity and eliminating some unit operations (especially hold steps). Continuous operation also enables production at smaller scales, which in turn reduces equipment size and facility footprint, generates opportunities to apply disposable technologies, and ultimately leads to reduced capital and operational costs, as highlighted in a number of recent economic analyses [10–14]. If integrated and continuous systems can be reliably executed in a consistent fashion over time, they also become prime candidates for increased automation, leading to reduced labor costs [8].

Second, continuous manufacturing can also increase facility responsiveness and flexibility. The low residence times and cycle times of a well-designed continuous process should allow for the manufacture of both stable and labile products, and companies with such processes could hypothetically build standardized platforms that serve a wide variety of molecule types. Standardization in combination with small-volume, intensified processing makes possible nimble, multi-product ballroom facilities [15, 16].

Third, continuous processing offers several positive implications for product quality. As mentioned previously, lower residence times and reduced holds should generally lead to better and less variable product quality. Additionally, because the regulatory definition of batch primarily depends on product uniformity and not the mode of manufacturing (21 C.F.R. § 210.3, 2015), a continuous process developed to perform consistently and steadily (with respect to product quality) both within and across runs can be leveraged to provide enormous operational freedom because pooling/splitting/batching strategies can be decoupled from run day [15, 17].

This review relates the progress made toward continuous biomanufacturing of therapeutic recombinant proteins. We first discuss approaches for continuous cell culture, with a focus on perfusion-enabling cell separation technologies. We follow with a review of various continuous downstream unit operations, covering categories such as clarification, chromatography, formulation, and viral inactivation and filtration. Relevant technologies and experiences from other related industries are also discussed. The chapter ends by summarizing case studies of integrated and continuous processing in the literature.

2 Current Continuous Technologies

2.1 Continuous Cell Culture

A holistic upstream/downstream integrated and continuous process requires a cell culture platform with a continuous (or at least semi-continuous) output of product-containing harvest. Fed-batch bioreactors are the current industry standard for recombinant protein production [3] but, because of their inherent batch nature, they are not readily amenable to a continuous process. However, several alternative cell culture strategies have been developed over the past 20 years that are capable of continuous product output and integration with downstream operations [4, 5, 18–20].

Continuous cell culture operations can offer more than just a continuous source of product. In many situations their longer durations and/or higher cell densities allow for process intensification relative to fed-batch approaches [14]. In some instances, continuous systems can also be controlled consistently over long durations, simplifying operations and guaranteeing consistent product quality attributes [15, 17, 21].

2.1.1 No Cell Retention

The most straightforward approach to continuous cell culture is the chemostat, where fresh medium is fed into the bioreactor and spent medium containing both cells and product is removed at the same rate. No separation devices are used to retain cells, so the cell concentrations in the harvest stream and bioreactor are equal. Although chemostats for mammalian culture have been studied for more than half a century [22, 23], they are still most commonly applied as experimental tools at small scales [24–27]. There is at least one example of a chemostat-like commercial process: Factor IX is made in a suspension cell culture using a semi-continuous batch re-feed process [28]. Because of its lability, the product cannot reside in the bioreactor for long durations. The batch re-feed process allows product quality to be controlled and maintained and reactor turn-around to be reduced.

However, the low growth rates of mammalian cells prevent such systems from achieving high cell densities and productivities, rendering chemostats infeasible and undesirable for manufacturing. To achieve productive continuous culture, some form of cell retention must be employed to intensify cell density in the bioreactor and decouple the cell growth rate from the media addition rate. Several categories of these perfusion-enabling cell retention devices, each with advantages and disadvantages, are discussed below.

2.1.2 Immobilized Cell Retention

Cells can be permanently separated from the perfusate if they can be immobilized to a fixed matrix. Culture medium can then be passed through the cellular environment and spent product-containing medium can be collected at the other end. Immobilized systems do not suffer from washout at any perfusion rate, cells are generally protected from shear, and high packed cell densities are possible. Common disadvantages of immobilization include cell-to-cell heterogeneity, representative sampling and accurate biomass monitoring, poor oxygen and nutrient transfer, and difficult scale-up [18]. Examples of immobilized systems include hollow-fiber membranes, flat plate membranes, gel encapsulation, ceramic matrices, and fluidized beds. Several comprehensive reviews of these strategies can be found in the scientific literature [29–33].

2.1.3 Density-Based Cell Retention

Viable, suspended mammalian cells have a density greater than that of their surrounding supernatant, and various techniques have been developed to exploit this difference to extract a relatively cell-free harvest, simultaneously retaining cells within a bioreactor. Devices that employ density-based separation include gravitational settlers, centrifuges, acoustic settlers, and hydrocyclones.

Gravitational Settling

Gravity can be exploited to separate cells from harvest if the culture can be pumped upward at a rate slower than the cell settling velocity. At standard conditions this approach only enables low perfusion rates although requiring long residence times for cells in the separator, away from the controlled conditions of the bioreactor. Early implementations of gravitational settlers included a conical sedimentation column [34, 35], an intra-reactor settling zone [36], and a Dortmund settler [37]. Although these systems could be maintained for long durations (25 days and beyond), they could not reach densities above 7 million cells/mL. Average viabilities were also fairly low (45–85%), presumably because of the long residence time of cells in the separator.

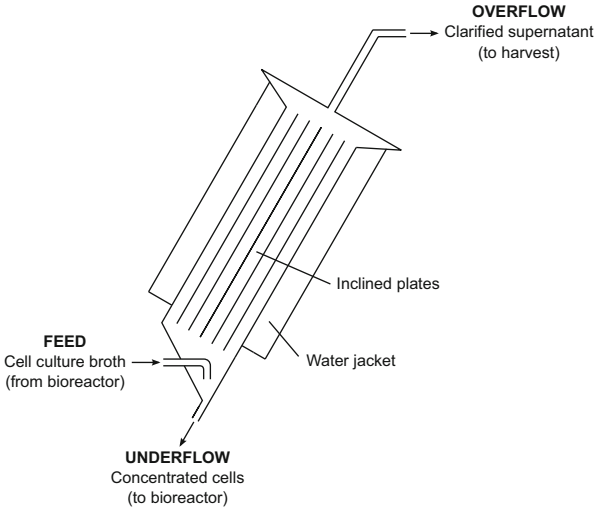

Fig. 1 Diagram of an inclined cell settler [43]

To increase cell settling velocity, enable higher perfusion rates, and decrease separator residence time, cells can be cultured on suspended microcarriers [38–40]. However, microcarrier-based cultures present a number of difficulties, including heterogeneity within the bioreactor because of stratification, cell-to-cell heterogeneity because of layered growth on microcarriers, and increased sensitivity to shear from agitation and sparging [41, 42].

Alternatively, settler geometry can be optimized. As described in Fig. 1, inclined settlers with sloped, parallel plates have been designed to create laminar flow and leverage the Boycott effect to allow cells to settle, accumulate, and descend down the lower plate with enhanced separation efficiency [44, 45]. Residence time can be further decreased by cooling and vibrating the settler, as well as flushing periodically with gas [46]. Mathematical and computational modeling can be used to explore various geometries and optimize inclined settler design [47, 48]. Methodology for empirically determining cell settling velocity has also been developed, further informing design decisions [49].

Gravitational settlers are well-suited to long-duration operation as there is no physical barrier prone to fouling or clogging. There are several examples of run lengths over 40 days [50, 51] and commercial examples running up to 6 months [21, 52]. Another commonly cited advantage of gravity settlers is their ability to retain viable cells and remove smaller dead cells via the perfusate [53].

Gravitational settlers have also been demonstrated to scale up relatively well in manufacturing settings. Inclined settlers have recently been used to develop high-density N-1 seed train operations to increase the inoculation density of the production bioreactor to reduce durations and increase facility utilization. After testing at laboratory scales, an inclined settler was implemented alongside a 3,000-L bioreactor and operated at a maximum perfusion rate of one reactor volume per day [54, 55].

Perfusion by gravitational settling does have disadvantages. Cell densities have only been demonstrated up to 20–30 million cells/mL; above that range, retention efficiency dramatically drops and cells pass through to the harvest. Separator residence times, even after optimization, are still fairly long [46]. Finally, because debris and some cells do exit the bioreactor in the harvest stream of a gravitational settler, an additional clarification step is required if downstream purification operations are integrated.

Centrifugation

Similar to gravitational settling, centrifugation relies on density differences for cell retention, but with an increased driving force that hypothetically should result in reduced residence times outside of the controlled bioreactor. Early experiments demonstrated both intermittent and continuous centrifugation using an autoclavable rotor connected to the reactor system via mechanical seals [56–58]. These cultures typically averaged 5–10 million cells/mL, peaking at around 15 million cells/mL, with durations up to 40 days. Perfusion experiments have also been conducted with disk stack centrifuges, where cells are separated from supernatant using a disk stack, collected in peripheral pouches, and sent back through the center of the rotor to the bioreactor [59, 60].

To eliminate the need for rotor sterilization and reliance on mechanical seals, the Centritech centrifuge (commercialized today by PneumaticScaleAngelus) was introduced. The Centritech uses continuous orthogonal flow and features a disposable insert (fixed onto a conical rotor) for cell culture collection and separation. The insert is connected to the bioreactor via a seal-free tubing bundle with an anti-twist mechanism allowing it to rotate at the centrifuge and remain stationary at the other end. Similar to gravitational settlers, the Centritech has been shown to retain viable cells preferentially and remove dead cells. Initial studies, however, did not lead to viable cell densities above 10 million cells/mL, and, at these higher densities, viabilities were quite low, averaging around 50% [61, 62]. More recent experiments have overcome these early challenges, resulting in high viabilities [54, 63]. One implementation in particular demonstrated average cell densities of 30 million cells/mL for over 30 days [64]. The Centritech has also been implemented commercially for the production of labile proteins [5].

Centrifuges that utilize continuous counterflow are also commercially available, including the Elutra cell separation system (Terumo BCT) and kSep (KPI Biopharma) [65]. Experience with these systems integrated to perfusion bioreactors is limited, but the kSep system has been demonstrated for up to 6 days at a peak of approximately 15 million cells/mL [66].

Acoustic Settling

Acoustic settlers trap cells in the pressure nodes of standing ultrasonic waves. Once trapped, cells aggregate and then settle back into the reactor by gravity [67]. Acoustic settlers do not require moving parts, can be easily cleaned and sterilized, and do not require any physical barrier for cell separation and therefore are not prone to fouling [68].

Different designs have been tested, including a dual-chamber device for integrated cooling [69, 70] and BioSep, a single-chamber approach marketed by Applikon [71, 72]. Periodic air backflush has also been incorporated to reduce pump stress and decrease cell residence time in the separator [73]. Investigators have explored the effect of various system parameters on retention efficiency and culture success, including power input, cell concentration, perfusion rate, bleed rate, cooling air flow, backflush frequency, backflush volume, duty cycle stop time, and recirculation ratio [74–77].

Acoustic separation has been successfully demonstrated at the pilot scale, enabling perfusion rates up to 200 L/day for 100-L bioreactor working volumes [76, 78]. Larger manufacturing devices are available and capable of handling 2,000 L/day, but no experience is reported in the literature. Similar to gravitational settlers, acoustic settlers are capable of long durations – experiments have persisted for 50 and even 100 days [73, 79]. However, densities have typically been demonstrated only at 10–15 million cells/mL, and the few examples that have transcended 20 million cells/mL [74, 80, 81] have also had very low viabilities between 40–60%, when reported.

Hydrocyclone Cell Retention

A hydrocyclone is a simple conical device that uses centrifugal forces to separate components by density. Figure 2 depicts this separator in a perfusion application: cell culture enters at high speeds and circles the cone. Cells are preferentially drawn outward and downward to the bottom outlet via a primary vortex as excess supernatant at low cell concentrations exits the top outlet via a secondary vortex [83]. Hydrocyclones are low-cost, have no moving parts, and can be readily applied to both disposable and clean-in-place/steam-in-place systems. These devices are also extremely size-efficient: a small hydrocyclone (1 cm diameter) can theoretically process up to 1,000 L/day [4]. Smaller volumes require intermittent perfusion because further scale-down of the hydrocyclone is not feasible [84].

Numerical models have predicted that hydrocyclones are capable of 90% cell retention efficiencies [82]. Short-duration experiments (up to 2 days) confirmed this estimate, showing 97% separation efficiency with no measurable impact on cell viability or productivity [85, 86]. More recently, longer cultures up to 8 days have been demonstrated, at bioreactor scales up to 300 L [87, 88]. Although hydrocyclone-mediated perfusion is a promising area, cell densities have yet to be pushed beyond 10 million cells/mL; additional study and work is required for

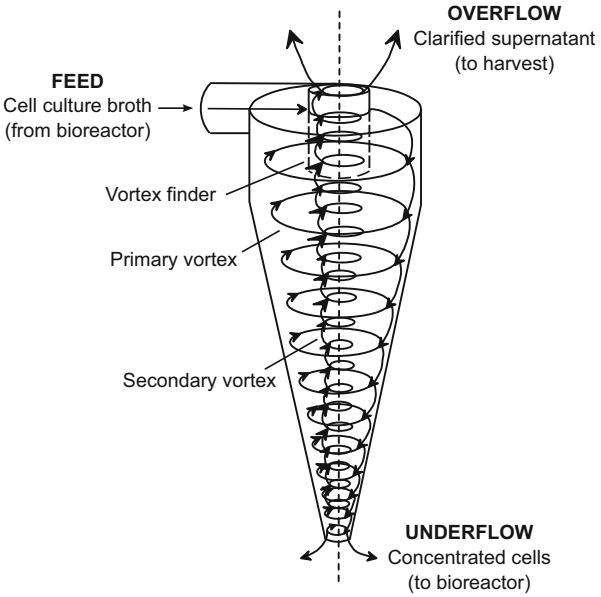

Fig. 2 Diagram of a hydrocyclone for cell retention [82]. The vortex finder is a short, removable section that prevents short-circuiting of feed directly into the overflow

this technology to compete with more conventional approaches such as gravitational settling or filtration.

2.1.4 Size-Based Cell Retention

Various forms of filtration have been tested in perfusion systems. By using a filter as a perfusion-enabling cell separator, two unit operations (cell retention and clarification) can be integrated to deliver significant process intensification. Filtration-based perfusion also significantly eases downstream integration: because the harvest stream is clarified, and continuous purification operations (such as continuous chromatography) can be directly connected if flow rates are matched. The primary challenge is filter fouling: at medium-to-high cell densities, dead cells and debris accumulate and standard filtration approaches lack longevity. A number of different filtration technologies have been developed in an effort to meet this challenge, and are described below.

Spin Filtration

Spin filtration (Fig. 3) was an early solution to the problem of filter fouling where cell culture supernatant is pumped into a cylindrical filter and out of the bioreactor. The spin filter rotates rapidly (either internal or external to the reactor), preventing

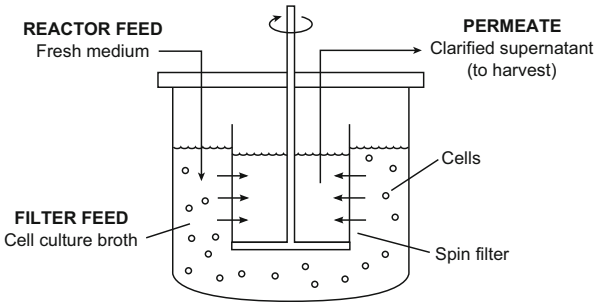

Fig. 3 Diagram of a spin filter within a bioreactor

cells from fouling the membrane. When internal, the filter is often integrated onto the agitator shaft [89–91].

Although spin filters certainly offer superior performance relative to static dead-end filtration, filter fouling is still a significant concern. Fouling is worsened by charged filter surfaces and serum-based media [92], antifoam addition [93], high perfusion rates, high cell concentrations, low filter rotational velocities, and low filter surface area [94]. Models have been developed (both numerically and using computational fluid dynamics) to indicate the existence of substantial exchange rates across the filter surface that can contribute to fouling [95, 96].

An early study avoided fouling concerns by coupling a large-pore (120 μm) spin filter with microcarrier-seeded cell aggregates (200–600 μm), enabling a culture duration of 30 days with cell densities as high as 60–70 million cells/mL [97]. Another experiment maintained 18 million cells/mL for 35 days, peaking around 25 million cells/mL [64]. For the wide majority of cases, however, spin filter cultures reach an average of 10–15 million cells/mL [54, 71, 93, 94, 98, 99].

Scale-up is also challenging, as the radius of the cylindrical screen needs to increase quadratically with the radius of the bioreactor to maintain a constant filtration flux. The technology has been scaled to enable perfusion at pilot-scale bioreactors with 175-L [100] and 500-L [94] working volumes. Additional scale-up may require moving the filter apparatus external to the reactor [98].

Hollow-Fiber Filtration

Hollow-fiber filters were introduced as cell retention devices that could alleviate some of the concerns around spin filters. Cylindrical cartridges packed with hollow-fiber filters scale much more readily because filter surface area increases quadratically with cartridge radius. Early implementations placed these cartridges directly within the bioreactor and cell densities as high as 90 million cells/mL were demonstrated [101, 102], though most examples are external to the reactor, allowing for easier access, monitoring, and replacement.

Hollow-fiber cartridges can be operated using tangential-flow filtration (TFF), where feed flow travels tangentially to the filter surface and permeate flows perpendicularly (Fig. 4A). This cross-flow action reduces the trapping of cell debris

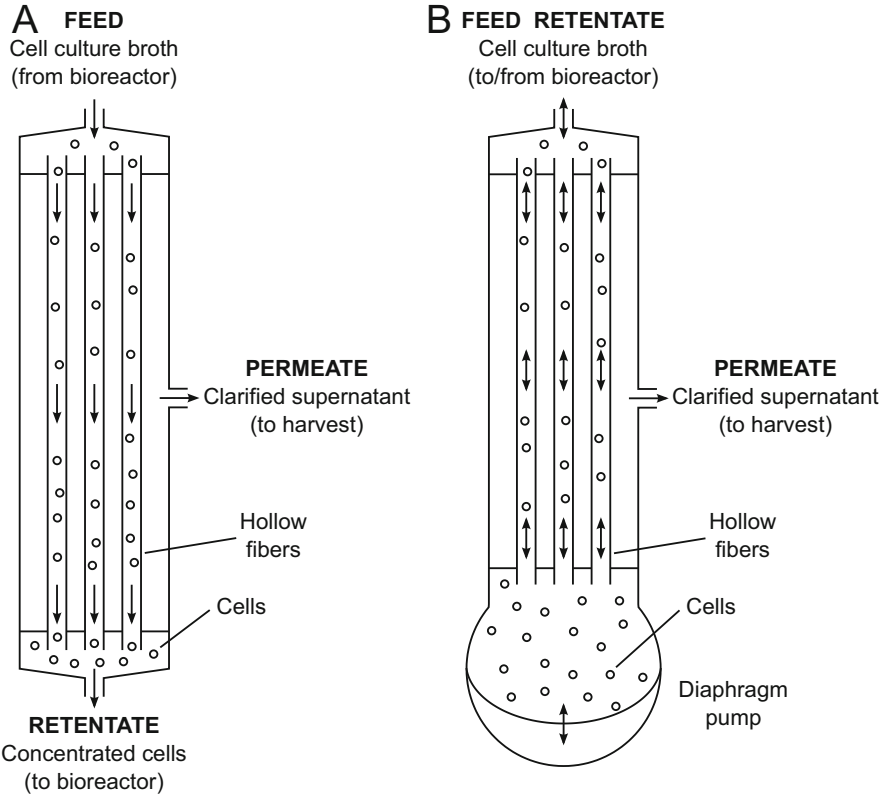

Fig. 4 Diagrams demonstrating cell retention via (**A**) tangential flow filtration and (**B**) alternating tangential flow filtration

and other solids into the filter, increasing system longevity. Early efforts established the feasibility of the technology across a variety of filter types and suppliers [103–105]. Filter fouling leading to product retention was noted across a membrane with 0.2 μm pore size [106]. One solution to increasing filter longevity was an increased recirculation rate and periodic pulsing through the hollow fibers [107]. Other researchers found that by avoiding high transmembrane pressures and washing the membrane surface daily at high flow rates with cell-free medium, fouling could be minimized and a continuous culture could be maintained for 30 days with a peak viable density above 30 million cells/mL [108].

More recently, cell densities and durations have been pushed increasingly higher and longer in TFF systems. Two different groups have demonstrated durations longer than 25 days peaking at 60–70 million cells/mL [109, 110] and Clincke achieved 45 days of operation with a peak density of 214 million cells/mL. At that ultra-high density, the viscosity of the culture and the dissolved pCO_2 became limiting [111].

Alternating tangential-flow (ATF) filtration is a specific implementation of TFF that has been widely applied to perfusion. A diaphragm pump is coupled to a hollow-fiber cartridge, and cell culture is alternatingly pumped into and out of the filter from the bioreactor (Fig. 4B). Advantages relative to standard TFF include a compact design and small footprint, gentler pumping, only one connection point necessary between the reactor and separation device, and significant back flow across the filter membrane which may reduce filter fouling. Despite these changes, filter fouling is still observed, and efforts have been taken to correlate and model filter performance [112].

High-density perfusion via ATF has been demonstrated for over 25 days [64, 109, 113], with one system operating for almost 70 days at greater than 40 million cells/mL [17] and another peaking at 130 million cells/mL [111]. ATF filtration has been frequently considered as a means toward high-density inoculation of the production bioreactor. Different groups have shown that inoculation densities of 5–10 million cells/mL are possible with no negative impacts on growth or product quality if the N-1 is operated with ATF perfusion [114–116].

Other Filtration

Disposable floating filters are available with some disposable bioreactor bag systems. These have been used to grow cells to densities above 20 million cells/mL, maintaining viabilities above 90% [117]. However, because of the lack of any cross flow, these filters are prone to clogging and are typically only used on the order of 10 days [118, 119].

Vortex flow filtration (VFF) consists of an internal, rotating cylinder surrounded by a concentric filter. Cell culture is sent to the annular space and cell-free supernatant is drawn through the filter and out of the annular space. The internal rotating cylinder forms Taylor vortices which prevent concentration polarization and particle deposition on the filter. Studies have been conducted both with hydrophilized polysulfone membranes [80] and a 10-μm stainless steel screen [120]. Low-density, long-duration runs have been demonstrated (5–10 million cells/mL for 75 days) as have shorter, higher density experiments (20 days with a 50 million cells/mL peak). As with spin filtration, scale-up could present challenges as the VFF diameter needs to increase quadratically with the bioreactor diameter.

2.1.5 Cell Density Control

Cell density can be controlled in perfusion systems to enable longer-duration cultures, increasing process consistency. If cells are left to proliferate unchecked, large swings in the extracellular environment can occur, potentially causing shifts to metabolism, productivity and product quality. The most common method for cell density control is to remove cell-containing fluid continuously or semi-continuously from the bioreactor at low rates [121]. This removal is commonly

referred to as "cell bleed" or "cell discard." If the bleed rate can be controlled to equal the apparent growth rate (the difference between the true growth and death rates), then the cell density remains constant [74]. In filtration-based perfusion, where dead cells and debris accumulate in the bioreactor, cell bleed can also play an important role in removing these types of materials from the system.

One approach consists of setting a constant volumetric bleed rate and then allowing the culture to adjust itself until the apparent growth rate equals the bleed rate [78, 106]. The primary advantage of this method is its simplicity – no cell density measurements (online or offline) are required for implementation. The method's downside is that, if cell growth rates change over time, the absolute cell densities fluctuate accordingly.

To incorporate feedback control, regular (e.g., daily) bolus bleeds can be manually executed after offline viable cell density measurements to bring the culture semi-continuously back to set point [110, 111]. Although this is relatively straightforward to implement from an infrastructure standpoint, the recurring requirement for manual intervention can be laborious. Additionally, because control is not continuous, by definition there are swings in cell density (and therefore cellular environment) between bleeds.

If a robust, online cell counting method can be developed, continuous bleeding for automated cell density control can be achieved. Various online proxies for cell density have been demonstrated, including optical density [122], metabolic rates [123–126], oxygen uptake rate [101], capacitance [17, 127, 128], and Raman spectroscopy [129, 130]. By linking the online measurement to a feedback-controlled bleed pump and recalibrating using offline measurements as necessary, cell densities can be maintained at consistent levels for long durations. Processes employing a feedback-controlled bleed need to be developed to optimize growth rate; if growth is too low, a consistent bleed cannot be maintained, and if it is too high, substantial product is sent to waste.

2.1.6 Historical Progress of Perfusion Technologies

Viable cell density profiles from various perfusion experiments with reported data in literature have been analyzed and compiled in figure form. In Fig. 5A, average viable cell densities in perfusion systems are plotted over time and categorized by separator type. Average viable cell density is defined as the integrated viable cell density (over the course of an experiment) divided by culture duration, and can serve as a rough indicator of a system's potential for productivity. (Note that this metric also rewards cultures with longer durations, as the impact of the lower-density growth phase is minimized.) The time profile of average densities reveals that, with only limited exceptions, average perfusion densities did not exceed 20 million cells/mL until 2010, after which a large number of demonstrations at or above 30 million cells/mL have occurred. Cell retention via filtration is the predominant enabler of these higher-density systems.

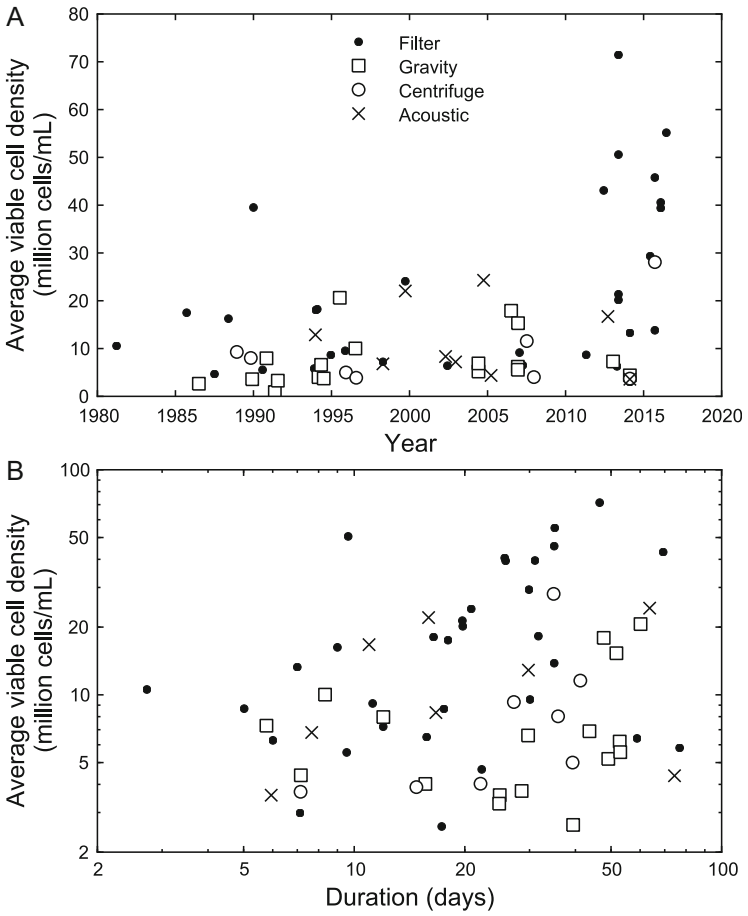

Fig. 5 Average viable cell densities reported in literature plotted by (**A**) year and (**B**) culture duration. Cell separator type is indicated by symbol (filtration – *closed squares*, gravitational settling – *open squares*, centrifugal settling – *open circles*, and acoustic settling – *crosses*). Data is based on over 60 experiments reported in the literature since 1980 [50, 53, 71, 74, 78, 88, 94, 97, 103, 111, 37, 54, 56, 73, 76, 93, 107, 122, 34, 62–64, 108, 109, 121, 131, 132, 51, 55, 80, 101, 110, 114, 124, 126, 133, 134, 17, 35, 36, 46, 57, 58, 81, 90, 91, 99, 102, 105, 113, 116, 117, 119]

In Fig. 5B, average viable cell density is plotted vs run duration. This figure reveals that most long-duration experiments (e.g., longer than 40 days) have occurred via density-based retention methods (gravitational, acoustic, and centrifugal settling). Because these methods do not utilize a physical barrier, they are not prone to fouling, and can more easily reach long culture times. However, when comparing systems by average density, it is clear that filtration-based perfusion has the advantage. At least within this curated data set, 75% of experiments with average densities over 20 million cells/mL and 100% over 30 million cells/mL used some form of filtration for cell retention (spin filtration, TFF, ATF, etc.).

2.2 *Continuous Chromatography*

Despite increasing competition from non-chromatographic techniques and pressure to reduce costs, chromatography has been and continues to be a dominant technique in biopharmaceutical purification. This prevalence is largely because of the high-resolution purification that can be achieved using chromatography, even for similar components. However, with improved cell lines and increasing titers in recent years, traditional batch chromatography has had some major drawbacks related to large-scale biopharmaceutical purification [135]. These include (1) the often inefficient usage of chromatography resin, (2) the large volumes of buffers needed, (3) the limited purity obtained for components with small adsorption differences, and (4) the discontinuity of the process. To overcome these limitations of batch chromatography, a number of continuous chromatography methods have been developed. Continuous chromatography operation can, in principle, be achieved by two modes of contacting the mobile and stationary phase: crosscurrent and countercurrent schemes. In certain cases, continuous operation can also be performed through use of flow-through chromatography with redundant units.

Continuous chromatography is particularly applicable to two main areas of protein purification: capture operations, for separating the product from cell culture harvest fluid, and polishing steps, where resolution between impurities and the protein of interest is important. For capture applications, integration of continuous chromatography systems with clarified harvest streams from a perfusion or fed-batch bioreactor can be envisioned. To enable continuous processing, the chromatography system must be capable of receiving a continuous feed stream. The output (eluate) can be continuous (such as with annular or flow-through chromatography) or periodic/cyclic (such as with periodic countercurrent chromatography). However, integration for prolonged periods of time (e.g., with a perfusion bioreactor) could be challenging, as it requires the continuous chromatography systems to be amenable to closed operation [15, 16, 136]. Some of the technologies that allow continuous chromatography are presented below.

2.2.1 Continuous Crosscurrent Chromatography

In crosscurrent chromatography, the resin bed moves perpendicular to the direction of the liquid flow within the bed. In continuous crosscurrent systems, a steady-state separation occurs in the axial and circumferential direction, in contrast to conventional batch systems where separation occurs only in the axial direction [137].

Continuous Annular Chromatography

Continuous annular chromatography (CAC) allows truly continuous separation of multicomponent mixtures using a rotating annular bed composed of a

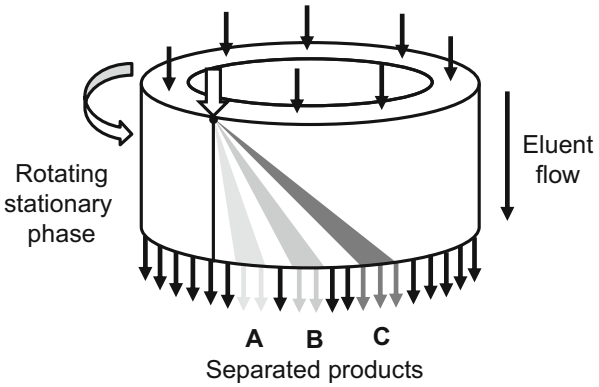

Fig. 6 Schematic view of P-CAC system showing isocratic elution of products A, B and C

chromatography matrix. This system was first conceived and proposed by Martin [138] and the theoretical feasibility was demonstrated by Giddings [139]. The first true CAC apparatus was constructed by Fox et al. [140, 141] and was tested for the purification of cow heart myoglobin and the separation of skim milk proteins from lactose and salt [142]. Since then, this technology has been applied to purify several biological molecules including sugars, amino acids, plasma-derived and recombinant blood clotting factors, plasmid DNA, antibodies, and vaccines.

The heart of the CAC system is a bed of conventional adsorbent which is packed between two concentric cylinders that forms the annulus (Fig. 6). The annular bed slowly rotates about its vertical axis. The feed mixture to be separated is introduced continuously through a stationary nozzle on top of the moving adsorbent bed. In the simplest case of isocratic chromatography, the rotating annulus is flooded with elution buffer which is flowing in the direction of feed (crosscurrent flow). Different components of the feed separate in the axial direction and develop helical bands from the fixed inlet point to the bottom of the annular bed. Several fixed outlets are kept at the bottom of the bed where the separated components are continuously recovered. The angular displacement of each component band from the feed point (i.e., the slope of the helical band) is dependent upon the flow rate, rotational speed of the annulus, and the component affinity for the stationary phase.

Step or gradient elution has also been applied for purification using CAC [136, 143–145]. In this case, the feed mixture and all necessary buffers (wash, elution, regeneration, etc.) are introduced through stationary nozzles along the circumference of the annulus. As the annulus rotates, each section of the stationary phase goes through load, wash, elution, and regeneration steps. Similar to isocratic chromatography, product can be continuously recovered from fixed outlet points.

In the late 1990s, CAC instruments became commercially available under the name P-CAC from Prior Separation Technology (Götzis, Austria). Several authors have successfully demonstrated transformation of conventional batch chromatography methods to CAC instruments [136, 144, 146]. Preparative continuous annular chromatography has been used for several different separation media, including ion exchange [143, 145, 147–149], displacement [150], affinity [144], and size

exclusion chromatography (SEC) [151–153]. The combination of two different separation media (SEC and ion-exchange chromatography) in one CAC column has also been demonstrated [154]. The potential for use of CAC for continuous integrated capture directly from perfusion cell culture was described by Vogel et al. [136] using an autoclavable P-CAC prototype. Despite several advances with CAC, this technology is yet to be implemented for large-scale protein purification. Obtaining uniform column packing and flow distribution and long term durability of the rotating mechanical parts are some of the remaining challenges with this technology.

Continuous Radial Flow Chromatography

Another adaptation of continuous annular chromatography is continuous radial flow chromatography (CRFC). CRFC operates similarly to CAC systems except feed and all necessary buffers flow in the radial direction (inward from periphery or outward from center in horizontal direction) instead of the axial direction (along the axis). The effective bed height of a radial flow column is the distance between the inner and outer cylinders and the cross sectional area is associated with the surface area of the tube. Short bed depth and large column cross-sectional area enable separations at high flow rates while maintaining low pressure drops [155]. However, radial flow columns have limited resolution because of the shorter bed depth. Thus, they are not ideally suited for applications where high resolution separation (e.g., analytical HPLC columns) or longer bed depth are required (e.g., SEC) [156].

Radial flow chromatography in batch mode has been investigated for several different separation media [157–160]. Both experimental results and theoretical modeling indicate that a radial flow column behaves similar to an axial flow column of shorter bed height (with similar resin volume) [157, 161–163]. Radial flow chromatography systems for batch operations are marketed by Sepragen (Hayward, CA) and Proxcys (Nieuw-Amsterdam, Netherlands). Recently, a Proxcys radial flow column packed with larger chromatography resin beads was utilized for capture of His-tag recombinant proteins from unclarified mammalian cell feeds [158]. Lay et al. [164] constructed a prototype CRFC column and demonstrated separation of bovine serum albumin (BSA) from lactoferrin. Similar to CAC [136], CRFC can be potentially integrated with a perfusion bioreactor; however, to date, commercial-scale CRFC systems do not appear to be available for bioprocessing applications.

2.2.2 Continuous Countercurrent Chromatography

Countercurrent processes inherently have superior mass transfer characteristics and thus high-purity products can be obtained, even at high flow rates. Likewise, countercurrent chromatography allows the stationary phase to be used more efficiently, improving productivity and decreasing solvent/buffer consumption as

compared to batch chromatography. The liquid-solid countercurrent movement is obtained by rotating the solid phase adsorbent (chromatography resin) in the direction opposite to the liquid phase flow. This, in principle, can be achieved by physically moving the solid adsorbent/columns in the opposite direction to the liquid flow (true moving bed) or by simulating the continuous movement of the solid phase using elaborate column and valve movements in a periodic or semi-continuous manner (simulated moving bed).

Simulated Moving Bed (SMB) Chromatography

The SMB process principle is a flow scheme that takes advantage of continuous and countercurrent movement of liquid and solid phase without actual movement of the adsorbent. Instead of a large packed bed used in traditional batch chromatography, SMB chromatography systems are characterized by the connection of multiple smaller packed beds. The simulated countercurrent flow is carried out through a complex valving system located between the columns, such that inlet and outlet positions are periodically switched from column to column in the direction of liquid flow.

The petroleum industry originally developed the SMB in the late 1950s to separate *p*-xylene from its isomers [165]. Later, its application scope was extended to the sugar industry and by the 1990s it was used by the pharmaceutical industry for manufacturing chiral and biological drugs [166–168]. The technology development in all these process industries was motivated by the need for cost reduction and large-volume production of low-value chemicals at high purity.

In the classical four-zone SMB process, the solid phase is divided into four zones (Fig. 7). Zones 2 and 3 are separation zones and zones 1 and 4 are used to regenerate the adsorbent and the eluent. Only one solvent/eluent is used and hence this operation is in an isocratic mode. The feed and eluent streams are simultaneously applied and withdrawn at appropriate points between the columns, which causes the slow and fast moving components to travel in opposite directions relative to the feed port. The component with lower affinity toward solid phase (faster moving component) travels with the liquid phase, and purified substance is collected at the raffinate port. The component with higher affinity toward the solid phase (slow moving component) travels in the direction in which solid phase movement is simulated, and purified substance is collected at the extract port. Successful SMB design and operation depends upon the correct selection of operating conditions, zone liquid flow rates, and switching time (which corresponds to solid flow rates). The use of SMB operation in isocratic/classical mode is most relevant for SEC-based applications [6, 169].

The original design of the SMB system was suited for the separation of binary mixtures. Over the years, several variants of SMB have been introduced that allow separation of ternary mixtures and application of gradients [6, 170–173]. Simulated moving bed chromatography has been used to purify a variety of biological molecules, including mAbs from cell culture supernatant [174, 175], influenza

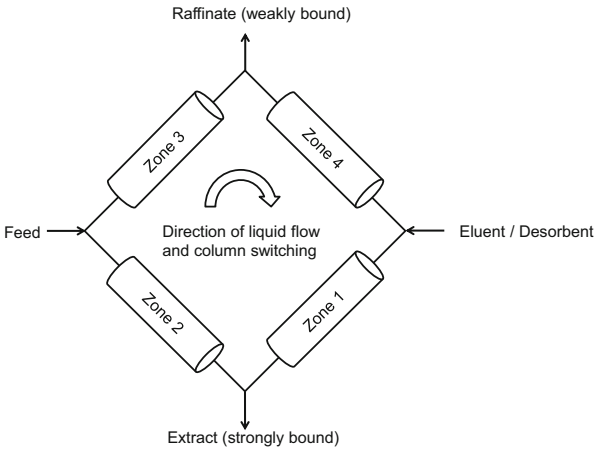

Fig. 7 Schematic of a four-column simulated moving bed (SMB) chromatography system

virus from cell culture supernatant [176], and lactoperoxidase and lactoferrin from whey protein concentrate [177]. A more detailed review of SMB chromatography and its applications have recently been discussed elsewhere [167, 178].

Periodic Countercurrent Chromatography

A simplified version of simulated moving bed chromatography is periodic countercurrent chromatography (PCC). This approach involves the use of three or more columns, where loading occurs in a countercurrent manner. A schematic of a cyclic three-column PCC system is shown in Fig. 8. The first column is loaded to saturation and the breakthrough is loaded onto the second column. The saturated column is washed on the third column and then eluted similar to a batch operation. Because such serial column loading loses very little of the target protein, even at high feed flow rates, column sizes can be considerably smaller than equivalent batch systems [17, 179]. The use of PCC is most suitable in capture applications where continuous feed stream is present (e.g., continuous clarified harvest from perfusion reactor), especially for labile molecules [17, 180]. To allow continuous operation, the duration of the recovery and regeneration tasks must not exceed the time for protein loading.

Bench-scale PCC units for biopharmaceutical application have been developed by GE Healthcare. These units employ a UV-based switching logic (where the key signal is the relative difference in UV at the column inlet and outlet) which allows monitoring and control of the column saturation level. This dynamic UV-based control makes the system highly responsive to variability in the feed stream or column performance. Successful integration of a perfusion bioreactor with PCC capture has been demonstrated using both stable (mAbs) and relatively less stable molecules (recombinant enzymes) [17, 180].

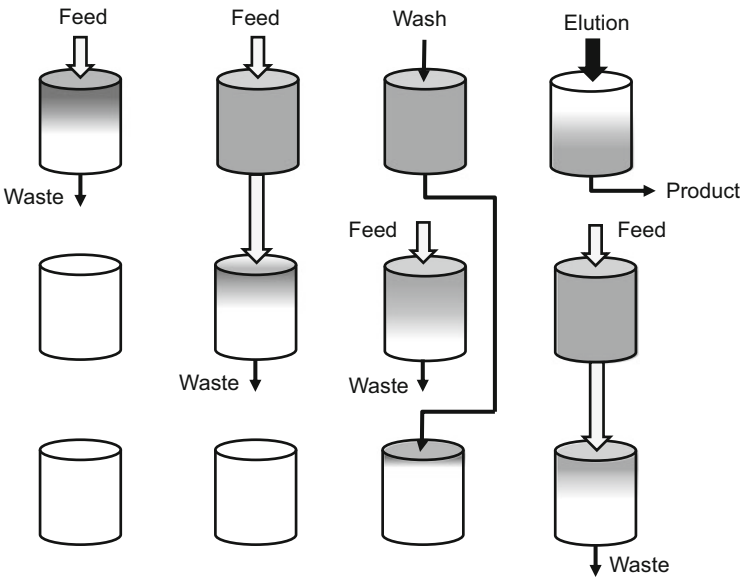

Fig. 8 Schematic diagram of the three-column PCC cycle

The use of CaptureSMB (ChromaCon), a two-column system analogous to PCC for mAb capture, has also been demonstrated [181, 182]. Instead of UV-based loading (used in GE PCC), the CaptureSMB system uses a time-based loading strategy to calculate breakthrough. To achieve sequential washing between two columns, the feed flow rate has to be zero; hence, a small reservoir bag between the continuous upstream processes and the CaptureSMB system is required. An advantage compared to other multi-column systems is that CaptureSMB requires less complex hardware, potentially leading to lower equipment costs and risks of failure. Other analogous multi-column chromatography systems currently available for biopharmaceutical applications are BioSC marketed by NovaSep [183], BioSMB by Tarpon Biosystems-Pall [184], and Octave by Semba Biosciences [185]. All these systems provide periodic (cyclical) operation, and hence are amenable to continuous operations.

Continuous Countercurrent Tangential Chromatography

Continuous countercurrent tangential chromatography (CCTC) is a recent development that allows truly continuous and countercurrent operation by enabling "true moving bed" technology, as opposed to simulated moving bed [186]. Instead of packing the resin particles in a fixed-bed column, the resin (in the form of a slurry) flows through a series of static mixers and hollow-fiber membrane modules. The microporous hollow-fiber membranes retain the large resin particles and let all dissolved species (protein and buffer components) pass through the membrane and

into the permeate. The buffers used in the binding, washing, elution, stripping, and equilibration steps flow countercurrent to the resin slurry in a multi-stage configuration.

In contrast to SMB, which operates in a cyclic mode, CCTC provides the opportunity to run at steady state without the need to switch valves after system start-up. As the resin is used in slurry form, the pressure drop is independent of particle size, in contrast to packed beds. Thus, smaller resin particles can be used in CCTC, allowing for higher mass transfer rates and improved productivity [187]. Another advantage is that all activities related to handling packed beds, including packing, cleaning, validation, and storage, could be eliminated. The concentration of product from a CCTC system is relatively dilute compared to packed bed systems. Hence, an inline concentration system may be needed to increase the final product pool concentration and decrease the loading volume for further downstream operations.

To date, proof of concept has been demonstrated for purification of BSA and myoglobin using an anion exchange resin [188], purification of an IgG4 from a mixture of BSA and myoglobin using a Protein A affinity resin [189], and purification of mAbs produced in Chinese hamster ovary (CHO) cells from clarified cell culture fluid [187, 190].

Multicolumn Countercurrent Solvent Gradient Purification

Multicolumn countercurrent solvent gradient purification (MCSGP) is a multi-column chromatographic process capable of using linear gradients for high-resolution separation of three component fractions [191, 192]. This strategy can be operated in a continuous [191] or semi-continuous [193] mode, with six columns or three columns, respectively. Similar to PCC and SMB, MCSGP uses multiple columns in sequence that are switched in position to get countercurrent contact. In contrast to traditional SMB, MCSGP can be used for more than just binary separations. For a complex mixture, where target product overlaps with early and late eluting impurities, three- or four-zone SMB cannot deliver pure target product in a single step; in fact, two sequential SMBs are needed to achieve this. Alternatively, MCSGP can be used for such difficult separations to obtain target product in a single step. Such separation challenges are typically faced in polishing steps of biopharmaceuticals. For example, Müller-Späth et al. [194] demonstrated the effectiveness of continuous MCSGP using cation exchange resins for the separation of charge variants of several commercial mAbs. Continuous capture via MCSGP has also been demonstrated. Müller-Späth et al. [195] demonstrated the use of a four-column MCSGP system with clean-in-place capabilities and fully continuous loading for the capture of mAb from clarified cell culture fluid using cation exchange resins with a gradient elution. Bench-scale MCSGP purification can be performed with a two-column system marketed as Contichrom (Knauer-ChromaCon AG).

2.2.3 Flow-Through Chromatography

In flow-through chromatography, impurities bind and the product of interest flows through. Anion exchange (AEX) products in flow-through mode have been widely used as a polishing step in mAb purification processes to remove residual impurities such as host cell protein, DNA, and a variety of viruses [196, 197]. Flow-through chromatography can be performed with conventional bead-based resin or membrane adsorbers [198, 199] and monoliths [200, 201]. In flow-through mode, membranes have shown advantages over traditional packed beds including faster operating flow rates, reduced buffer requirements, and disposability [202].

Use of flow-through operations in batch processes have improved productivity in feeds where impurities are less abundant than the product (polishing steps) or in processes with feeds at high product concentrations. The application of flow-through chromatography to continuous operations can be easily imagined with parallel or redundant units used in an alternating fashion; that is, one unit is binding impurities and the other column is being regenerated for the next cycle [16]. Such an approach can also be applied to other dead-end filtration systems.

2.2.4 Expanded-Bed Chromatography

Expanded-bed chromatography (EBC) utilizes a fluidized chromatographic adsorbent bed, which allows desired proteins to be purified directly from crude (particulate-containing) feedstock [203–206]. The primary difference in setup of EBC compared to traditional chromatography columns is that the top flow adapter is appropriately positioned such that the bed is allowed to expand upward in the direction of liquid flow. The bed expansion increases the bed voidage, which allows unhindered passage of cells, cell debris, and other particulates during application of crude feed to the column. Thus, a combination of clarification, concentration, and adsorptive purification can be achieved in a single step, allowing significant cost savings [158, 207, 208]. The flow distributor and the adsorbent/beads are the two main factors influencing the EBC operations [209, 210]. Batch EBC has been successfully used for protein purification directly from *Escherichia coli* homogenate, yeast, whole hybridoma fermentation, mammalian cell culture, milk, and animal tissue extracts [203, 206, 211–213].

Continuous countercurrent EBC has been used for direct purification of lysozyme from egg whites and enriched bovine milk [214]. The mode of operation consisted of four countercurrent contactors arranged in a series and in a loop for continuous loading, washing, elution, and regeneration. The process buffers and unclarified feed entered the respective columns at the base and moved upward through the adsorbent. The adsorbent bed was fluidized so that it behaved as an expanded bed instead of a well-mixed system. Continuous operation was obtained by constantly removing adsorbent through the base of the bed and simultaneously applying it to the top of the next stage, just above the level of the expanded bed. In

conventional batch EBC, each adsorbent particle remains approximately stationary at a point governed by parameters such as liquid flow rate and particle dimensions. Conversely, in the continuous countercurrent EBC system, removal of adsorbent from the base of the bed causes suspended particles to fall with respect to the column, thus resulting in a net downward flow of the adsorbent material [215]. Similar to CCTC, a truly countercurrent continuous operation can be achieved with continuous product streams generated from unclarified feeds. Batch EBC columns have recently been made available (DSM RhobustTechnology) but application of continuous EBC for routine biological production has not been reported.

2.3 Other Technologies for Continuous Purification

2.3.1 Continuous Clarification of Cell Culture Harvest

Many of the perfusion-enabling cell retention methods previously mentioned can also be used for stand-alone continuous clarification (e.g., gravitational settling, centrifugation, acoustic settling, and tangential flow filtration). Continuous depth filtration has also been demonstrated in a manufacturing environment using redundant filter trains. Instead of directly processing cell-containing bioreactor material, the downstream system was designed to process continuously harvest material that had passed through a gravitational settler. Parallel filter trains were integrated into an automated control system that switched to a new train when the current train reached a high pressure limit [16, 52].

2.3.2 Continuous Precipitation and Flocculation

Precipitation and flocculation have been explored to improve purification efficiency and impurity clearance and to simplify the process by decreasing the number of purification steps [196, 216, 217]. Compared to chromatography, these techniques provide greater flexibility across a range of protein titers as scale-up is volume-based and independent of titer. Precipitation methods rely on lowering the solubility of target molecules to create solid particles. This can be achieved by altering factors such as pH and conductivity (salting-in or salting-out) of solution, or through addition of precipitants such as ethanol, ammonium sulfate, polyethylene glycol (PEG), caprylic acid, or divalent ions [218–222]. Flocculation relies on flocculating agents such as polyelectrolytes that cause adhesion of dispersed particulates into larger-sized clusters, resulting in an increase in the average particle size [223, 224]. The floccules formed can be collected by filtration, sedimentation, or centrifugation at low speed. Precipitation/flocculation methods are designed either to precipitate or flocculate the protein of interest, leaving impurities (DNA, host cell proteins, cell debris, etc.) in solution or to precipitate impurities and leaving the protein product in solution [205, 225].

Precipitation has been used at industrial scale for blood plasma protein fractionation. However, this application has been typically performed in batch mode in several stages where ethanol is added to aqueous solutions at specified conditions for pH, temperature, and ionic strength [226]. Continuous fractionation of human blood plasma using tubular reactors [227, 228] and a series of mixed-suspension mixed-product removal reactors (MSMPR) [229] has been demonstrated. Along with tubular reactors and MSMPR, continuous precipitation of proteins can also be performed using centrifugal precipitation chromatography [219].

Hammerschmidt et al. [230] converted a two-stage batch precipitation system based on calcium chloride flocculation and cold ethanol precipitation into a continuous purification process for recombinant antibodies from clarified cell culture supernatant. Tubular reactors with cooling (via a double pipe heat exchanger) and helical static mixers were used for continuous operation. The continuous system not only provided uniform precipitate yields and improved productivity but also offered uniform cooling, which can become a limiting factor during cold ethanol batch precipitation processes. Similarly, continuous sequential precipitation of impurities followed by a target monoclonal antibody (mAb) in clarified cell culture supernatant with PEG as precipitant has also been demonstrated [231]. Inexpensive precipitants (ethanol, calcium chloride, PEG, etc.) can be cost-effective replacements for the Protein A capture step, providing an opportunity to develop inexpensive fully continuous mAb processes [10, 232].

2.3.3 Continuous Aqueous Two-Phase Extraction (ATPE)

Aqueous two-phase extractions (ATPE) are liquid-liquid biphasic systems that have been used for downstream processing of several biological products including cells, proteins, enzymes, hormones, viruses, and plasmid DNA [233–238]. Aqueous two-phase extraction can be applied for clarification purposes [239] and can also serve as an alternative to more traditional chromatography operations [240]. ATPE relies on the formation of two immiscible aqueous phases such that the target protein partitions preferably into one phase and impurities partition preferably into the other. Organic solvent mixtures are not suitable as they can induce protein denaturation. Hence, ATPE for biological compounds is usually carried out with two polymers or a polymer and a salt dissolved in water above a critical concentration [240]. Polymers (such as PEG, polyvinyl alcohol, dextran, and starch) and buffering salts (such as phosphates, sulfates, and citrates) have been used to create the two phases. To improve the specificity of the protein of interest to a particular phase, functionalization of the phase-forming component is also possible [241].

The mechanism of phase separation in aqueous two-phase systems and in conventional organic–aqueous systems is similar; hence, most of the standard extraction equipment used in the chemical industry can be used for ATPE [242, 243]. Continuous ATPE can be performed using appropriate column contactors, spray columns, rotating disk contactors, or mixer-settler units [244–246]. Use of multi-stage ATPE devices such as multi-plate column contactors and

mixer-settler configurations can overcome the limitations of single-stage extraction through improved recovery and/or purity [247, 248]. Multi-stage ATPE has been successfully evaluated for the purification of mAbs [245, 248–250]. Continuous ATPE devices provide a promising non-chromatographic alternative for use in downstream mAb processing, allowing simultaneously clarification, concentration, and partial purification in one unit. One of the concerns about ATPE at industrial scale is the high consumption of polymers and salt leading to negative impacts on water treatment [247].

2.4 Continuous Formulation

Formulation of therapeutic proteins is developed based on clinical needs, patient compliance, delivery method, stability of the drug, storage and distribution, and market competitiveness. Liquid formulations have generally been preferred as they are less expensive, faster to develop, and generally more convenient for administration. However, dried formulations (e.g., lyophilized or crystallized) have been used successfully for protein drugs that may not be stable enough to be handled as a liquid formulation.

2.4.1 Continuous Ultrafiltration and Diafiltration

Ultrafiltration/diafiltration (UF/DF) is routinely used in the biopharmaceutical industry for high concentration liquid formulation of therapeutic proteins. Highly concentrated protein formulations are desirable for therapeutic uses as they allow for dosages with smaller volumes, and are more economically packaged and stored. The use of a UF/DF system allows both concentration of the product and diafiltration into desired buffer (e.g., formulation buffer) to be performed with the same unit. Typically, UF/DF uses tangential flow filtration technology, where feed flows parallel to the membrane surface rather than perpendicular to the surface. Traditionally these steps are operated in batch mode. During ultrafiltration, the feed is recirculated through the membrane module and multiple passes are required to achieve the desired concentration. During diafiltration, the feed is recirculated through the membrane module as the formulation buffer is added at a rate equal to the permeate flow rate to maintain constant volume. Approaches to continuous UF and continuous DF operations have been described in the literature.

Single-pass tangential flow filtration (SPTFF) concentrates protein solutions in a single pass, without the need for a recirculation loop and tank [251]. The feed is directly pumped through the SPTFF module and concentrated product is obtained from the retentate port. The basic principle of SPTFF is increased residence time in the feed channel, which leads to increased conversion in a single pass through the module. Increased residence time can be achieved by reducing the flow rate, increasing the path length in a serial configuration or by simply adding more TFF

membranes in series. Single-pass tangential flow filtration is designed to have multiple conventional TFF stages in a single unit, which can potentially minimize capital investment and footprint area compared to traditional TFF systems. The use of SPTFF can easily be implemented in integrated and continuous processes as inline concentrator for dilute streams [252–254] or in the final formulation step for concentrated liquid formulation.

Continuous DF using TFF can be achieved with a multi-stage cascade system where DF occurs in either a concurrent or countercurrent manner [6, 255]. Multi-stage cascade UF systems have been successfully used for continuous protein purification [256–258]. Similar to cascade UF systems, for multi-stage concurrent DF processes multiple filtration units are connected in series. In the first stage, feed and buffer are added at a rate equal to the permeate flux. In the subsequent stages, only fresh buffer is added at a rate equal to permeate flux. The countercurrent DF process also consists of several units in series, but enables lower buffer utilization. Here, feed is continuously added to the final stage and fresh buffer is continuously added to the first stage. The retentate from each stage is recycled and serves as a feed to the previous stage in the cascade. As the numbers of stages increase, the impurity removal improves and the buffer requirement decreases [9]. One of the challenges to using membrane cascades is the delicate control of interacting flows [259, 260]. In addition, with an increasing number of membrane stages, extra buffer tanks and pumps are usually required, complicating the process and increasing the initial capital cost. Therefore, for a membrane cascade system to be practical, the number of cascade units must be minimized and the control of the system needs to be simple.

Other approaches to industrial-scale buffer exchange systems are through use of SEC and countercurrent dialysis [261]. Size-exclusion chromatography for continuous buffer exchange can be envisioned using a simulated moving bed system with multiple columns. Klutz et al. [16] used a dialysis membrane module, where continuous buffer exchange was performed in a countercurrent flow. The feed stream was continuously fed through the hollow fibers/capillaries, whereas the wash fluid/diafiltration buffer was passed through the shell side. The flow rate of the feed and retentate stream (fed through the capillary) and of the diafiltration buffer was controlled through use of pumps. Gambro Revaclear 300 capillary dialyzer (Baxter) was used for performing continuous diafiltration; however, use of other hollow-fiber devices is also possible [262].

2.4.2 Continuous Lyophilization

Lyophilization (i.e., freeze-drying) is the method of choice to increase the shelf life of sensitive proteins. By removing water from the formulation and sealing the resulting cake in a vial, the drug can be stored, shipped, and later reconstituted to its original form for injection. Lyophilization is inherently conducted as a batch operation. The starting liquid solutions are filled in vials or trays, frozen, dried (the remaining “bound” water is driven out), and then handled as a batch.

To date, continuous lyophilization for biological products has not been demonstrated. The food industry, which routinely uses freeze-drying, has taken steps to move from batch to semi-continuous or continuous freeze-drying approaches because of the large production needs and cost-effectiveness. GEA Niro has developed a continuous lyophilization system (CONRAD) for food products (such as coffee, fruit, and meat) in which the product is moved through a long tubular lyophilization chamber on a tray.

Efforts to develop continuous freeze-drying for pharmaceutical industry are ongoing. De Meyer et al. [263] evaluated spin freezing instead of conventional freezing as a part of a continuous freeze-drying step. In spin-freezing, the vials with liquid formulation are rotated rapidly along their longitudinal axis. The cooling and freezing of the solution are achieved by using a flow of sterile gas with a controllable temperature around the rotating vial. Such frozen vials could be continuously transferred into separate drying units (primary and secondary dryer units) through appropriate load-locks which maintain pressure and temperature within each unit [263]. Weisselberg [264] has described a continuous lyophilization process in which the product solution cascades through a series of vertically stacked trays. Rey [265] has discussed the possibility of using similar systems for lyophilization of biological products, although this has not yet been demonstrated in practice.

2.4.3 Continuous Crystallization

Protein crystallization, which has been mostly applied in protein structure analysis, has also been recognized as a method of protein purification [266, 267] and formulation [268]. Protein crystals form a regular lattice that can exclude impurities and misfolded protein, and hence can be used in purification applications. Feasibility of protein purification by crystallization has been demonstrated with lipase [269], ovalbumin [270], mAbs [271], and at industrial scale for insulin [272].

For formulation applications, the crystalline state of proteins offers several advantages for delivery: (1) longer shelf life and more stability than liquid formulations, (2) controlled release because of better dissolution characteristics, and (3) subcutaneous or inhalation therapy-based delivery instead of intravenous infusion [271, 273]. Protein crystallization for delivery and formulation application has been extensively reviewed [268, 274].

Crystallization is often operated in batch mode in chemical and pharmaceutical applications [275]. The most dominant crystallizer type used in pharmaceutical industry for batch operations is based on a stirred tank design [276]. Continuous crystallization offers an opportunity to provide enhanced product quality, improved process efficiency, and better control of crystal morphology [277, 278]. In recent years, continuous crystallization has attracted great interest in the pharmaceutical industry [279–281]. As shown in Fig. 9, there are two main types of continuous crystallizers that have been investigated: (1) mixed-suspension mixed-product removal (MSMPR) reactors and (2) tubular crystallizers such as plug flow reactors (PFR) and continuous oscillatory baffled crystallizers (COBC) [276]. MSMPR

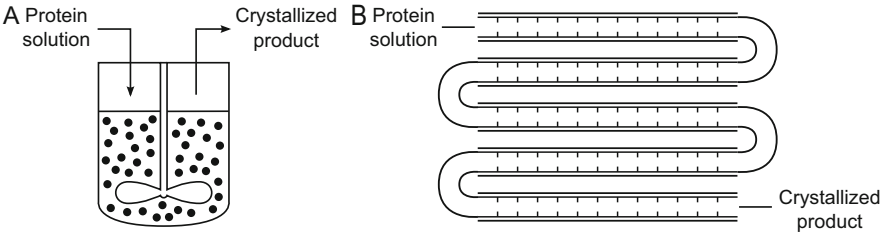

Fig. 9 Schematic description of (**A**) a mixed-suspension mixed-product removal (MSMPR) reactor, and (**B**) a continuous oscillatory baffled crystallizer (COBC), which serves as a specific example of a tubular flow reactor [277]

reactors have been widely studied in the pharmaceutical industry. This system operates similar to continuous stirred tank reactors where product is continuously introduced and crystals are continuously removed. Converting batch operations to continuous mode by operating the stirred tank designs in MSMPR mode is considered to be an easier and more cost-effective approach than replacing existing batch equipment with completely new continuous crystallizer designs [276]. Several configurations of MSMPR reactors have been developed (single-stage, multi-stage, with and without recycling operations, etc.).

Plug flow reactors rely on tubular reactors with static mixers, and offer an alternative means of continuous crystallization. Such crystallizers can provide continuous crystallization with much more uniform product quality (i.e., narrow crystal size distribution) [275]. Plug flow crystallizers are ideally suited for mechanically fragile systems such as proteins over tank-based crystallizers; however, long tubes maybe required to attain sufficient residence times. A COBC is a tubular crystallizer containing periodically spaced orifice baffles, which allows uniform mixing because of the creation of oscillatory motions in each interbaffle zone [277]. Scale-up is potentially easier in tubular crystallizers as high product quantities can be obtained by installation of parallel tubes and/or running the systems for longer times. Recently, Neugebauer and Khinast demonstrated a continuously operated tubular crystallizer for the production of lysozyme protein crystals [282]. However, commercial application of continuous crystallization for recombinant protein production has yet to be established.

2.5 Continuous Viral Inactivation and Removal

To ensure viral safety, regulatory guidelines require that manufacturers of biological products for human use demonstrate the capability of the manufacturing process to remove and/or inactivate known or adventitious viruses. Typically, removal is established through virus reduction filters and chromatography steps, and inactivation is established by use of heat, irradiation, chemical, or pH treatment.

2.5.1 Virus Removal by Filtration

Virus reduction filters (i.e., nanofilters) remove viruses from the product stream using size exclusion [283, 284]. The main advantage of this process is that, under optimal conditions, both enveloped and non-enveloped viruses can be robustly removed without affecting the quality of the derived products. Traditionally, viral filtration has been operated in batch mode where each batch of feed material is processed sequentially through the viral filter. To date, viral filtration technology has not been able to process a continuous input of feed stream for prolonged periods of time. However, similar to flow-through membranes and flow-through chromatography, continuous viral filtration is possible using redundant and parallel filters. The feed can be switched to the second filter when the capacity of the first filter is achieved [285].

2.5.2 Continuous Viral Inactivation

In a traditional monoclonal antibody process, inactivation of enveloped viruses is achieved by incubation at low pH [286, 287]. This is inherently a batch process, where a process intermediate is collected in a vessel and held at low pH for the required inactivation time of 30–120 min [286, 288]. To date, commercial application of continuous low pH viral inactivation has not been reported. Several configurations of tubular reactors have been proposed for continuous viral inactivation, and Klutz et al. [285, 289] have demonstrated the most promising among these to be the coiled flow inverter (CFI). A CFI reactor consists of several straight helix modules, where the tube reactor is coiled around a coil tube. This reactor provides close-to-plug-flow behavior in a laminar flow regime and long residence times. The successful proof-of-concept was demonstrated with Protein A capture eluate continuously processed through a CFI [285]. CFI reactors have compact design, narrow residence time distribution, low investment costs, easy scale-up, wide operating window, and compatibility with single-use technology [285, 289]. Similarly, use of CFI could be adopted for continuously operated inactivation using chemical treatment (e.g., solvent/detergent).

Ultraviolet-C (UV-C) light at a wavelength of 254 nm is an effective method for inactivation of non-enveloped viruses [290, 291]. Continuous flow UV-C reactors for virus inactivation have been designed [292, 293]. The effectiveness of continuous flow UV-C in viral inactivation of protein solutions has also been demonstrated [294–296]. A continuous UV-C system, UVivatec, was developed by Bayer Technology Services and is marketed by Sartorius Stedim Biotech. Similar to continuously operated inactivation, in continuous UV-C, fluid streams move helically along the lamp. Secondary circulating flows (Dean vortices) are generated which provide highly efficient mixing and allow for uniform and controllable irradiation of the entire volume.

3 Case Studies for Integrated Continuous Biomanufacturing

Several approaches to integrated continuous biomanufacturing are possible, including hybrid systems (i.e., some unit operations are integrated and continuous whereas others are conventional batch operations) and fully integrated continuous systems [7]. In most cases, integration and continuous operation provide increased productivity and flexibility, decreased cost of operations, and improved product quality (especially for labile molecules). Fully continuous systems potentially offer the maximum benefits among these approaches. To date, commercial implementation of fully integrated continuous processes has not been established. However, several proof-of-concept approaches have been attempted which provide opportunities for the implementation of end-to-end continuous processes in the biopharmaceutical industry.

Vogel et al. [52] demonstrated a hybrid integrated manufacturing platform, where an upstream perfusion bioreactor was integrated with a continuous cell separation process based on disposable flow paths, followed by membrane chromatography operated in a rapid cycling mode. Further purification/processing was performed in a batch mode. The continuous perfusion reactor was equipped with an inclined plate settler for cell retention, returning a majority of the cells back to the reactor. Harvest (along with some debris) was further processed through continuous cell separation via two parallel disposable filter assemblies operated in alternating fashion. Rapid capture of the target proteins (recombinant blood coagulation factors) in clarified harvest was achieved through large scale anion exchange membrane adsorbers. Overall, purification performance with the integrated platform process was comparable to the conventional process, with a significant reduction in downstream cycle time. A similar integrated approach (upstream perfusion, continuous cell separation, and membrane chromatography capture) has been successfully used in GMP manufacturing for other molecules at Bayer.

As depicted in Fig. 10, lab-scale end-to-end fully continuous production of mAbs has been demonstrated by Godawat et al. [15]. This process included a perfusion bioreactor with a cell retention system (ATF) and two periodic countercurrent chromatography systems (PCC1 and PCC2). This work built upon a previously demonstrated integrated continuous capture system consisting of a bioreactor and PCC [17, 180]. In the end-to-end approach, the bioreactor/PCC1/PCC2 system was run in an automated and continuous manner across the entire upstream and downstream operations, ultimately producing drug substance. The continuous operation was enabled by balancing the rate of mass and flow across various unit operations. PCC1 was used for continuous capture using Protein A resin. The low pH inactivation was performed in a cycled batch mode. The intermediate column eluate was designed such that the protein eluted in appropriate formulation buffer at the required drug substance concentration. The eluate was further polished using a membrane adsorber in a flow-through mode, resulting in minimal changes to either the protein concentration or buffer formulation, thus eliminating the requirement

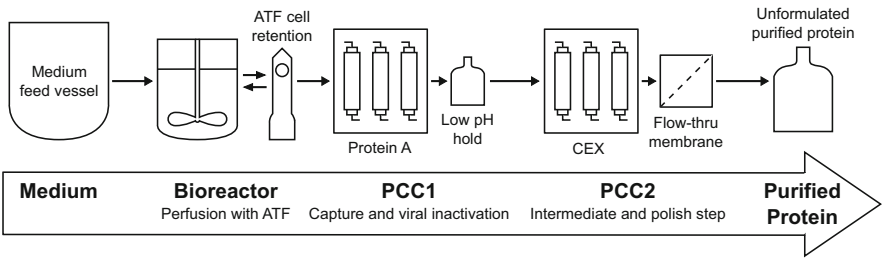

Fig. 10 Architecture for one example of end-to-end continuous bioprocessing from Godawat et al. [15]

for terminal UF/DF. However, this approach might not be feasible for all protein drugs, and incorporation of UF/DF in the downstream architecture may be required. This end-to-end bioprocessing architecture did not include a nanofilter in the process train because the pumps and the valves available on the two PCC systems did not allow for addition of another unit operation. The data in the article indicated that the end-to-end process increases process throughput and significantly decreases the equipment footprint, eliminating several non-value added unit operations.

Another proof-of-concept for fully continuous and automated production of mAbs has been demonstrated by Klutz et al. [16] (Fig. 11). This process included a perfusion bioreactor with continuously operated filtration achieved by redundant filters. Protein A capture chromatography was performed using a multi-column chromatography system (BioSMB). A continuous flow reactor with close-to-plug-flow behavior, the CFI reactor, was used to provide the necessary incubation time of 60 min for viral inactivation at low pH. Continuously operated intermediate purification and polishing was performed with two subsequent chromatography steps in flow-through mode. Viral filtration was operated with two alternating filters. The formulation was achieved with a continuous concentration and diafiltration. An ultrafiltration membrane was used for concentration, whereas countercurrent diafiltration using dialysis membrane modules was used for buffer exchange. Disposable bags were used as a reservoir between every unit operation to increase process robustness and to balance short-term differences in process flow rates between successive unit operations. The upstream unit operations prior to multi-column Protein A chromatography were operated continuously for 28 days; however, downstream unit operations were only operated for 60 h (because of sterility concerns). Though robust long-term operation was not achieved, this study demonstrated a successful technical proof-of-concept for a fully continuous monoclonal antibody process.

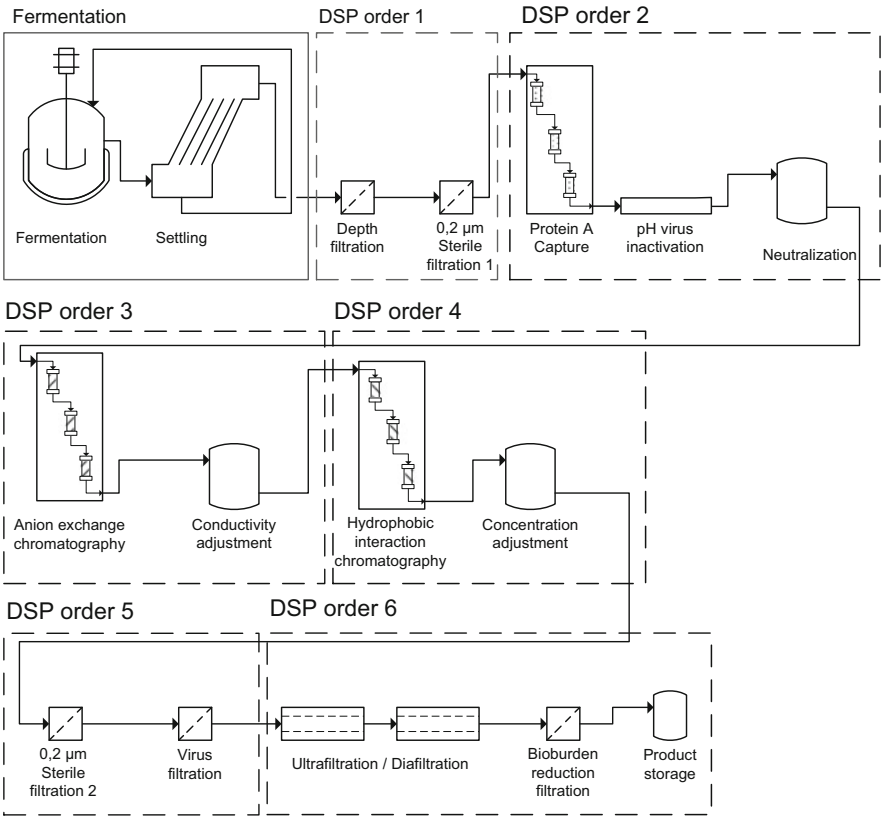

Fig. 11 Process scheme for continuously operated production of monoclonal antibodies as described by Klutz et al. [16]. Picture adapted from Klutz et al. [11]

4 Conclusion and Outlook

Continuous manufacturing has been well-developed in the petrochemical, chemical, and food industries. Recently a growing number of pharmaceutical companies have been working toward building continuous manufacturing into their processes. The biopharmaceutical industry is gradually maturing and continuous manufacturing of recombinant therapeutic proteins is still in its infancy. Economic pressures from increased competition and government regulation, coupled with the desire to gain access to emerging markets, are driving process innovation and implementation of continuous bioprocessing in this industry. As discussed in this review and by others in the literature, most of the necessary building blocks to implement a fully integrated and continuous bioprocessing platform are already established. The case studies highlighted in this review are significant advances toward realization of fully continuous integrated processes for monoclonal antibodies. However, there are still technical challenges that must be overcome before successful commercial

implementation. These challenges include better online process analytical technologies, cell line stability, reliability of hardware for long term operability and sterility, reliability of scale-down models, robust automation, and global process control for all unit operations. These technical challenges can be addressed with joint effort and collaboration between industry, academia and, regulatory agencies. Although it may not be straightforward to transition from batch to continuous manufacturing, there are significant long term benefits that could prove crucial as the biopharmaceutical industry evolves.

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Platforms for Manufacturing Allogeneic, Autologous and iPSC Cell Therapy Products: An Industry Perspective

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Abstract As cell therapy processes mature from benchtop research protocols to industrial processes capable of manufacturing market-relevant numbers of doses, new cell manufacturing platforms are required. Here we give an overview of the platforms and technologies currently available to manufacture allogeneic cell products, such as mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs), and technologies for mass production of autologous cell therapies via scale-out. These technologies include bioreactors, microcarriers, cell separation and cryopreservation equipment, molecular biology tools for iPSC generation, and single-use controlled-environment systems for autologous cell production. These platforms address the challenges of manufacturing cell products in greater numbers while maintaining process robustness and product quality.

Keywords Allogeneic, Autologous, CAR-T, Cell therapy, iPSCs, Mesenchymal stem cells

Contents

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1 Introduction

Cell therapy is the practice of using living cells, either from a donor (allogeneic) or from the patient (autologous), as a therapeutic modality. Different cell types and modes of action are used in cell therapy, ranging from allogeneic mesenchymal stem cells (MSCs) that are delivered intravenously or intramuscularly to treat stroke or peripheral artery disease, to autologous genetically engineered immune cells delivered intravenously to eliminate cancer, to donor-derived induced pluripotent stem cells (iPSCs) differentiated into insulin-producing cells which are encapsulated and injected subcutaneously to treat diabetes. Although there is great promise in cell therapy and the related field of tissue engineering, manufacturing the required cells can be daunting. This new therapeutic modality is not only complex to manufacture but the cellular product is often more sensitive to ostensibly minor process changes or variations, which may result in an ineffective therapy. A deep understanding of cell biology, clinical mode of action, and process and manufacturing considerations are all critical for success. A significant amount of R&D and process development, as well as choosing the appropriate commercially relevant manufacturing platform are imperative for success. In this chapter we discuss challenges and potential solutions in the area of cell therapy manufacturing and how these therapies can be made available to the patients that need them.

The focus of this chapter is on three manufacturing/therapy modalities which are currently leading the field and are distinct in terms of their manufacturing methods and challenges. These are allogeneic cell therapies, autologous cell therapies, and induced pluripotent stem cell (iPSC)-based therapies.

2 Allogeneic Cell Manufacturing

2.1 Introduction

Multiple cell types are used as allogeneic therapies, including mesenchymal stem cells (MSCs), hematopoietic stem cells, iPSCs, and cancer cells. These cell types are used with the aim of treating a variety of clinical indications including cardiovascular disease, neurodegenerative disease, diabetes, autoimmune diseases, graft-versus-host disease, and tissue replacement, to name a few. A fundamental risk of allogeneic cell therapy is the potential to elicit an immune reaction which could destroy the donor cells, thus rendering them ineffective. However, there are some ways to overcome this risk; for example, by using cell types that are hypoimmune (i.e., do not elicit a significant immune response in the recipient), such as MSCs, and by encapsulating cells in such a way that protects them from the host immune system.

From a manufacturing perspective, the allogeneic approach holds significant advantages such as the ability to scale up manufacturing to reduce therapy cost, the ability to choose the donors with highest cell potency, and the ability to have an off-the-shelf frozen therapy that can be administered at any time to an incoming patient. However, the manufacture of allogeneic cell therapies (as with other CT modalities) is not simple and is still evolving. The main considerations for manufacturing are to achieve high quality cells (potent) in sufficient quantities to treat eventually millions of patients and at a cost per dose that is sustainable for a specific indication. In this section we review and discuss the following two main manufacturing paradigms: 2D manufacture and 3D bioreactor manufacturing. We discuss the advantages and disadvantages of each, as well as considerations regarding downstream processing and facility design.

2.2 2D Manufacturing

The standard method of growing cells in academic labs is in 2D plastic flasks. This method of growing both adherent and non-adherent cells, although differing from the natural in vivo environment, is well-accepted and generates sufficient cells for most lab uses, such as biological assays and small animal experiments. As early cell therapies were mostly developed in translational academic labs, this meant that early cell therapy products were developed in 2D platforms. However, because small culture flasks, such as T-75 and T-175, are too small to produce a sufficient number of cells for even small clinical trials, these cell culture methods were scaled up.

The 2D flask concept was expanded into 10-layer and 40-layer Nunc® Cell Factory systems, which have surface areas much larger than T-175 flasks (36- and 144-fold larger surface area per vessel, respectively). An additional improvement to the 2D culture method was the introduction of the Hyperstack® Cell Culture Vessel (Corning®, Tewksbury, MA), which incorporated a gas permeable surface, eliminating the need for headspace and allowing an incremental improvement in

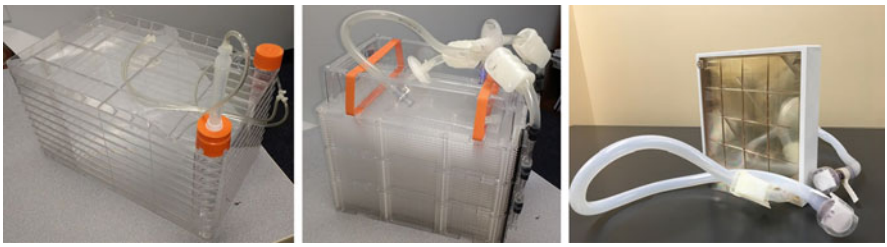


Fig. 1 2D platforms; Nunc®10 layer Cell Factory (*left*), Corning® 32 layer Hyperstack® (*center*) and CellCube® (*right*)

efficiency. Ten-layer cell factory processing and manipulation is almost entirely manual, whereas 40-layer Cell Factory systems, because of their size and weight, are partially manipulated for fluid exchange and detachment (Thermo Fisher Scientific, Waltham, MA). There have also been attempts to design large-scale 2D manufacturing solutions, products such as the CellCube® Module (Corning®, Corning NY) and the Xpansion® Multiplate Bioreactor System (Pall Corporation, Port Washington, New York), which are similar to cell factories and Hyperstacks but include perfusion capabilities and limited environmental control (Fig. 1). Several of these platforms are used today to produce cell therapies for clinical trials.

The manufacturing process using a 2D platform varies between different cell types, but usually involves seeding and passaging of cells with a seed train involving increasing numbers of 2D culture units which are kept in 5% CO_2 incubators at 37°C. Media are exchanged by removing the 2D vessel from the incubator, placing in a grade A clean space (e.g., laminar flow hood), and manually replacing the media. The timing of this media exchange is typically based on a predetermined interval, although the timing of passage and harvest for adherent cells is usually determined based on percent confluence of the cells on the surface as observed under a microscope (although only one of the cell factory layers can be viewed). At the end of the expansion process, cells are manually removed from the multiple vessels that constitute the batch and are pooled for downstream processing.

In general, scaling up 2D platforms is both fundamentally inefficient and logistically impractical. At the fundamental level, 2D expansion is relatively inefficient in terms of cell growth surface area to vessel-volume ratio. It is also not cost effective because the only way to produce more cells is to purchase more cell factories, cost increases linearly with manufacturing scale, undermining the primary economic motivation of large-scale manufacture (economies of scale). In addition, of course, the general lack of pH/DO (dissolved oxygen) monitoring or environmental control makes it difficult to envision using these platforms to grow more sophisticated cell therapy products.

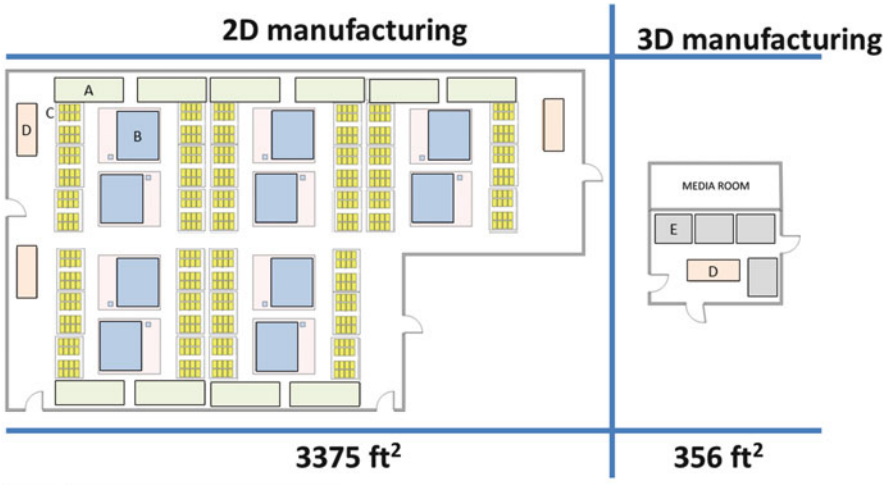
The logistical 2D challenges, however, are more acute. Large-scale 2D cell manufacturing requires large GMP clean rooms equipped with many biosafety cabinets and incubators, which are expensive to build and maintain. The culture vessels need to be manipulated primarily manually using open processes, requiring large teams of highly-trained workers who can be difficult to find and retain. For these reasons, these platforms are limited in their ability to meet allogeneic manufacturing requirements in terms of quantity and cost. Using current methods, the size of a

2D-based manufacturing lot is capped at approximately 100 10-layer cell factories per batch, primarily because of clean room space and downstream processing time [1].

2.3 *Bioreactor-Based Manufacturing of Adherent and Non-adherent Cells*

Because bioreactors address many of the shortcomings of 2D manufacturing, their use in allogeneic cell therapy manufacturing is becoming increasingly commonplace. There are many bioreactors available and each has advantages and limitations for specific cell types and processes. Some of the more common bioreactor configurations are stirred-tank, packed-bed, and rocker-based. These bioreactors are manufactured by multiple companies at various scales, ranging from 250 to 2,000 L and above. In most cases the vessels used are single-use and are available in both bag configuration as well as hard plastic. Bioreactors can be used to culture single cells, cell aggregates, and adherent cells on different commercially available microcarriers.

The primary advantage of using bioreactors is increased efficiency in terms of the number of cells obtained from a given vessel volume (up to 80-fold increase over 2D). For example, the proposed floor space required to build a 2D manufacturing suite capable of producing batches of one trillion cells is nearly ten times as big as a comparable 3D suite (Fig. 2). Additional advantages include a reduced need for



A	Staging Area
B	Gas flow unit
C	Incubators
D	Biological Safety cabinet
E	200L Bioreactor system

Fig. 2 Manufacturing footprint necessary for a batch size of one trillion cells; 2D vs 3D

clean room and incubator space, closed processing, automation, and environmental control (temperature, DO, pH, etc.). As opposed to a flask or cell factory, the interior of a bioreactor is easily accessible to probes and sampling lines which allow the user to monitor closely the cells and their environment [2]. In addition, media exchange, whether performed in batch mode or via perfusion, can also be automated. Finally, bioreactors are inherently scalable; large-scale reactors with volumes in the hundreds and thousands of liters and are relatively commonplace in the manufacturing of biotechnology products such as proteins, biologic drugs, and viruses.

The disadvantages of bioreactors are that they are expensive to purchase, they require skilled personnel to set up and monitor, and in some cases can introduce undesired fluid shear stress on cells. One of the main considerations when using a dynamic bioreactor culture, as opposed to a static 2D culture, is agitation and shear. Although cell expansion occurs in the bioreactor, earlier process steps such as isolation and seed train operation are often still done in 2D. As an example, 2D culture is generally required for isolating MSCs, which are selected based on plastic adherence, and one or two 10-layer Nunc® Cell Factory systems are needed to expand enough cells to seed a 50-L bioreactor [3]. However, it is certainly possible to develop bioreactor-based seed trains in which cells are cultured in successively larger bioreactors, either by harvesting and re-seeding cells, or by transferring microcarriers to larger reactors and adding new carriers and media. It is also possible to seed frozen cells directly into a bioreactor from an intermediate cell bank.

In some cases, cell therapy companies initiate clinical trials with cells from a 2D culture, and then wish to move to 3D bioreactor culture to supply the required cell numbers. In these cases, biological comparability between cells grown in both systems need to be shown. The effect of suspension culture on the biological activity of cells is a key question to consider before switching from 2D to 3D bioreactor platforms. There are several key differences between 2D and suspension culture, such as shear stress, altered culture conditions, and cell–cell interactions. Because of these differences, cells cultured in these two platforms may have a somewhat divergent biological profile. Particularly for MSC therapies, in which the mechanism of action is largely based on the secretome, changes to culture conditions may change critical attributes of the product. An example is increased secretion of VEGF in response to suspension culture conditions [4, 5].

2.4 Process Variables for Bioreactor Cell Culture

Considerations in developing and implementing a 3D bioreactor culture manufacturing system include:

- Choice of carrier type (adherent cells)

  Cell carriers can be primarily classified into static carriers, such as Fibra-Cel® carriers used in packed-bed bioreactors, and mobile microcarriers, which move in suspension in both stirred-tank and rocker bioreactors. Microcarriers are usually

several hundred microns in diameter, and are further classified into porous and nonporous microcarriers. Nonporous microcarriers are essentially solid plastic beads upon whose surface the cells adhere, akin to 2D surfaces. And as with 2D surfaces, these microcarriers are often coated with materials that promote adherence. Unlike 2D cell culture, however, in microcarrier cell culture the cells can form bridges between microcarriers, leading to microcarrier aggregation. Also, unlike 2D cell culture, the cell is in a constant state of motion and subject to shear stress that could potentially stress it, dislodge it from its surface, or change its biological behavior. Porous microcarriers offer cells additional surface area for attachment, and some degree of protection from shear forces in the bioreactor. Independent of the porous/nonporous classification, microcarriers can be made from either degradable or non-degradable materials, which primarily affects downstream processing.

- Choice of system and vessel (stir tank, packed bed, wave)

  Stirred-tank reactors are characterized primarily by their central impeller, which keeps the bioreactor fluid in motion. This keeps microcarriers in suspension and promotes the even diffusion of gases and nutrients throughout the reactor. Packed-bed reactors are a subset of stirred-tank reactors. With the cells confined to a packed bed, media perfusion is relatively easy, although cell sampling and harvesting is more difficult. WAVE bioreactor systems are cheap to produce and scale up, and are good for suspension culture. However, perfusion and monitoring is more difficult, and the rocking motion of a wave reactor is insufficient to suspend microcarriers.
- Media feeding strategy

  Media changes in bioreactors are usually done either through nutrient addition, total or partial media replacement, or perfusion. If a cell culture produces low and non-damaging levels of waste products, concentrated levels of nutrients (e.g., glucose) can be added over time to feed the growing culture. Waste metabolites such as lactate and ammonia often begin to accumulate, and either media replacement or perfusion is required. In stirred-tank reactors, media replacement is done by allowing carriers to settle, pumping media out and pumping it back in. This can be a lengthy process on a large scale, and also results in abrupt changes to the cells' environment. Perfusion, in which fresh media is gradually fed in as old media is gradually fed out, is the ideal way to feed/drain and still maintain a stable environment. Perfusion rate can be set based on a theoretical rate of nutrient consumption per cell, or based on real-time process variables (e.g., glucose concentration).
- In-process sampling strategy

  Bioreactor samples consist of cells, the reactor media, or both. Bioreactor media are accessible in all bioreactor configurations, but in packed-bed bioreactors the cells are usually inaccessible for sampling. Cell samples are taken for tracking the reactor cell concentration over time, and sometimes for imaging and characterization (e.g., bioreactor processes which include differentiation). Bioreactor media are analyzed for metabolite concentration, both as an in-process control and for setting the perfusion rate. However, sampling the bioreactor has certain drawbacks. First, to get a representative bioreactor sample, an operator often needs to take a large volume, which can impact on the final yield. Sampling needs to be performed at least once a day, and processing of

sampled material can be time-consuming. Finally, repeated sampling can increase the risk of contaminating the bioreactor. For these reasons, the industry is trending away from sampling and toward in-process analytics, which can replace sampling. Examples include biomass probes which correlate with cell density and spectroscopy probes (Raman, NIR), which can relay the real-time concentration of multiple metabolites.

- Method of oxygen delivery

  The goal of oxygen delivery is to keep up with cellular oxygen demand. Oxygen can be fed into a bioreactor system either via the headspace above the liquid or via a sparging tube at the bottom which bubbles gas up through the bioreactor. The volumetric mass transfer coefficient k_La describes the efficiency of oxygen transfer in a system, and is a function of vessel size/volume, media, temperature, agitation speed, and oxygen delivery (headspace, sparging, or both). The k_La of a given combination of conditions can be measured without cells, and that data can be used to choose an optimal set of conditions for cell culture.
- Optimization of seeding and agitation

  Bioreactor agitation rates are sometimes set based on k_La measurements, as mentioned above, but are also optimized to keep microcarriers in suspension. Bioreactor seeding can be done either under agitation, without agitation, or with a combination thereof. Agitation is important to distribute the seeded cells evenly, but too much agitation kills off a large number of them. Protocols that work for one cell type can be used as a starting point for developing a protocol for a new cell type, but the work must largely be done empirically.
- Determination of optimal DO and pH levels

  Given the level of control bioreactors have over DO and pH levels, it is important to test the effect of both on the cells. These tests should not be conducted in 2D because in 2D an initial DO or pH level generally drifts in one direction over the course of culture. In bioreactors, a PID loop is used to maintain DO or pH at a given set point. It is important to find not only the optimal set point but also the boundaries within which the cells are unaffected.

2.5 Downstream Methods and Challenges

Downstream processes for allogeneic products include cell harvesting, washing, concentrating, formulating, final fill finishing into vials or bags, verification of lack of visible particulates, and finally cryopreservation. Although each of these process steps in itself can be considered straightforward, the nature of the cell product combined with all these steps makes the downstream process quite complex. Two main considerations for downstream processing of allogeneic cell therapies are the time window, which is limited because the cells are adversely affected by prolonged periods in a suboptimal environment (temperature, nutrients, substrate, etc.) and that the current methods for conducting the multiple downstream unit operations for large numbers of cells are not fully developed and available. A

Fig. 3 (*Left*) TFF system for cell therapy downstream processing (source: Lonza Walkersville Inc.). (*Right*) Sep continuous centrifugation systems

discussion of some of the considerations for each of the downstream unit operations follows.

- Cell harvesting

  Harvest methods differ depending on cell culture method (single cells, cell aggregates, cells attached to microcarriers). Adherent cells on plastic microcarriers need to be detached from the substrate using enzymes in a manner akin to 2D cell harvest, although the cells then need to be separated from the plastic carriers. Cells grown on degradable microcarriers are harvested by dissolving the microcarriers themselves, leaving behind a cell suspension which is then collected for further processing. Cell aggregates might need to be dissociated using various enzymes as well. Because enzymes are often used in the harvesting step, it is critical to have a good understanding of their effect on cell viability, and whether they cleave surface markers critical to the product's function or identity.
- Concentration and washing

  Washing is required to remove enzymes, fetal bovine serum (FBS), and other unwanted residuals; cells need to be washed in a way that does not harm them. Cells need to be concentrated to bring them up to the therapeutic dose density. This will vary primarily based on the rout of delivery (e.g., I.V., I.M.), but typical densities include 10–20 $\times$ 10^6 cells/mL. Use of a standard centrifuge is an option, although this is a volume-limited manual open process that very quickly becomes unfeasible at large scale. Two available technologies are tangential flow filtration (TFF) and continuous centrifugation (Fig. 3). In TFF, cells pass over a filter, the medium passes through, and the cells are retained. This can be done both to concentrate and to wash the cells. The downside is that the cells are required to circulate repeatedly over the filter, and this can kill cells and introduce particles. In continuous centrifugation the cells are pumped into a flow chamber which suspends cells using centrifugal force and allows the medium to flow out; this can also be done both to concentrate and to wash.

This is the principle of the kSep® system (kSep, Morrisville, NC). This step can be challenging for larger volumes or higher numbers of cells.

- Formulation

  Cells must be formulated with the appropriate excipients, including appropriate cryopreservant fluid to prevent damage when freezing cells. In many cases DMSO at 5–10% is used for this purpose, although there is also a trend toward DMSO-free cryopreservants. Human serum albumin is also a common ingredient in cryopreservation media, used as a regulatory-approved replacement for FBS.
- Final fill finish

  Homogeneous delivery of cells to bags or vials is also a challenge. At high concentrations, cell solutions become sticky and cell aggregation occurs. There are currently no real robust turnkey solutions for this processing step, which means that it can be a difficult matter. The main risks are inaccurate dosing and excessive hold time. Cells need to be homogeneously maintained in solution and a system that allows sufficient bagging or vialing speed must be used. Bags or vials also need to be appropriate for cryopreservation, in most cases in liquid nitrogen.
- Visual test

  As per regulatory requirements, each dose must be "essentially free" of visible particulates (50–100 μm). Therefore each vial/bag needs to be visually inspected by a human, usually using a black and white background for observation, to ensure that no such visible particles (e.g., cellulose, plastic, metal) are present in the dose. Automated systems that are capable of such visual analysis do not yet exist.
- Cryopreservation

  Cryopreservation, typically at −196°C in liquid nitrogen or nitrogen gas, is accomplished using a controlled rate freezer (CRF) to allow optimal freezing of cells. A CRF has the flexibility to run custom protocols to optimize the freezing protocol for any cell type.

All of these downstream steps need to happen within a given time window, which may vary between cell types but usually does not exceed 6–8 h to minimize cell damage. In addition, the ability to conduct some unit operations for large numbers of cells or doses is extremely challenging because of the lack of automated and scalable hardware.

2.6 Summary: Allogeneic

Allogeneic cell therapies, with their potential for scaling-up, off-the-shelf availability, and reduction in CoGS, represent an extremely promising approach to a

wide host of diseases and conditions. However, current methods of manufacturing need to evolve and the correct approaches must be adopted by translational researchers and pharmaceutical companies to enable future commercial manufacturing at scale. Clearly, the use of 3D bioreactor scalable platforms for manufacturing is a central aspect in this move toward commercialization. However, hand-in-hand development of downstream and point-of-care delivery methods must be developed. A thorough understanding of critical quality attributes (CQAs) of the cell therapy, mode of action, and commercial goals (dose, patient number), and matching these as early as possible to the appropriate manufacturing methods, are absolutely critical for success. Moreover, one must consider that changes to the manufacturing process, once in the clinic, especially a move to a different manufacturing platform (e.g., 2D to 3D bioreactors), are a significant hurdle that may pose comparability issues.

3 Autologous Cell Manufacturing

3.1 Introduction

Autologous cell therapies utilize multiple cell types (e.g., Mesenchymal Stem Cells (MSC), Natural Killer (NK) cells, Dendritic Cells (DC), T cells, hematopoietic stem cells, and myoblasts) to treat a variety of clinical indices [6, 7]. Although the following is not an all-inclusive list, MSCs have been utilized to improve liver function, regenerate tissue, and regulate immune response, and have been shown to be effective in treating liver cirrhosis, liver failure, multiple sclerosis (MS), osteoarthritis, and Graft vs Host Disease [8–13]. NK cells are used in various autologous immune enhancement therapies to regulate cellular immune responses against malignant tumors and treat recurring gliomas, breast cancer, and other types of cancerous tumors [14–16]. Dendritic cells pulsed with tumor-associated antigens (TAAs), thus becoming antigen-presenting cells (APC), are able to target and kill specific tumors through cell-mediated cytotoxicity by stimulating an antitumor immune response. There are also several dendritic cell (DC) studies to determine the effectiveness of using DCs as a vaccine against various types of cancers and other autoimmune diseases, such as Type 1 diabetes [17]. In addition, there have been many recent successful treatments of critical patients suffering from advanced leukemia using autologous CAR-T cells [7, 18–22], which have increased demand for such therapies across the world.

3.2 Autologous Processes: Isolation, Expansion

The majority of autologous immunotherapies are first investigated at a small research scale for a select few individuals (less than 20) with less than 500 mL of culture per patient. Thus, many autologous procedures involve steps to isolate, modify, activate, expand, harvest, and test cells in traditional 2D tissue culture vessels, such as well plates, dishes, flasks, or Nunc® Cell Factory systems, with manufacturing timelines ranging from 1 to 3 weeks.

Current CAR-T manufacturing is labor intensive, requiring a large number of manual, open process steps [23, 24] including Ficoll gradient cell separation, cell activation, vector introduction (which can be viral or non-viral), cell expansion of target cell types, optional removal of undesirable cell types, and finally harvest. MSCs used in autologous cell therapies are typically derived from three main sources: fresh bone marrow of a patient's Iliad crest (BM-MSCs), adipose tissue through liposuction (AT-MSCs), or previously cryopreserved umbilical cord (UBC-MSCs). BM-MSCs and UBC-MSCs are commonly isolated using density gradient separation (e.g., Ficoll separation), whereas isolation of AT-MSCs involves the digestion of the fatty tissues, usually in a collagenase digestion procedure. Isolation of cells of interest from peripheral blood mononuclear cells (PBMCs) occurs via density gradient isolation or magnetically labeled antibody selection. In all these processes there are several "open steps," which are labor-intensive, time consuming, and introduce a risk of contamination into the process.

Methods for the in vitro expansion of these cells include co-culturing of the cells of interest with an irradiated feeder cell line, the use of protein-rich media formulated with human plasma/platelet lysate or FBS, and cytokine media supplementation. Ideally, an optimized, chemically defined medium specific to the cell process is used to avoid issues with serum and platelet lysate, including lot-to-lot variability, serum availability, quality assurance, and quality control standards. The amount of additional media supplements added to chemically defined media should also be limited, as many autologous cell processes require the use of costly cytokines, activation agents, and growth hormones. Determining the optimized media and feed strategy in the early stages of a product's life cycle streamlines future scaling up or scaling out processes for commercial manufacturing.

3.3 Challenges for Commercializing Autologous Cell Therapies

Although there are some closed and even automated cell expansion systems used in autologous cell therapies, which are discussed later in this chapter, the activation and expansion of many autologous cell processes occur in standard, open tissue culture vessels, including well plates, cell culture dishes, T-flasks, cell factories,

cell bags, and other gas-permeable rapid expansion cultureware such as G-Rex™ vessels (Wilson Wolf, St Paul, MN). Depending on the therapy, a few of these lab-scale vessels are adequate to provide over one billion cells per batch of the population of interest. For example, after approximately 2–3 weeks, the G-Rex system is able to produce up to 2×10^9 viable donor-derived virus-directed cytotoxic T lymphocytes targeted against the Epstein–Barr virus (EBV-CTLs) from 1×10^7 PBMCs [25]. However, these systems require multiple manual manipulations during the inoculation, activation, feeding, splitting, washing, and harvesting steps of a process. With each step in these systems, the risk of contamination and the overall costs associated with labor, lab usage, and materials increase.

As the number of patients requiring treatment increases, the overall autologous batch size for each patient is not expected to exceed more than a few liters because of the limited amount of starting material obtained from a patient to initiate the therapy and the time sensitivity of the cells to retain their optimal levels of functionality and efficiency. Thus, scaling up autologous immunotherapy processes to larger batch sizes is typically not needed and scaling out the process for multiple batches (i.e., one patient per batch) at a given time requires a thorough assessment of space, labor, and financial abilities and restraints. Many small-scale vessels (e.g., flasks, dishes, plates, cell factories, etc.) are limited by the available clean room area needed, and costs associated with required labor, increased materials, and risks of contamination or lot failure. In addition, the need to segregate strictly between patient materials requires extensive cleaning procedures between patient samples when common equipment is used. Therefore, when considering the best strategy for increasing throughput of patients treated as well as dose size per batch, the answer likely lies not in the use of larger vessels but rather in closing and automating manufacture. In addition to being labor intensive, 2D manufacturing methods are not robust and present a high risk of product contamination because of both the length of culture time and the number of manual washes, feeds, and cell manipulations that occur within the manufacturing process.

In addition to contamination risks, current autologous cell manufacturing methods are also cost-prohibitive for all but a select few patients [21, 26]. One of the main reasons for this high cost is that the production of engineered autologous cells for therapy differs significantly from traditional manufacturing of biological products such as monoclonal antibodies or recombinant proteins. Traditional commercial biological manufacturing models center on process scale-up such that a single but larger batch of product can be packaged and shipped to treat many patients [26]. This scale-up allows for a decrease in manufacturing cost through economies of scale.

However, in the case of autologous cell therapy, the product is only manufactured for a single patient with a limited number of doses, making the requirements for the commercial manufacturing of these cell products out-scalable, not up-scalable. Therefore, the concept of increasing the batch size to allow for increased financial efficiency via reduction in cost/unit of product does not apply [27]. Thus, to make these life-saving therapies universally available, other

cost-saving methods must be explored. Switching manufacturing from a manual process to an automated process can allow much-needed cost reduction [6, 24, 27]. Automation would allow preprogrammed bioreactor systems to perform the necessary steps of manufacturing with minimal labor input. This would greatly reduce labor costs by reducing both the number of personnel needed and the space and time required to manufacture a dose.

Autologous therapies must also be extremely robust. Patients seeking autologous therapies have often exhausted all traditional therapies, thus leaving autologous therapy as a last option for successful disease treatment. As these powerful therapies become more mainstream and thus more widely available, they are likely to become viable treatment options for patients during earlier phases of their disease. In both instances, because cells being produced as autologous therapies are being manufactured "on demand" for patients in need, there is little margin for error. A contamination or mistake in the culture process means that a patient in critical need has to both donate more cells for manufacture and wait longer to receive treatment. Moreover, there is variation in starting material based on biological and clinical differences between patients. The ability to have a robust process increases the chances of manufacturing a high quality and potent therapy for each individual patient. Clearly, an automated and controlled process is desirable from both a clinical and a cost perspective. An automated process would allow for consistent execution of process steps, resulting in decreased process-to-process variation [6]. Additionally, it follows that automation would be paired with a closed system, thereby making manufacturing both more affordable and more robust.

3.4 Commercialization Solutions for Autologous Cell Therapies

3.4.1 Streamlining: Closed Systems, Disposable Components

One method to reduce the risk of contamination of autologous cells is shifting as much of the procedure as possible from an open process to a closed system. A closed system is a sterile environment where multiple steps of a manufacturing process can occur without disrupting the sterile environment. The number and type of steps which can be transferred to a closed system is determined by the culture system and by the components being used.

There are a few devices used to help decrease the risks associated with the isolation of cells, by performing the process steps in a closed system, as opposed to "open" processes in which a sample is handled in vessels which have removable lids/caps. The Pall Purecell Select™ system is a closed system isolation method designed to isolate mononuclear cells from whole blood though gravity filtration. With this system, a patient's blood sample is injected by syringe into the input bag, the entire device is suspended from an IV pole or hook, and the sample then passes through a filter which retains cells of interest and removes unwanted cells to waste.

Subsequently, the filter is washed with reverse flow, and the cells of interest are collected into a cell collection bag with an aseptic syringe luer lock adapter. Another example of a closed isolation system is the Miltenyi CliniMACS™. The CliniMACS™ system utilizes magnetic microbeads labeled with various types of antibodies for either positive or negative selection of target cell populations. The Miltenyi Prodigy™ also uses magnetic bead selection and is capable of cell expansion.

One of the simplest closed method systems for cell expansion involves the use of sterile cell expansion bags. Many companies sell cell expansion bags along with weldable tubing and sterile connections that allow cell inoculation, feeding, expansion, and harvesting under sterile conditions. Such systems allow the reduction in the number of open process steps, thereby reducing contamination risk. Cell expansion bags meant for static culturing are typically made of gas permeable materials and can be tissue treated for 2D cultures. Other cell expansion bags may be untreated and non-gas permeable for use in controlled suspension bioreactor culture systems (e.g., GE WAVE). Use of such a system was utilized in 2013 for the manufacture of a CD19 autologous CAR-T cell under Good Manufacturing Practices (GMP) which were used in a Phase I clinical trial treating a pediatric B cell malignancy [28].

Clearly, more advanced culture systems which would allow increased automation and closing of all process unit operations, thus allowing out-scaling, reducing Cost of Good (COGS), limiting manpower needs and clean room space, as well as better monitoring and control over the whole process are desirable. Such culture systems are currently being developed.

In small-scale, scale-out manufacturing, the use of disposable components is preferable. This allows scale-out and minimizes the risk of contamination. Although the use of disposable components would at first glance involve additional cost for the process, the elimination of costs to clean and sterilize components as well as to clean rooms would offset the cost of disposable process components. Furthermore, utilizing disposable components would significantly decrease the downtime of the autologous manufacturing facility, which is one of the most cost-prohibitive aspects of autologous manufacturing.

3.4.2 Biofeedback

Another caveat of autologous cell manufacturing revolves around the patients themselves and the cells which they supply. Patients seeking treatment have likely undergone many alternative treatments and therapies previously. They may have undesirable medication or undesirable cell types in their system when cells are harvested for manufacturing, which may make cell manipulation and/or expansion difficult. The ability to monitor critical process parameters such as culture temperature, pH, dissolved gasses, nutrients, metabolites, confluency, and biomass provides valuable insight into better cell health and more efficient cell growth [26, 29].

There are several offline devices and assays that provide these types of measurements through absorbance and florescence measures or conversion of electronic signals from the free flow of ions when presented with a sample of the culture supernatant. However, to reduce the risk of contamination from manual manipulation and sampling of a culture, other methods, such as PreSens® microsensor optodes, Lonza CytoSMART™ Live cell Imaging System, or similar non-invasive culture monitoring devices, are recommended.

A system which incorporates biofeedback based on culture health and other real-time monitored analytes would create a dynamic culture system which could potentially improve expansion and yield of difficult-to-manipulate cells.

3.4.3 Available Systems for Commercializing Autologous Therapies

Autologous cell manufacturing must address three main issues: (1) the ability to scale-out manufacture to be able to treat significant numbers of patients, (2) improved process control and robustness both to minimize risk of contamination and to minimize failure caused by poor performance of cells, and (3) cost reduction of the autologous manufacturing process to make these therapies available to more than just a select few. Because traditional scale-up strategies cannot be utilized to reduce the cost of manufacturing for therapeutic autologous cells [27], other options must be explored. Utilizing an automated and closed bioreactor system would allow scale-out as well as significant reduction both in personnel and facility requirements, translating into cheaper manufacturing costs. Multiple companies are working to develop such a system specifically to address the needs of autologous manufacturing.

The ideal autologous cell manufacturing system would allow multi-step processing sequences that include a maximum of process steps including initial cell isolation (Ficoll, magnetic bead, or adherence), automatic feeding and washing, ability to incorporate downstream processes such as magnetic bead selection and/or electroporation of cells, harvest of cells, and finally concentration of cells. Additionally, a system that includes biosensors coupled with biofeedback-based process adjustment would allow for valuable process control. The ideal autologous manufacturing system would also accommodate multiple cell types, including not only suspension CAR-T cells but also adherent cell types such as mesenchymal stem cells and dendritic cells. Finally, a method to quantify cell concentration and/or confluence such as via an in-system camera would be very beneficial to gain valuable insight into cultures without repeated disruption through sampling or removing the culture chamber or bag for visualization under a microscope.

Two systems which incorporate many of the desired components of the future autologous cell manufacturing system are the CliniMacs Prodigy® by Miltenyi and the Cocoon™ system by Octane Biotech Inc. The CliniMacs Prodigy® features a closed system capable of automatic cell separation via density gradient, cell washing, positive and negative cell selection using magnetic beads, expansion of suspension cells, and final cell concentration [30]. The CliniMacs Prodigy® system

has several fixed components of the system such as the fractionation chamber and magnetic separation capabilities. There are multiple tubing sets which have been designed specifically for isolation and expansion of certain cell types. The technician would manually attach the selected GMP tubing set to the system using sterile welding and run a predesigned program to generate the cells of interest. This system incorporates a small microscope for visualization of cells within the system during culture. One drawback of the system is that, although it does incorporate sampling ports for offline biofeedback sampling, it does not incorporate such sampling automatically and throughout the run. Furthermore, although development is underway to translate processes for adherent autologous cell types such as MSCs to the system, traditionally the Prodigy has been limited to suspension cell types only.

A second system which is gaining attention in the automated cell culture realm is the Cocoon™ system by Octane Biotech Inc. This system features a closed and fully automated cell culture system that can successfully culture and expand both adherent and suspension cells. The Cocoon™ system incorporates a small input chamber which can be used both for sample loading and for low volume processes that are volume dependent, such as viral transduction for CAR-T cell generation. After loading or other minimal volume-requiring activities, cells are then automatically transferred to proliferation chambers designed specifically for the cell type being cultured. The Cocoon™ system features inline monitoring and control of fluid oxygen, pH, and CO_2. Feeding, washing, and concentration of cells are accomplished through completely automated software, enabling the technician to invest little to no hands-on time after sample loading until time of harvest. One feature of the Cocoon™ system which makes hands-off perfusion possible is the fact that, in addition to a 37°C culture chamber, the Cocoon™ system also has a 4°C chamber incorporated which allows up to six different media and reagents to be pre-loaded and fed via the preprogramed expansion protocol. Each protocol can be designed specifically to user specifications, making this technology flexible yet robust.

3.5 Centralized Manufacturing vs Point-of-Care

As autologous cell therapies increase in prevalence, one strategic question that arises is the concept of whether these cells should be manufactured at centralized/regional facilities or whether manufacturing should be shifted to "point-of-care," meaning the cells would be harvested bedside in the hospital where the patient is being treated, engineered and expanded, and then returned to the patient with all manufacturing occurring at the same facility where the patient is seeking care. Both centralized manufacture and point-of-care manufacture have benefits and drawbacks.

Centralized manufacturing would allow the cell engineering to occur in a state-of-the-art facility designed and constructed specifically for the manufacture of autologous cell therapies. Additionally, the staff that support such a centralized

location would be well-experienced in performing techniques critical for engineering and production of the therapeutic cells and would have full quality control and quality assurance support. One major drawback of centralized manufacturing revolves around the logistics of how the cells themselves would be transported, both from the patient for ex vivo manipulation and then back to the patient post-manufacture [29]. Same-day or next-day shipping is a viable option in developed countries such as the United States. However, expedited shipping of cells would not make this therapy available to patients who are in need worldwide because of the limitations of viable shipping options. A second method to address the shipping of cells would be to evaluate cryopreservation of cells for shipment to patients. This could prove to be a viable option; however, additional studies to evaluate the stability and efficacy of cryopreservation for some autologous cell types is necessary [6]. One final consideration is that centralized manufacturing facilities would likely be limited to the number of patients that can be treated at a given time, which could limit the total number of patients treated [6, 7].

The second manufacturing strategy revolves around point-of-care autologous cell engineering. In short, this would make the production of the cells possible in many hospitals worldwide. A key component around which this strategy revolves is the use of a closed, disposable system equipped with automation of a maximum number of process steps. Having a system with disposable components would reduce the downtime of manufacturing by reducing the requirement for cleaning and sterilization of tools. In addition to a reduction of downtime, the facility would not require dedicated space for cleaning and sterilization of reusable system components. Moreover, the use of a closed system would significantly reduce facility needs by allowing open cell manipulations to occur within an approved biosafety cabinet, which would need to be cleaned after use and then utilized for the next patient. Reduction of facility needs would greatly decrease the financial burden on the hospital utilizing the closed system, thus resulting in more hospitals or point-of-care sites capable of performing autologous cell therapy treatments on-site. Finally, a fully-automated system would decrease the need for intensive training of on-site technicians in specialized ex vivo autologous cell engineering. This would both decrease costs by decreasing specialized labor requirements and increase the number of sites willing to incorporate these powerful autologous cell therapies into their repertoire of available life-saving therapies, thus making them available to patients within their region.

No matter which manufacturing method is implemented, full sample and product traceability is crucial. There should be multiple fail-safes to ensure the patient sample is properly handled from time of collection through time of patient treatment. Some of the closed systems previously discussed in this chapter, including the CliniMacs Prodigy® and Octane Cocoon™ system, are equipped with barcode scanners that allow the user to link information about all materials used in a process to the batch generated. This is one example of how the equipment used in a process can increase lot traceability across a process, as well as prevent potential user error.

3.6 Regulatory Considerations

One final consideration for the expansion and use of autologous cell therapies is how this therapeutic, cellular product is regulated by the Food and Drug Administration (FDA) and equivalent regulatory agencies worldwide moving forward. Currently, the FDA is responsible for ensuring that autologous cell therapy products are safe, pure, potent, and effective [31]. Historical FDA oversight involved products which are mass produced in large batches or lots of a single product which can be used by multiple patients. A small portion of this lot can be tested for safety, purity, and effectiveness, leaving a large portion of the product available for treatments. Autologous cell therapies by definition are problematic under current regulatory definitions (21 CFR 1271 [31]) for a variety of reasons, one of which is that variations in the starting material, a specific patient's cells, makes a pre-determined standard of purity of final cellular product defined by frequency of specific cellular markers difficult because of inherent variations in these markers among different patients [7]. Furthermore, quantifying a baseline for potency of therapeutic autologous cells is also difficult because the cells themselves are dividing and growing rather than remaining static [32]. Specifics of how certain aspects of Phase I, II, and III clinical trials for certain autologous therapies would be accomplished given the limited number of starting materials and the difficulty with administering an effective placebo further complicate the regulatory situation of autologous cell therapies [6]. As this powerful and quickly-developing therapy modality moves forward, it is clear that one size does not fit all with regard to regulation of all cell therapy products.

3.7 Summary

Autologous cell therapies hold much promise for successful treatment of many diseases and conditions including multiple forms of cancer. To make these life-saving therapies accessible to all patients in need, the processes must become more standardized, robust, and cost efficient. One method of achieving these goals is to develop autologous cell therapy processes with the following attributes:

(1) A closed system, with limited/no open process steps from initial inoculation through final formulation
(2) An automated process to increase process robustness and limit the risk of human error in a process with limited starting materials
(3) Incorporated dynamic biofeedback
(4) Disposable system components
(5) Cost effectiveness

Finally, it is also important to assess the benefits and disadvantages of manufacturing any cell therapy product in either centralized or point-of-care

facilities. Although centralized facilities have dedicated space and staff focused on delivery of a high quality product, complications surrounding delivery of both the initial tissue sample and final cell product may arise. Moreover, manufacturing cell products at the point-of-care may increase the risk of human error in the handling, documentation, or process troubleshooting for the product.

4 Induced Pluripotent Stem Cell (iPSC) Manufacturing

4.1 Introduction

The isolation of human embryonic stem cells (hESCs) from the inner cell mass of 8-day-old blastocysts [33] introduced the concept of pluripotency (i.e., the ability of cultured cells to form all cell types of the body that are derived from ectoderm, endoderm, or mesodermal lineages). This remarkable accomplishment dramatically changed the fields of developmental biology, in vitro differentiation, and regenerative medicine. In 2007, Dr. Shinya Yamanaka successfully converted adult human cells to induced pluripotent stem cells (iPSCs) [34]. The iPSCs have similar characteristics to embryonic stem cells (ESCs) and by definition have the ability to self-renew indefinitely and become any cell type in the body. Initially, retroviruses expressing four transcription factors (Oct3/4, Sox2, Klf4, c-Myc) were used in the reprogramming process, which was readily replicated worldwide and improved upon by numerous investigators. Similar to ESCs, human iPSCs are pluripotent and can be readily derived from any individual. iPSCs have become an important scientific tool and are spurring advancements in basic research, disease modeling, drug development, and regenerative medicine. Equally important, this discovery unlocked many new opportunities for using iPSCs in both allogeneic and autologous cell therapy applications. iPSC-based therapy is a newly developing field and builds on several key technical advances that have enabled the widespread use of embryonic stem cell (ESC)-based technology [35–38] for drug discovery and basic biology. Companies such as Geron, Asteris, Ocata (formerly known as Advanced Cell Technology), Biotime, Viacyte, and Johnson &Johnson have developed products from ESC and several have initiated early-stage clinical trials [39], and several patients have been treated with no deleterious side effects [40]. These results have led companies such as Healios and Megakaryon to initiate plans to generate products using iPSCs. Recently, a study involving one patient treated with retinal pigment epithelium (RPE) cells derived from iPSCs was carried out using cells manufactured in a cGLP environment using autologous cells (http://stemcellstm.alphamedpress.org/site/misc/News159.xhtml). The huge potential of iPSCs for therapeutic purposes stems from the fact that differentiated cells (from blood or skin) can be taken from a donor, turned into iPSC, expanded as needed, and then differentiated into the required cell type. This means that a future in which tissue replacement (e.g., cardiomyocyte replacement after acute myocardial infarction) or

even organ replacement (e.g., kidney replacement) can be facilitated by use of this method. The cells utilized can be either from the patients themselves or from a donor. The advantage of using a patient's own cells is that there is no risk of immune rejection, but a disadvantage is an extremely expensive and not off-the-shelf therapy. An advantage of securing cells from a donor is that this is a less expensive approach which is potentially also off-the-shelf but requires lifelong immunosuppression for the patient (similar to donor organ transplantation).

Here we briefly highlight some of the key considerations regarding the manufacture of iPSCs, differentiation of iPSCs into cell therapy products, and characterization of iPSCs and their derivatives during the manufacturing process.

4.2 iPSC Generation

Initially, iPSCs were generated through reprogramming with retrovirus constructs [34, 41] which permanently integrated into the cell genome. This method is not preferred for clinical cell therapy applications. Moreover, these cells were usually generated and expanded using a feeder layer system which has lot-to-lot variability, regulatory and safety concerns, and scalability issues. Later, alternative reprogramming methods were established including: (1) non-integrating SeV reprogramming where Sendai-viral particles were used to transfect the target cells with replication-competent RNAs that encode the original set of reprogramming factors (OCT4, SOX2, KLF4 and cMYC), (2) non-integrating episomal-based reprogramming using plasmids (e.g., Epstein–Barr virus-based episomal plasmid DNA replication system) encoding reprogramming factors OCT4, SOX2, KLF4, LMYC, and LIN28A in combination with different enhancers (e.g., P53 knockdown (shP53)), and (3) mRNA reprogramming where the cells are transfected with in vitro-transcribed mRNAs encoding OCT4, SOX2, KLF4, and cMYC with additional reprogramming factor LIN28A [42–44]. The main factors used to compare each of these reprogramming methods are safety, efficiency, cell line stability, reliability, and ease of establishing a GMP-compliant process [42]. SeV reprogramming, although efficient and reliable, lacks GMP compatibility because a cGMP grade reprogramming reagent is not available. In comparison with SeV reprograming, episomal-based reprogramming is an integration-free, reliable, and cGMP-compliant method that can be used for different starting materials (bloods cells and fibroblasts). RNA-based reprograming has been shown to be fast, highly efficient, and have zero footprint. However, this method suffers from difficulty in successful reprogramming of fibroblast cells and, most importantly, insufficient reproducibility by different groups. Although the method of derivation, starting materials, and morphology of iPSCs can be different, a method-specific difference in the quality of iPSC lines with respect to marker expression profiles, differentiation capacity, DNA methylation, or genetic instability has not been observed [42].

As highlighted by Daley and colleagues [42], there are currently safer alternative reprogramming methods compared to the original viral transfection, but the choice

of reprogramming method depends on the specific applications or requirements of each research lab. As the field of pluripotent stem cells is rapidly growing and further methods and technologies are evolving (e.g., using a gene-free, small molecule-based reprogramming method), it is important to shift the focus to establishing methods to manufacture clinical quantities of pluripotent stem cell-derived products. We have recently reported the development of a robust, reproducible, and cGMP-compliant manufacturing process to generate clinical-grade iPSCs from cord blood CD34+ cells for use in further manufacturing of therapeutic cellular products [45]. The next section briefly describes some of the main design considerations in establishing this iPSC manufacturing process [46].

4.3 iPSC Manufacturing Process Design Consideration

The use of non-integrating plasmid DNA to carry the reprogramming transcription factors into the somatic cells (e.g., CD34+ cells derived from newborn umbilical cord blood or adult peripheral blood mononuclear cells) has been previously reported [43, 44]. However, switching to integration-free methods and potentially clinically-compliant methods to generate cGMP-compliant human iPSCs is often inefficient and technically challenging. To establish a robust and reliable cGMP iPSC manufacturing process, we took three major stages [46], focusing on: (1) establishing an iPSC generation process using a non-integrating episomal-based technology (stage 1.0 – proof of principle), (2) process optimization and protocol development based on the critical attributes of the process (stage 2.0), and (3) tech transfer of the manufacturing process into a cGMP cell therapy suite (stage 3.0).

A number of challenges must be considered in the development of a cGMP iPSC manufacturing process. These challenges include: (1) iPSC derivation challenges (including safety of the reprogramming method, efficiency, donor-to-donor variability, and choice of starting materials), (2) iPSC manufacturing challenges (including development of a cell culture system for generation and expansion of iPSCs, sensitivity and robustness of the iPSCs, cryopreservation, and revival of the iPSCs), and (3) safety and QC challenges (including standard safety concerns such as sterility, normal karyotype, residual plasmid clearance, in-process controls to evaluate the quality of iPSCs, and critical attributes of the final iPSC products). Other challenges are labeling and packaging, storage and warehousing of the final product, facilities, human resources, and training. Equipment and utilities requirements should also be considered during the design considerations for developing a cGMP manufacturing process. Finally, regulatory issues applicable to the tissue acquisition and iPSC manufacturing and testing need to be carefully evaluated from the early stages of the process [45, 46].

4.4 iPSC Directed Differentiation Processes

From the cell therapy applications perspective, human pluripotent stem cells, including iPSCs and hESCs, have the potential to be used in allogeneic applications. However, iPSCs are the only source of pluripotent stem cells that can be used for autologous cell therapy applications by taking a patient's own tissue (e.g., peripheral blood or skin biopsy), isolating the appropriate population of cells (peripheral blood mononuclear cells – PBMCs or fibroblast cells from skin biopsy), generating patient-specific iPSCs, and differentiating the iPSCs into specialized cells, which could undergo a gene correction method prior to transplantation into the patient. The concept of autologous iPSC transplantation has been tested in animal models [47, 48], demonstrating the feasibility of this approach. The first clinical trial involving one patient treated with retinal pigment epithelium (RPE) cells derived from iPSCs was recently carried out using cells manufactured in a cGLP environment using autologous cells (http://www.cdb.riken.jp/en/news/2014/researches/0915_3047.html).

Pluripotent stem cells are not directly transplanted into human subjects because of their proliferation and tumorigenic capability. iPSCs must undergo a differentiation process, which is usually stage specific and guided by specific chemicals and cytokines that direct the cells through the differentiation process based on the appropriate signaling pathway identified in research labs. Functional insulin-secreting beta islet cells have been generated from iPSCs using a multistage directed differentiation process [49, 50]. As reviewed recently by Li and colleagues, significant progress has been made toward the differentiation of pluripotent stem cells into highly homogeneous neural progenitors with a larger proportion of mature dopaminergic neurons with improved survival and integration after transplantation [51]. Other diseases have also been targeted by investigating the potential of pluripotent stem cells to generate specialized cells with transplantation capacities [52–55]. The directed differentiation process often requires very accurate control of cell fate in each differentiation stage. Varying parameters, including the type of cell culture system prior to the start of differentiation, induction time at each step of the differentiation, type and concentration of the cytokines or chemicals, differentiation medium composition, mode of culture (2D vs 3D), and physiological conditions (e.g., oxygen concentration) play important roles in the outcome of the differentiation process.

4.5 Characterization of Pluripotent Stem Cells and Their Derivatives

In parallel to developing a process to generate human iPSCs or iPSC-derived products, it is critical to establish an appropriate final product testing platform to evaluate identity, safety, purity, and viability of the final product. However,

establishing a characterization platform for iPSCs or iPSC-derived products may be very challenging, considering the absence of specific guidelines for characterization of these cells. The field of pluripotent stem cells and their application for cell therapy is still emerging, but there are growing efforts to address these unmet needs. We have established a platform for characterizing iPSCs by focusing on the criticality of the assay (i.e., indicating safety, identity, or purity) according to the existing regulatory guidelines for cell therapy products [45, 56]. Importantly, one critical feature of a release assay is the ability to qualify the assay or availability of an existing standard, GMP-compliant quality control assay. The assay qualification is performed according to the current Good Manufacturing Practices, the International Conference on Harmonization Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation guidelines [57]. Depending on the nature of the assay, accuracy, precision, specificity, limit of detection (LOD), and limit of quantification (LOQ) are determined during the qualification studies. Aside from standard safety assays, including plasmid clearance, karyotype analysis, sterility, mycoplasma, and endotoxin tests, we have developed and qualified some of the iPSC-specific assays including: (1) flow cytometry to evaluate the expression of four PSC-specific markers(SSEA-4, Tra-1-60, Tra-1-81, and Oct3/4), (2) quantitative PCR for evaluation of residual plasmid clearance used for reprograming, and (3) cell count and viability. Short Tandem Repeats (STR) have also been incorporated into the release assays to confirm that the final iPSC product matches the initial donor cells used in the reprogramming process. According to FDA regulations, release of allogeneic master cell banks for clinical use requires extensive testing for the presence of viral contaminates. Therefore, master cell bank viral testing needs to be included in the release testing, but the viral testing panel for hiPSCs should be adjusted based on the cellular characteristics of pluripotent stem cells and should be comprised of both in vitro and in vivo assays [45]. In addition to the release testing, we have also incorporated additional characterization assays (classified as For Information Only (FIO)) in the testing panel for iPSCs, including evaluation of hiPSC colony morphology, plating efficiency of hiPSCs post-thaw, and embryoid body (EB) formation. The EB formation has been used to demonstrate the identity and potency of hiPSCs by investigating spontaneous differentiation into three germ layers (i.e., ectoderm, mesoderm, and endoderm) and evaluating the results through immunofluorescence at the protein level or qPCR analysis at the transcript level. Post-thaw plating efficiency was evaluated based on alkaline phosphatase (AP) staining. AP, a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions, has been widely used for evaluation of undifferentiated pluripotent stem cells including both embryonic stem cells and iPSCs [34, 58–60]. Considering that iPSCs could very likely be used as starting material for derivation of a variety of cell therapy products, an additional subset of analytical methods should be incorporated into a routine testing process to provide data in an unbiased way, such that if collected in a database over time the users would be able to monitor potential variability of critical characteristics of iPSCs. This variability lies in the biological changes associated with the manufacturing process at or after

implantation as they respond to the environment. We propose the use of a transcriptome analysis, a SNP-CHIP/CGH array, and whole genome sequencing as three basic tests to complement the standard tests for pluripotency, differentiation ability, and composition that are routine [56].

In the case of iPSC-derived specialized cells and products, it is crucial to develop two critical assays associated with safety and potency of the final product. Considering that iPSCs have the potential to proliferate almost indefinitely as well as the potential to generate tumors, it is necessary to develop a safety assay to ensure that the iPSCs are eliminated from the final product lot through the directed differentiation process. The assay needs to be sensitive enough to detect very small quantities of iPSCs at the gene level. Moreover, a safety assay must be developed to detect the functionality of the final product developed from iPSCs. For instance, Pagliuca et al. use a glucose stimulated insulin secretion (GSIS) assay to evaluate the functionality of iPSC or hESC-derived beta cells generated in a 3D-differentiation process. This test evaluates the capacity of the PSC-derived beta cells to respond to multiple, sequential high-glucose challenges as well as depolarization with KCl [49].

4.6 Summary

In summary, human pluripotent stem cells and iPSCs, in particular, hold great potential to be used as starting material for derivation of a variety of cell therapy products through directed differentiation processes. The directed differentiation process is usually a stage-specific process requiring tight control of the differentiation process from pluripotent stage into multi-potent and eventually into specialized cells with specific functions. The manufacturing of cGMP-grade iPSCs and their products requires compliance with cGMP regulation and implementation of appropriate in-process controls and final characterization tests to ensure that safe and high quality materials are generated. Recent advances in the development of cGMP manufacturing processes for the generation of clinical quantities of iPSC products as well as the outcome of ongoing clinical trials using PSC-derived products should have a major impact on the commercialization and routine use of iPSC-derived cell therapy applications.

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Gene Therapy

Barb Thorne, Ryan Takeya, Francesca Vitelli, and Xin Swanson

Abstract Gene therapy refers to a rapidly growing field of medicine in which genes are introduced into the body to treat or prevent diseases. Although a variety of methods can be used to deliver the genetic materials into the target cells and tissues, modified viral vectors represent one of the more common delivery routes because of its transduction efficiency for therapeutic genes. Since the introduction of gene therapy concept in the 1970s, the field has advanced considerably with notable clinical successes being demonstrated in many clinical indications in which no standard treatment options are currently available. It is anticipated that the clinical success the field observed in recent years can drive requirements for more scalable, robust, cost effective, and regulatory-compliant manufacturing processes. This review provides a brief overview of the current manufacturing technologies for viral vectors production, drawing attention to the common upstream and downstream production process platform that is applicable across various classes of viral vectors and their unique manufacturing challenges as compared to other biologics. In addition, a case study of an industry-scale cGMP production of an AAV-based gene therapy product performed at 2,000 L-scale is presented. The experience and lessons learned from this largest viral gene therapy vector production run conducted to date as discussed and highlighted in this review should contribute to future development of commercial viable scalable processes for vial gene therapies.

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Contents

1 Introduction

The field of gene therapy refers to a broad area of pharmaceutical development in which the therapeutic agent enables the introduction of genetic materials (either RNA or DNA) into cells to modify or restore gene function for the treatment or prevention of disease. Two fundamental strategies have evolved to restore or modify target cell function: ex vivo or in vivo gene delivery (Fig. 1). Ex vivo gene therapy requires the harvest of cells from the patient or a donor. The therapeutic gene is transduced into the cells in a cell therapy manufacturing setting and the cells are subsequently re-introduced into the patient. Conversely, in vivo gene therapy relies on functional modification of targets by direct transgene injection into the patient. There are multiple ways to deliver the therapeutic transgenes, including the use of both viral and non-viral vectors (Fig. 2), each offering exciting advantages and some key limitations. As the field evolves, exploiting the biological properties of each transgene delivery method continues to expand the vector choices for gene delivery and advance further the gene therapy pipelines. The focus of this chapter is on the production and manufacture challenges of the viral vectors, whether used for ex vivo or in vivo gene therapy.

Since the first gene therapy trial was conducted nearly three decades ago, the field has experienced periods of rapid growth despite significant and highly

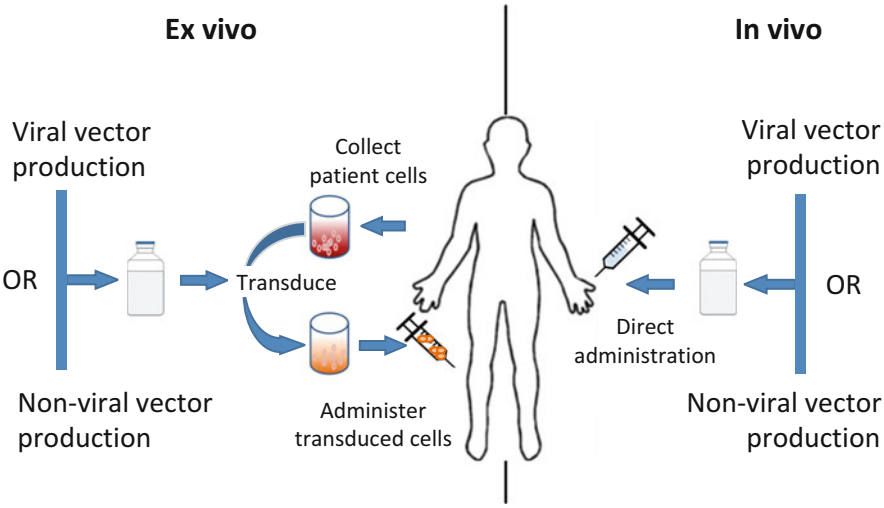


Fig. 1 Ex vivo and in vivo approaches to gene therapy

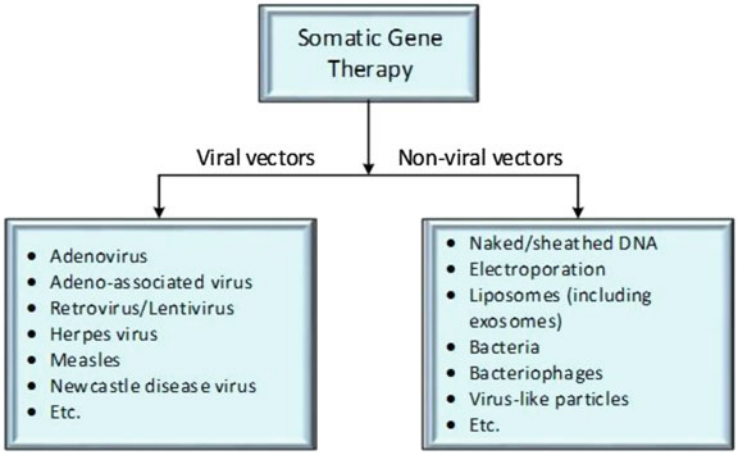


Fig. 2 Viral and non-viral vectors for somatic cell gene therapy

publicized setbacks early on, such as a death from a severe immune reaction to an adenovirus vector in an OTC clinical trial [1] and development of leukemia from insertional oncogenesis by a retroviral vector in a SCID trial [2]. Recent notable positive clinical outcomes in late stage clinical trials have been reported for a wide range of disease indications including rare, monogenic diseases such as Leber's Congenital Amaurosis Type 2, X-linked severe combined immunodeficiency, adrenoleukodystrophy and common oncological indications such as acute lymphocytic leukemia and chronic lymphocytic leukemia [3]. Since 1989 there have been more than 2,200 gene therapy clinical trials approved globally, with over 200 in the last

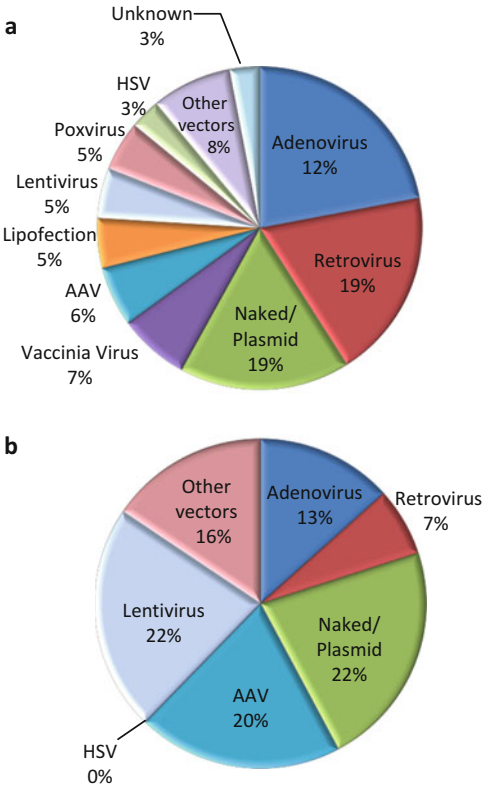


Fig. 3 Percentage of gene therapy trials by type of gene delivery vector. (**a**) All trials between 1989 and July 2015 ($n = 2{,}210$). (**b**) Trials initiating in 2014 through July 2015 ($n = 175$) (Journal of Gene Medicine database, accessed Jan 2016, http://www.abedia.com/wiley/vectors.php)

2 years (Fig. 3). However, despite the large numbers of recombinant viral vector therapeutic products in various stages of clinical trials, the field is still in its infancy in terms of approved commercial products and to date there are only six licensed products in major developed markets (North America, EU, Japan) and nine globally. Of these, five are viral-based therapies (Table 1).

Based on the clinical efficacy demonstrated in multiple late-stage clinical trials using viral gene therapy vectors, it is anticipated that more programs can advance through licensure stages and emerge as attractive therapeutic options for many devastating indications that currently have no cure in coming years. Although many of these are for orphan indications with small market sizes, the requirement for vector dosage would still be potentially high, especially for therapeutic indications where high vector doses based on per kilogram of body weight are required [4]. The increasing demand of vector dosage in clinic inevitably poses challenges for the current viral vector manufacturing capacity and critical issues related to vector safety profiles and manufacturability need to be adequately addressed before viral-based gene therapy is considered as a routine therapy. Given the diversity of pathologies that are potential targets of gene therapy and the heterogenic nature of viral vectors that are applied as gene delivery vehicles, it is unlikely that any

Table 1 Approved gene therapy products

Product	Type	Indication	Approved
Strimvelis	Viral ex-vivo: gammaretrovirus	ADA SCID	2016 – EU
Imlygic	Viral: oncolytic HSV-1	Inoperable melanoma	2015 – US
Kynamro	Non-viral: antisense oligonucleotide	Homozygous familial hypercholesterolemia	2013 – US
Glybera	Viral: AAV1	Familial lipoprotein lipase deficiency	2012 – EU
Macugen	Non-viral: RNA-aptamer	Wet age related macular degeneration	2004 – US, 2006 – EU
Gendicine	Viral: Ad5-p53	Head and neck cancer	2003 – China
Vitravene	Non-viral: RNA oligonuclotide	Retinitis by cytomegalovirus	1998 – US (withdrawn 2002)
Oncorine	Viral: oncolytic Ad5	Head and neck cancer	2005 – China
Neovasculgen	Non-viral: naked VEGF plasmid	Peripheral arterial disease	2011 – Russia

single viral vector is best suited for all indications [5] and therefore the development of many different types of vectors is justified and warranted. When choosing suitable viral vectors for intended therapeutic indications, a wide range of factors influence the choice and these include the inherent biological properties of the virus such as its cell and tissue tropism, transduction efficiency, ability to infect dividing or non-dividing cells, ability to integrate into host genomes and other features such as cloning capacity and vector productivity in cell culture systems. Although the earliest gene therapy trials predominantly used adenoviral and retroviral vectors, the emergence of the aforementioned serious adverse clinical events that are attributed to vector administration prompted the study of additional vehicles for gene delivery. Although more optimized adenovirus and retrovirus vectors with improved safety and efficacy features are still being developed for certain clinical applications, an increased proportion of the viral vector products currently entering the clinical development pipeline are based on adeno-associated virus (AAV) and lentivirus (Fig. 3). Other viral vectors, including herpes simplex virus, alphavirus and pox viruses, have also been used in the clinic. However, their usage as gene delivery vectors are more limited and many clinical successes observed with these vectors are in the oncolytic virotherapy field. Some of the key properties of the most commonly used vectors are summarized in Table 2 and manufacturing considerations for these vectors are discussed in more detail below.

The diversity of the different types of viral vectors, each with its own requirements for production, poses an operational challenge for manufacturers, especially for an organization such as a contract manufacturer, and demands inherent flexibility to accommodate the diversity in manufacturing needs. Despite the difference in vector biology, the common manufacturing feature that impacts the success of all viral gene therapy products is the need for reproducible manufacture of vectors in sufficient quantities and with titer and potency to support their clinical usage. This

Table 2 Key biological properties of common gene therapy vectors (modified from www.genetherapynet.com)

Vector	Adenovirus	Adeno-associated virus	Retrovirus	Lentivirus	HSV-1
Particle characteristics					
Genome	ds DNA	ss DNA	RNA	RNA	ds DNA
Diameter	70–100 nm	20–25 nm	80–120 nm	80–120 nm	150–200 nm
Protein capsid	Naked	Naked	Enveloped	Enveloped	Enveloped
Insert size limit	Generation: 1st: ~5 kb, 2nd: ~10–14 kb, 3rd: ~30 kb	~4.7 kb	7–10 kb	~10 kb	~40–50 kb
Gene therapy properties					
Ability to infect cells	Dividing and non-dividing	Dividing and non-dividing	Non-dividing	Dividing and non-dividing	Dividing and non-dividing
Interaction with host genome	Episomal	Episomal (>90%)	Integrating	Integrating	Episomal
Expression	Transient	Long lasting in non-dividing cells	Long lasting	Long lasting	Long lasting in non-dividing cells
Key advantage	High titer, efficient transduction	Broad tropism, low immunogenicity, long term expression in non-dividing cells	Integrating, for long term expression	Pseudo-typing generates broad tropism	Lytic/latent modes; broad tropism and strong neurotropism
Main limitation	Capsid mediates strong immune response	Small payload capacity	Insertional mutagenesis	Insertional mutagenesis	Fragile; requires aseptic processing

chapter introduces some of the key issues and considerations faced by a manufacturing organization serving this diverse, growing industry. The underlying biology and recombinant vector engineering is beyond the scope of this chapter, and the reader is directed to many excellent, recent reviews in the citations throughout the text [6]. Here, we provide a brief background relating to manufacturing considerations for some of the major classes of gene therapy viruses.

1.1 Adenovirus

The adenovirus viral genome contains two major transcription regions, early and late, each encoding essential viral functions such as DNA replication and viral

packaging. The biosafety of recombinant adenovirus vectors greatly improved with the generation of replication-defective adenovirus which are deleted for key early genes required for virulence, E1 and E4, yet still contain and express most other viral genes required for function. For first-generation adenoviral vectors, the E1 region is deleted to allow therapeutic transgene expression and often the non-essential E3 region is also deleted to accommodate larger gene inserts. Second-generation adenoviral vectors contain additional deletions in the E2 and E4 regions to permit further increase of transgene capacity to ~10–14 kb and reduce viral protein mediated host immune response. Production of both first-generation and second-generation helper-independent adenoviral vectors is typically straightforward and achieved by inoculum expansion of a complementing packaging cell line which is then infected with recombinant adenovirus seeds from a cGMP bank. Viral production is a terminal process, as adenovirus is a lytic virus. The cell lines most commonly used for first-generation vectors are human embryonic kidney 293 (HEK293) or human primary embryonic retinoblasts (PER.C6) [7] which contain stably integrated portions of the adenoviral early viral genome to provide the E1 gene products in *trans* and drive viral replication and packaging. For second-generation vectors, packaging cell lines that further complement other missing functions are used. Over the last two decades, significant progress has been made in adapting adenovirus production to suspension-based, serum-free, fully scalable systems and manufacturing on the 500 L scale and beyond has been successfully achieved [8]). For the production of replication-competent oncolytic adenovirus that does not require complementing E1 functions from the host, alternative cell lines such as HeLa and A549 cells have been used. A key advantage of adenovirus is that its relatively high virus productivity in culture systems and $>10^{13}$ vector particles per liter (vp/L) can typically be achieved for E1-deleted adenovirus [9]. This corresponds to significantly less than 1 L of production culture per dose for many somatic cell therapy, vaccine and oncology indications where doses often range from 10^{10} to 10^{12} vector particles [7].

Third-generation adenoviral vectors have been further engineered by removing nearly all viral genes to generate a helper-dependent (HDAd) or 'gutless' adenovirus. Gutless vectors show low or no immunogenicity, prolonged transgene expression and can accommodate a high payload capacity (up to ~30 kb in practice) making them an appealing delivery tool for transgenes with large coding regions [10, 11]. Manufacturing gutless adenovirus is complicated by the requirement for replication-competent Ad to provide deleted yet essential replication and packaging functions for the HDAd construct. The helper virus genome typically contains loxP sites flanking the viral packaging sequences such that conditional expression of Cre recombinase during helper-dependent adenovirus (HDAd) production excises the helper virus' packaging signal and allows the HDAd to be preferentially packaged. Although efficient for packaging, this method introduces a downstream processing challenge, as purification processes must discern and separate the HDAd product from residual helper.

1.2 Adeno-Associated Virus

AAV is a dependoparvovirus, first identified as a co-infecting agent in an adenovirus sample, requiring a helper virus such as HSV or Ad5 to replicate within host cells. It is structurally one of the simplest viral vectors for gene therapy and the AAV virion is a small, 20–25-nm, robust capsid composed of three related viral proteins VP1, VP2 and VP3, enclosing a single strand DNA genome. Similar to wild-type AAV, recombinant AAV vector generation in mammalian cells requires helper functions to promote vector production and packaging. Most of the early rAVV vectors used in the clinic were derived from AAV2, but many of the products currently under development use a wide range of human or primate capsids adapted or engineered for specific tissue tropism or to avoid pre-existing neutralizing antibodies present in the population [12, 13]. As an added safety feature to minimize wild type AAV production, wild-type *rep* and *cap* are provided in *trans*, and the vector genome harbors only the therapeutic gene and *cis*-acting inverted terminal repeats (ITRs) for replication and packaging during production.

Multiple manufacturing methods have been developed for rAAV vectors, and the variety of production systems and capsid variants available in some ways make this one of the more complex classes of virus for a CMO to manage. As each system has its own advantages and disadvantages, no industry consensus has surfaced. Three different mammalian production system are commonly used. A fourth system, including several variants, is based on baculovirus in insect cells.

1. Transient transfection of multiple plasmids into HEK293 cells containing an integrated copy of Ad5 E1a gene. The vector genome, transgene *rep*/*cap* and all of the Ad5 genes required for AAV production except E1a, are provided transiently at the time of production [14–17].
2. Herpes virus (HSV-1)-based systems rely on recombinant HSV to introduce both the AAV genome and *rep*/*cap* genes into mammalian cells such as BHK21 [18–20].
3. Producer cell lines are permissive cell lines such as HeLa or A549 that have been stably transfected with an integrated rAAV genome and the AAV *rep*/*cap* genes. Production is induced by infection with a helper virus, such as wildtype Ad5 [21–24].
4. Baculovirus-based systems re-engineer *rep*/*cap* genes to express the different *rep* and *cap* isoforms from baculovirus promoters, and co-introduce these along with the AAV vector genome into Sf9 insect cells, usually using multiple baculovirus constructs [25–29].

The upstream productivity of AAV systems has increased to the region of 10^{14} vector genomes per liter (vg/L) for many of these systems, but dose requirements for AAV gene therapy in some indications has increased faster to levels above 10^{15} vg, placing additional challenges and opportunities on production capabilities.

1.3 Gamma Retrovirus and Lentivirus

Gamma retroviral and lentiviral vectors are derived from related classes of enveloped retroviruses, and are both physically more fragile and less stable than either adenovirus or AAV. Although the genome organization, number of encoded proteins and virus biology differ between a gamma retrovirus (simple retrovirus) and lentivirus (complex retrovirus), they share many attributes. The exterior of these viruses consists of a lipid bilayer, derived from the membrane of the cell substrate used for production, in which envelope protein is embedded. Within the membrane are structural proteins derived from the *gag* gene, enzymes required for viral infection, reverse transcriptase and integrase derived from the *pol* gene and viral protease, along with two copies of the RNA genome. Multiple aspects of the assembled particle have been shown to contribute to viral instability, including envelope protein, reverse transcriptase, and membrane lipid composition [30–32]. The recombinant vector genome contains only *cis*-acting elements and the transgene expression cassette. The genes for *trans*-acting viral proteins are introduced separately into the production system, and current methodology separates the different genes across multiple plasmids to minimize risk of recombination which could generate a replication-competent species. For further safety, many vectors are based on a self-inactivating (SIN) design, with a deletion in the U3 region of the 3′ long terminal repeat (LTR). During reverse transcription in the target cell, this deletion is reproduced in the 5′ LTR, rendering the integrated provirus transcriptionally inactive [33]. An internal promoter is engineered into these constructs to drive expression of the transgene in the intended target cell type. Although any viral vector intended to be replication defective must be designed to minimize risk of generating replication-competent recombinants during production, the focus on this aspect of design and lot release testing is particularly acute for all classes of retroviral vectors.

Both gamma retroviral and lentiviral vectors may be produced either from stable producer cell lines or by transient transfection of multiple plasmids. As these classes of virus are amenable to pseudotyping to alter vector tropism, the production system often replaces the native envelope protein with one from a different virus [34, 35], which is incorporated into the lipid envelope as the vector buds from the surface of the cell into the culture medium [36]. Producer cell lines for gamma retroviruses with non-toxic envelope proteins share a similarity with typical monoclonal antibody production cell lines in that vector and budding is constitutive throughout cell expansion and production, allowing for the possibility of continuous manufacturing. Because of the toxicity of certain envelopes and lentivirus genes, producer cell lines are more challenging to develop for these vectors and are therefore less common. Although some stable producer cell lines have been successfully obtained by using inducible promoters [37–41], the most common method for lentivirus production is through transient transfection of multiple plasmids into adherent HEK293T cells [42].

For this class of therapeutic vector, comparing productivity in the literature can be challenging because of the lack of a universal method of product quantitation, with a prevalence of reports based on cell-based assays for transducing or infectious units (TU/IU), which are assay-dependent relative measures of infectious particles. However, the productivity of these vectors is typically lower than adenovirus or AAV and it is not uncommon for a dose used in a clinical trial to be produced from tens of liters of production culture or more [43].

1.4 Herpes and Other Large Enveloped Viruses

Herpes simplex virus (HSV) is a large, enveloped, double-stranded DNA virus and has multiple advantages for use as gene therapy vector as it displays a broad host cell range, is highly infectious, capable of infecting both dividing and non-dividing cells, and can achieve stable long-term transgene expression in neurons through establishment of latent infections. In addition, the HSV vector also has the unique ability to accommodate large foreign DNA fragments which could include multiple copies of the transgene because of its large genome size of ~152 kb [44, 45]. Both replication-defective and replication-competent HSV-based gene therapies have been developed.

The majority of the replication-defective vectors target neurological disorders in central and peripheral nervous systems such as chronic pain, epilepsy, multiple sclerosis, and Parkinson's disease. Predominantly, replication-competent or attenuated HSV vectors have been developed as an oncolytic virus for cancer treatment. The recently approved Talimogene laherparepvec (T-VEC) for the treatment of melanoma is one example of an attenuated HSV-1 vector engineered to destroy cancer cells selectively and to secrete GM-CSF to enhance anti-tumor immune response further.

Similar to oncolytic HSV, many other large enveloped DNA or RNA virus vectors, such as vaccinia virus, vesicular stomatitis virus, measles virus, poliovirus, and Newcastle disease virus, have also been developed in recent years as oncolytic viruses with or without genetic modifications and demonstrated meaningful clinical outcomes in multiple trials [46].

Despite the clinical appeal and recent advance of applying HSV and other large enveloped vectors, either as gene transfer vectors or oncolytic virus therapy, development of large-scale manufacturing for these types of virus is lagging because of their technically challenging nature. Based on the availability of the complementing cell lines, upstream production typically relies on an adherent-based system using Vero, HeLa, HEK293, or MRC-5 cells, which are inherently difficult to scale-up. Furthermore, the sensitivity of large virus vectors to common in vitro inactivation condition often results in inconsistent batch-to-batch yield or low viral productivity per cell. In addition, large enveloped viruses are often fragile and require processing in gentle conditions in order to minimize shear stress, shorten processing time, and limit exposure to high-salt or low pH buffer

compositions, further introducing manufacturing limitations in both upstream and downstream unit operations [47]. Furthermore, most large enveloped viruses have large particle sizes that would be retained by 0.2-μm filters if subjected to a terminal sterilization filtration step during manufacture. Specific requirements of either aseptic processing in grade B cleanroom environment or in completely closed systems or implementation of specific testing strategies for in-process intermediates or final products sterility need to be considered in order to satisfy GMP requirements of these products.

2 Challenges Facing Viral Vector Manufacturing

Although the success of gene therapy relies on the safe and effective delivery of genetic material to target cells, whether in vivo or ex vivo, the commercial feasibility of implementation of these therapies relies on development of robust, scalable, and cost-effective manufacturing. Early stage clinical development tends to utilize scaled-up laboratory processes to accelerate to first-in-human studies until an established commercial platform process can be leveraged for subsequent programs. The gene therapy field is only just approaching approval of products, and thus most products in the development pipeline are still transitioning to optimized scalable methods. As clinical trials progress into late stage clinical evaluation and as companies explore large market indications or target indications needing even higher doses, manufacturers are challenged to produce sufficient product to supply a trial or meet projected commercial demand.

To address the need for clinical and commercial vector supply, the gene therapy field has adopted methods borrowed from more mature manufacturing such as monoclonal antibodies, recombinant proteins, and small molecules, where the principles and practice of scale-up are well-established. As most viral vector manufacturing is performed in mammalian or insect cell hosts, the fundamental principles of cGMP manufacturing for biologics, biosafety, and adventitious agent control used for CHO-based processes hold true. However, large-scale production challenges unique to viral vector manufacture remain because less mature manufacturing processes tend to be cumbersome, expensive, and inefficient as compared to recombinant protein platforms, which can result in high lot-to-lot variability. Of note, gene therapy products may require additional measures or pose special challenges for implementation. These include the use of complex and custom biologic raw materials such as DNA plasmids for transfection or master and working viral banks for infection which must be managed as part of a product campaign and meet GMP standards and product quality control, adding to the complexity, time, and costs associated with therapeutic product manufacture. In general, raw material control is required for critical and high-risk raw materials and poor understanding of raw materials causes decreased production yields, variable product quality, and increased cost of goods. Significant technology and process development effort has made production systems scalable, but the ability to scale

the manufacture of biologic raw materials is equally essential. In the case of AAV, the producer cell line approach discussed in the case study at the end of this chapter has enabled scale-up to 2,000 L.

An additional unique challenge faced by the gene therapy products uncommon in recombinant protein production is the enhanced need for viral containment in a manufacturing facility. The majority of viral vectors and cell lines used in gene therapy require the use of practices and procedures for biological agent containment that meet Biosafety Level 2 (BSL2), or in rare cases enhanced BSL2+. To determine the appropriate biosafety level, the critical first step is a risk assessment process that takes into account risk group classification, mode of transmission, viral virulence, titers and concentrations, large volumes, and type of recombinant transgene, among others. Biosafety level can significantly impact facility design elements such as directional airflow and waste decontamination methods.

From a facility perspective, viral manufacturing challenges require flexibility at the operational level, especially in contract manufacturing organizations (CMOs) where multi-product production is required. With the various viral vector platforms and the multitude of approaches for producing GMP-grade product, a facility needs to be adaptable to accomodate client to client production process requirements. At times this demands rapid and complete replacement of equipment to an entirely different production method, with associated line clearance. Additionally, as the cross contamination risk is greater for viral products because of the transmissibility of viruses, methods to contain products within the production area are essential, and greater assurances are required to ensure there is no product carryover on equipment or suites, requiring extensive cleaning validation studies. As the vast majority of products are in the clinical development stage, cleaning validation may not be justifiable for a batch or two of product and equipment may be used only once.

To address this and other challenges posed by the heterogeneity of the field and the need for containment, the adoption of single-use disposables has brought significant savings in terms of capital expenditure and product changeover time, translating to increased productivity, efficiency, and flexibility for manufacturers. Single-use equipment has also increased flexibility for contract manufacturers to work with many clients with different processing requirements because of the shorter lead times for equipment and ancillary supply procurement. By virtually removing the need for clean-in-place validation, steam-in-place sterilization, and significantly reducing the additional use of water and caustics, manufacturing flexibility has increased, essentially eliminating cross contamination occurrences. These are critical operating conditions for a multi-product clinical facility, where batch size, production, and purification methods vary widely between products, and rapid turn-around time provides operational and economic advantages [48]. As single-use equipment, such as a bioreactor or holding vessel, is typically not hard plumbed or permanently connected, even a 2,000-L vessel is reasonably mobile and can be transported in and out of a cleanroom to accommodate process equipment requirements for an upcoming production batch. With the option of custom design bioprocess bags, a bioreactor shell can be transformed for an entirely different production process in a relatively short period of time and lesser expense compared

to fabrication of stainless steel vessel. Currently, single-use alternatives are available for the majority of unit operations, from media and buffer preparation to final product fill, as integrated process solutions such as bioprocess containers, bioreactors, filtration components, chromatography skid flow paths, connections systems, and even single-use, pre-packed chromatography columns [49]. Integrated implementation of single-use solutions across unit operations is particularly critical for the gene therapy industry where single-use bioprocessing equipment may not be optional but rather required by many multi-product viral facilities and the evolving regulatory science.

In the following sections we provide an overview of the most common upstream and downstream solutions in use for gene therapy manufacturing and present a case study of the largest industry-scale production of AAV gene therapy products.

3 Upstream Production of Viral Vectors

Viral gene therapy cell culture production processes have borrowed from – and evolved essentially in parallel to – cell culture processes utilized for decades in the recombinant protein industry. However, because of the diverse landscape of viral vectors systems and therapeutic indications, the industry has not coalesced into a dominant viral production method, as it has for the protein industry, where production using stable cell lines such as Chinese Hamster Ovary cells (CHO) in stirred tank bioreactors is common. Although all upstream viral vector processes start with the thaw of a cell bank typically sourced from mammalian or insect cells, and end with harvesting for downstream production, essentially all cell culture formats and expression systems are utilized (Fig. 4). The differences between adherent- and suspension-based cell culture systems are some of the most fundamental from an upstream unit operations perspective, requiring different equipment and operational know-how. Either cell culture platform can support several methods of viral production, which is dictated in turn by the biology of the chosen production system (i.e., transfection- vs infection-based production), each requiring different types of raw materials (plasmids vs viral stock).

We introduce below the most common methods for viral vector production, and highlight key advantages and challenges faced by each.

3.1 Cell Culture Systems

Most mammalian cells are anchorage-dependent. In the field of recombinant protein production these have been readily adapted to growth in suspension. Many of the processes in viral gene therapy were originally developed for adherent cells cultured in flasks using transient transfection processes. Because these early methods have been challenging to transition to suspension-based culture, adherent

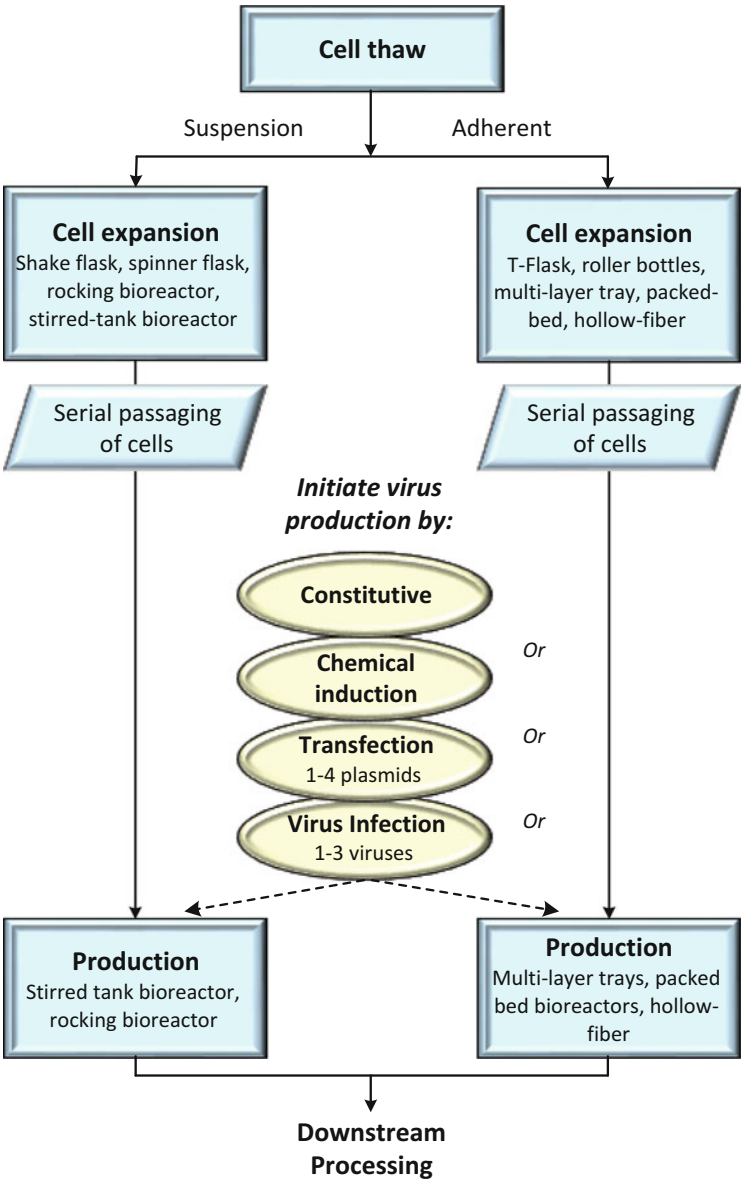


Fig. 4 Generalized example cell culture process flow for viral gene therapy products

systems are still widely prevalent in both early and late clinical scale manufacturing. Scalability of adherent cells is limited by the surface area available and adherent cell culture in conventional flasks is often labor-intensive and cumbersome, requiring many open processing steps. Roller bottles have been effectively used for adherent cell transfection, with robotic-assist devices used to scale-out

labor-intensive manipulation of many bottles, such as RollerCell40 processing systems. However, this is a large capital expense, particularly for facilities which are not dedicated to production by this method.

To address the need for less-capital-intensive adherent scale-up solutions, industry developed flat multi-layered disposable cell culture systems such as Nunc™ Cell Factories™ or Corning CellSTACK® which have served as a workhorse in transient transfection processes. Currently, several gene therapy products in phase I/II employ transient transfection in multilayered systems as a more rapid and cost-effective method of drug product manufacture. However, open steps are still needed and, because of the weight and size of the system, manually operated support equipment is needed to manipulate the larger-scale units (40-stack), requiring several operators even for simple operations such as media change. In addition to personnel constraints, scale-out of an adherent process using multi-layered systems is also limited by available incubator space. Because of the large footprint incubators occupy in a cleanroom suite, where space is at a premium, many facilities contain at most a few incubators. To reduce incubator space requirements, technology has made progress and systems such as Corning HYPERStack have been developed to maximize the cell growth surface area per incubator volume by eliminating the gas headspace requirement of adherent cell culture systems. Oxygen is provided to cells through a gas-permeable membrane which also serves as the substrate for cell attachment, essentially providing more than twice the surface area of a typical 10-stack system in the same volumetric footprint. For any of these systems, a challenge of scale-out across multiple vessels is the increased risk of contamination caused by the increased number of manipulations, particularly for open steps. Although adventitious agent contamination is a concern in production of all biologics, risk of contamination with viral agents must be managed even more closely for viral gene therapy products, as many have limited opportunity to incorporate general viral clearance mechanisms during purification. Manufacturers of scalable flat stock production vessels have responded to this risk by offering single-use vessels that can be operated as a closed system using sterile tube welders rapidly and efficiently. For even greater cell number and process parameter control, adherent packed bioreactors, which provide high cell density and active pH and oxygen control in a perfusion culture format, have been adapted to gene therapy manufacturing. The integrated iCELLis® bioreactor has emerged as a scalable single-use platform for packed-bed production of viral vectors, with manufacturing scale surface areas of up to 500 m^2, and a 125× scale-down model available for development. Studies have shown that process-critical plasmid transfection using the polyethyleneimine (PEI) method works efficiently in this system, where total viral yield exceeded production in standard multilayer vessels [16, 50]. Early in the development of gene therapy manufacturing, traditional packed-bed bioreactors were used. The world's first marketed gene therapy product, Gendicine (Shenzhen SiBiono Genetech Co, Shenzhen, China), is manufactured in a packed-bed bioreactor using Fibra-Cell disks [51], a cell-attachment matrix constructed of polyester and polypropylene. Another viable option to scale adherent cells is the use of microcarriers as cell substrates, allowing for the use of suspension vessels and

conditions. For example, Fibra-Cell disks have also been successfully used in a single-use Wave bioreactor to produce lentivirus by transient transfection of HEK293T cells [52] and Cytodex microcarriers have been used to produce vectors derived from canine adenovirus in MDCK cells [53].

Despite the advancement made with adherent systems, the ultimate objective for scale-up of most viral production systems is a single-cell suspension process, which is generally easier to expand through the seed train, monitor for cell growth and viability, and adapt to animal derived component free (ADCF), chemically defined media. Adenoviral vectors were one of the first classes of viral vector to progress to production in suspension, as the virus is biologically able to infect cells in suspension, does not require cell-cell contact for transmission, and multiple E1-complementing cell lines used as hosts such as HEK293 and PERC-6 were adapted early on to suspension serum-free conditions. The earliest single-use bioreactor design based on wave-like motion in bioprocessing bags was adopted by the Ad5 vector manufacturers more than two decades ago and is still commonly used today, typically up to 100-L scale, although up to 500-L scale is also currently possible. Single-use stirred tank bioreactors have recently become more prevalent, with capacities up to 2,000 L commercially available. Some of the reasons for the transition from rocking motion bioreactors to stirred tank systems include general applicability of well-developed scale-up methodology from fixed stirred tank bioreactors, as well as the increasing availability of small footprint bench top bioreactors for process development.

The next hurdle being addressed to optimize manufacturing processes is production at high cell density in single-cell suspension cultures. This has long been a challenge for adenoviral vectors, where a significant drop in specific productivity is seen when infecting over ~10^6 cells/mL in batch culture with HEK293 and PER.C6 cells [54]. Recently, a new amniocyte-derived cell line has been reported to have less loss in specific productivity up to almost 10^7 cells/mL [55]. Of note, baculovirus-based systems in insect cells represent one area where higher density is more common, such as production of AAV at approximately 5×10^6 cells/mL [56].

3.2 Virus Production Systems

3.2.1 Producer Cell Lines for Retro and Lentiviral Vectors

Operationally, one of the simplest methods of virus production is constitutive production from producer cell line (Fig. 5). This approach mirrors CHO-based systems for secreted proteins, as the only biologic bank required for the production system is a cGMP cell bank. Gamma retroviruses are the only common type of gene therapy vector for which this method is used, as stable cell lines can be made in a straightforward manner when the vector contains a non-toxic envelope protein such

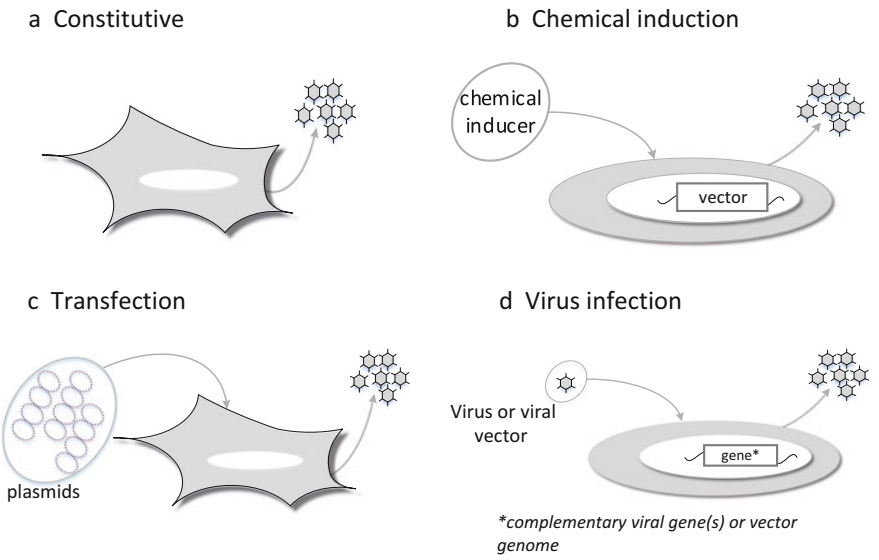


Fig. 5 Approaches used for upstream production of viral gene therapy products

as from murine leukemia virus (MLV). The virus buds continuously from the cell surface into culture medium, ready to be harvested.

Packaging and producer cell lines are less simple for lentiviral vectors because of the toxicity of components such as viral protease and commonly used envelope proteins such as VSV-G [35], which lead to cell line instability [37, 38]. For this reason, expressions of the toxic lentivirus genes are placed under the control of regulated promoters, such as tetracycline inducible promoters alone or in combination with a cumate switch [35]. Although the positive Tet-on system is simpler logistically to induce, requiring addition of doxycycline to trigger production, cell lines are less stable than the more tightly regulated Tet-off system, where removal of doxycycline is required to induce production, posing a notable challenge for large-scale operations. It is anticipated that once ongoing efforts reliably overcome the hurdles of generating a high producing stable cell line [40, 43], this approach can result in a scalable lentiviral production platform.

3.2.2 Transient Transfection of Plasmid DNA

Production by transient transfection of plasmid DNA is still one of the most widely used methods to generate pre-clinical and clinical supplies of many viral vectors: AAV, lentivirus, and retroviruses with toxic envelope proteins [50, 57–59]. One advantage of this approach is the rapid turnaround time between vector design and production, which can be on the order of days to weeks for smaller scale preclinical research purposes. Plasmid production and lot release to supply even early phase

clinical cGMP manufacturing can still be on the order of only 3–4 months, with optimal scheduling at third-party vendors, compared to the complex process of creating a high producing stable cell lines for cGMP banking, which typically takes greater than 6 months [43, 57]. As all components of the vector and packaging elements are introduced into the cell only at time of virus production, there is no concern of selective disadvantage produced by toxicity from any of these components.

Typically, three to five different plasmids are used to supply all components necessary for vector production, maintaining plasmid size small enough to be produced as raw materials with reasonable yield. Thus, a key consideration of this production system is the need to obtain custom high quality DNA as part of the preparation for manufacturing. Typical transfection requirements are on the order of up to a few milligrams of plasmid per liter of production culture, and a gram or more of purified plasmid may be needed to supply early stage clinical manufacturing campaigns consisting of multiple 100-L scale viral vector production lots. Plasmid supply is one of the considerations for practical limits of scalability of transient transfection for commercial manufacturing, although efforts are ongoing to maximize efficiency of plasmid utilization [15]. Additionally, a consequence of the high levels of plasmid used in this production system is that it becomes a significant process impurity which must be effectively degraded and removed by downstream operations.

Multiple methods have been used to introduce plasmid DNA into cells, including precipitation with calcium phosphate, polyethyleneimine (PEI), cationic lipids, and electroporation; the first two are mostly used for large-scale viral vector production [58, 60]. Calcium phosphate precipitation is low cost and has been successfully and reliably used for high specific productivity of AAV, but it can be challenging to implement consistently without very strict control because it is sensitive to many factors, including pH, temperature, method of mixing, and reaction time [35, 58, 61]. Also, because of the toxicity of calcium phosphate complex, a medium exchange is necessary after transfection to maintain viability of the production cells, adding to processing complexity. PEI, on the other hand, has gained popularity as a transfection reagent because the functional formation of the DNA complex is less sensitive to environmental factors and manipulations may be less complex than with calcium phosphate. Additionally, no medium exchange is required following PEI transfection [59, 62]. Transfection by PEI is reported be equivalent to calcium phosphate in terms of efficiency and productivity, and it is currently the predominant reagent used for suspension transfection [35, 57, 63]. Recently, flow electroporation methods have also been shown to be comparable to calcium phosphate and feasible for a scalable suspension culture process [64].

To achieve high productivity of a viral vector, both an optimized process and a highly transfectable host cell are required. The most commonly used cells are isolates of HEK293T cells, which express the SV40 large T antigen [65, 66]. Most transient transfection processes are based on adherent cell culture systems [57] as lower productivity is often observed in suspension transfection systems [16]. However, significant progress has been made in transfecting serum-free suspension cultures,

and a recent report using a PEI reagent has demonstrated specific productivity for AAV greater than 1×10^5 vg/cell and greater than 1×10^{14} vg/L at harvest [15], which is comparable to the best reported adherent production. Much progress has been made in optimizing production of viral vectors by transient transfection, and the method has been successfully used to supply many early stage clinical trials. However, as product requirements for clinical trials continue to escalate, manufacturing sufficient quantities of gene therapy vector by plasmid-based methods continues to be a challenge.

3.2.3 Virus-Based Production Systems

A number of clinical and commercial production systems use virus seeds as raw materials in the production of the therapeutic vector, but the production mechanism varies widely. Certain common features are shared, such as the need to produce master and working virus banks as critical raw materials, and the terminal nature of production, by virtue of the viral infection of the culture.

The simplest use of a virus seed is for expansion of the therapeutic viral vector itself, whether by virtue of being a replication-competent virus or a vector that lacks genetic elements required for replication that can be provided by a complementing cell line. A typical example is recombinant E1-deleted adenoviral vectors. Careful design of the viral vector and matching complementing cell line is critical for minimizing the risk of recombination during virus production to generate replication-competent revertants, which must be strictly avoided and was one of the reasons for development of the PER.C6 cell line [67].

Other production methods have used one type of virus as a raw material to produce a different type of viral vector as the therapeutic product. The underlying rationale behind using virus as a raw material varies, and motives may include the efficiency in which raw material viruses deliver genetic material to cells for transient production systems, the relative speed at which recombinant viruses can be generated compared to stable cell lines, or the need to provide helper functions for some production systems. For example, the use of multiple baculoviruses in place of plasmids for the transient production of lentiviral vectors has been described [68]. The baculovirus raw materials are expanded by simple infection of insect cells and used to co-transduce HEK293 cells to produce lentivirus with a human cell-derived lipid envelope. No baculovirus byproduct is generated because of its inability to replicate in mammalian cells. However, clearance of residual input baculovirus by the downstream process is a consideration, requiring methods that would not also harm the fragile lentiviral vector product.

Furthermore, three of the common methods for AAV production are also based on using other virus types as raw materials: recombinant herpes simplex virus (HSV), baculovirus, or adenovirus-based systems. In the HSV system, separate recombinant HSV constructs deliver AAV vector genome and *rep*/*cap* packaging components to a mammalian cell, as well as providing necessary helper functions for AAV replication [20]. AAV production with recombinant baculovirus is

performed in insect cells, with the AAV genes re-engineered in various ways to express the overlapping isoforms of *rep*/*cap* using baculovirus regulatory elements [25, 26, 69]. Many variations are currently in use, with the most established methods typically using two or three baculovirus constructs to introduce all AAV vector components to naïve SF9 cells in serum-free suspension culture [59, 68–70]. Glybera, an AAV1 vector developed by uniQure biopharma B.V. (Amsterdam, The Netherlands) for the treatment of familial lipoprotein lipase deficiency (LPLD) and the first approved viral gene therapy product in the western world, is manufactured using a baculovirus-based production method [71]. The baculovirus constructs do replicate along with AAV production in these established systems, but a recent modification has been developed to reduce levels of baculovirus byproduct that must be cleared from the AAV harvest by downstream processing. In this system, the recombinant baculovirus requires a complementing cell line for production of the raw material, and these complementing factors are not present in cells used to produce AAV. Another variant of the baculovirus-based AAV production system currently in development is based on a stable capsid-specific host cell with integrated *rep* and *cap* constructs under control of baculovirus promoters. Production is induced by infection with a single recombinant baculovirus that also introduces the product-specific vector genome [27, 69]. As with all baculovirus-based AAV production systems, the correct ratio of the three VP1, VP2, VP3 capsid proteins required for optimal virus infectivity can be difficult to achieve, but the system holds promise for rapid, flexible production of AAV without concerns for the stoichiometry of co-infecting multiple independent viruses in a cell.

The main challenges for both baculovirus- and HSV-based AAV production systems are twofold: the high virus volume required for large-scale manufacturing and the need for downstream operations to clear the virus byproduct. Production and storage of these somewhat fragile enveloped virus raw materials can become a primary consideration in a manufacturing campaign and these viruses are often produced only shortly before AAV production. An interesting alternative to the baculovirus system, however, is the titerless infected cell (TIPS) method for production of AAV. This system involves using cryopreserved concentrated baculovirus-infected insect cells (BIICS) in place of baculovirus seed stocks. BIICS are prepared for each baculovirus construct and are added together to uninfected insect cells in the production bioreactor during the cell expansion phase at a ratio of approximately 10^{-4} BIIC:producer cells [56]. The baculovirus within the BIICS continue through the replication process and uninfected producer cells continue to propagate until the baculovirus completely infects all cells after several rounds of amplification. The advantage of this system is the elimination of producing, testing, and releasing seed stocks of baculovirus inoculum just prior to AAV production. Enough BIICS for a number of lots of AAV production can be produced in advance and stored in a manageable volume for an extended period in the vapor phase of liquid nitrogen without degradation. For raw material clearance after harvest, a simple detergent-based methods can be used as an inactivation step without adversely affecting the rugged, non-enveloped AAV product [19, 72], as both the HSV and baculovirus are fragile enveloped viruses susceptible to lysis.

A readily scalable virus-based approach to AAV production relies on helper virus to induce production from a stable recombinant mammalian cell line. Two variations of this approach are based on either a producer cell line or a packaging cell line, both of which use adenovirus as the helper virus. In an AAV producer cell line, both the vector genome and the AAV packaging genes are stably but quiescently integrated into the host cell, until vector production is induced by infection with a helper virus [22]. AAV producer cell lines have been successfully developed with both HeLa and A549 as the cell substrate [21–24] and with HEK 293 cells when the *rep* gene is very tightly regulated [73]. The packaging cell variation of this system only contains the AAV *rep/cap* genes and a recombinant E1-deleted adenovirus brings in the AAV genome; co-infection with wild type adenovirus is also performed to provide the required E1a helper gene [74]. A key benefit of the producer cell line approach is the relative simplicity of inducing production at large scale with a virus raw material that is stable, and easy to produce and store. Although detergent-based methods cannot be used as the inactivation step for viral clearance with the non-enveloped process-relevant virus, heating is a simple and robust method for adenovirus inactivation based its lower thermostability than AAV [75].

As HeLa producer cell lines adapt well to suspension, serum-free conditions, scale-up, and seed trains in bioreactors can closely mimic the processes utilized in the protein industry. Successful scale-up of a suspension-based producer cell clone to 250 L in single-use bioreactors has been reported in the literature [21] and the case study presented at the end of this chapter describes in more detail a producer cell line process scaled to 2,000 L in single-use bioreactors at Lonza Viral Therapeutics in Houston, TX. A significant consideration for this approach, however, is that wild type adenovirus is a replication-competent human pathogen, which impacts process, facility, and testing strategy and design. The methods by which viral safety for an adenovirus-based production system have been addressed in large-scale cGMP manufacturing are discussed as part of the case study.

4 Downstream Unit Operations

The objectives of recovery and purification operations are generally the same for viral gene therapy vectors as for recombinant proteins produced in mammalian cell culture – removal of process-related impurities, product concentration to targets intended for dose administration, consistent product quality and characteristics, and high recovery. Although there are examples of clinical ex vivo cell therapy applications with fragile gamma retroviruses where crude harvest is used directly to transduce target cells, and the only significant downstream goal for is removal of the cells used for production [76, 77], this is not the standard for most gene therapy. Even for ex vivo cell therapy, most lentiviruses produced are purified to both concentrate vector and remove cellular components that may inhibit transduction

[30, 78, 79], and regulatory agencies generally expect therapeutic viral vectors to be purified [80].

4.1 General Considerations for Developing Purification Processes for Viral Vectors

Operational solutions for clinical viral vector purification processes can often be found in existing pilot-scale protein manufacturing equipment and technology, as typical gene therapy manufacturing scales in clinical development are tens to hundreds of liters, with the 2,000-L case study below the largest to date. However, viral vectors have additional constraints or challenges for downstream process design as compared to most traditional biologics, for example because of large particle size, temperature or shear sensitivity, low total mass of product relative to process impurities, and traits which can be confounding for typical adventitious agent control strategies. Methodologies and equipment must also be compatible with often harsh disinfection procedures and rigorous containment and cross-contamination prevention measures. As with upstream manufacturing, a wide range of downstream purification solutions are in use across the industry, which is an additional challenge for manufacturing facilities such as CMO which serve a broad cross section of the gene therapy field.

Purification challenges specific to virus size are more significant for very large viruses, such as HSV and poxviruses (~0.2 μm diameter) which cannot pass through sterilizing grade filters and thus may require completely aseptic processing [81]. Retrovirus, lentivirus (~0.1 μm diameter), and baculovirus (rod-shaped) can be sterile filtered, but are prone to high product losses at such filtration steps [82–84]. Thus 0.2-μm filtration of these viral vectors may be limited only to final drug product, requiring other methods of bioburden control during drug substance manufacturing. Although smaller non-enveloped viruses, such as AAV (~20–25 nm diameter) and adenovirus (~90 nm), are routinely sterile filtered [9, 59], particle sizes are still sufficiently large to limit diffusion into pores of most traditional chromatography media, reducing binding capacity relative to typical protein processes. To address this challenge, membrane and monolith-based chromatographic methods are commonly used for virus purification [85–88]. Large and complex virus particles can also be sensitive to operational stresses. For example, the half-life of retroviruses is only hours at 37°C and 1–2 days at ambient temperatures [30, 89, 90], requiring rapid downstream processing. Shear can also be a concern, and hollow-fiber filters may in some instances be preferred over flat sheet filters for this reason during ultrafiltration and/or diafiltration (UFDF) [91] operations. Retroviruses and lentiviruses are also particularly sensitive to pH extremes and high salt concentrations. Process buffers for these viruses all tend to be close to pH 7 and a rapid dilution step is often included after high salt elutions [35, 76].

Many types of viral vectors can incorporate cellular components within the viral particle itself, such as cellular DNA within an AAV capsid [92–94] or cellular

proteins in retroviruses [95, 96]. This defines intrinsic limits to clearances that can be achieved for such process impurities and poses analytic challenges for developing downstream processes where maximizing clearance of unincorporated impurities is desired and mandated by regulatory guidelines. Developing purification processes to remove product variants can be particularly challenging, as virus particles are complex structures consisting of protein, nucleic acids, and, in the case of enveloped viruses, lipid membranes with intrinsic heterogeneity. Variants without the interior nucleic acid component, such as "empty" AAV and adenovirus capsids differ in density from the intended product, but differences that can be exploited for chromatographic separation are subtle [97–99].

Concentration of viral products is also often very low on a mass basis compared to recombinant proteins (e.g., micrograms per milliliter), which can result in losses to non-specific adsorption at high purity [14], and limit options for analytic tools that are routine for protein process development. Quantitation is usually performed by methods with greater inherent variability and lower throughput than direct physical measurements, such as qPCR to measure encapsidated vector genomes, ELISA for capsid proteins, or by cell-based infectivity assays [79, 100, 101]. This imprecision can be challenging for evaluating differences in yield, particularly in process optimization experiments when individual variables may have only modest effects. Although UV absorbance can be used for quantitation of AAV and adenoviruses [102, 103], this measurement is typically performed only near the end of purification when the virus particles are at close to maximum achievable concentration and purity, and it is a destructive process using SDS to reduce artifacts of light scattering by intact capsids.

A general consideration for process design is compatibility with systems for waste stream decontamination, which must be particularly robust for virus production facilities. Sodium hypochlorite (bleach) rapidly and effectively inactivates a wide range of viruses [104] and is thus widely used from development labs to large-scale liquid waste treatment tanks for cGMP manufacturing facilities. An example of a chemical used in many protein purification processes that is not compatible with this type of waste treatment system is ammonium sulfate. Besides generating noxious fumes, explosions can result from mixing ammonium sulfate and hypochlorite [105]. Although viral manufacturing facilities using hypochlorite-based waste treatment tanks should clearly avoid ammonium sulfate, it is generally advisable to design processes that account for the prevalent use of bleach to decontaminate spills or development lab waste streams in any virus facility.

4.2 Downstream Unit Operations

Downstream processes vary significantly across the gene therapy industry. However, for clinical manufacturing focused on scalable methodologies, process flow is often some variant of the following (Fig. 6): harvest clarification, nuclease digestion of free DNA, and concentration with optional buffer exchange, followed by

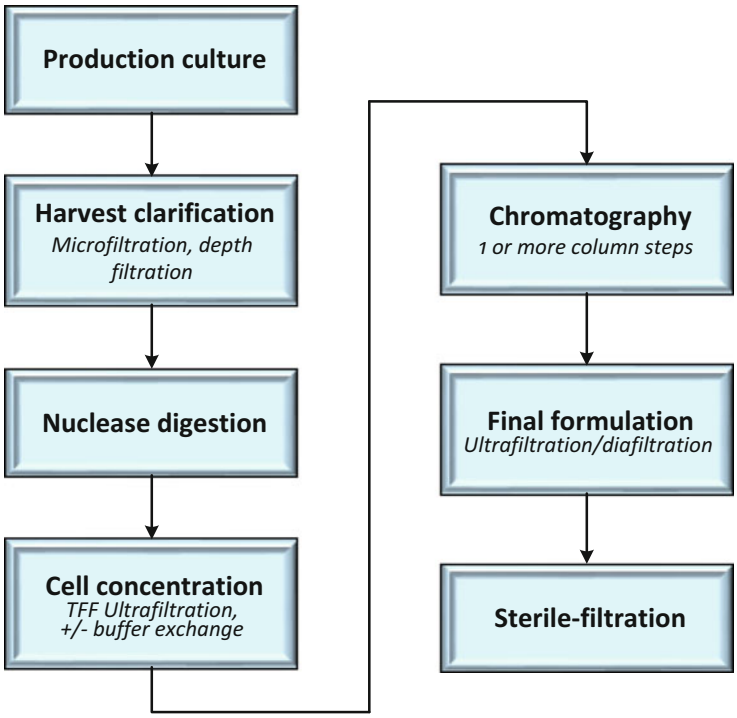


Fig. 6 Generalized example purification process flow for viral gene therapy products

one or more chromatography steps, final buffer exchange step, and sterile filtration [106, 107].

Harvest operations are influenced by a number of fundamental factors, including the size and type of the production bioreactor, final cell density and viability, whether the production is performed in suspension or adherent culture, and whether viral particles are cell associated or are released into culture supernatant fluid. Gene therapy recovery methods typically do not yet have to address the same challenges of high biomass as recombinant proteins. Although high density production systems approaching or exceeding 10^7 cells/mL are being developed for mammalian cell cultures for some vaccines [9, 108] as well as for baculovirus-based production systems in insect cells [56], in general production cell densities for gene therapy manufacturing are closer to 10^6 cells/mL. Non-enveloped viruses, such as adenoviruses and AAV, are often cell associated, and recovery may require cell lysis. Mechanical methods of cell disruption such as microfluidization have been used successfully, as well as detergent lysis, and autolysis by the virus itself [99, 107]. The pattern of vector partitioning between cell and supernatant fractions can vary based on details of the production system, even for a given virus. For example, AAV has been recovered from culture medium, lysed cell pellets, or in combination because of the presence of product in both cell and supernatant fractions [15, 56, 109]. Enveloped

viruses which bud from the surface of cells such as lenti- and retroviruses are collected directly in culture supernatant [30, 76]. Clarification is commonly performed by single-use filtration systems, including depth filters or with a series of membrane filters to minimize risk of filter fouling, which can reduce recovery of lenti- and retroviral vectors [35, 79, 107, 110, 111]. Although centrifugation is performed at smaller scales, continuous centrifugation methods are not yet essential for cell densities and production volumes in most gene therapy processes. Thus the capital equipment required for large-scale centrifugation operations and process development is not commonly available in manufacturing facilities serving this industry [107].

A step frequently associated with harvest in viral purification processes is nuclease digestion [106, 107]. For processes where cells are lysed, particularly with detergents, DNA digestion is performed early in the harvest where it reduces viscosity and improves recovery in clarification [112]. It is often a primary method for reducing levels of plasmid carryover from transient transfection production methods and achieving host cell DNA clearance to regulatory targets of <10 ng/dose. For vaccine indications, reduction of residual host cell DNA size reduction to <200 bp is also a specific goal to meet regulatory expectations [113]. In those processes where nuclease digestion is incorporated solely to meet DNA clearance objectives, the step is often positioned downstream after partial purification and volume reduction for cost savings by reducing the total amount of enzyme required [83]. Further efficiency can be achieved through optimized buffer conditions, such as low salt concentration with Benzonase®, the most commonly used industrial nuclease, which is inhibited by monovalent captions. The DNA digestion step has also been performed with Pulmozyme, used for treatment of cystic fibrosis [114], and a number of other industrial nucleases have recently become available after the Benzonase® patent expiration[107].

Concentration steps in current scalable manufacturing processes are typically performed by ultrafiltration using tangential flow methods. Molecular weight cutoff is determined by the virus, with 100–500 kDa being common [78, 107]. At small scale, high speed centrifugation is still often used for concentration, particularly for retroviruses [30]. Precipitation methods to allow concentration by low speed centrifugation have also been developed and can be scaled up [78] but this approach requires capital investment in suitable large-scale equipment, and would typically have the constraint of single-use product-contact surfaces in multiproduct CMO facilities. Methods that precipitate viruses with ammonium sulfate [115] have the additional caveat of being chemically incompatible with sodium hypochlorite.

Historically, purification of viral vectors has exploited properties of large particle size and high density because of nucleic acid content. Size exclusion chromatography and ultracentrifugation with many types of density gradients are routinely used in research settings, as well as cGMP operations for smaller clinical trials [78, 116]. In general, these methods are not considered scalable, and as gene therapy field has advanced to later stage development and higher dose products, more emphasis has been given to designing processes based on unit operations that have been well-established in the manufacture of recombinant proteins. However,

advances have been made, particularly in scalable size-based methods of separation to take advantage of this distinguishing product attribute. For example, chromatography media with an inert porous shell and inner core capable of high capacity protein binding allow very large virus particles to be purified in flow-through mode based on size exclusion principles. Viral particles are excluded from the matrix and protein impurities bind and are retained in the particle core [53, 117, 118]. Steric exclusion chromatography uses polyethylene glycol (PEG) to provide a size-based advantage for binding to hydrophilic chromatography media [119]. Additionally, traditional SEC chromatography can be reasonably efficient for large-scale processing with up to 0.2–0.3 CV load volumes when separating viruses that elute in the void volume from low molecular weight retained impurities. For example, effective purification of adenovirus was achieved with high loads in a quasi-continuous SEC process [88, 120].

Most other common chromatography techniques used for protein purification, including ion exchange, affinity, and mixed mode and hydrophobic interaction, have also been effectively applied to virus purification [107]. Unsurprisingly, ion exchange has long been a workhorse for much virus purification, as anion exchange flow-through methods have historically been used for viral clearance in recombinant protein processes because of the tight binding of many types of viruses [121]. Examples of purification schemes that include anion exchange steps exist for most classes of gene therapy viral vectors and anion exchange is the one chromatographic method shown to be capable of separating vector-genome containing AAV capsids from those devoid of DNA [97, 98]. Many viral vectors require high salt for elution from anion exchangers, which can be a risk for inactivating retroviruses and may require rapid forward processing [35]. Progress has been made in developing affinity chromatography for viral vectors with the potential to achieve a high degree of purification in a single step. Historically, the ability of many viruses to bind heparin has been exploited [90, 122], but avoiding animal-derived components such as heparin is generally preferred for cGMP manufacturing processes. More recently, regulatory-friendly immunoaffinity chromatographic media have been developed for multiple serotypes of AAV [106]. Given that multiple gene therapy products can be based on the same virus serotype, viral vectors may be an ideal candidate for development of synthetic affinity ligands designed for chromatographic purification.

For manufacturing processes which also include the upstream use of viruses as raw materials, such as recombinant baculovirus, HSV, or Ad5 helper virus for various AAV production methods, clearance and inactivation steps for these process-relevant viruses must be included in the downstream manufacturing process. Although the stated scope of ICH Q5A guidance on viral safety for biotechnology products derived from human and animal cells does not include viral/gene therapy products, many of its principles do still apply. Because AAV is a small, robust, non-enveloped virus, multiple strategies for clearing other viruses can be used, such as detergent for inactivating enveloped viruses, viral filters which can discriminate between large and small viral particles, and differences in thermostability [19, 21, 75]. However, for larger, more fragile enveloped viral vector

products, robust clearance mechanisms for other viruses are generally not available, impacting process development considerations.

Formulation is an area of particular potential for future development, as the majority of gene therapy products are simply stored frozen at $<-60^{\circ}C$ in isotonic buffers. Although an ultra-low storage temperature is practical for bulk drug substance, it can be a logistical challenge for drug products at hospital pharmacies. Although these logistics may be manageable for limited numbers of treatment centers for ultra-orphan indications, development of formulations that do not require frozen storage is undoubtedly a focus as gene therapy products are commercialized. Trends to increasing dose levels of AAV vectors to 10^{13} vg/kg or higher [4] provides incentive for developing higher concentration drug products. An impediment to achieving these objectives has been the large amount of material required to support such formulation development studies relative to current manufacturing scales and supply requirements for clinical trials. Advances in formulation are greatly facilitated by improved methods for reducing the scale at which buffer exchange and formulation development experiments can be effectively performed, as well as larger scale and/or higher yield production.

5 Adventitious Agent Control

As most viral vector manufacturing is performed in mammalian or insect cell culture, the fundamental principles of adventitious agent control for cGMP manufacturing of biologics apply, although demonstrating viral safety can pose special challenges. For recombinant protein manufacturing in cells of human or animal origin, the three pillars of adventitious virus control described in ICH Q5A are (1) selection of source materials and testing those for viral contaminants, (2) testing the capacity of the production process to remove and/or inactivate viruses, and (3) testing for viral contamination at appropriate stages of production.

Host production cell banks and most other raw material sourcing and testing is comparable between gene therapy and other biologics. However, it is more common for gene therapy processes to include animal-derived components, such as serum, with the additional stringent control measures those entail, such as sourcing from BSE-free countries and irradiation prior to use. Testing plans for cGMP cell banks are influenced by the species of origin, which is often a human cell line for viral vectors. When a virus seed stock is used in manufacturing, these viral banks can pose the most challenges for testing. Replication-defective viral vectors manufactured by amplifying seed stocks in complementing cell lines can usually be tested for adventitious agents in standard assays using non-complementing indicator cell lines, but some modifications may be required. For example, recombinant first-generation adenovirus vectors still show cytopathic effects when inoculated on cells at high concentrations, even without replication. In this situation, indicator cells may be serially passaged after inoculation with test articles to dilute out the product-based cytotoxicity although still permitting detection of potential

propagating replication-competent adventitious viruses at later passages. For banks of replication-competent viruses, strategies used routinely by viral vaccine manufacturers may apply [123]. For example, uninfected control cultures can be a tool for detecting adventitious agents. Cells and culture media from the production lot are segregated just prior to infection and are propagated in parallel with production of the virus bank. Samples from the control culture are then used to perform non-specific cell-based adventitious agent assays, and a panel of PCR assays for specific adventitious viruses is performed at an appropriate sampling point for testing the bank itself.

The ability to evaluate a viral vector manufacturing process for the capacity to remove and/or inactivate viruses varies significantly by type of gene therapy vector. For products that are large, fragile enveloped viruses, such as lentivirus or retroviruses, it may not be possible to design general mechanisms for viral clearance into a process. However, small, robust, non-enveloped viruses such as AAV can withstand more rigorous purification and tolerate some viral inactivation methods [19, 75]. If a process-relevant virus is present (e.g., baculovirus, HSV, or Ad5 helper virus for various AAV production methods), demonstrating its clearance and time-dependent inactivation based on principles in ICH Q5A is advisable.

In-process safety testing for gene therapy products adheres to the same principles as for recombinant proteins, although frequently the testing strategy must account for the recent or current use of animal components such as FBS or trypsin, with virus testing panels relevant to human cell lines. For production systems which include replication-competent or cytotoxic viruses, many of the same issues described above for viral banks or seed stocks also apply, such as use of virus-specific molecular tests and uninfected control cultures to provide samples for cell-based assays. Of special consideration to viral vectors is the potential of the production system itself to generate a replication-competent variant of the recombinant viral vector being manufactured. This is relevant for all viral vectors, but is of particular concern for those based on human pathogenic viruses, such as adenovirus, herpes virus, and retroviruses. including HIV. The risk of generating replication-competent recombinants is managed through careful design of the recombinant vector and its production system. Expectations for testing strategies including sample points and assay sensitivity requirements are described in detail in regulatory guidance [124, 125], and can be particularly significant for retro- and lentiviruses. For these products, routine testing for RCR or RCL requires 5% of a production batch or up to 300 mL of harvest, in addition to 10^8 end-of-production cells.

6 Facility Considerations

For operator safety, Biosafety Level 2 (BSL2) procedures are required for many production processes, either based on the type of viral vector or by the use of immortal human cell lines, with additional considerations for large-scale

(BLS2-LS) and/or enhanced BSL2 for certain viruses or process volumes [126–128]. Containment becomes a priority, and facility design elements may differ somewhat from CHO-based manufacturing, such as an air pressure sink in a cell culture production room where virus levels are high.

Principles of segregation between steps of a given manufacturing process may be more variable across gene therapy products. When viral clearance steps can be designed into a manufacturing process, segregation between key pre- and post-clearance operations may be desirable. Likewise, there may be advantages to segregating virus-free cell culture operations from virus production cultures. However, given the predominantly clinical development stage of the gene therapy industry, the prevalence of biotech companies advancing gene therapy products without internal manufacturing capabilities, and a preponderance of products being developed for orphan or small market indications, use of multiproduct contract manufacturing facilities is common. Therefore processes must often be designed for flexible facilities, and may need to incorporate concepts such as temporal rather than physical segregation.

For these reasons, the move toward closed and single-use systems for unit operations, already evident in the general biotherapeutic manufacturing industry, is even more prevalent for gene therapy products. This has led to rapid adoption of new technologies, such as single-use bioreactors up to 2,000 L scale, equivalently sized disposable-bag based storage vessels, and chromatography skids with single-use flow paths based on peristaltic pumps and pinch valves. However, as larger scale single-use manufacturing is achieved, handling the physical volume of the virus-contaminated waste streams from these facilities and flow-rate limitations through connections in current bioprocess bag configurations has identified new bottlenecks to address in manufacturing facility and process design. Where single-use technology cannot be applied, many manufacturing facilities require product-contact equipment to be dedicated to a single product. Cost considerations for such equipment, particularly for early stage development and small market indications, can have a significant influence on process design.

7 Case Study

The following case study describes the path to the first known 2,000-L scale manufacturing for an AAV product, performed at Lonza Houston cGMP manufacturing facility using a client's late stage clinical process. The process transferred in was intentionally designed to model recombinant protein manufacturing methodology as much as possible, including use of a stable producer cell line in serum-free suspension culture, scalable industry-standard unit operations, and multiple robust orthogonal viral clearance steps. However, multiple aspects differed from a typical monoclonal antibody or protein manufacturing process, the most significant being the use of a live helper virus, wild type Ad5, to induce production. The HeLa cell substrate is also unusual but is based on source material

rigorously evaluated in safety studies [129]. Because manufacturing would be performed in the multi-product Lonza Houston facility, the entire 2,000-L scale process was by necessity based on single-use technology.

The primary objectives for this case study were to demonstrate successful process transfer to Lonza Houston existing gene-therapy manufacturing facility and feasibility of the anticipated commercial manufacturing scale, although product supply for various uses was also desired. Demonstrating feasibility of commercial scale was a critical milestone for the client as this scale was unprecedented for an AAV-based gene therapy product, and reduction to practice was considered essential by the biotechnology investment community.

7.1 Process Description

An overview of the manufacturing process is shown in Fig. 7. The process begins with the thaw of a vial of a master or working cell bank into a shaker flask suspension culture, and expanding through shake flask and bioreactor cultures to a 400-L perfusion culture in a 500-L stirred tank bioreactor. Perfusion is used for the N-1 inoculum culture to maintain cells in exponential growth phase to a viable cell density approximately one log higher than the target for seeding the production culture. Cells are then transferred to the 2,000-L stirred tank production bioreactor, diluting into a serum-free production medium distinct from the cell expansion medium. As the cells are infected with Ad5 helper virus and do not grow appreciably during production, the majority of the final production culture biomass is provided by the N-1 inoculation culture. However, just prior to infection, a sample is taken from the production bioreactor to seed a parallel uninfected control culture, analogous to many viral vaccine manufacturing processes [113]. This control culture is supplemented from the same source of feed medium as that to be used during vector production to ensure exposure to this raw material, and then serially passaged for a minimum of 14 days prior to sampling for routine cell-based adventitious agent testing. Meanwhile, the Ad5 infected 2,000-L production culture is operated in fed-batch mode for 4 days, adjusting control set-points on day 3 to reduce cell viability and increase vector partitioning into the culture supernatant prior to harvest on day 4. The culture is harvested by clarifying through a series of depth and membrane filters, and then digested with Benzonase® nuclease for host cell DNA size reduction. The clarified harvest is then passed over anion exchange membranes in series to clear the majority of the byproduct Ad5 helper virus, and the AAV product remains in the flow-through. Most of the Ad5 is retained by the first of the two membranes. Thus the downstream membrane is challenged by relatively low concentrations of Ad5 and provides a good safety margin for the primary clearance step, increasing overall process robustness. The final recovery step is harvest concentration by ultrafiltration, using flat sheet tangential flow filters. Purification is performed using a series of chromatography steps with traditional packed columns and step elutions. As a significant majority of capsids produced by

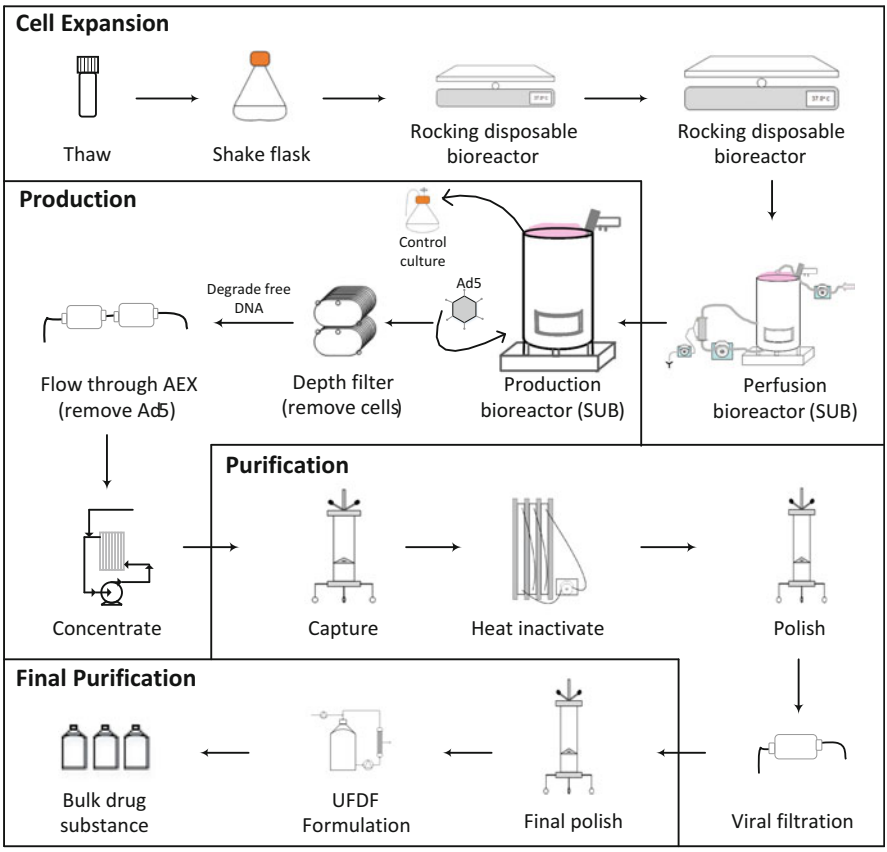


Fig. 7 Overview of client 2,000-L scale manufacturing process for an AAV1 gene therapy vector

the upstream process contain vector genomes, no removal step for empty capsids is required. Two additional robust viral clearance steps are incorporated in the process. The first is heat inactivation, designed to inactivate the process-relevant Ad5 virus, based on the well-established difference in thermostability between AAV and adenovirus [75]. The second is nanofiltration, similar to what may be performed with large proteins. As the product is itself a parvovirus, the parvovirus-rated filters standard in the recombinant protein industry cannot be used and a filter rated for clearing retroviruses is required. Both of these viral clearance steps are based on mechanisms which can be verified on a run-to-run basis: product temperature for heat inactivation and post-use filter integrity for nanofiltration. Thus the process was designed to position either downstream of the point where Ad5 is already situated or below the limit of detection in a sensitive assay (after the capture column) to provide verifiable safety margin. The final step in manufacturing bulk drug substances is diafiltration for buffer exchange using a hollow-fiber TFF filter. The process was designed to incorporate principles of segregation between unit

operations occurring at several points – after the primary Ad5 clearance step during recovery and after viral filtration.

This client process had been previously developed and operated at the 250-L bioreactor scale in a facility which had been purpose-built. In contrast, the Lonza clinical manufacturing facility was designed to serve a gene therapy industry characterized by a diversity of manufacturing processes and technologies, and thus for maximum flexibility. Additionally, because of the frequent changeover between products, many of which are infectious, heavy emphasis is placed on closed systems and single-use technology. Where single-use is not feasible, product-contact equipment is generally client-dedicated. However, fewer restrictions are placed on product-contact equipment in the process development laboratory, where initial stages of process transfer were achieved, initially at bench scale and then at 250 L.

7.2 Raw Materials

One of the major considerations for process scale-up and cGMP operations in a single-use facility is raw material specifications and sourcing. For example, such processes use a large number of custom tubing and connector sets, bioprocess bag configurations, and other containers, many with long lead times for design and production. Planning for custom biologic raw materials in particular starts very early in process transfer. Unlike many current gene therapy manufacturing processes, no large-scale DNA plasmid manufacturing was required for this project, as the process was based on a stable recombinant producer cell line. However, a cGMP Working Virus Bank (WVB) of the wild type Ad5 helper virus was a critical and uncommon biologic raw material, which was needed at a relatively large scale to supply AAV manufacturing at 2,000 L. Although not product-specific per se, as the helper virus could be used with any similar AAV producer cell line, this project did require production of a suitable WVB to supply this first large-scale AAV manufacturing process. The goal was to establish virus banks which would be suitable for late stage clinical and commercial AAV manufacturing, and the client provided the two biologic starting materials: a cGMP master cell bank of the serum-free adapted HeLa S3-subclone used as the host cells and the initial seed stock of Ad5 that had been recently re-cloned by limiting dilution under ADCF conditions to address exposure of the previous phase 1/2 virus stocks to serum and trypsin much earlier in their history.

Ad5 amplifies efficiently in HeLa cells, and a simple 5-day ADCF suspension batch culture of a non-recombinant HeLa S3 subclone infected at low MOI produces sufficient virus to infect several hundred- to a thousand-fold larger AAV production culture. For the intended use as an upstream raw material, limited downstream purification was acceptable for an Ad5 manufacturing process, with the primary objective being concentration to allow a few hundred milliliters of WVB or less to infect a 2,000-L AAV production bioreactor. This goal not only

simplifies both initial freezing and thawing aliquots at the time of use but also allows logistically-friendly long-term storage of sufficient raw material to supply several years of commercial AAV manufacturing on one or two shelves of a −70°C freezer. The production scale chosen for the WVB was 250 L, as one batch at this scale would supply sufficient raw material for at least a year of continuous AAV manufacturing at 2,000 L. Before scale-up, a Master Virus Bank (MVB) was first produced in a similar process at a 10-L scale.

The most critical consideration for a cGMP Ad5 bank was adventitious agent control, both because it is used as a raw material in a mammalian cell culture process – AAV production – and because the lytic Ad5 virus is confounding in many standard cell-based adventitious agent tests. The cGMP banks of the cell substrate undergo standard testing according to regulatory guidance, and an uninfected control cell culture is maintained in parallel with each Ad5 production batch. For this, cells are sampled from the production bioreactor just prior to Ad5 infection and seeded in a 1-L suspension culture using a shaker flask capped with a weldable dip tube assembly to maintain a closed system during manipulations. The culture is serially passaged for 14 days to maintain cell health and then provides samples to perform standard in vitro and in vivo adventitious agent testing, including a cell-based portion of mycoplasma tests. PCR-based testing is performed on the unprocessed bulk harvest of the virus production culture. An additional line of defense in the process design was to incorporate a viral barrier for both the production media and the downstream process/formulation buffer. Although high temperature short time (HTST) and UV-C are commonly used as viral barriers for large-scale recombinant protein manufacturing processes, a simpler method to develop for the 10- and 250-L process scales and the limited number of runs was to filter medium and buffer through a single-use parvovirus rated nanofilter [130, 131]. The release and adventitious testing of Ad5 stocks is summarized in Table 3.

The majority of raw materials in this 2,000-L scale process are single-use components such as filters, bags, tubing, connectors, specialized pump tubing, cell culture vessels, and chromatography system flow-kits. Suppliers of single-use cell culture and storage vessels generally stock particular configurations of many of the components, which is convenient for users as these raw materials are usually available with short lead times and at lower cost than customized components. However, standard configurations of vessels for the 2,000-L process did not scale inlet and outlet tubing proportionally to the vessels used at 250 L, and would result in longer processing times because of port and tubing flow-rate limitations. For the two runs planned by the client to demonstrate the ability to scale-up, this tradeoff was acceptable for many of the components, as customizing bags for a small number of units was not cost-effective, and some of the standard configurations could accommodate work-around by connecting multiple ports in parallel for the same transfer operation. For longer term use, customization would likely be advantageous for most of the larger volume single-use vessels, particularly those greater than 500 L.

Table 3 Release testing for Ad5 virus banks

Unprocessed bulk harvest	Uninfected control culture	Ad5 MVS/WVS[a]
Mycoplasma – broth and agar cultivatable portion PCR assays for adventitious viruses: AAV Hepatitis A, B, C HSV 1, 2 HIV 1, 2 HTLV 1, 2 HHV 6(A, B), 7, 8 CMV EBV Parvovirus B19 Bovine polyoma virus Human polyoma virus (BK, JC, KI, WU) Mycobacterium tuberculosis Retroviral reverse transcriptase	In vitro assay for adventitious viral contaminants (indicator cells: MRC-5, Vero, HeLa) Mycoplasma USP 〈63〉 and Ph. Eur 2.6.7	Sterility Appearance Identity HPLC for structural proteins qPCR for E1A/E2A Genome concentration (qPCR) Infectious titer (TCID50) Particle to infectivity ratio Endotoxin

[a]Testing performed vialed MVS/WVS or bulk prior to vialing

Nonetheless, a significant number of customized single-use connections and containers were required for this 2,000-L process, requiring detailed planning and preparation. Typically, stainless steel facilities supporting a 2,000-L production bioreactor would contain transfer lines and processing skids integral to the facility, with little run-specific planning needed for connections and fluid transfer between vessels and equipment. With the availability of sterile tubing welders and an assortment of single-use connectors (e.g., Pall Kleenpak, GE ReadyMate, Colder AseptiQuik), even in large bore sizes, all connections needed for a 2,000-L process are possible in single-use format, but the compatibility of connectors between components, particularly those provided by different suppliers, is not guaranteed. For a single-use, predominantly closed process, it was essential to diagram every connection and transfer line during the planning phase, accounting for relative equipment placement, process flow rates, and sterilization method. Some single-use connectors do not have rotational freedom for joining the two parts of the connection, such as a hinged anchor point that must be aligned. This can be significant when connecting single-use manifolds, as the connectors must all be aligned in compatible orientations prior to sterilization, and this level of detail is needed in specifications for component assembly.

In addition to a large number of connectors and connection adaptors, a few vessels also required customization, including the 500-L single-use bioreactor (SUB) bag used for the N-1 perfusion, the 2,500-L bag used as a holding vessel at various points in the upstream process, the 200-L retentate reservoir used in harvest concentration by tangential flow ultrafiltration (harvest TFF), and the bag

used in the downstream viral clearance heat inactivation step. To accommodate cross-flow rates for perfusion and a concentration step at the end of the N-1 culture expansion, customized ports were required for the recirculation loop in the 500-L SUB bag. High cross-flow rates of 75–80 L/min were a similar limitation for the harvest TFF. In general, a custom-designed bioprocess bag system with outer support container is desirable, which could in principle be customized for 200 L capacity with large-bore tubing. However, there was concern that such high flow rates in typical bioprocessing bags and tubing connections could pose a risk of leaks or rupture, which was considered unacceptable because of the presence of large amounts of infectious virus. Therefore a custom 200-L polypropylene tank with standard triclamp fittings was designed for sanitization with hydroxide and operated as single-use. The primary consideration for designing the 2,500-L bag used as a holding tank was the need for ports to accommodate multiple uses with a wide range of operational flow rates, ranging from 250 mL/min at some stages of cell culture perfusion to 60 L/min for pre-use flushes of the harvest depth filters. Some of these uses were dictated by design of the virus production facility for fail-safe containment – all process liquids are first collected within the suite for controlled transfer to a chemical decontamination tank where sodium hypochlorite is added. The heat inactivation step required integration of disposable sensors and a custom-designed recirculation loop. Third party suppliers were supportive of designs of custom solutions, but significant advance planning was required to ensure timely availability of these raw materials.

A final but significant raw material consideration was warehousing requirements. The total volume of single-use consumables for a 2,000-L scale process is considerably larger than that required for the typical gene therapy manufacturing process operated at 200 L or below. Additionally, the strategy for supplying process buffers and media for a two-run campaign was to leverage the capabilities of Lonza's -Walkersville manufacturing facility and third-party suppliers to allow manufacturing operators to focus on the extensive preparation required for a large-scale process based on single-use technology. Thus storage of pre-manufactured buffers and media was also required. To accommodate all raw materials for a 2,000 L production-process with sufficient back up, the Lonza Houston cGMP warehouse area was relocated and significantly expanded, and mobile GMP-qualified refrigerated units were obtained for storage of the large volumes of media. The mobile units offered a simple and flexible solution without the need for construction and prevented having under-utilized refrigeration units after completion of the large-scale runs, when the facility is engaged with the more typical gene therapy clients who operate at scales in the hundreds of liters.

7.3 Principles of Scale-up

The client's 250-L scale production process was designed to leverage proven scale-up methodologies by using equipment standard in the monoclonal antibody

industry, and scale-up to the 2,000-L process was performed directly, without any intermediate scale. The stirred tank bioreactors were obtained from the same vendor as the original scale, agitation rates conserved power, and sparge gas flow rates maintained the same vessel volumes per minute with a dual sparge scheme directing oxygen and carbon dioxide through a microporous sparger and air through an open pipe. As a conservative safety measure for a plastic bag containing 2,000 L of infectious virus, one compromise was made in slightly reducing the air exchange rate of the vessel headspace (overlay rate) to reduce pressure in the bag further below operating limits. The maximum size available for hollow fiber filters used for perfusion culture is smaller for the fully single-use format than for filters used in a reusable housing. Thus, to scale proportionally the perfusion bioreactor process, a manifold of three single-use TFF filters was used, requiring extreme attention to detail in designing the pre-assembled sterile connections. As mentioned previously, some of the larger volume upstream fluid transfer steps did require longer processing times because of limitations in single-use container ports and tubing sizes. Chromatography steps conserved load ratios and residence time, although peak collection criteria were optimized to reduce the collection of excess buffer. Dead-ended filters were scaled up by surface area-to-volume and flow rate by flux rate when possible. Again, at high higher flow rates (20 L/min and above), the standard vessel ports and tubing sizes restricted flow rates, so these flow rates were often lower than desired. The viral filtration maintained feed pressure, and surface area was scaled up to the nearest filter size available, which was oversized because of the limited standard options for retrovirus-rated filters.

The one step for which proven methods for scale-up were not available was the heat-inactivation step, used in the downstream process to provide a safety margin for clearance of the Ad5 helper virus. The 250-L production scale process was performed in a bioprocess bag on a rocking mixer with heating capability, similar to a WAVE bioreactor. The process incorporated a recirculation loop, both to avoid low-temperature dead-legs at ports in bag and to include an in-line temperature sensor for better control in achieving the 52°C target. At the original scale, process volume at the heat inactivation step was 6 L, which was heated in a 10-L bag in less than 1 h. For the 2,000-L scale, the process volume for heat inactivation was reduced to 20 L because of more efficient chromatography peak-collection criteria. A larger rocking mixer was available from the vendor, which allowed proportional operational scale-up for 20 L, although heating time was almost three times longer. Performing the process on the larger mixer in two cycles reduced total duration of product exposure to elevated temperatures to times similar to the original process, and was the method performed for the 2,000-L engineering run. For routine use, a more efficient system was desired, and a flow-through heat exchanger with disposable product contact surfaces was evaluated for the second 2,000-L batch. The standard single-pass system was adapted to a closed recirculation loop with a small surge bag to accommodate expansion and the target temperature was achieved within 30 min (Fig. 8). For both the rocking mixer and the recirculating flow-through heat exchanger, heat-loss through exposed tubing and sensors was a significant factor, requiring insulation in designing the systems.

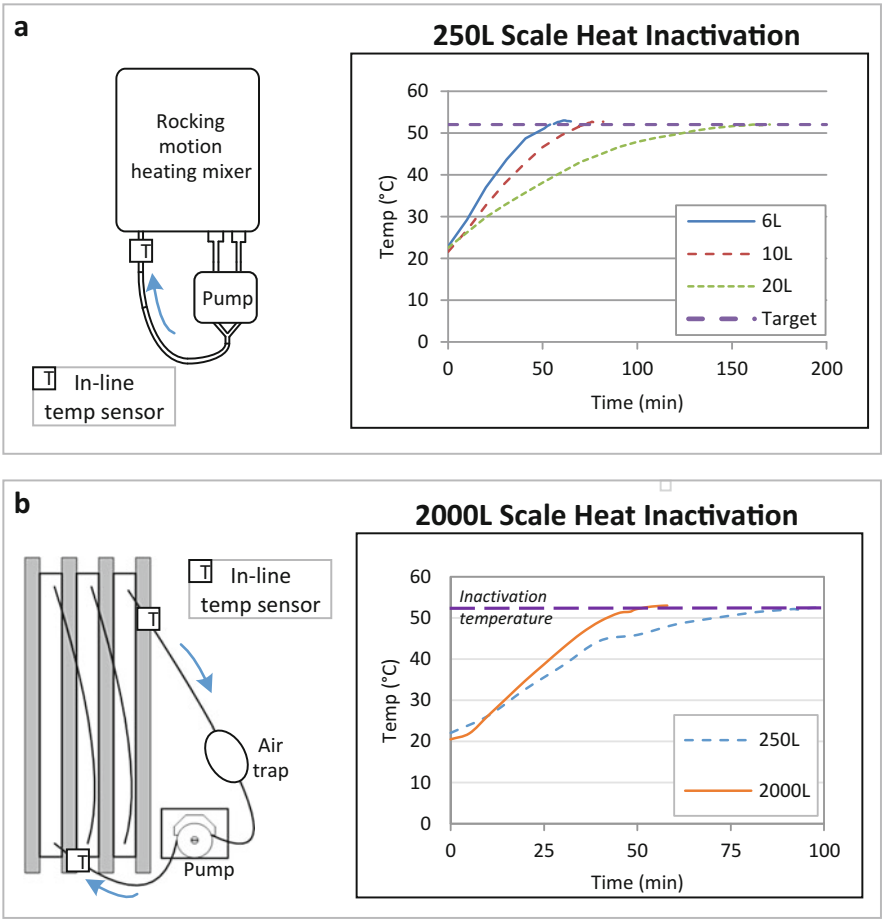


Fig. 8 Scale-up of heat inactivation step. (**a**) Original process using Wave mixer, with temperature profiles for heating 6-L (250-L scale) or 10-L (2,000-L scale divided in two cycles) in a Wave20 bag and heating 20-L in a Wave50 bag (2,000-L scale in one cycle). (**b**) Recirculating flow-through heat exchanger, comparing temperature profiles of heat-exchanger process (2,000-L scale in one cycle) and original 250-L process in Wave mixer

7.4 Execution of the Batches

The client's production campaign consisted of two 2,000-L AAV production batches, an engineering run performed under cGMP conditions followed by a clinical product supply batch. Example photographs of individual unit operations during production are shown in Fig. 9. Both runs were successful, but both, particularly the first, varied from routine manufacturing by diverting material at various points to supply a feedstream for ancillary studies, such as viral clearance, or to allow prospectively for troubleshooting if necessary. Several months were

Fig. 9 Unit operations from 2,000-L AAV production run

scheduled between the runs to assess and apply lessons learned. Two cleanrooms in a modular cGMP facility were used, one for all upstream operations, including the uninfected control culture, and one for downstream. This strategy was suitable for clinical stage manufacturing by incorporating temporal segregation and change-over cleaning between pre- and post-viral filtration steps.

The first 2,000-L run demonstrated the feasibility of commercial scale AAV manufacturing, but processing times were longer and labor requirements were

higher than expected because of the complexity of an entirely single-use process at large scale coupled with the impact of flow rate limitations in some components. In general, manual setup and intervention are more extensive in single-use operations, and require more logistical planning of work-flow. For example, processing times were exacerbated by ancillary manual equipment that is inefficient at this scale, particularly with manifolds, such as screw clamps for large ID (>0.5 inch) lines used to divert process fluids. Additionally, single-use flow-path chromatography systems based on peristaltic pumps and pneumatic pinch clamps for directing flow have time limits for exposure to hydroxide and the duration for which tubing can be clamped, which must be accounted for in scheduling and logistics. A predominantly single-use process requires extensive movement of raw materials from the warehouse to the production floor, and output of solid waste at large scale was such that after a major processing day nearly another shift was needed to handle proper decontamination and disposal. The decontamination autoclave was heavily utilized and backlogged at multiple points. The first 2,000-L batch demonstrated that opportunities to level workload throughout the process should be sought. For example, workload is relatively light during the initial cell expansion steps, and this time can be used to complete as much component staging and preparation for the labor-intensive large-scale N-1 culture through harvest and recovery operations as space in the facility allows.

The second 2,000-L production run was able to incorporate a number of lessons learned during the first run. Execution and transitions between unit operations were simpler by better resource and activity leveling, allowing for time-consuming large volume fluid transfer steps that could not be addressed with new components in the time and budget allotted. Between the two runs, a number of considerations for designing a commercial manufacturing for this process were identified. For example, four cleanrooms would be preferable to segregate operations and provide ample time for cleaning and setup of single-use systems without interfering with other operations: (1) cell expansion (no virus), (2) production and harvest, including the primary Ad5 removal step, (3) purification to the viral filtration step, and (4) post-viral filtration and drug substance. The uninfected control culture performed as part of each production batch would ideally be maintained in a separate virus-free space, which could potentially be a QC cell culture laboratory if an operationally simple closed culture system with weldable tubing were used. For repeated and routine manufacturing, single-use components throughout the process should be customized to fit the process and equipment spacing, and fabrication and sterilization of assemblies should be outsourced. Additional automation should be incorporated into single-use processes, particularly for simple operations such as fluid diversion with large bore tubing. Increased use of wall penetrations to transfer buffers and media would reduce movement of materials into the cleanroom, which is already high for single-use processes. Finally, a hybrid process based on both CIP and single-use components may be preferable for routine operations, particularly if a commercial viral production facility were product dedicated. Although a completely single-use large-scale process is possible, and even practical where product changeover is frequent, chromatography skids, valves for large volume

fluid transfer, and certain components of the harvest/recovery train with high flow rates are places where traditional stainless steel technologies are still most efficient. In such a purpose-built commercial facility, experience in this case study suggests that a stable producer cell line process could be operated efficiently at the 2,000-L scale at a rate of about once per week.

7.5 Performance

A volumetric productivity of just over 10^{14} vector genome-containing AAV particles was achieved at the 2,000-L scale, consistent with process performance observed at 250 L and in bench scale bioreactors. Overall yield was impacted by other uses for in-process feedstream, but results are consistent with the expected ~30% overall recovery previously observed in routine operations. Test results for the first 2,000-L batch were comparable to 250-L reference material and the lot was released for clinical use (Table 4). Purification across the process performed as expected (Fig. 10), and relative potency at 94% as measured in a dose–response transgene protein expression assay was indistinguishable from reference (Fig. 11). For business reasons unrelated to the performance of the run, the full panel of lot release testing was not performed on the second batch.

Table 4 Release testing performed on first 2,000-L AAV batch

Uninfected control culture	Eluate from capture column[a]	Bulk drug substance[b]
Mycoplasma USP 〈63〉 and Ph. Eur 2.6.7 In vitro adventitious virus testing	Mycoplasma USP 〈63〉 and Ph. Eur 2.6.7 In vitro adventitious virus testing	rAAV genome concentration (qPCR) AAV1 capsid (ELISA) rAAV infectivity (TCID50) Genome:infectivity ratio Relative potency SDS PAGE/purity Residual infectious Ad5 (cell-based limit assay) Residual Ad5 protein (western blot) Replication-competent AAV Host cell protein (ELISA) Host cell DNA (qPCR for 18S and E6 DNA) Residual Benzonase (ELISA) Appearance pH Osmolality Bioburden Endotoxin

[a]The eluate from the capture column is the farthest upstream point in the process where Ad5 levels will not be confounding to cell-based assays

[b]Significant additional characterization testing is also typically performed, and includes analytic ultracentrifugation (AUC) for assessing percentage full capsids and aggregation

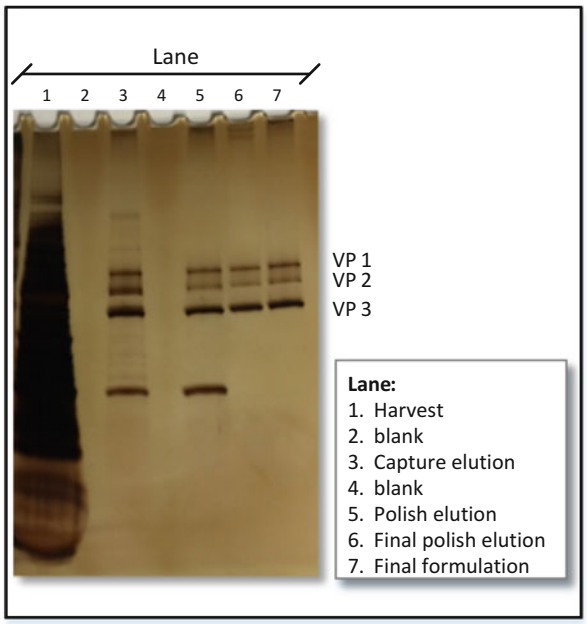


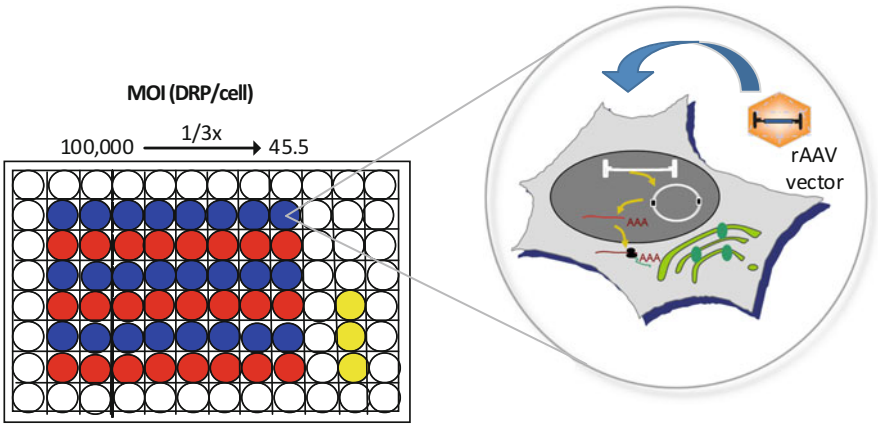
Fig. 10 Silver stain SDS polyacrylamide gel electrophoresis in-process analysis of 2,000-L AAV1 engineering run, monitoring purity of the three AAV capsid proteins (VP1–3)

7.6 Summary

The first successful scaled up production of an AAV production system to 2,000 L as described in this chapter is a milestone for gene therapy, clearly demonstrating the feasibility of commercial scale viral vector manufacturing in a multiproduct facility based on single-use technology. With further operational improvements and manufacturing in a purpose built facility based predominantly on single-use technology, the 2,000-L scale-up process described is capable of producing more than 40 batches/year from a single production train, corresponding to an anticipated annual production capacity approaching 10^{19} vector genomes. Supplying Ad5 helper virus for this level of production is straightforward and can be managed with a short manufacturing campaign every few years with very modest storage requirements to maintain years of inventory.

8 Conclusion

As the field of gene therapy continues to mature and increasing numbers of clinical programs move toward late phase trials and market approval, the demand to improve existing manufacturing processes to meet the need to produce well-characterized and larger quantities of viral vectors continues to grow. The rapid advance of gene therapies in the clinic and their promise to be applied as the standard treatment options for multiple disease indications in the not too distant



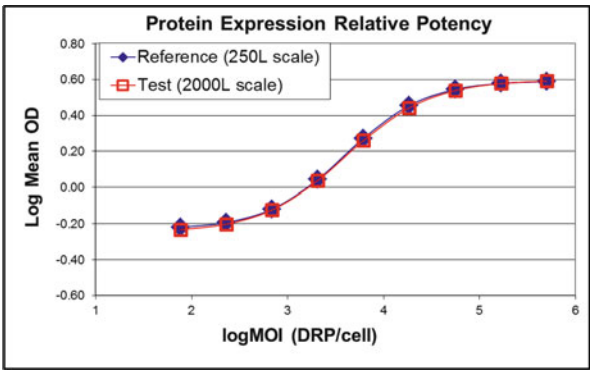


Fig. 11 Dose–response cell-based relative potency assay with ELISA readout for transgene expression

future also present unique challenges for manufacturing. As discussed in this chapter, viral vector production is complex and large-scale production methods and facilities to accommodate many different production systems are needed to meet product demands. Higher yielding and more robust production systems such as developing more efficient packaging cell lines and improving high-cell density culture systems and continuous processing methods are needed as dose levels escalate, and more efficient methods for using product-specific biologic raw materials can be developed, such as transient transfection at higher cell densities with less than proportional increases in plasmid concentration [15]. Operational trends can continue to focus on closed-system single-use technologies for multiproduct manufacturing facilities, given the prevalence of both early stage clinical gene

therapy programs and products being developed for small commercial market orphan indications. Scale-up for late stage indications can also likely accelerate the transition to production bioreactors that scale-up in vessel size rather than scaling out to more production vessels. However, as process volumes increase, single-use fluid transfer technology is one area in need of future innovation for cost-effective solutions for higher process flow rates, such single-use pumps, valves, ports, and manifolds, as well as integration of automation technologies for fluid process controls. Opportunities also exist for creative customization of single-use systems to reduce process complexity by simplifying manipulations specific to each of the many different gene therapy production systems. In parallel with manufacturing process improvement, considerable effort is needed to identify clinical phase and production process appropriate testing strategies and to develop reliable testing methods to assess the identity, purity, potency, safety, and stability of the final products in addition to demonstrating lot-to-lot consistency and comparability. Development, qualification, and validation of product release and quality control testing methods through the product development life cycle are critical, and specifications for process intermediates and final product release should be refined and tightened gradually as products move through clinical developments through licensure. Because of the complex biologic nature of the viral vectors and various manufacturing processes being developed for different vectors, it is clear that many existing test methods continue to evolve. Early industry-wide efforts such as establishing adenovirus reference standards and rAAV reference standards continue to help establish international standards for vector quantitation and dosing [100, 132, 133]. Opportunities exist for further development of future reference standards and related assays, and the need for harmonized standards is urgent. In addition, advancement in newer assay technology such as wider adoption of massive parallel sequencing (MP-Seq) methods for assessing unknown adventitious agents as well as vector genetic stability, rapid PCR based testing for RCL and RCR, real time vector quantitation using virus-counter and others can add more options to the existing product release testing methods and help to ensure that therapeutic products are meeting the safety and quality criteria, as well as streamlining critical in-process testing. The gene therapy industry is maturing, and development of truly scalable, robust, commercially feasible production methods is ongoing to replace the more brute-force manufacturing commonly used to support early stage clinical trials. Multiple platforms and approaches for manufacturing certainly continue to be required to accommodate the wide range of products under development in this field. However, we anticipate that scale-up successes can start to define efficient gene therapy production platforms that can accelerate early stage clinical manufacturing to be more robust, cost-effectively and regulatory compliant, much in the way that optimized CHO platforms have helped streamline monoclonal antibody development. The promise of gene therapy is just starting to be realized, and continued improvements in the manufacturability of these complex products are crucial for their commercial success.

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High-Throughput Process Development for Biopharmaceuticals

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Abstract The ability to conduct multiple experiments in parallel significantly reduces the time that it takes to develop a manufacturing process for a biopharmaceutical. This is particularly significant before clinical entry, because process development and manufacturing are on the "critical path" for a drug candidate to enter clinical development. High-throughput process development (HTPD) methodologies can be similarly impactful during late-stage development, both for developing the final commercial process as well as for process characterization and scale-down validation activities that form a key component of the licensure filing package. This review examines the current state of the art for HTPD methodologies as they apply to cell culture, downstream purification, and analytical techniques. In addition, we provide a vision of how HTPD activities across all of these spaces can integrate to create a rapid process development engine that can accelerate biopharmaceutical drug development.

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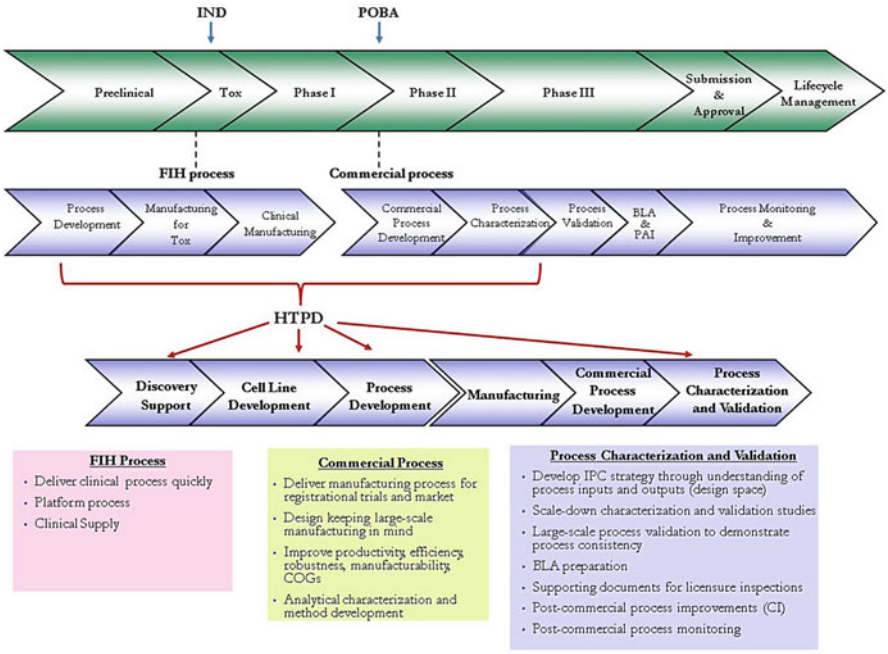
Graphical Abstract



Keywords ambr, Caliper, High throughput, Octet, Process characterization, Process development, Rapid screening and development, Tecan

Contents

1 Introduction

The rapid development of biopharmaceutical manufacturing processes remains a significant area of focus for the entire biotechnology industry. Clinical trial timelines are typically the longest item during the drug development cycle, which can span a decade. However, despite the fact that the clinical development time frame is the longest, or perhaps because of it, there is significant emphasis on reducing the time required to get a drug into clinical development. Before clinical entry, process development and manufacturing are the gating items that dictate when clinical trials can start. A number of strategies have focused on reducing the time these processes take. Additionally, rapid experimentation can aid the development of a robust and high-producing commercial process, and generate the supporting data sets required for regulatory approval. A key strategy has been the development of high-throughput process development (HTPD) tools that can enable the execution of large numbers of experiments in parallel, thus reducing the total time needed for process development.

HTPD tools have significant applicability throughout all phases of clinical development of a biopharmaceutical. Figure 1 shows a typical biopharmaceutical development pathway. Two primary opportunities are available to develop a manufacturing process for a biopharmaceutical. The first opportunity is before clinical entry, when a first-in-human (FIH) process is developed. The emphasis at this time is rapid clinical entry. As a result, this process is developed to the extent that it provides drug substance of a quality commensurate with that of clinical entry, but is not necessarily a fully optimized process for that molecule. In general, it is desirable to use a cell line that has sufficient productivity and stability to be used for commercial manufacturing. However, the upstream and downstream processes typically follow a platform. A platform approach leverages similarities within a

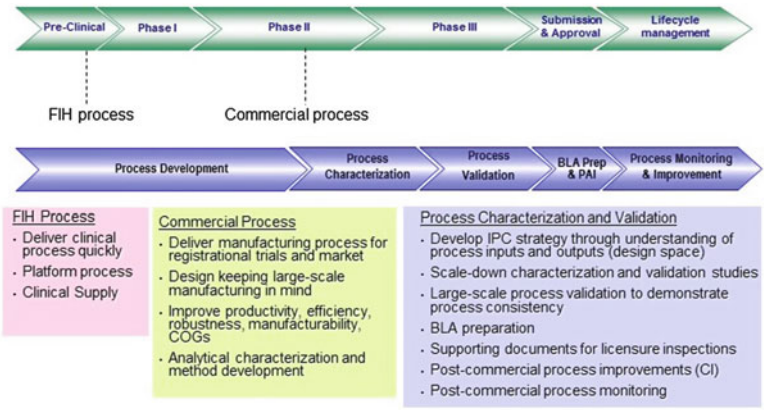


Fig. 1 Typical development pathway for a biopharmaceutical

certain class of proteins such that a common approach can be adopted to produce and analyze them. Platform approaches have been most successfully developed for monoclonal antibodies (mAbs) and related proteins and have significantly reduced the timeline from having the mAb sequence to initiating clinical trials from 2 years or more to about 1 year [1–3]. This time savings has been accompanied by a reduction in the number of staff needed for process development and thus has been unprecedented in terms of its savings for the biotechnology industry. In addition to the clinical versatility of mAbs, the platform approach has enabled the entry of hundreds of mAb drug candidates into clinical development and commercial approval for over 50 mAb therapeutics [4, 5]. Although process platforms have reduced the amount of experimentation for mAbs, other classes of biomolecules still require significant experimentation before an FIH process can be developed. Additionally, even when these products fall into a class with biochemical similarities that may allow for a platform approach to be used, significant experimentation is needed to develop a robust platform approach that is applicable to a new product class. As a result, the need for HTPD techniques still exists in FIH process development.

The next opportunity to develop a process comes when the commercial manufacturing process is being developed. During the commercial process development phase, there is a strong desire to bridge in with drug from the final manufacturing process and final scale as early as possible during the pivotal clinical trials. This reduces unknowns in terms of product quality comparability and ensures that the results seen in clinical trials translate directly into efficacy seen once the product hits the market. Another key factor is the decreased ability to predict the duration of clinical development, because what constitutes pivotal clinical data can change significantly depending on the results seen in the clinic. Phase II clinical data can be used as pivotal data to seek licensure approval for biopharmaceuticals, particularly in the oncology area. Additionally, worldwide health authorities have created several accelerated licensure pathways, such as the US Food and Drug Administration's (FDA's) breakthrough therapeutic designation and other accelerated approval pathways. Furthermore, the advent of biosimilars necessitates the development of robust processes capable of matching the innovator's product quality profile even before clinical trials start. This requires the ability to conduct a large number of experiments in parallel and in an accelerated fashion early during cell line and process definition.

Typically, commercial process development is initiated after some proof of biological activity of a molecule is obtained from early clinical trials. The focus of this phase of development is economic supply of the drug substance to fulfill projected market demand for the drug. As explained earlier, it may be necessary to skip this phase of development in the case of a biosimilar or an expedited clinical development path. For those programs, the emphasis has to be on making the FIH process robust and productive enough to support commercial launch; hence, more effort is expended up front to develop a robust, scalable, and productive process. However, when there is time to engage in this phase of development, it can result in a process with improved productivity and economics that can be used for long-term

commercial supply. The commercial process development cycle emphasizes improving cell culture titer, resolving any issues with scalability/repeatability, and improving the loading capacities of downstream chromatographic steps. This development phase does require a large number of development experiments and is the most likely to benefit from high-throughput (HT) experimentation.

The next phase before commercial launch of a biopharmaceutical is that of process characterization and validation. During the process characterization phase, deliberate perturbations to the process are made using a qualified scale-down model (SDM) to study their impact on process outcome [6–8]. This is in line with the Quality by Design framework proposed by the the FDA in ICH Q8-R2 in 2009. The premise here is that the multidimensional space of operating parameters and other input variables to a process need to be fully explored at a small scale to ensure that the large-scale manufacturing process does not go into any unintended zones during long-term operation. The SDM can be used to study the impact of operating parameters and their ranges in combination with each other. This phase does require a large number of experiments to be conducted, and these can be facilitated by HT experimentation. The limitation so far has been whether the current methodologies for HT experimentation meet the criteria for a rigorous SDM of a large-scale manufacturing process. Increasing advances in scale-down equipment, particularly in the cell culture space, are setting the stage for HTPD models to be used to explore the design space for a process.

Even after commercial launch, SDMs are useful for troubleshooting specific issues seen in large-scale manufacturing or for root-cause analysis in the event of a deviation from the batch records.

Clearly, HTPD tools and the ability to generate large data sets of experimental data that are directly relevant to the large-scale manufacturing process for a biopharmaceutical are a central area for continued development by the bioprocessing community. This chapter delves into the current state of the art in terms of HT tools for upstream, downstream, formulation, and analytical areas during process development. We also examine potential areas for further development in the future.

2 High-Throughput Cell Culture Process Development

The cell culture process is typically the longest part of the process development cycle. Mammalian cell culture processes typically have the longest experimental duration, with 2–3 weeks being typical for the production bioreactor step, with an additional 2–3 weeks spent on the expansion of seed cultures. This is mainly a result of slow growth rates intrinsic to mammalian cells.

Shake flasks and bench-top bioreactors remain the traditional platforms used for the development of cell culture processes. Shake flasks allow high-throughput experiments to be performed, but it is not possible to control important process parameters such as agitation rate, dissolved oxygen (DO), and pH. These

parameters play key roles in developing a robust, efficient cell culture process, which dictates product quality and yield. Thus, shake flasks have limited ability to replicate bioreactor conditions, which limits their usefulness for cell culture process optimization experiments.

Over the last decade, several attempts have been made to develop miniaturized bioreactor systems that mimic the performance of stirred tank systems. The intent has been to develop a high-throughput miniaturized cell culture system that mimics culture conditions in a larger bioreactor. These systems have used microtiter plates, spin tubes, and microfluidics for cell culture. Several systems have been commercialized based on these developments including the Bioprocessors SimCell microbioreactor array [9], Applikon 24 deep well plates [10, 11], Excellgene TubeSpin Satellites [12], Hexascreen multiple minibioreactor system, Pall micro24, and m2p Labs BioLector™. However, an efficient model with appropriate controls similar to those in a traditional bioreactor has remained elusive, despite these important advances. For example, TubeSpin satellites are configured with vented caps that allow the exchange of gases via the headspace. This system does not permit the in-situ measurement and control of pH and DO. The Hexascreen multiple minibioreactor allows six experiments to be run in parallel. The SimCell microbioreactor array and m2p Labs BioLector™ are not equipped with any impeller or design characteristics that mimic traditional bioreactors. Moreover, the small volumes (1–1.5 mL or less) make it difficult to conduct analytical assays, offline pH, DO, and metabolite analyses necessary for process optimization.

The other approach in the use of HT technologies relies on miniaturization of conventional stirred tank bioreactor systems that can generate greater information about bioreactor production steps in shorter periods of time, while consuming less material and significantly less resources. Several systems have been commercialized based on this approach, such as the DASGIP Parallel Bioreactor System from Eppendorf containing multiple bioreactors in the range of 35 mL–4 L that can be operated on a parallel basis with a single integrated controller. Biopod from Fogale Nanotech is capable of running eight minibioreactors (80–800 mL of working volume) at once. More recently, the ambr™ system has elicited significant interest in the biopharmaceutical industry because it comes closest to reproducing the performance of stirred tank bioreactors in a microscale system [13, 14].

The ambr system now comes in two formats, ambr15 and ambr250. The ambr15 system has 24–48 single-use bioreactors controlled by an automated workstation. Each bioreactor has a 10–15 mL working volume, an internal impeller, and capability of gases being individually supplied and controlled to each vessel. The bioreactors have individual monitoring and control for process pH and DO. The temperature and agitation are controlled for each culture station, containing 12 bioreactor vessels in ambr15, reducing the flexibility of experimental design on this system. Ideally, these parameters could be controlled for each vessel individually, providing more design flexibility. Another limitation with the ambr15 system is that all the feed/base additions in a process are pipette tip-based bolus additions with an automated liquid handler. Thus, feed rate optimization or continuous feed paradigms cannot be established with this system. The manufacturer, Sartorius Stedim

Biotech, has addressed these concerns in a next-generation system called the ambr250. In this system, each vessel has an individual temperature and agitation control with four dedicated displacement pumps for each bioreactor vessel. The ambr250 system has 12–24 single-use bioreactors controlled by an automated workstation. The ambr250 provides for slightly larger working volumes (100–250 mL). In addition, larger bioreactors enable the assessment of gassing in the headspace, antifoam, and other parameters that are not accessible in the ambr15 format. The temperature and agitation are controlled individually, which increases the range of experiments that can be performed during cell culture optimization from a single run. This system can also integrate with analyzers such as cell counters and pH meters. These systems are valuable for transitioning processes from the microreactor scale into a format that is fully transferable to production scale, while still enabling some final evaluation and definition of critical process parameters.

In recent years, numerous studies [13–18] have been conducted to determine the ability of ambr™ systems in high-throughput cell culture process development. The study by Lewis et al. [16] focused on demonstrating reproducibility of the ambr15 system and compared cell viability and titers between this system and 7 L bioreactors. The study by Hsu et al. [15] compared the cell culture performance of four CHO cell lines in ambr15 with 2 L bioreactors and shake flasks. The study by Moses et al. [17] compared the performance of three mAb-producing cell lines in ambr15 and 3 L bioreactors. The study by Neinow et al. [18] investigated the physical characteristics of the ambr15 bioreactors. The authors also compared the cell culture performance of a CHO cell line in ambr15 with 5 L bioreactors and shake flasks. The study by Rameez et al. [14] demonstrated the ability to employ the ambr™ system to make key process decisions during the development of a biopharmaceutical manufacturing process. The capability to fine-tune process controls with 24–48 single-use miniature bioreactor vessels provides a platform for fractional factorial and minimum-run designs to enable identification of key process parameters and interactions of those process parameters. The processes used in this study involved complex feed formulations, perturbations, and strict process control within the design space, which are in line with processes used for commercial scale manufacturing of biopharmaceuticals [14]. Changes to important process parameters in ambr™ resulted in predictable cell growth, viability, and titer changes, which were in good agreement with data from conventional larger scale bioreactors. The ambr™ system successfully reproduced variations in temperature, DO, and pH similar to the larger bioreactor systems. Additionally, the miniature bioreactors were found to react well to perturbations in pH and DO through adjustments to the proportional and integral (PI) control loop [14].

The studies listed above support the utilization of ambr for clone screening and for development of a cell culture process. Janakiraman et al. [13] used ambr15 to perform process characterization studies and develop an input control strategy. The SDM established with ambr15 was comparable to both their 15,000 L manufacturing scale and 5 L bench-scale bioreactors. The control strategy generated from the ambr™ system was comparable to the bench-scale SDM and confirmed the utility

of the ambr™ system for process characterization studies and control strategy development. However, despite this study, the ambr15 system still meets skepticism in being accepted as a SDM for a large-scale production bioreactor during process characterization and validation studies. The ambr250 system has the potential of overcoming that perception. The study by Tai et al. [19] demonstrated that ambr250 could be useful for process characterization by being an effective SDM.

Overall, these studies support the capability of the ambr™ system as an efficient SDM in high-throughput cell culture process development and during process characterization. Use of ambr™ systems has the potential to significantly alter the time spent during process development studies, process characterization, and scale-down validation preceding a program's entry into large-scale conformance lot manufacturing. It is anticipated that the ambr™ system will continue to have a significant impact on process development over the next few years. The next sections illustrate this capability with cell culture data for several molecules.

One important area that needs significant improvement when employing multibioreactor systems is handling a high number of generated samples for fast analysis of key metabolites, daily pH and cell count measurements. Data analysis can be a bottleneck in the HTPD approach. Accurate HT metabolite analyzers and integrated automated analyzers for pH and cell count measurements are needed to yield the required data at a time adjacent to the process, enabling key changes and corrections to be made. Similarly, it is crucial for the success of the HTPD approach that a data analysis module is capable of exporting data and combining data from various analyzers and bioreactors to perform timely and cost-effective data analysis. To some extent, ambr™ systems have addressed these issues and there are continuous improvements being made in terms of analyzer and data integration capabilities every year. However, these steps largely remain labor intensive and require more time, especially for data analysis.

The ambr™ system as SDM for bioreactors across various scales: comparison of cell growth, cell viability, key process indicators, productivity, and product quality

SDM development is an iterative process, requiring comparable performance of several predetermined key process indicators across the various bioreactor scales. SDM development relies on engineering principles whereby process parameters are divided into scale-dependent and scale-independent parameters. Scale-independent parameters such as pH and DO set points, process temperature, and seeding density are maintained across different scales. On the other hand, SDM establishment is based on scale-dependent parameters, such as power per unit volume (P/V), volume of gas per volume of liquid per minute (vvm), tip speed, mixing time, and the volumetric mass transfer coefficient (k_La). Most of these principles are discussed in various studies of SDM establishment using ambr™ systems [13–15, 18, 20, 21]. P/V and k_La are the most frequently used criterion for SDM. However, the P/V number for an ambr15 vessel at a given tip speed will always be 10- to 12-fold greater than for conventional stirred tank bioreactors (i.e., not numerically "equivalent"). The reason is mainly the different physical characteristics of these miniaturized bioreactors compared with conventional bioreactors. The vessel geometry

in an ambr15 system is different from that of a conventional vessel. The vessel has long blades (11.4 mm) relative to the x and y footprint dimensions, which account for a high P/V. Moreover, physical characteristics such as flow regime and O_2 gas velocities in ambr15 are different from those in conventional stirred tank bioreactors. The higher P/V numbers in ambr15 are necessary to achieve sufficient k_La values to satisfy the O_2 demand during cell culture. The O_2 transfer requirements of the cells are generally met by agitation intensities (expressed as mean specific energy dissipation rate, $T\varepsilon$ W/m^3) of the order 10–20 W/m^3, but similar cell growth is seen when cells are subjected to $T\varepsilon$ values up to 250 W/m^3 [18]. The high P/V in ambr15 cannot be correlated with the shear forces on cells within these small-scale bioreactors. Other studies also bear out the fact that correlations developed for conventional large-scale reactors tend to be inapplicable to small-scale systems such as micro- or minibioreactors [22, 23]. Thus, when considering scalability based on ambr15 vessels, P/V should not be chosen as the correlation factor to determine agitation rates in conventional stirred tank bioreactors.

On the other hand, ambr250 has multiple geometrical similarities to conventional bioreactors: it has two three-blade pitched impellers, four baffles, and three gas ports. One gas line is an open pipe (2 mm diameter), which can deliver processes gases N_2, air, O_2, and CO_2. The second gas line is for headspace aeration, and the third is used for venting purposes with the potential to analyze offline gases, if needed. Headspace aeration increases oxygen transfer but, more importantly, can be used to remove excess CO_2 from the culture. Xu et al. [20] have shown k_La to be a suitable scaling criterion for the ambr250.

Figure 2a, b shows a comparison of time courses for viable cell growth and viability for two different recombinant CHO cell lines in ambr™ vessels and conventional bioreactors of different scales (3 and 15 L glass bioreactors and a 200 L single-use bioreactor producing a mAb and a non-mAb protein). The cell growth characteristics were similar between replicates for the ambr™ vessel (less than 5% relative standard deviation). The cell growth and viability were comparable to the other bioreactors. The time courses for growth profiles between ambr™ and all other bioreactors in this study were not significantly different. The absolute values for cell growth, viability, and protein concentration between ambr™ and other bioreactors were within 10–15% of the mean values. Table 1 shows the growth rate and cell-specific productivity comparisons across scales. Cultures showed similar growth rate in ambr™ and bioreactors of various scales (±2% when compared with 3 L bioreactors and ±8–10% compared with 15 and 200 L bioreactors). The growth rates from this study were similar those reported by Hsu et al. [15] (0.350–0.390), who compared the cell culture performance of four CHO cell lines in ambr™ with 2 L bioreactors. On the other hand, the average cell-specific productivities differed when comparing ambr™ across scales (Table 1). The cell-specific productivity in ambr™ was found to be higher than in conventional stirred-tank bioreactors. The study by Hsu et al. [15] reported that the average cell-specific productivities differed by less than 13% between ambr™ and 2 L bioreactors.

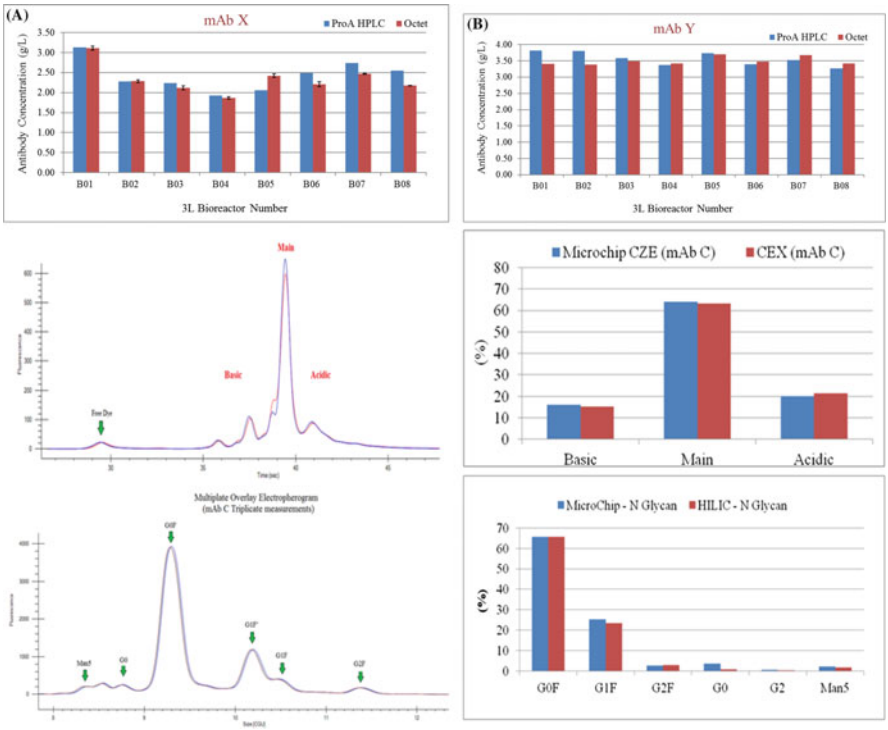


Fig. 2 Comparison of time courses for viable cell growth for recombinant CHO cell lines in ambr™ vessel and other scales of traditional bioreactors: 3 L and/or 15 L glass bioreactors and 200 L single-use bioreactor for (**a**) mAb and (**b**) non-mAb. The experimental data for ambr™ show an average of 3 and 2 vessels in (**a**) and (**b**), respectively

During the development of SDM, special attention should be paid to one or a combination of process indicators, depending upon their importance in controlling critical quality attributes. For example, the study by Janakiraman et al. [13] focused on matching pCO_2 profiles in the ambr15 SDM, which dictated process yield and product quality. On the other hand, the study by Xu et al. [20] focused on matching cell growth and metabolite profiles, which dictated process yields for that particular cell culture process. Figure 3a, b shows a comparison of time courses for two different recombinant CHO cell lines producing two mAbs in ambr250 SDMs. Matching cell growth and lactate profiles for these two CHO cell lines were key process indicators that dictated process yield and product quality. Table 2 shows the product quality comparison for two different recombinant CHO cell lines producing mAbs from ambr250 SDM across scales. In conclusion, data from the ambr™ SDMs can be translated across scales in terms of cell culture profiles, key process indicators, productivity, and product quality to perform process optimization and characterization studies investigating the design space of several process parameters and, eventually, to develop an input control strategy.

Table 1 Comparison of cell culture performance in various bioreactor systems (ambr™, 3 and/or 15 L glass bioreactors and 200 L single-use bioreactor) for viability at harvest (%), titer (normalized), cell-maximum growth rate (1/day), and cell-specific productivity (pg/cell/day) for a monoclonal antibody and a non-monoclonal antibody

Bioreactor system	Viability at harvest (%)	Titer (normalized to 200 L titer values)	Cell maximum growth rate[a] (1/day)	Cell-specific productivity (pg/cell/day)
Monoclonal antibody				
ambr[b]	90.27 ± 0.14	0.96	0.37	16.20
3-L[c]	98.70	1.06	0.37	10.60
15-L[d]	91.38 ± 2.19	0.88	0.34	10.80
200-L[c]	90.20	1.00	0.34	11.70
Non-monoclonal antibody				
ambr[e]	81.20	0.99	0.46	
15-L[c]	61.40	0.94	0.51	
200-L[c]	84.20	1.00	0.47	

[a]Measured from days 0 to 8 for monoclonal antibody and from days 0 to 7 for non- monoclonal antibody
[b]$n = 3$
[c]$n = 1$
[d]$n = 4$
[e]$n = 2$

Integrated utilization of high-throughput bioreactors and high-throughput analytics for rapid and robust cell culture process development.

HTPD tools create the need for HT analytics to realize the benefit of the rapid experimentation. This section describes a case study showing one such integration that we achieved in our laboratory. In addition to the ambr™ system, we integrated two HT analytical technologies to create a HTPD platform where the effect of media, feeds, feeding frequency, and process parameters on various product quality attributes can be studied from the early phases of cell culture process development. The two HT analytical technologies are ForteBio's Octet® for rapid and accurate analysis of antibody concentrations and LabChip® separation system that utilizes reusable microfluidic chips for rapid screening of molecular weight, N-glycan, and protein charge profiles. Octet utilizes biolayer interferometry (BLI)-based biosensors for antibody quantification. These biosensors are coated with a biocompatible matrix to analyze specific biomolecular interactions. Both these analytical technologies provide particular value in applications where conventional methods such as HPLC, enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and capillary electrophoresis (CE) have limitations in throughput, performance, workflow, and ease of use.

Figure 4 shows a schematic for the HTPD approach, which utilizes HT microbioreactors and HT analytics to accelerate product development. The HTPD approach can be utilized all the way, starting from selection of a clone during the

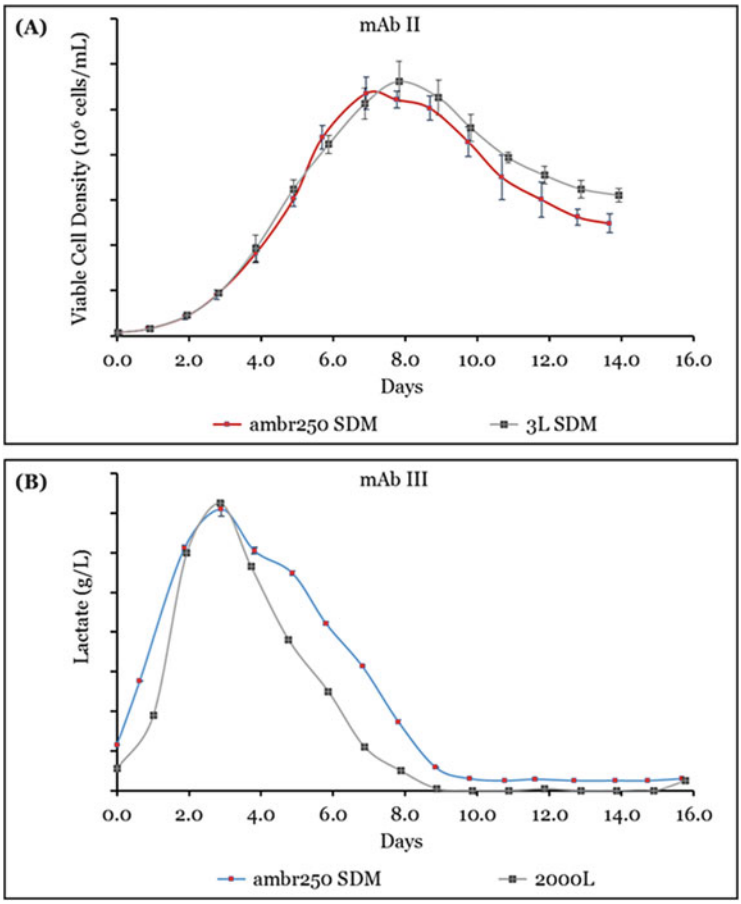


Fig. 3 Comparison of time courses for viable cell growth and lactate profiles for two recombinant CHO cell lines in ambr™ SDMs for two different mAbs. Matching (**a**) cell growth and (**b**) lactate profiles for these CHO cell lines were key process indicators and, in turn, dictated the process yield and product quality

Table 2 Comparison of product quality (size, charge, and glycan) for ambr250 size-down model and a large production bioreactor system (≥2,000 L) for a monoclonal antibody

Product quality attribute (%)	Large scale (≥2,000 L)	ambr250
Aggregates	0.49–1.00	0.60–0.69
Acidic	0.69–1.00	0.90–0.94
Main	0.93–1.00	0.95–0.96
Sialylated	0.62–1.00	0.62–0.76

All values are normalized to the large scale data

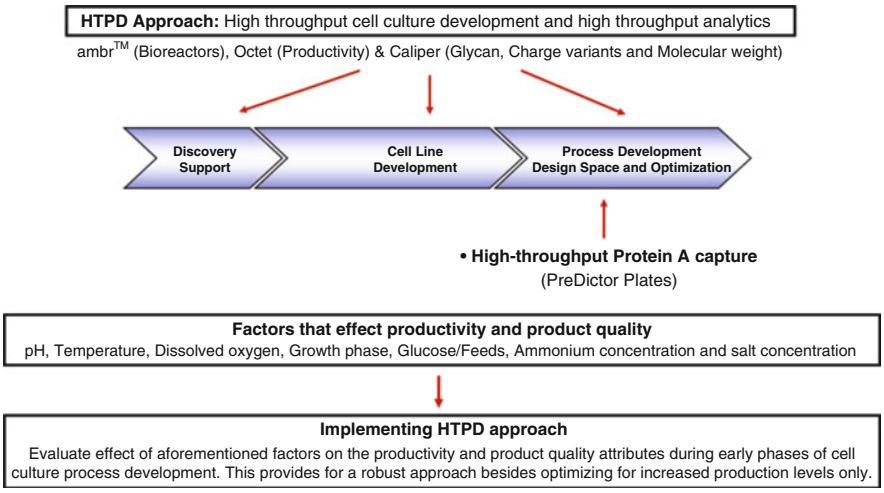


Fig. 4 Utilization of high-throughput cell culture development and high-throughput analytics (HTPD approach) in accelerating product development during the first-in-human (FIH) phase of the biopharmaceutical development life cycle

cell line development. Due to limitation of time and resources, relatively few clones (top 1–4 clones) are evaluated in conventional bioreactors, which decreases the chance of identifying a high-producing clone with desired quality attributes. HTPD overcomes this limitation of time and resources while offering the ability to evaluate a larger number of clones (top 24–48 clones) in parallel under representative stirred tank bioreactor conditions. This broader screening benefits biosimilar programs in which the aim is to identify a clone that is capable of producing specific product quality attributes.

Figure 5 shows comparative data from HT and conventional analytical methods. Fortebio Octet data is compared to traditional Protein A high-performance liquid chromatography (HPLC) for titer measurements for a mAb. Additionally, Caliper LabChip® data is compared with cation exchange (CEX)-HPLC data for charge profile and hydrophilic interaction liquid chromatography (HILIC) HPLC data for glycan mapping. As can be seen from Fig. 5, the HT analytical methods gave comparable information to the traditional analytical methods, but with a significantly higher throughput capability.

We now give details of two case studies that demonstrate the integrated HTPD approach employed during cell culture process development for a biosimilar mAb. Case study I aimed at evaluating eight different feeds for a CHO cell line producing a biosimilar mAb. Case study II was a design of experiments (DOE) study evaluating the effect of process pH and four different feeding frequencies (FDS A, B, C, and D) for the selected feed on the same biosimilar. We monitored the productivity and product quality attributes (charge and N-glycan) and compared them with those of the innovator drug product. As an example, Fig. 6a, b shows one specific glycan structure (G0F) from these case studies, a critical quality attribute in this biosimilar,

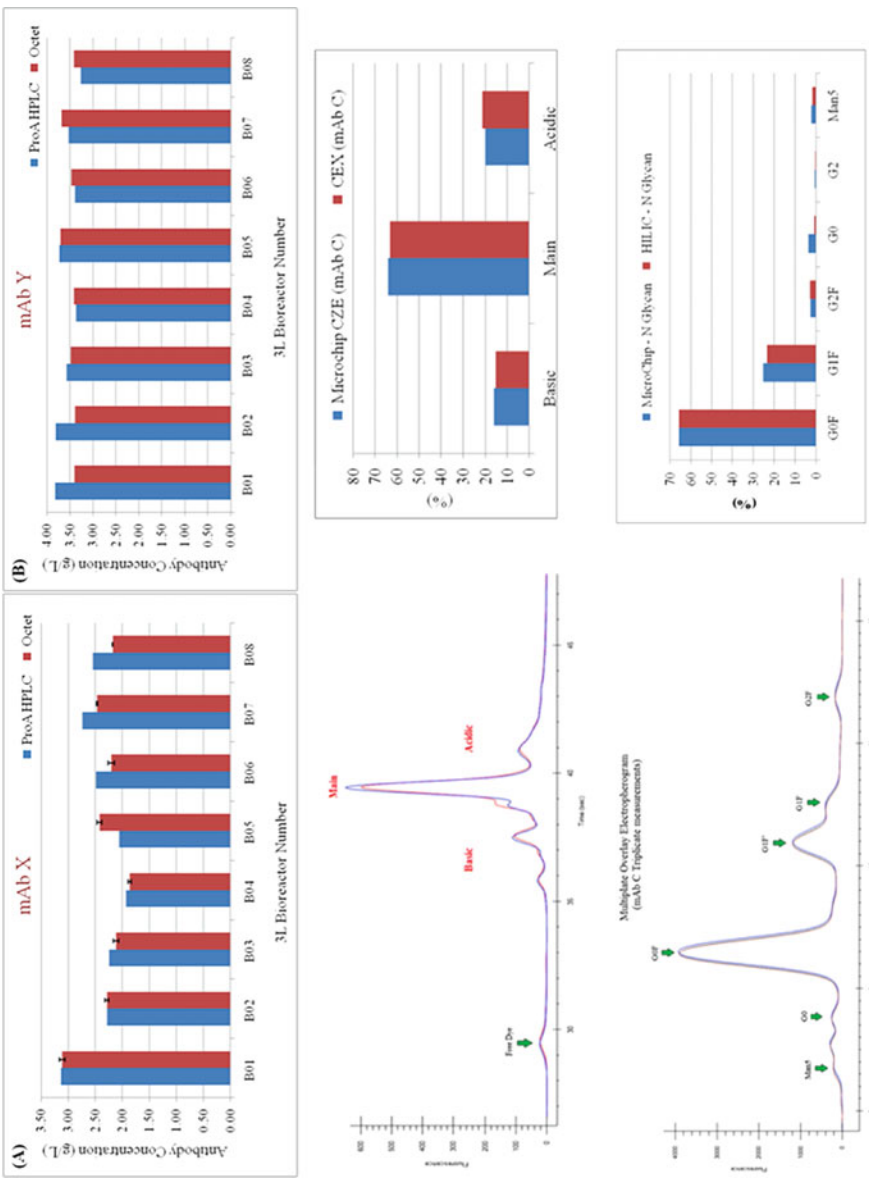


Fig. 5 Comparison of (*top*) (**a**) mAb X and (**b**) mAb Y concentrations in eight 3 L glass bioreactors using Octet™ and Protein A HPLC methods; (*middle*) mAb C charge variants using LabChip™ and conventional cation-exchange chromatography (CEX) methods; (*bottom:* mAb C N-glycan species using LabChip™ and conventional fluorescence-based hydrophilic interaction liquid chromatography (HILIC) methods. The variability between HTPD and conventional methods was less than 5%

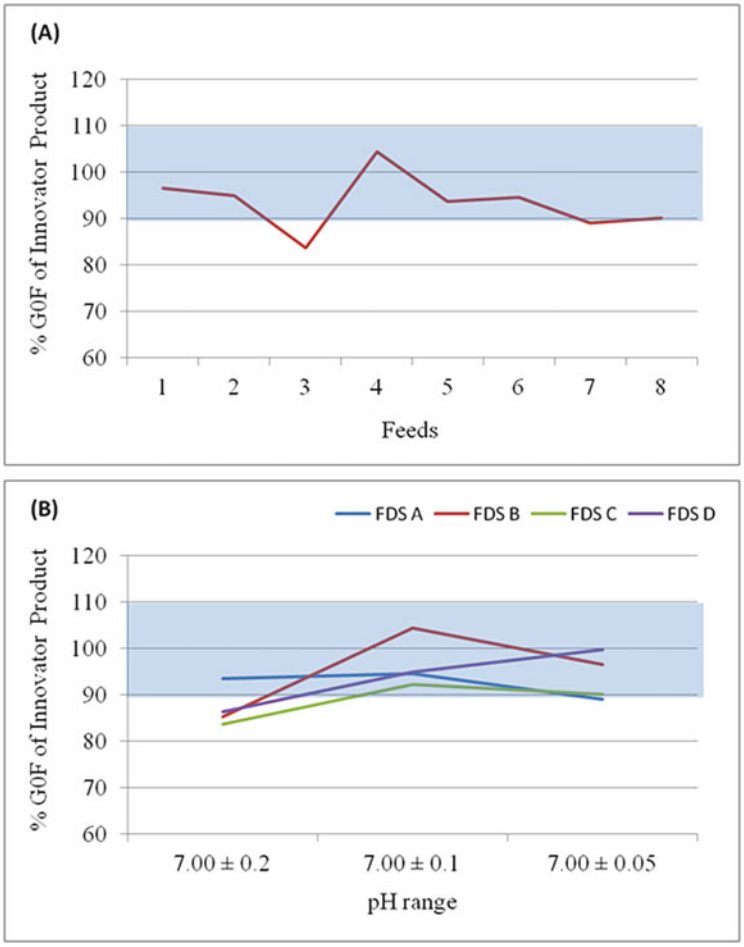


Fig. 6 Percentage (normalized to innovator value) of specific glycan structure (G0F) in case studies I and II, a critical quality attribute in the Biosimilar, and change it undergoes under various tested process conditions (**a**) with various feeds and (**b**) with pH deadband. Based on the results, the conditions which do not allow the G0F to remains within the innovator range (*blue region*) of the originator molecule were not carried forward

and shows the change it undergoes under various test process conditions. Based on the results, the conditions that did not allow the G0F to remain within the value ± variability of the originator molecule were not carried forward. Thus, feeds 3, 7, and 8 (Fig. 6a) were not evaluated further. Moreover, the selected feed showed strong interaction with respect to process pH to control the critical quality attribute in this biosimilar (Fig. 6b). Both these studies helped assess product quality metrics from cell culture process development and identify the best conditions for producing a molecule with product quality attributes matching those of the innovator.

3 High-Throughput Downstream Process Development

Development and characterization of the downstream process during a biopharmaceutical's development life cycle has historically relied on univariate, trial-and-error laboratory-scale chromatographic experiments. Portions of this traditional approach have recently been replaced with HTPD techniques to accelerate development timelines and enable the generation of large datasets across a wide range of operating conditions [24–26]. Although conventional column evaluations are still necessary and beneficial for material generation, process confirmation, and scaleup, HT techniques can be employed early in development to narrow the development window with reduced effort and cost. Furthermore, implementation of HTPD approaches during later process characterization phases can increase speed, enhance process understanding by generating more data, and reduce material requirements. Implementation of a HTPD approach for process characterization has not been accepted by the regulatory agencies to date, although data demonstrate that comparable results can be achieved for minicolumns and the traditional process characterization approach [27].

In addition to HTPD methods used to screen and optimize chromatography conditions, 96-well plates have also been implemented to investigate optimal conditions for unit operations including, but not limited to, protein refolding, PEGylation, and protein precipitation [28]. An automated inclusion body refolding screening study was described by Vincentelli et al. in which a range of refolding buffers were evaluated for 24 proteins. A suitable refolding buffer was identified for 17 of the 24 proteins studied using this approach [29]. Several vendors have launched products to support the screening of protein refolding and precipitation conditions.

3.1 Tools for High-Throughput Downstream Process Development: Chromatography Studies

Several tools have been developed to support HTPD for downstream activities. For chromatographic experiments, there are three basic HTPD approaches: microtiter plates micropipettes, and minicolumns (Table 3). Each tool and its applications are described in the following sections.

Table 3 Comparison of traditional chromatographic approaches with HTPD tools

Variable	Traditional lab-scale columns	Microtiter plates	Micropipettes	Microcolumns
Time	High	Easy	Easy	Easy
Cost	High	Low	Low	High
Automation	Difficult	Difficult	Easy	Easy
Experimental design capabilities	Broad	Narrow	Narrow	Modest

3.1.1 Microtiter Plates

A common technique used early in development involves microtiter plates. For the described application, microtiter plates are 96-well filter plates filled with a fixed volume of resin. Process solutions are added to each resin-containing well using a multichannel pipette or robotic liquid handling system in an iterative manner. Each phase of a chromatographic unit operation can be employed by addition of the process solution (i.e., equilibration buffer, load, wash buffer, elution buffer, etc.). Typically, the process solution and resin are mixed, followed by an incubation period. The process solution is then removed by centrifugation or vacuum filtration. Effluent is collected in an additional 96-well plate suitable for its intended use. For example, eluates can be collected and analyzed using a plate reader to ascertain step yields. After protein content assessment, HT analytics can be employed to assess other product quality attributes (i.e., content of high molecular weight species or host cell protein).

Many vendors provide microtiter plates prefilled with their resins for initial resin and condition screening studies (i.e., GE Healthcare, MilliporeSigma, Sartorius Stedim, Pall Corporation). Alternatively, empty microtiter plates can be manually filled with any chromatography resin or combination of multiple resins. When preparing microtiter plates in house, it is important to consider the method used for addition and ensure that a uniform distribution of media is reproducibly achieved [24]. In addition to evaluating chromatography resins in microtiter plates, vendors have also developed 96-well plates for evaluation of membrane adsorbers.

There are a broad number of uses for microtiter plates in the process development life cycle. Most applications involve studying conditions in the semi-equilibrium state to help narrow the operating window of a traditional column format. Kramarczyk first described the application of batch-binding in a 96-well filter plate for the development of ion exchange and hydrophobic interaction chromatography (HIC) unit operations [30]. Since then, the approach has been employed to evaluate dynamic binding capacity on Protein A [31], explore the selectivity of HIC resins for removal of high molecular weight aggregates [26], and evaluate the effect of pH, sodium chloride, and phosphate on ceramic hydroxyapatite performance [32]. The main advantages of microtiter plate screening are that many conditions can be evaluated in a short period of time and the material requirements are low. With this approach, one can narrow the operating window for evaluation using a laboratory-scale column. Sanaie et al. employed this approach to identify operating windows on CEX for a glycoprotein [33]. The automation of experiments utilizing microtiter plates was described by Coffman et al. for the separation of impurities from target mAbs for HIC and CEX unit operations [24]. Similar experimental designs have been reported for the evaluation of membrane adsorbers [34, 35]. More recently, McDonald et al. developed a HT batch-binding screen for the optimization of CEX elution conditions for optimal removal of impurities [36].

The use of microtiter plates for downstream development purposes has been extended to the evaluation of defined unit operations to clear model viruses. Connell-Crowley et al. [37] utilized microtiter plates and Q Sepharose Fast Flow or Capto-adhere in a full factorial DOE to evaluate the impact of pH and salt concentration on clearance of the virus XMuLV. Data were also generated using traditional laboratory-scale columns packed with Q Sepharose Fast Flow. Comparable data were generated between the two formats, suggesting that HT techniques can be utilized to understand the capabilities of a step for virus clearance [37]. This strategy could be useful in cases where material availability or viral clearance study design could be impacted by the viral clearance design space. Given that all process-specific parameters are not conserved in the microtiter plate format, this HT technique would not serve as a reasonable scale-down model for a viral clearance validation study.

A major limitation of microtiter plates is the inability to evaluate conditions where dynamic movement impacts performance. A fundamental assessment of this was performed where mass-transfer principles were applied along with published experimental data to identify instances where microtiter plates are not translatable to laboratory-scale columns. In all cases, the adsorption process should be considered and, in instances where adsorption only occurs on the surface of a particular resin, microtiter plates probably do not generate data translatable to laboratory-scale columns [38]. Furthermore, the noncontinuous operation limits the types of experiments that can be performed. For example, the batch format does not support separations based on size because only one stage of separation is possible. In general, microtiter plates are useful early in development to narrow the operating space before performing studies with laboratory-scale columns.

3.1.2 Case Study: Mixed-Mode Resin Screening Using Microtiter Plates

We have used microtiter plates in a recent head-to-head comparison of four mixed mode resins. The impact of load pH, elution pH, and elution sodium chloride concentration were evaluated for three different mAbs. For each resin and antibody, operating zones where low yields were obtained were easily identifiable (Fig. 7). Identification of favorable operating conditions can contribute to reducing the effort required for scaling to traditional laboratory-scale columns to finalize a unit operation. These data were used to narrow the load and elution operating pH window to evaluate the ability of each resin to remove host cell protein and high molecular weight aggregates while still maintaining an acceptable step yield.

In addition to the downstream specific experiments outlined above, microtiter plates are a useful tool for the capture of cell line development and upstream process development samples for further analytical analysis. High-throughput capture and concentration of a large number of samples can reduce the time and labor required to prepare these samples for analysis by techniques where cell culture media may interfere with the assay. In the case of mAbs, PreDictor™ plates

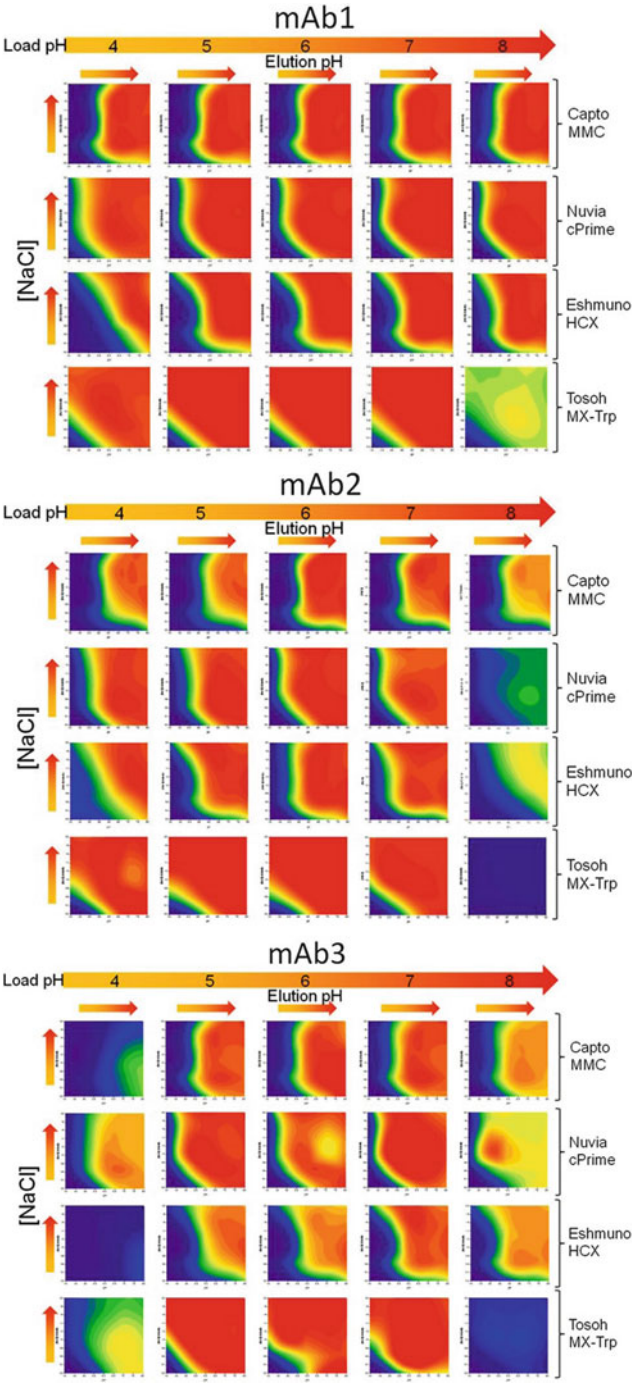


Fig. 7 Evaluation of four mixed-mode resins using high-throughput methods. Microtiter plates were used to evaluate the impact of load pH, elution pH, and elution sodium chloride concentration on each of four mixed-mode resins for three monoclonal antibodies

(GE Healthcare) prefilled with ProA media are a useful tool for antibody capture and concentration prior to analysis.

3.1.3 Case Study: Head to Head Comparison of PreDictor™ Plate Capture Versus Traditional Laboratory-Scale Capture

The representativeness of samples generated from microtiter plate capture for product quality assessment is a concern given the operational differences compared with traditional column capture operation. Because all unit operation parameters cannot be maintained during microtiter plate capture (i.e., linear flow rate, continuous operation), the population of product captured can differ and result in nonrepresentative product quality data. To assess the feasibility of using microtiter plate capture for sample analysis in lieu of traditional capture approaches, we captured an mAb using both methods. Both PreDictor™ plate capture and the defined operating parameters using a laboratory-scale Protein A column utilized identical buffers. The resulting samples were assayed by size-exclusion chromatography (SEC)-HPLC, CEX-HPLC, nonreduced and reduced CE-SDS, and glycan. The PreDictor™ plate captured samples were immediately neutralized. Comparative samples from traditional column capture were reported after viral inactivation and neutralization. The difference in the viral inactivation unit operation may contribute to the 2% difference observed in the SEC-HPLC analysis. The glycan data for the column approach was reported after complete purification to bulk drug substance (BDS) through the defined downstream process. Minimal differences were observed between the PreDictor™ plate captured material and the BDS generated. Table 4 summarizes the data generated using both methods and demonstrates the validity of the microtiter plate approach for use in assessment of samples coming from cell line development or upstream process development.

3.1.4 Case Study: Microtiter Plate Capture to Support Cell Line Development and Upstream Process Development

For biosimilars, and in certain cases for innovator molecules, a specific product quality profile is desired from the upstream process. A biopharmaceutical's glycan profile, for example, is a product quality attribute that is typically not influenced by the downstream process, meaning that any desired profile must be achieved during the upstream process. Implementation of PreDictor™ plate capture and analysis during the production phase of the upstream process can help define critical parameters that influence the product quality profile. The data reported in Table 5 exemplifies a case where the glycan profile was monitored from day 12 to day 16 of the production. Data are reported as percentage change from the day 12 sample. During this time, a dramatic increase in percentage change of high-mannose glycans and a decrease in percentage change of galactosylation were observed. The time course data were used to define an acceptable harvest window to meet the target glycan profile.

Table 4 Comparison of data generated using PreDictor™ plate capture and traditional chromatography columns

	SEC				CEX		
	%HMW	%Main	%LMW		%Acidic	%Main	%Basic
PreDictor™ plate	3	97	0		13	59	27
Column—cycle 1	5	95	0		11	54	35
Column—cycle 2	5	95	0		11	55	34
	Non-reduced CE-SDS						
	%LC	%HC	%HL	%HH	%HHL	%NG	%Main
PreDictor™ plate	1	0	0	0	3	5	90
Column—cycle 1	1	1	0	1	4	5	88
Column—cycle 2	1	0	0	1	4	5	89
	Reduced CE-SDS						
	%LMW	%LC	%MMW	%NGHC	%HC	%Post HC	%HMW
PreDictor™ plate	0	30	0	5	65	0	0
Column—cycle 1	0	29	0	5	65	0	0
Column—cycle 2	0	29	0	5	65	0	0
	Glycan						
	%G0	%G0F	%M5	%G0F+N	%G1F	%G2F	
PreDictor™ plate	1	71	1	6	13	3	
BDS	0	74	1	6	13	3	

With the exception of glycan, all samples generated with PreDictor™ plates are compared with column-captured material after viral inactivation. The glycan sample generated by PreDictor™ plate is compared with a bulk drug substance (*BDS*) sample
HMW high molecular weight species, *LMW* low molecular weight species, CEX cation exchange chromatography, *LC* light chain, *HC*heavy chain, *HL* 1 HC, 1LC, *HH* 2 HC, *HHL* 2HC, 1LC, *NG* non-glycosylated, *MMW* mid molecular weight, *NGHC* non-glycosylated heavy chain

Table 5 Production bioreactor glycan time course

	% Fucosylation	% aGalactosylation	%High mannose	% Afucosylation	% Galactosylation
Day 12	0	0	0	0	0
Day 13	0	2	5	2	−10
Day 14	0	5	24	8	−22
Day 15	0	7	26	8	−29
Day 16	−1	6	45	14	−30

Values are reported as percentage difference from the day 12 sample

3.1.5 Micropipettes

PhyNexus, Inc. (San Jose, CA) has developed the PhyTip®, a packed resin bed housed within the end of a pipette tip. The intention of the PhyTip® is to serve as a protein purification and enrichment tool that can be used to concentrate feed streams or perform initial screening studies. The PhyTip® can be coupled with a multichannel pipette or robotic system to increase throughput for resin and

condition screening. Resin volumes vary from 5 to 320 μL. Tip-based methods rely on pulling sample up into the tip and repeatedly aspirating sample back and forth for a fixed period of time to mimic residence time. This maximizes product binding and allows the system to reach equilibrium.

Most PhyTip® products focus on affinity capture, targeting antibodies (ProA, ProG, and ProPlus, a mixture of ProA and ProG media) and other tagged proteins (His, GST, streptavidin, etc.). The product line was expanded to include gel filtration media as well as ion-exchange media. A set of custom micropipette tips were employed for developing a microscale purification process to evaluate various yeast fermentation conditions for the generation of human papillomavirus virus-like particles [39]. Furthermore, PhyTips® were used to evaluate the impact of pH and salt concentration on antibody capture. After identifying ion-exchange conditions, two selected conditions were scaled up 500-fold, and it was confirmed that similar trends were observed between scales [40].

In addition to the products available from PhyNexus, custom pipette tips have been described in the literature [41]. MilliporeSigma offers the ZipTip®, which are designed to aid in sample preparation for mass spectrometry (MS) and other analytical techniques that require desalting, purification, and concentration of small volume samples.

3.1.6 Minicolumns

The development of a microscale, packed-bed minicolumn has allowed many traditional downstream process development experiments to be scaled to the microscale. Atoll GmbH, now a part of Repligen, offers products with packed resin beds ranging from 5 to 600 μL for use in HTPD experiments. The MediaScout® line comes in formats compatible with centrifugation, pipettes, and automated robotic systems. In addition to the minicolumns, Atoll provides the MiniChrom line that allows evaluation of column volumes ranging from 0.2 to 10 mL.

CentriColumns come in a 96-well plate format and encompass a series of individual small columns housed in a base plate. The small columns can be procured in resin bed volumes of 50–200 μL and can be reorganized within the base plate as needed, based on experimental design. Above the packed resin bed is a reservoir for process solutions to be added via pipette or an automated liquid handling system prior to exposure to the packed bed by centrifugation. A receiving plate nests underneath the base plate for collection of column effluent. Sequential liquid addition and centrifugation steps are performed to mimic a chromatographic unit operation.

MediaScout® PipetColumns are similar to the CentriColumns; however, they come in strips of eight columns and are intended to be operated with a positive liquid displacement pipette. The use of a positive liquid displacement pipette allows a controlled flow rate to be evaluated because the dispensing speed of the pipette can be modulated. The eight-column strip can be moved across a 96-well plate to collect fractions as resins or operating conditions are evaluated.

The RoboColumn® platform is fully compatible with automated liquid handling systems for hands-free operations. Up to 96 RoboColumns® can be organized on a

specialized plate, depending on experimental design. Tecan and Atoll have collaborated to make the Tecan Freedom EVO® platform compatible with operation of RoboColumns®. The characterization and optimization of this platform has been essential in understanding critical considerations for ensuring the scalability of this platform [42].

Several studies have focused on assessing the scalability of data generated using minicolumns. Experiments were executed comparing the use of PipetColumns and RoboColumns® with traditional laboratory-scale operations to evaluate the ability of minicolumns to generated data that is predictive of performance at the laboratory scale. Although there are inherent differences in the operations of minicolumns versus traditional chromatography (i.e., intermittent flow versus continuous flow), the minicolumns generated data that was suitable for use in simulations of benchtop performance [43].

MiniChrom columns are intended to be a second level of miniature scale evaluation during process development activities. MiniChrom columns are compatible with most liquid chromatography systems and have been proven to generate scalable results. Brenac Brochier et al. [44] evaluated MiniChroms ranging from 2.5 to 10 mL for column packing performance, dynamic binding capacity, and elution conditions across a series of mixed-mode resins. Their study highlights the advantage of using minicolumns early in development for savings in material and time [44].

3.1.7 Application of UNICORN Method Queues and Scouting Functionality

When traditional HT methods are not desired or available, alternative approaches can be implemented to gain efficiency. GE Healthcare, as part of their UNICORN software and ÄKTA Avant platform, has embedded functionalities that enable users to employ automation.

Method queues can be written or the scouting functionality can be used to allow initial setup of the ÄKTA system, followed by sequential execution of multiple chromatography experiments. The standard ÄKTA Avant system configuration allows 18 buffer inlets, 7 sample inlets, 5 column positions, a fraction collector, and 10 outlet lines. A custom configurable system, the ÄKTA Pure is also available if the full functionality of the ÄKTA Avant is not required. In addition to GE Healthcare, other vendors provide chromatography systems and software that allow similar automation.

3.2 Applying High-Throughput Tools for Chromatographic Process Development

Several tools are available to help reduce the time and material required for early downstream process development studies. Each of these tools is best applied to

Table 6 HTPD techniques and applications for downstream process development

Process variable	Micropipettes	Microtiter plates	Minicolumns
Static binding capacity	✓	✓	–
Dynamic binding capacity	–	✓	✓
Adsorption isotherms	–	✓	–
Resin screening	✓	✓	✓
Screening of binding conditions	✓	✓	✓
Screening of elution conditions	✓	✓	✓
Gradient elution studies	–	✓	✓
Separations based on size	✓	–	✓
Resin lifetime and cleaning efficiency	–	✓	✓
Capture and concentration of samples from cell culture media	✓	✓	✓

specific situations in downstream processing. In contrast to cell culture processes, none of these HT downstream tools is a perfect SDM for large-scale column operation. Nevertheless, important data can be rapidly collected by applying these techniques for the appropriate purpose. Table 6 compares some of the experimental capabilities of the methods outlined above.

Microtiter plates and micropipettes are useful tools for experiments that can be performed in a state of equilibria, whereas minicolumns provide the ability to assess dynamic properties at the miniature scale. This conclusion was experimentally demonstrated by a comprehensive evaluation of the three HTPD approaches, focusing on analysis of yield, aggregate clearance, and host cell protein clearance [45]. RoboColumns® provided the most representative results compared with conventional chromatography columns; however, all three methods provided trending information and predicted worst case conditions. The broad implementation of HT approaches across the industry highlights the advantages in cost, effort, and material requirement, which reduce the time needed to define downstream processes in an effort to bring biopharmaceuticals to the clinic as rapidly as possible.

4 High-Throughput Analytical Methods

The emergence of HTPD upstream and downstream technologies for process development has drastically increased optimization and development time, which can result in significant cost savings and increased productivity [28]. One byproduct of HTPD is the generation of significantly more samples that require rapid analytical assessment. During early phase process development, critical quality attributes have not been fully identified. However, it is crucial to develop a process that reproducibly produces a product with similar quality attributes. It is also crucial to understand product quality attributes as they relate to process conditions because this will assist in process development [46]. To assist in this demand for rapid

Table 7 Comparison between conventional and high-throughput analytical methods for supporting various process development activities

Quality attribute	Historical	Supporting high-throughput process development
Titer	ELISA, HPLC	BLI, BIO-HT
Charge	vIEF-gel, clef	iCE
Potency	Binding ELISA, SPR	Liquid-handling robotics, BLI, semi-automated SPR
Aggregates/ fragments	SDS-PAGE, CGE, SEC-HPLC	Chip-based microfluidics, cartridge-based systems, UPLC
Oxidation	HPLC, PMP	UPLC
Residuals (host cell protein)	ELISA, HPLC	Flow-through microfluidics, liquid-handling robotics, BLI
N-glycan	PNGase F followed by reductive amidation, intact mass	Kit-based sample preparation with instant labels, UPLC, or microfluidics

analysis, many technologies have been developed for fast analytical characterization of protein-based therapeutics. No single analytical method is capable of determining all product quality attributes because no single method can interrogate primary, secondary, and tertiary structures [47]. During the early years of protein analytics, many technologies could fully characterize proteins; however, these methods often required significant hands-on time and/or instrument time. Later technologies evolved higher throughput platforms that were able to sample 20–-30 samples/day, with some analyst setup time. However, the number of samples submitted and required turnaround time associated with HT development cannot be supported by these technologies [48]. Fortunately, technologies have evolved beyond these second-generation technologies to deal with over 50 samples/day with minimal analyst setup time. This section describes the different technologies for evaluation of protein quality attributes in support of HTPD (Table 7). The analytics evaluated during upstream process development include evaluation of titer, charge variants, potency, aggregates and/or fragments, oxidation, and glycan variants. Typically, downstream process development focuses on evaluation of aggregates, charge, potency, and residuals [49, 50].

4.1 Analysis of Upstream Samples

Host cell proteins, DNA, and media additives typically interfere with the analysis of upstream samples. Although some traditional analytical methods can be used to interrogate protein product quality of upstream samples without sample manipulation (e.g., SEC, Western blot), most methods require significant development and execution effort or the results provide only qualitative assessments [51]. To alleviate this problem, some recent advances allow the rapid development and assessment of protein quality attributes without purification or rapid small-scale (offline) purification, as previously outlined in Sect. 3.

4.1.1 Capillary-Based Western Blots

Western blots are a powerful tool for analyzing protein quality attributes (size, titer, charge) in complex mixtures such as upstream samples. However, running Western blots often requires significant development time and is low throughput. ProteinSimple™ has developed multiple platforms that allow for the rapid development and assessment of capillary-based Western blots in their Simon® system and the newer generation system, Peggy Sue®. These systems are able to determine the charge or size profiles of specific proteins in cell culture supernatant, even if the protein is in low abundance or only a small amount is available. Although the instrumentation can be costly, it is able to provide results for up to 96 samples in one run.

4.1.2 Small-Scale Purification of Upstream Samples

Although certain product quality or titer measurements can be made directly on upstream samples without purification, the majority of analytical measurements still require some degree of purification. As outlined in Sect. 3, small-scale (offline) purification can be carried out in a 96-well plate format. For antibodies, several commercially available kits exist that contain equilibration, wash, and elution buffers. One example is the Agilent™ AssayMap® affinity purification cartridge. The platform is able to purify up to 96 50 µL (about 100 µg) aliquots of antibody in about 45 min. Although the system is rapid, robust, and easy to use, the included elution buffer is not ideal for all antibodies, because the low elution pH can impact protein product quality. GE™ PreDictor® plates are another option for small-scale protein purification. These plates can be modified to contain any resin available in a slurry, and the downstream equilibration, wash, and elution conditions can be used to produce small quantities of partially purified protein that facilitate analysis.

4.2 Product Quality

HTPD can be extremely effective for producing protein in an abridged timeline. Analytics that can assess product quality need to be rapid as well as accurate, robust, and precise [52]. This section evaluates rapid methods for analysis of various product quality attributes and compares them with traditional methods.

4.2.1 Charge

Protein charge profile can be impacted by asparagine deamidation, aggregation, N-terminal pyroglutamate formation, isomerization, protein sialyation, fragmentation, or C-terminal lysine variants. The impacts of these amino acid modifications

on in-vivo efficacy, clearance, and safety can vary dramatically and are difficult to predict [53]. Deamidation of antibodies in the variable regions can lead to loss in potency, whereas deamidation in other regions may not impact potency at all [54, 55]. Similarly, in mAbs, C-terminal lysine variants are unlikely to impact potency [56, 57]. However, with the increase in the number of bispecific antibodies, Fc fusions, and non-mAb proteins being introduced to the clinic, it is important to study charge variants in all regions. Traditionally, charge variants have been studied using isoelectric focusing (IEF) gels or CEX-HPLC [50]. The information produced by these methods is often more qualitative then quantitative. The advent of CE (typically evaluated using a Beckman Coulter PA800® Instrument) allowed e quantitative assessment of acidic and basic variant isoelectric points. Although capillary zone electrophoresis (CZE) and capillary isoelectric focusing (cIEF) allow accurate and robust determination of acidic and basic variants (and isoelectric points using cIEF), method development can be complex and sample preparation and analysis is often complicated. Imaged capillary electrophoresis (iCE) significantly reduces sample acquisition time and allows rapid method development. The ProteinSimple™ Maurice® and iCE3® systems are two platforms that can accurately and precisely assess the protein charge profile of sample in less than 10 min. This allows rapid optimization and quick data turnaround times. As observed in Fig. 8, iCE is able to provide detailed information regarding pI, whereas CEX-HPLC typically reports a main peak with relative acidic and basic variants. Although CEX-HPLC typically has the shortest sample preparation time and can be very robust, it often requires significant instrument time. Furthermore, CEX-HPLC fractions can be collected in large quantities from several injections and analyzed for specific variants. CZE or cIEF are typically the least robust methods and require more sample preparation with medium run times. CZE and cIEF have also demonstrated the ability to be multiplexed with MS for direct analysis of peaks [58]. Although CE-MS is useful, its widespread implementation has not been as rapid as other MS-based methods because of sensitivity and buffer compatibility issues.

4.2.2 Oxidation

Oxidation imparts minimal impact on protein molecular weight and can be difficult to evaluate. Protein oxidation has been linked to differences in efficacy [59]. Traditional methods to evaluation oxidation have used reverse-phase HPLC methods to evaluate either intact proteins on a C4 column or protease-fragmented proteins on a C18 column. Although peptide mapping methods are information-rich, they often impart sample preparation artifacts and can exhibit significant run-to-run variability. Analyzing intact proteins is significantly more reproducible because there is no sample preparation; however, the information provided is often not sensitive enough to determine small changes in oxidation. The development of ultraperformance liquid chromatography (UPLC) HIC and reverse-phase columns has improved the sensitivity of oxidation determining methods, decreased acquisition time, and decreased mobile phase requirements by decreasing column size and particle size. Proteases

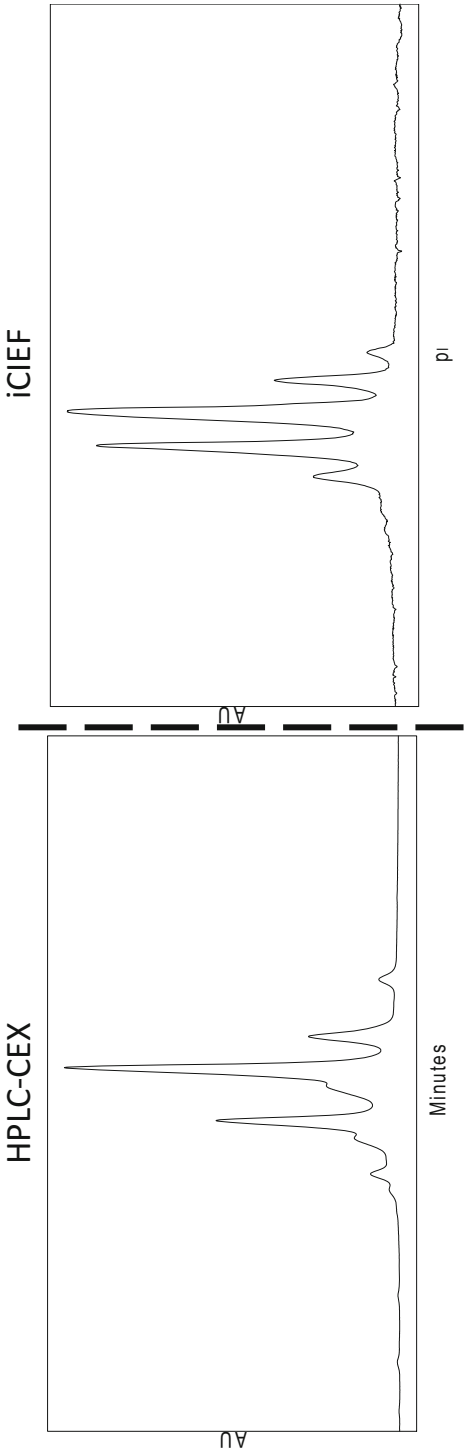


Fig. 8 Comparison of charge profiles for a monoclonal antibody analyzed by CEX-HPLC and icIEF

that cleave very specific regions in a protein have been developed to break proteins into large fragments that can be clearly separated by UPLC. One example of this is the enzymatic digestion of antibodies into Fc and Fab regions. The smaller proteins fragments often yield an increase in oxidized versus nonoxidized fragment resolution, which can be confirmed by LC/MS [60]. Although this approach does not identify site-specific oxidation, the straightforward sample preparation has been adapted to Genovis™ small spin columns , which can be used to support HTPD. In combination with reverse-phase or HIC UPLC methods that have short run times (less than 10 min), oxidation can be quickly evaluated. Both reverse-phase and HIC methods are prone to low resolution. As a result, where possible, it is best to avoid Fab oxidation through selection of a protein sequence (using sequence liability analysis) with minimal modification in the Fab region. Fc region oxidation is not as important to avoid; however, in some cases Fc oxidation has been shown to impact Fc binding [61, 62].

4.2.3 Aggregates/Fragments

Protein aggregation and, to a lesser extent, fragmentation/deglycosylation are critical quality attributes in nearly all protein production processes. Aggregates can lead to significant side effects and fragmentation can lead to loss in potency [63–65]. Traditional methods for analysis of fragments include SDS-PAGE, which evolved into capillary gel electrophoresis (CGE), and SEC-HPLC [66]. CGE and SEC-HPLC have become the gold standards, resulting in the US Pharmacopeial Convention (USP) providing compendial SEC and CGE methods for the analysis of mAbs. The USP SEC method uses a 30 min analysis time with minimal sample preparation. This method can be adapted to UPLC and the run time can be reduced to less than 7 min; however, the USP HPLC method is generally sufficient for supporting the number of samples provided by HTPD. One simple improvement is to use HPLC configured to sample from 96- or 384-well plates. CGE is typically used to analyze fragments and deglycosylation because it provides higher resolution then running SDS-PAGE and data can be analyzed using chromatography software. CGE is preferred to SEC-HPLC because SEC-HPLC is often unable to resolve protein fragments or smaller impurities. Analyzing protein fragments by CGE requires significantly more effort than using SEC-HPLC; therefore, many technologies have focused on increasing CGE throughput. Some companies have developed technologies around disposable chip or cartridge platforms that can analyze samples significantly faster. Caliper®, Maurice®, and Peggy-Sue® are platforms that take advantage of chip or cartridge technologies to analyze samples. The Caliper® is a powerful microfluidic and chip-based platform than can be used to evaluate protein fragments in hundreds of samples per day. The Caliper® system is excellent for screening upstream or downstream conditions where significant numbers of samples are produced. However, compared with the gold standard (CGE), the Caliper® tends to yield results indicating higher purity. This may be a direct result of the reduced sample preparation associated with the Caliper® compared with the USP CGE method, or the result of different interactions because

the Caliper® is a microfluidics based system. The newly developed Maurice® platform uses cartridges, thus eliminating many of the moving parts associated with the Beckman Coulter PA800 systems that can result in breakage of the capillaries or instrument errors. Although the cartridges can only run 200 samples per cartridge, changing the cartridge is significantly easier then replacing a broken capillary. Although there is only a time advantage for sample preparation associated with Maurice® compared with the more traditional Beckman Coulter PA800 systems, the larger benefit lies in the larger number of samples that can be run in a single Maurice® run than in Beckman Coulter runs. Similar to the Maurice®, the Peggy-Sue® (Simple Western family) uses a capillary to separate proteins. However, instead of using UV or fluorescence for direct detection of protein, the Peggy-Sue® uses fluorescent antibodies to label proteins of interest. This offers two benefits over the Maurice®. The first benefit is that the system is able to analyze proteins of interest in complex mixtures such as cell culture supernatant. The second benefit is that the detection threshold is significantly lower, which allows quantification of significantly smaller amounts of protein. This is especially important to consider when dealing with limited amounts of material. Furthermore, the system can handle up to 96 samples per day, allowing rapid throughput of upstream samples for analysis.

4.2.4 Potency

A manufacturing process can produce protein that is clear of all aggregates and residual contaminants; however, if the protein is not active, the process is irrelevant. For this reason, potency is the most important quality attribute [47]. Potency can be evaluated using cell-based assays or by testing binding to proteins of interest. This section evaluates protein binding assays. For a more complete evaluation of cell-based assays please refer to published reviews [67–69]. Traditionally, protein-based binding assays utilize high-binding 96-well plates for determining EC_{50} values relative to a control sample [70]. Alternatively, surface plasmon resonance (SPR) is a label-free and real-time method used to provide protein–protein kinetic information [71]. However, both of these methods often require significant development time and lengthy sample preparation and evaluation times. Typically, an ELISA-based protein binding assay requires overnight incubation of a protein of interest with a high-binding plate, followed by several rounds of incubation for blocking, protein interaction, and detection steps. Furthermore, using a traditional eight-point standard curve on a 96-well plate automatically limits the plate to 11 samples with a control. Replicate preparations of samples are often required to ensure data reliability, thereby significantly reducing the number of samples that can be run on a plate. Using SPR to evaluate K_d values is one of the most powerful methods for interrogating protein–protein interactions. However, the method development aspect and technical expertise required is very high. In recent years, many technologies have been developed to reduce the development and/or sample run or significantly increase the sample throughput while reducing operator input.

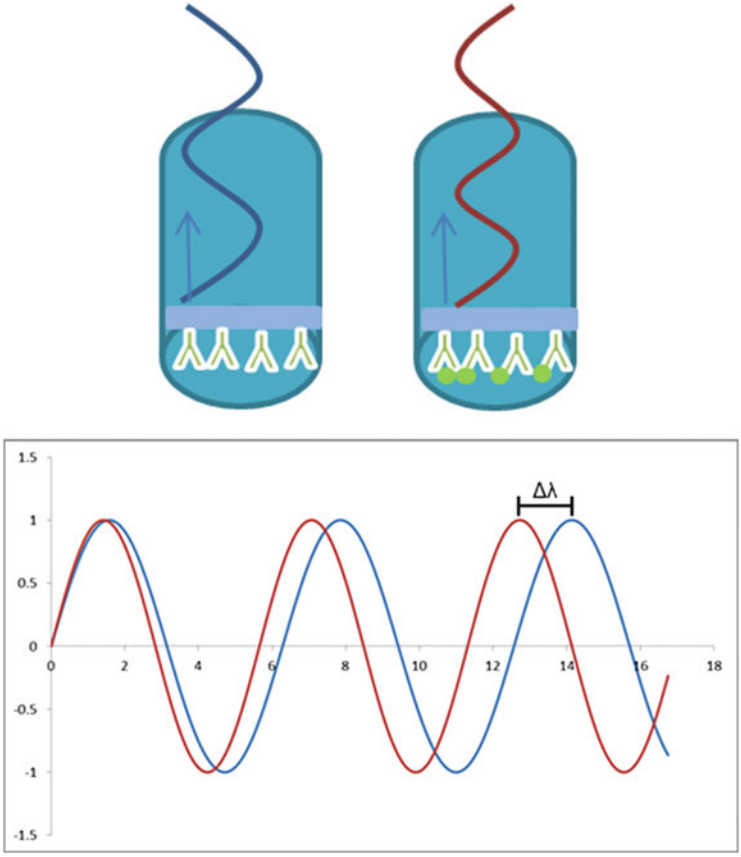


Fig. 9 Theoretical shift in wavelength observed when incident light interacts with antibody and with antibody bound to antigen at a sensor surface

Biolayer interferometry (BLI) is a method that has come to the forefront for determination of apparent K_d s or determination of relative potency values. BLI measures the change in optical density at a sensor tip, which is directly correlated to protein binding. A commonly used platform that utilizes BLI to measure binding is the Octet™ platform. As shown in Fig. 9, incident white light is sent through an optical fiber to the tip, where it is partially reflected back through the optical fiber to a detector. When the optical density changes at the tip as a result of interaction of immobilized molecules with bound molecules, there is a corresponding shift in wavelength. The detector measures the change in wavelength of the reflected light. One significant advantage of the optical fiber sensor approach is that different chemistries can be used to immobilize a wide range of molecules on the sensor tip. Some of the commonly used sensor tips include Ni-NTA, streptavidin, and

Table 8 Comparison between plate-based, BLI-based, and SPR methods for potency analysis

Parameter	ELISA	BLI/Octet	SPR/Biacore
Typical run time (one sample)	Overnight plate coating +6.5 h	*Potency ≤30 min kinetics ~35 min	~100 min
Maximum samples per day (8 h run time)	***6	**Potency = 176 kinetics = 26	≤5
Binding potency precision	%RSD ≤30%?	%RSD ≤10%	N/A
Kinetics precision	N/A	%RSD ≤10%	%RSD ≤20%
Cost per sample	~$10	≤$5	~$150
Maintenance	Minimal	Minimal (15 min/month or after heavy use)	Moderate (20 min/week + 90 min/month)
384-well capacity	×	✓	×

RSD relative standard deviation

Protein A. Although the sensors can be used for more than one analysis, this often requires more development and can lead to a loss in signal. As a result, the sensor tips are often used only once, leading to increased material cost. However, the increased material cost is balanced by reduced analyst time. Furthermore, K_d values between SPR and Octet™-based BLI methods have been shown to be comparable (Table 8). With the significantly increased sample throughput and reduced method development time, BLI is crucial to success in supporting HT analytics.

Another approach for improving sample throughput is to use automation. Significant advances have been made in the fields of liquid handling in both plate-based automation and SPR automation. Automating the SPR development process can significantly improve development time by allowing more samples to be evaluated and tested in a single sequence using multiple conditions. Some vendors, such as SensiQ®, have developed platforms to automate SPR analysis using microfluidics (the microfluidics portion also reduces run time). This approach requires more development time than BLI, but the sensitivity is dramatically improved. Measured K_d values can range from 10^{-4} to 10^{-11} M for BLI, as opposed 10^{-4} to 10^{-9} M for Octet. Furthermore, the microfluidic approach allows analysis when sample volume is extremely limited and can analyze samples volumes as low as 10 μL. Automation in combination with liquid handling has become widely adopted in industry to handle many plate-based ELISAs [72]. Companies such as Hamilton and Tecan have developed specialized robots that allow users to develop programs to run and evaluate many types of plate-based assays. Although the up-front setup time is significant, the payoff is the ability to run samples 24/7 with minimal analyst interaction. Converting a potency binding ELISA, which can take a trained analyst as long as 2 days, into a 1 h setup assay can significantly improve work flow.

Potency assays have historically required significant analyst time with low sample throughput; however, the addition of automation and new analysis technologies can dramatically reduce this time. This approach makes it possible to analyze

hundreds of upstream or downstream samples for potency in a short time, thus allowing quick process decisions.

4.2.5 Residuals

Clearance of residuals is crucial to the success of downstream process development. Residual host cell proteins, residual DNA, or process-related impurities can lead to significant safety concerns [46, 73]. The process for assessing residuals has traditionally been limited to HPLC methods or ELISA. HPLC methods are able to support the number of samples associated with HTPD, but many residuals cannot be tested with HPLC methods because of specificity or sensitivity issues. Plate-based ELISAs are able to detect very low amounts of impurities; however, they can be technically challenging to develop and optimize because of lack of dilutional linearity, specificity, or accuracy. Furthermore, plate-based ELISAs are labor intensive. As with potency assays, many technologies have been developed to overcome some of these limitations, including use of automated liquid handling systems, microfluidic-based systems, or BLI. The BLI approach and robotic approach are similar to those outlined in Sect. 4.2.4 on potency [74]. Robotic preparation systems have also been developed for analysis of residual DNA. These systems automate sample preparation (nucleic acid extraction), allowing the analyst to focus efforts in other areas. The microfluidic approach represents a significant advance in the field of residuals analysis. Gyros Protein Technologies™ has developed a microfluidic system for analysis of residual host cell protein or Protein A. The Gyrolab™ platform is able analyze 96 samples in 75 min with minimal hands-on time. One important note is that these methods cannot directly assess coverage. Addressing coverage of host cell proteins requires 2D SDS-PAGE in combination with the associated Western blot and/or analysis of residuals by MS. The initial investment in these methods can be high. However, the technologies described above can be used to infer coverage by looking at dilutional linearity to ensure no hook effect is observed. Additionally, once MS analysis has been completed and host cell proteins identified, it can be operated in detection mode to look for specific proteins. Using tandem MS, samples can be rapidly analyzed for problematic host cell proteins [75]. BLI, robotic liquid handling, and advanced microfluidic systems can all significantly reduce the amount of hands-on time needed for sample analysis, which is crucial for successfully supporting downstream HT development.

4.2.6 N-Glycan

Protein glycosylation is an important quality attribute that is crucial to study early in process development and optimization. Protein glycosylation can impact antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), potency, and safety properties [76–78]). Developing an upstream process without understanding the glycosylation properties can lead to significant problems

in the clinic. Furthermore, because the downstream process is not likely to impact this process, protein glycosylation should be closely monitored during upstream process development. Typically, glycan analysis involves denaturing the purified protein, deglycosylating using PNGase F, and labeling with a fluorescent reagent. Although the overall process has not changed much, the process has been optimized to speed up deglycoslyation, clean-up, labeling, and analysis times. Commercially available kits (Prozyme or Waters) in combination with UPLC analysis or use of the Caliper system allow up to 96 samples to be analyzed with 1 day turn-around. This process can be used to analyze samples collected from ambr® bioreactors with minimal turnaround time, allowing rapid selection of optimal conditions.

4.2.7 Multi-attribute Methods

Combining methods to achieve as much information as possible with one sample preparation is of significant interest. One approach gaining momentum is MS-based multi-attribute monitoring (MAM). It is possible to use MAM to acquire information about fragments, ID, glycosylation, and charge variants in a single method [79]. MAM development can be a lengthy process, however, with second generation mass detectors that are robust and can be easily implemented into a traditional HPLC stack; the approach is becoming more readily feasible. Implanting a MAM approach during HTPD results in more knowledge gained about the product and the process without the need to run continuously multiple methods that cannot provide information about site specific modifications.

4.3 Integrated Workflows for Rapid Assessment of Product Quality and In-Depth Analysis of Quality Attributes

Studying a single product quality throughout upstream or downstream may not present a rate-limiting step for analytical analysis using traditional methods. However, studying only one attribute does not significantly reduce risk to the development strategy or demonstrate control of product quality. To study all attributes using traditional methods, as shown by the workflow in Fig. 10, would take a single analyst over 2 weeks of laboratory time and many hours of instrument time. As an example, in Fig. 10, 50 samples are submitted for product quality analysis.

When samples were submitted using the faster throughput methods outlines above, the analyst time was reduced to less than 3 days, with complete data collection in less than a week (Fig. 11). The overall savings in instrument time and analyst time became significant by combining all of the higher throughput methods. This savings in time can be applied to more in-depth analysis of each individual method and can be used to implement orthogonal assays.

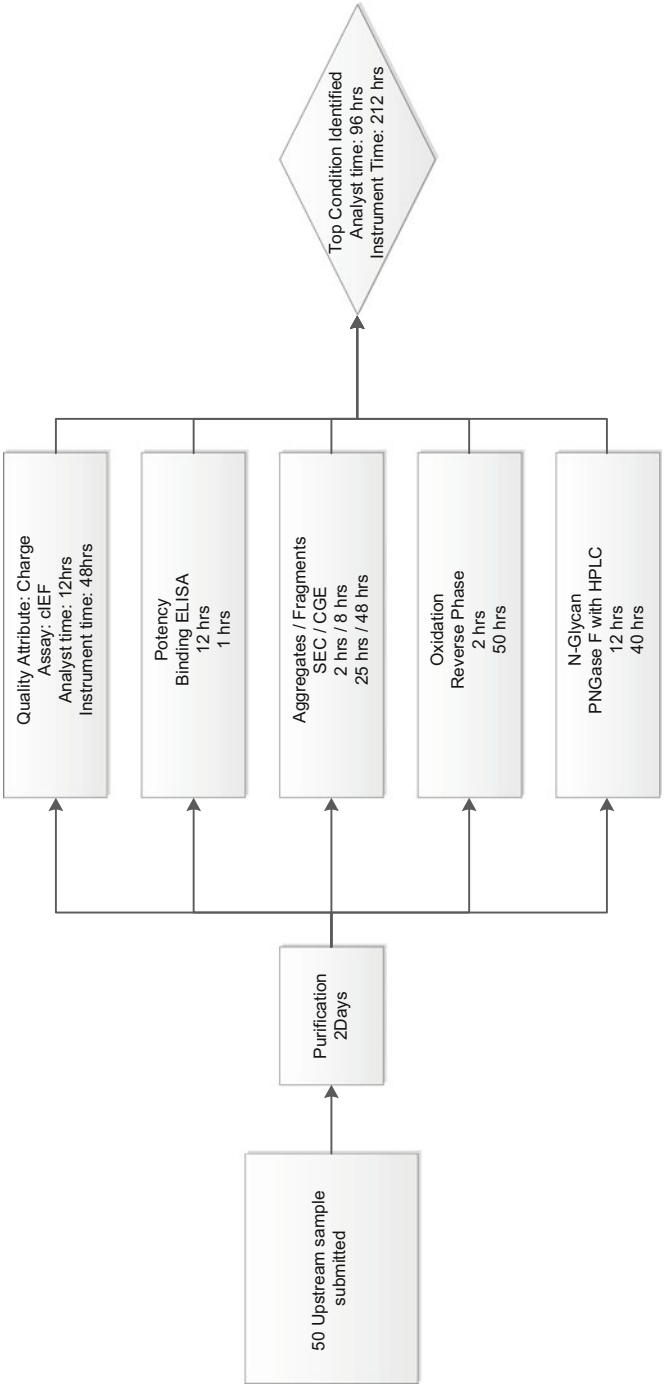


Fig. 10 Workflow for analysis of upstream samples for product quality

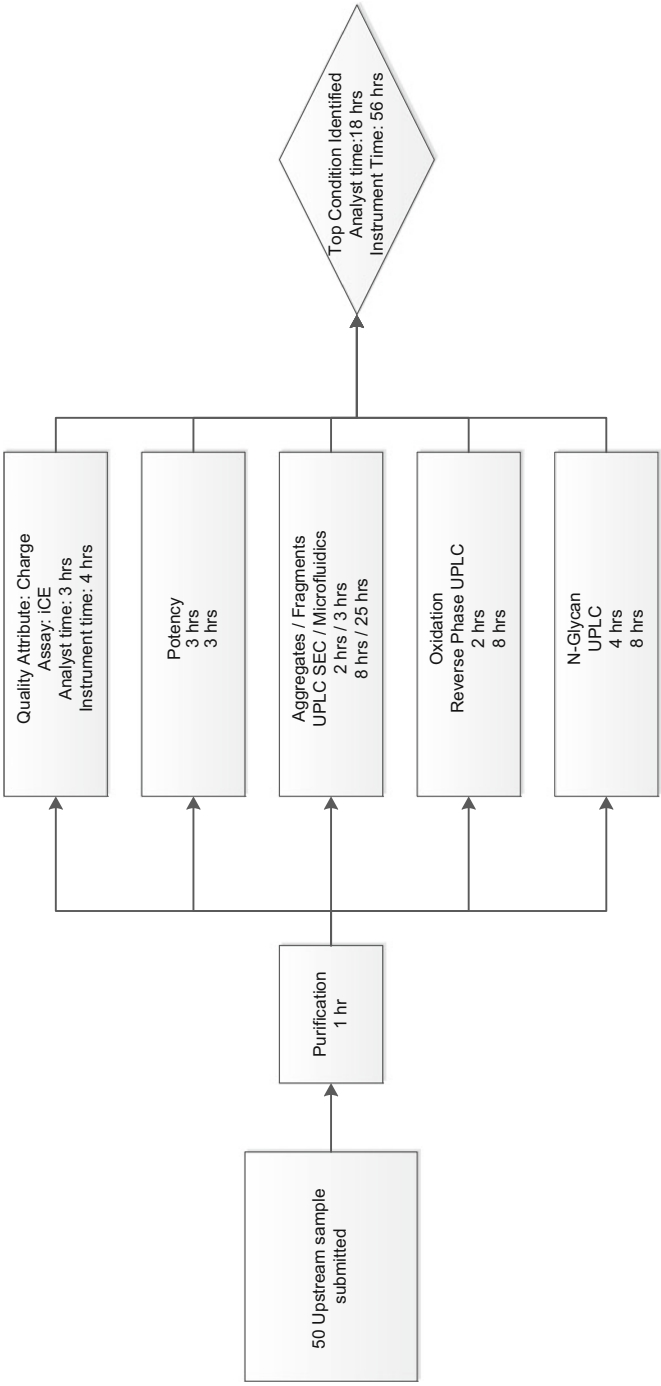


Fig. 11 Integrating higher throughput analytics into an upstream sample analysis workflow

Supplemental platforms can be integrated into the workflow for added product quality information. HPLC and UPLC fraction collectors can allow supplemental peak investigation. These fractions can be analyzed by potency assays or MS to help identify those attributes that are critical to the protein. However, many of the new platforms do not rely on LC for product information; therefore, fraction collection is generally not possible. However, direct injection onto MS after separation by microfluidics in the case of CE has been successfully implemented (not including CGE). By combining these orthogonal assays, more information can be collected using the same sample preparation; therefore, significantly more information can be collected by using a little more instrument time.

5 Conclusions

The development of mAb processes have been greatly facilitated by the use of platform approaches in the industry. However, the same has not been possible for other classes of proteins that do not have the same degree of biochemical similarity. As a result, there is urgent need for HTPD tools to speed up the development of bioprocesses. Less time spent in development translates directly into faster introduction into clinical trials at the FIH stage. Later in the development pathway, there is a need to conduct rapid process characterization and scale down validation experimentation to study the manufacturing process at the laboratory scale and create a robust control strategy that can be implemented at a large scale. The tools that have been developed for cell culture, chromatographic purification, and rapid analysis are making a significant difference in how process development experiments are conducted throughout the biotechnology industry. These tools are increasingly enabling rapid and more efficient experimentation in a shorter timeframe.

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A Different Perspective: How Much Innovation Is Really Needed for Monoclonal Antibody Production Using Mammalian Cell Technology?

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Abstract As biopharmaceutical companies have optimized cell line and production culture process development, titers of recombinant antibodies have risen steadily to 3–8 g/L for fed-batch mammalian cultures at production scales of 10 kL or larger. Most new antibody products are produced from Chinese Hamster Ovary (CHO) cell lines, and there are relatively few alternative production hosts under active evaluation. Many companies have adopted a strategy of using the same production cell line for early clinical phases as well as commercial production, which reduces the risk of product comparability issues during the development lifecycle. Product quality and consistency expectations rest on the platform knowledge of the CHO host cell line and processes used for the production of many licensed antibodies. The lack of impact of low-level product variants common to this platform on product safety and efficacy also builds on the established commercial history of recombinant antibodies, which dates back to 1997.

Efforts to increase titers further will likely yield diminishing returns. Very few products would benefit significantly from a titer greater than 8 g/L; in many cases, a downstream processing bottleneck would preclude full recovery from production-scale bioreactors for high titer processes. The benefits of a process platform based on standard fed-batch production culture include predictable scale-up, process transfer, and production within a company's manufacturing network or at a contract manufacturing organization. Furthermore, the confidence in an established platform

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provides key support towards regulatory flexibility (e.g., design space) for license applications following a quality-by-design strategy.

These factors suggest that novel technologies for antibody production may not provide a substantial return on investment. What, then, should be the focus of future process development efforts for companies that choose to launch antibody products using their current platform? This review proposes key focus areas in an effort to continually improve process consistency, assure acceptable product quality, and establish appropriate process parameter limits to enable flexible manufacturing options.

Keywords Cell culture production, CHO platform, CHO technology, Continuous processing, Innovation, Mabs, Mammalian cell technology, Monoclonal antibodies, Perfusion

Contents

1 Introduction

Industrial mammalian cell culture technology used for the production of recombinant protein therapeutics was established in 1987 with the licensure of recombinant tissue plasminogen activator. Since that landmark, mammalian cell culture has become the production methodology of choice for most biopharmaceutical products. The growing success of antibody-based therapies has driven advances in process technology and production facility design and management, with concomitant reductions in the cost of goods and improved process reliability. With novel protein

products becoming a larger fraction of the product pipeline in many companies, continued cost pressures for innovator companies, and the introduction of biosimilar products, it is important to consider where to invest process development resources into innovative technologies.

2 Current Platform Cell Culture Production Processes

2.1 Cell Line Development

Chinese Hamster Ovary (CHO) cells are the most common choice of cell line for the production of recombinant protein therapeutics. They are generally capable of high productivity, demonstrate consistently good growth phenotypes, can be adapted to chemically-defined media, and typically do not generate product variants whose post-translational modifications present a concern for product safety.

Prior to 2010, many companies developed an improved cell line to be used for pivotal clinical trials and commercial production, replacing the cell line used for initial clinical trials. Now, it is more common to use the same cell line for all clinical phases leading to commercialization. This single cycle of cell line development is a much more efficient development strategy, provided the initial cell line is sufficiently productive and product quality is acceptable. Improvements in expression vector design combined with screening technologies allowing the examination of thousands of clones has led to high and consistent titers; for recombinant antibodies, it is typical to achieve titers of 3–8 g/L from initial cell lines using standardized media and process conditions. This optimizes speed to the clinic and simplifies the development lifecycle, as there are fewer issues of product comparability arising from cell line changes during clinical development.

The optimization of expression vectors continues to yield improvements in titers, while selection markers remain relatively standardized. These improvements can be employed efficiently within typical platform process media and conditions.

One area of general interest is the use of targeted integration for production cell line generation. By constructing a parental cell line with a "hot spot" identified for the integration of heterologous product genes through gene swapping technology such as Cre-lox [1, 2] or other techniques, it is possible to generate a productive cell line with one (or very few) gene copies consistently and quickly. This offers the potential for improvements over the established technique of random integration of multiple copies throughout the CHO genome. Evidence presented at conferences has indicated there would likely be reduced sequence variants, improved stability of expression, and more consistent expression and growth phenotypes from product to product. Several companies are implementing targeted integration for Current Good Manufacturing Practice (cGMP) cell lines, and this is one example of a recent innovation in cell culture technology that may have broad applicability. It is noteworthy that this technology has been in various stages of feasibility and subsequent optimization for over a decade in some laboratories, which gives a sense of how the complexity of major changes in cell culture technology can give rise to relatively long implementation phases.

However, issues with the long-term stability of production expression remain a complication for some cell lines. Occasionally, gene expression may be very stable, even for seed cultures that are carried in repeated passaging for over 100 generations. Other cell lines may show an expression decline of 50% or more in similar time frames. The mechanism of decline may be traced in many cases to loss of copy number, but not always. The ability to predict which cell lines will display instability, and which will not, is currently not understood. The outcome of extended culturing on product expression levels is typically the sole determinant of a cell line's stability. This results in either a lengthy aging study prior to selection of the final clone, living with the consequences, or switching cell lines if an unstable phenotype is observed later in development.

Recent health authority feedback has emphasized assurance that production cell lines are derived under appropriate conditions, which strengthens the confidence that a single clone is present at the time of cell deposition. The driver for this feedback presumably is concern over the potential for shifts in product quality attributes over time if the line was not clonally derived. Companies have generally responded by either implementing two rounds of limiting-dilution cloning into the production cell isolation approach or validating image analysis to provide assurance of a single cell being present in the isolation well during a single round of limiting-dilution cloning. Industry has also provided feedback on the importance and value of process and product data demonstrating consistency over the typically expected range of cell age employed in the manufacturing process [3, 4].

The impact of high-throughput screening for bioreactor conditions and media optimization enables further improvements in yield and consistency as larger regions of bioreactor and medium operation spaces are evaluated [5, 6]. With a high degree of miniaturization comes the challenges of defining a high-fidelity model of a production bioreactor, including pH control, aeration strategies, and culture feeding management. Although these miniature reactors are not necessarily intended to be qualified scale-down models sufficient for process characterization or validation, key differences could give rise to confounded conclusions influencing the selection of the optimal production clone.

2.2 Production Bioreactors and Facilities

The design basis for suspension mammalian cell production cultures primarily relies on stirred tank bioreactors (although airlift bioreactors are in use). These bioreactors have changed relatively little since the establishment of deep-tank CHO bioreactor technology in the 1980s. Of the three primary modes of bioreactor management (batch, fed-batch, and perfusion), fed-batch cultures are the most common. Feeding strategies can vary from bolus to continuous with one or multiple feed solutions; feeding on demand occurs with feedback control loops or simpler prespecified feeding schedules. Feeds can include nutrient concentrates, glucose, trace elements and vitamins, or other media components. The flexibility of this simple process design combined with the

many parameters available for the optimization of product titer and quality have resulted in it being the workhorse in the biotechnology industry today, with the demonstrated capability of high titers, consistent product quality, scalability, and ready transfer to multiple facilities, including contract manufacturing organizations (CMOs).

Mammalian cell production bioreactors range in size from 25,000 L down to hundreds of liters depending on the production scale required for commercial and clinical supply demands. Small-scale laboratory bioreactors used in process development, characterization, and validation studies are often 2–5 L in volume. This 10,000-fold scaling factor is a key advantage for conventional stirred tank bioreactors, enabling many experimental conditions to be tested efficiently, including complex statistically designed experiments. The scale-up/scale-down heuristics and principles are now well-established (although they may vary slightly from company to company or between bioreactor types). Many companies have transferred multiple processes from small-scale clinical to large-scale commercial facilities, between plants in their commercial network, or to CMOs (one or multiple sites). The body of knowledge used to assess the risks, complications, and solutions needed for scale-up and technology transfer for stirred tank fed-batch bioreactor processes is quite extensive. The track record of successes speaks both to the robustness of this core bioprocess technology and the accumulated wisdom of nearly three decades of experience [7, 8].

One recent advance in the field of bioreactor design is the development of disposable (single-use) bioreactors that are capable of cultivating cells similarly to the stainless steel vessels, with highly consistent behavior for nearly all key performance indicators. The differences in the methods of agitation and mixing are one obvious difference, but these differences have not proven to be a significant complication in most cases. The production scale of the disposable reactor can reach 2 kL (recently, a 3.5-kL disposable bioreactor was launched by one company), but it is unlikely to ever reach the volumes of many common large-scale commercial facilities (10–25 kL). Complications have been observed with the low-molecular-weight leachables from the bioreactor polymer film(s) slowing or halting cell growth. However, improved supplier understanding of bag film chemistries combined with rigorous control and testing of vendor-initiated changes should fully prevent this problem from recurring [9–11]). These systems have made significant inroads into clinical or small-scale (often dedicated to a single product) commercial production.

A common facility design for a very large-scale commercial plant employs one or two seed trains, a few inoculum trains, and four to eight production bioreactors, all serving one purification train downstream. Additional flexibility is gained if appropriate segregation and the number of seed and inoculum trains enables concurrent cultivation of two different cell lines; in this case, a second purification train would be needed to process the harvests. The ratio of bioreactors to purification trains may limit the duration of the production culture or vice versa, with a typical production culture duration for antibody production being 10–16 days. These are highly efficient and productive facilities for the commercial production of large volumes of therapeutic proteins. The production of two or more products is key to maximizing plant utilization; it encourages platform processes that minimize downtime due to equipment swaps or significant re-programming of controllers. With disposable bioreactors and

liquid handling equipment, facility designs can be freed of some of the requirements for facilities supporting stainless steel bioreactors. Utilities for clean steam, clean in place (CIP), sterilization in place (SIP), or water for injection may be reduced or even eliminated, greatly reducing the complexity of equipment, piping, and facility layout. These "factories of the future" will have reduced footprints and can be built and brought online faster than conventional stainless steel facilities.

In the past 20 years, biotechnology manufacturers using mammalian cell production systems have begun to implement additional virus barriers as business risk mitigation to ensure a continued supply of product as well as the freedom to operate a facility. Cases of viral contamination have occurred in clinical or commercial facilities, some of which have led to extended periods of time for remediation and resumption of manufacturing [12–14]. High-temperature short-time (HTST) heat treatment of media is used in many facilities and has been proven effective [15]. Although other techniques have also been employed as barriers for some raw materials that are incompatible with heat treatment, such as gamma irradiation for serum and virus retentive filtration for lipids solubilized in alcohols, HTST is used for the bulk of viral barrier applications in commercial processes (see [16] by Shiratori and Kiss on virus barriers). The use of animal-derived raw materials for new cGMP antibody production processes is rare. Avoiding their use in any stage of preclinical and clinical development reduces the risks of adventitious agent contamination or product quality changes in the development lifecycle.

2.3 Media and Feeding Strategies

Cell culture media formulations may be developed in-house or selected from several suppliers of media for cGMP use. The optimal formulations of seed, inoculum, production, and feed media are key to ensuring consistent product quality and high titer processes. Chemically-defined media are now commonplace and are replacing complex media, including hydrolysates.

The use of a chemically-defined medium allows for much greater understanding of the effects of specific media components on both process performance and product quality. The refinement and optimization of amino acid ratios (e.g., cysteine/cystine, asp/asn/gly/gln) and absolute levels is now possible in the absence of interference from hydrolysate or serum contributions via peptides and uncharacterized levels of trace metal forms. This allows for targeted studies that can better control metabolic behavior consistency. On the other hand, the absence of potentially significant quantities of trace metals from complex materials requires a much broader understanding of the importance of these trace metals on cell growth, metabolic responses, and product quality. Additionally, metal impurities in other raw materials may significantly impact the total levels in meaningful ways and must be accounted for in medium formulation designs. Formulations are often blended based on platform knowledge in designing feeds that can further boost performance. The improvement know-how around component impact influences the feeding strategy

development for modulating product quality attributes, including preventing misincorporation, excessive proline amidation, or trisulfide formation [17–21].

2.4 Harvest

For very large-scale (≥1 kL) mammalian cell cultures, disc-stack centrifuges dominate for the initial cell removal step of the harvest operation. Non-hermetically sealed centrifuges that are successfully used for many industrial applications, including microbial cell separation, can cause significant mammalian cell disruption due to the energy dissipation associated with the air-liquid interfaces. To avoid this type of cell disruption (lysis), the associated debris, and other potential issues that can arise, most companies employ either hydrohermetic or fully hermetic centrifuges.

As biotechnology companies developed improved cell culture processes that delivered improved growth and viability profiles and higher antibody titers, it was discovered that cell lysis during the harvest operation released reducing enzymes and energy sources that could trigger a catastrophic antibody disulfide bond reduction event [22–24]. This behavior was experienced and reported by multiple companies. Mitigations for preventing antibody reduction were developed, including control of the dissolved oxygen level in the harvested cell culture fluid (HCCF) by sparging air to prevent the establishment of reducing conditions. Depending on the specific cell culture process and the levels of reducing power present at harvest, varying amounts of air sparging may occur. Assessment of product stability in the presence of air sparging of the HCCF has generally shown minimal impact with antibody molecules, but other novel formats susceptible to disulfide bond reduction may require other mitigations.

For existing large-scale facilities, the retrofitting of non-hermetic centrifuges to hydrohermetic units may be possible at reasonable costs and downtime. Given the numerous observations of antibody reduction across multiple companies, it is highly recommended that new facilities employ fully hermetic centrifuges for mitigation purposes.

Clarification (often by depth filtration) and sterilizing-grade filtration of centrate fluids is a standardized operation to deliver low turbidity and low bioburden to harvest pool storage prior to initiating purification operations.

2.5 Downstream Processing Limitations

Each combination of a product's unique downstream process and the intended manufacturing facility has a limit in its downstream processing train, where higher titers cannot be completely recovered. Typical downstream bottlenecks include in-process pool tank volumes, buffer make-up volumes, or chromatographic or ultrafiltration capacities. Some simple process fixes include the use of isocratic flow-through chromatography steps instead of product bind/elute steps or single-pass tangential flow

filtration to provide modest concentration of in-process pools. With an assessment of facility fit limitation during Phase III process development, antibody titers as high as 6–8 g/L can often be processed in existing facilities without substantial equipment retrofit. These high titers, combined with demonstrated production bioreactor scales of 10–25 kL, suggest that (except for unusual products with multi-ton scale annual demands) there may be no substantial benefit to pushing titers higher than this purification bottleneck, particularly if it requires the development of novel downstream processing unit operations to handle the increased mass from the cell culture process.

2.6 Summary of the State of the Art

The state of the art of industrial mammalian cell culture for cGMP production of therapeutic proteins has arrived at a rather mature production technology base [25]. The majority of active companies in this sector have converged on fed-batch cultures in large bioreactors, and a network of CMOs supporting this design are available for development or contract production for clinical or commercial supply. The capacity and cost of goods for production with existing very large-scale facilities is also quite favorable because economies of scale combined with multiproduct operations can optimize plant utilization. This leads to the following question: What new innovations are needed for the future of mammalian cell culture production technology, whether for monoclonal antibodies or other recombinant protein products?

3 Key Focus Areas for the Current Process Platform

The following areas offer significant opportunities for investment in process knowledge, product quality, process consistency, and robust operations in clinical and commercial production.

3.1 Raw Material Variability and Sourcing

Process consistency is a key objective for commercial production. One known source of cell culture process perturbations is the raw materials, which can have minor (or occasionally major) impacts on a cell culture process. Complex and undefined medium components may be one source of variability. However, even chemically-defined media may be prone to uncontrolled variations arising from the composition of trace components, which may vary from media lot to lot, particularly as a result of impurities in another raw material. A well-designed medium will dampen this variability, typically by adding the trace components as a specified raw material themselves. One specific example is with low levels of essential trace elements (e.g., metals), which

may have subtle effects on product glycans arising from their role as co-factors for glycosyltransferases [26]. Even simple and commonly used raw materials can affect process performance. For example, some polymeric shear protectants have been shown to suffer from lot-to-lot variations in protective function, resulting in variations in cell viability or the extent of cell growth and subsequent titers [27].

If raw materials can influence product quality or process performance, it is worth investigating the root cause and considering actions that can return the process to stable operations with the highest product quality capability offered by a mean-centered and consistent critical quality attribute (CQA) output. These actions could be implemented using a design space if there are other process parameters that also influence the quality attribute that is drifting. Alternatively, basal or feed media can be supplemented with the variable components, either to a consistent level that factors in the contribution of each raw material lot or to a level high enough to minimize the impact of media lot variation.

In one case study, a depth filter used as a pre-filter for a cell culture medium was determined to be leaching manganese into the filtered medium. This contribution to the total manganese in the culture medium was originally unknown, yet its fraction of the total manganese influenced the resultant antibody glycan profile. When the filter manufacturer made a change to the source of diatomaceous earth used in the filter matrix, the amount of leached manganese was significantly reduced, and a shift in the antibody glycan profile resulted. Once the root cause was determined to be this "absence" of the inadvertently supplemented manganese, the medium formulation was adjusted to restore the total manganese levels to the historical levels, and the glycan profile was restored.

Although disposable bioreactors and media storage bags are not a raw material used in media formulations, they can have an influence on cell growth or product expression due to leachables. There have been examples of serious impact to cell growth after media storage in bags, which was exacerbated by gamma irradiation prior to use [9–11]. This issue was resolved by the disposable bag supplier after identification of the component and mechanism of toxicity. This is another cautionary case study that should be kept in mind after vendor-initiated changes of disposable materials that contact cells or media.

Dual sourcing of cell culture raw materials such as media or key media components (e.g., hydrolysates) can be a valuable risk mitigation strategy to guard against supply interruption or uncontrolled variability. Media formulations that are primarily defined chemicals should be easily sourced from two or more vendors. However, the subtle impacts of blending, handling, storage, or environmental exposure may alter the levels of trace or slightly reactive compounds (thiol compounds, iron, etc.) or influence media stability, which may only be revealed upon second sourcing or scale-up [28–30].

A solid understanding of potential raw material lot variation impacts at the time of a product licensure application is a key element of a cell culture license application. These experimental study designs and interpretations can be complicated, but very informative, in evaluating process consistency and potential commercial performance.

3.2 Advanced Process Controls and Facility Management Across a Network of Production Sites

Several companies and CMOs manage a network of cell culture production facilities for multiple commercial products. Differences in equipment and facility design are frequently encountered as networks grow by acquisition or are expanded long after the first sites are licensed. The alignment of process controls and bioreactor management (e.g., pH, aeration) can be complicated because the subtleties of online or offline calibration may differ from site to site, emphasizing the benefits of standardization around best practices.

Process monitoring and cross-site comparisons of processes running in multiple plants provide an opportunity for continued advancements in process knowledge. When raw material variation affects process performance, a multiple-site network data review can provide critical information quickly to jumpstart root cause analysis. Common-cause investigations (when appropriately coordinated and executed) can accelerate corrective action and prevention compared to single-site production.

Advanced statistical analysis methods, such as multivariate analysis (MVA) [31], have become more readily available to biotechnology process scientists and engineers at affordable costs and with user-friendly interfaces. They should continue to be exploited for the value they can bring in advancing process understanding. In many situations, mechanistic models are simply unavailable to interpret process performance, and MVA approaches may be the only practical approach available. In addition, the use of advanced process controls can provide improved process consistency and performance while also enhancing process knowledge that can be leveraged across processes. Further development of strategies for improved control of glycosylation profiles will be needed given the continued learning about the importance of glycan structures on the biological activity of some antibodies and recombinant proteins [32, 33]. Additional examples of such advanced controls include the use of online Raman spectroscopy to estimate nutrient and/or waste product concentrations as well as institute closed-loop actions to better manage metabolism [34, 35]. Such online sensing solutions should be pursued further, including consideration for the prediction of product concentration and product quality attributes in addition to the aforementioned metabolic profiles.

3.3 Process Parameter Control Ranges and Targets

At the time of a commercial license application, process characterization studies will have identified critical process parameters and established acceptable process parameter control ranges for the cell culture unit operations [36]. The definition of criticality is based on a parameter's impact on product quality, rather than on key performance indicators such as titer or cell density. There may be opportunities for optimizing a process by moving one or more parameter targets within acceptable ranges. This is the

concept behind a design space as envisioned by the International Conference on Harmonization Q8 [37], which enables post-licensure movement of multiple parameter targets without requiring regulatory approval. Commercial processes have now been approved with a design space [38], and descriptions of how they may be established have been described [39]. One opportunity for future cell culture license applications is to establish sufficiently wide parameter ranges to enable operational flexibility, process robustness in light of raw material variability and equipment design differences between sites, and optimization of process performance. Of course, sufficient attention must be paid to the appropriate qualification of the scale-down models used to generate the data supporting the claimed process ranges [40, 41].

In some cases, the tuning of process parameters within an acceptable range can have a significant impact. It is not uncommon to have production culture pH or temperature targets demonstrably influence cell metabolism, including the production or consumption of lactate. The means of aerating the culture with blends of air and oxygen, sparger geometry, and agitation rate may also affect metabolism and titer through variations in the ventilation, or stripping, of carbon dioxide from the production bioreactor. These and other parameters represent fine control elements that are worth studying in development, commercial production, and between facilities in a network.

The types of flexibility in key parameters that would benefit manufacturing within a design space include pH, temperature, culture duration, limit of in vitro cell age, feeding strategies, simple vs. complex process control strategies, and parameter excursion studies that cover temporary deviations in some control parameters. Individual plants in a network or at a CMO may have different preferences for targets of some of these process control elements, and establishing wider multivariate ranges at the time of licensure could be quite valuable in supporting commercial production over the long term.

Products in the pipeline will also benefit from the use of a consistent process platform, if applicable, building on knowledge from process characterization and commercial production using very similar processes. Although every cell line and product are unique, many elements and learnings established from the initial licensure of a platform process will inform subsequent products' risk assessment, enabling streamlining and simplification of the final licensure phase of product development.

3.4 Novel Product Formats

Novel protein constructs or formats are becoming a larger fraction of the pipeline of many biopharmaceutical companies. These include bispecific antibodies, receptor fusion proteins, antibody-drug conjugates with site-specific toxin loading, or antibody cytokine fusions. Most of these products fit into the current cell culture production platforms with no required modifications. There may be some adjustments needed in media components or bioreactor parameters based on unique product quality considerations posed by either novel product variants or product-related impurities. In general, the cell culture process platform used for antibody

production is an excellent starting point for process development for novel product formats, offering a rapid entry into Phase I clinical studies and a platform knowledge base that informs subsequent Phase III development and commercialization.

4 New Process Technologies: A Cautionary Note

Novel process technologies that are a radical shift from the established platform process design described above are being considered in academic and industrial laboratories, with some larger-scale implementations. Although there are certainly innovations and increased process understanding needed for the current platform process, there needs to be a balance between the investment placed in "revolutionary" versus "evolutionary" process technologies. Major shifts in a production basis would carry many uncertainties regarding scale-up, robustness, production costs, and development timelines, among others. In some cases, the magnitude of the rewards is also uncertain and may be overestimated as being critical for future competitive markets (it is unlikely that the cost of goods sold [COGS] of recombinant protein therapeutics will be a determinant of a competitive market). Are investments in many of the new technologies under evaluation warranted? What problem(s) are they solving or creating? The history of bioprocess technology over the last 30 years indicates that many novel technologies burst onto the scene, then faded as the challenges of implementation were faced.

4.1 Perfusion Culture

Perfusion culture is an active area of investigation, as well as some controversy. Perfusion cell cultures use a cell retention device (centrifuge, spin filter, filter or inclined settler) to retain cells in the bioreactor during the inoculum or production phases. In some recent advances, the product is also retained in the bioreactor through the use of an ultrafilter [33, 51, 52]. A number of perfusion processes for biopharmaceuticals have been licensed, accounting for a small proportion (<10%) of all commercial mammalian cell culture processes [42]. In most of these cases, the product is an enzyme, blood factor, or other product that may exhibit instabilities when exposed to extended fed-batch culture conditions (i.e., residence time).

Many companies that ran perfusion cultures for early products in their portfolios moved away from perfusion to fed-batch for antibody processes. However, for a few companies, an installed production base using perfusion has been an important driver to continue this basis for pipeline programs. Recently, there has been renewed enthusiasm toward the further evaluation of perfusion cultures. This appears to be driven by two factors: (1) the potential to combine perfusion with a disposable bioreactor to drive process intensification and maximize plant productivity, and (2) the ability to enable a fully continuous production train when coupled with a continuous downstream process (see the next section).

There are potential advantages to perfusion processes [43, 44]. Often, the volumetric productivity (g/L/day) from a perfusion-based production bioreactor is higher than fed-batch operations, thus allowing a reduction in some aspects of the production plant size and necessary capital. Because scaling up perfusion bioreactors beyond 1 kL is difficult due to limits of the cell retention device, "scaling out" with multiple suites or facilities is sometimes claimed as an advantage. This comes at the loss of economies of scale, but potentially with shorter lead times to build out increased capacity.

Although there are claims that continuous cell culture processes will have more consistent product quality, there is little evidence in the published literature that this is the case. Benchmarking highlights experiences with commercial perfusion processes that have had quality attributes drift with extended cell age, requiring the pooling of multiple harvests to maintain product consistency. Other complications include elevated contamination rates for some cell retention devices, a slow approach to steady state, and raw material impacts on many batches produced over several months with complex batch genealogy. Many issues in manufacturing (e.g., technical failure, deviations, microbial contaminations) cause an immediate impact on productivity because troubleshooting and maintenance cannot be performed between batches as with conventional processing. Finally, extended culture durations lead to longer development, characterization, and validation cycles, as well as greater expenses and the generation of less knowledge in understanding the process.

In addition, the portability of perfusion processes to CMOs may be lacking if surge capacity is needed. Although the use of smaller-volume bioreactors enables perfusion operations with disposable bioreactors, they still require large-volume tanks for feed media and harvest operations. Many plants are not set up for this type of operation and lack the specialized equipment needed for cell retention and continuous harvest. Very high cell densities in perfusion cultures may push the limits of oxygen transfer and process control in disposable bioreactors. A significant capital investment would be needed to convert or build continuous processing capacity for multiple plants in existing facilities. Indeed, authors have cautioned against pursuing this type of platform change for companies that have established large-scale fed-batch infrastructure [45, 52].

4.2 Fully Continuous Processes

Fully continuous processing for biologics drug substance production would require the previously described continuous (i.e., perfusion) cell culture, as well as a downstream processing train (simulated moving bed or countercurrent chromatography with no process pool hold tanks) that is capable of producing a purified drug substance from a continuous cell culture harvest. Today, the products made by perfusion cell culture use batch purification operations.

For highly unstable proteins, a perfusion culture plus low-temperature purification trains have been a common processing solution; a continuous downstream train

is not automatically required for these products. Other potential advantages have been claimed, including a reduction in plant footprints, open ballroom facility design, compatibility with disposables, benefits of a fully automated process, and improved product consistency.

Continuous purification processes are neither well established, scalable, transportable, nor particularly valuable when coupled with perfusion culture [46]. Simulated moving bed or countercurrent chromatography using three to six (or more) columns per step are the most advanced of the continuous options. However, to our knowledge, they have yet to be scaled up or used for the cGMP production of proteins. Furthermore, they may have limited utility for multicomponent separations.

In some cases, there may be value to "connected" processes, where only two unit operations are run in series without a pool tank. A virus filtration step could be connected to the outlet of a flow-through chromatographic step, for example, if there were facility fit limitations for a four-column process needed for a nonstandard antibody-like product. Another example would be in-line concentration using single-pass tangential flow membranes. However, these are not fully continuous processes as envisioned by some.

Although the combination of a perfusion cell culture process and a continuous purification train would be a technological tour-de-force, it is not clear what problem it would solve (or what advantage it would bring) to the existing innovator facility networks or large-scale CMOs. The perfusion culture complications listed previously still exist, and the scale-up and validation of totally novel purification unit operations/equipment is no small feat. Connecting the two would present real and significant engineering, control, and quality assurance challenges. Complex, interacting control loops require significant automation and monitoring to prevent scheduling issues when process upsets occur. Furthermore, the debugging of many novel unit operations would require a significant investment prior to clinical production, let alone licensure/inspection and commercial operation. This added complexity can translate into increased failure rates, as up to six chromatography columns run as a simulated moving bed for each of three process steps (18 columns, not 3) will likely have a higher overall failure rate than batch downstream processing. In addition, managing the ensuing interruptions would be a significant challenge to the entire production train.

Note that some of the advantages of continuous processing for small-molecule active pharmaceutical ingredient (API) synthesis or drug product (DP) tableting (reduced solvent usage, near-infrared radiation monitoring of API production, use of small plug flow reactors) are sometimes mentioned in the literature, but they have little relevance to biologics. In some cases, comments from regulatory authorities speak broadly on the advantages of continuous processing, but they do not differentiate between proteins and small molecules.

The current platform for mammalian cell-derived products is durable, predictable, cost-effective, and efficient. Claims about the superior performance of fully continuous processing are, at this point, aspirational. Given that, what is the benefit of perfusion cell culture coupled to a batch downstream? This was the design basis that several companies had established in the past, but generally moved away from.

4.3 Novel Production Hosts

New mammalian cell hosts would bring significant hurdles for implementation compared to the more established CHO cell. For several decades, investments have been made into alternate production hosts such as transgenic animals, plants, yeast strains with engineered glycosylation pathways, and human cell lines (such as the PER.C6 line). Despite these efforts, very few products have been launched with totally novel hosts in the last decade.

If the host under development were uniquely enabling of certain product quality attributes that could not be effectively produced or controlled using conventional cell lines, that may be a driver for an alternate host, as in the case of specifically tailored glycoforms produced from engineered yeast strains. Otherwise, an alternate host is unlikely to have a significant impact on COGs, especially at very large scale of production. In the ton-per year processing, the downstream processing costs are a much larger fraction of the overall COGs than the cell culture or upstream costs [47]. Therefore, reducing the upstream costs through the use of an alternate host with reduced cost compared to CHO cell culture would have diminishing effects on the COGs.

Even a well-established production host like *Escherichia coli* (licensed for the production of the first recombinant protein therapeutic, Humulin, in 1982) is chosen primarily for production of niche smaller non-glycosylated proteins such as hormones, cytokines, or antibody fragments, despite the capabilities to express complex, correctly-folded multi-subunit disulfide-bonded proteins to high concentration in the periplasm [48]. The production COGs of antibodies produced in CHO using existing technology is estimated to be as low as $20–30 per gram [25]. Although the COGs of insulin produced by *E. coli* or yeast would be lower, much of the benefit is derived from the very large scale of production; at a more modest production scale of less than 1 ton of product per year, there might not be much difference in the COGs of the two hosts (in part because the downstream processing train for intracellular *E. coli* proteins has more unit operations and lower yield than purification trains isolating a secreted product from CHO cells). The common perception that mammalian cell-derived proteins are inherently more expensive to produce than microbially-derived proteins is not always true.

4.4 Biosimilars

The development, licensure, and marketing of biosimilar products is an emerging opportunity for many companies planning to enter this competitive space in the decade ahead. The ability to match all of the innovator's product attributes within the innovator's historical product ranges presents a challenge. Some firms are seeking to solve this problem with an innovative approach to complex process control strategies in order to maintain CQAs within the innovator's goalposts [49], including feedback

bioreactor control using process analytical technology (PAT) techniques. If the innovator's product was made by fed-batch culture, would production using a fundamentally different process technology (e.g., continuous culture) make matching innovator CQAs difficult? Although this may be a problem that can be solved by extensive process development, it would seem that a biosimilar manufacturing process that matches the innovator's production process would likely have a simpler path forward in matching product quality attributes.

The development of the commercial cell line for biosimilars would likely follow a conventional state-of-the-art effort, including a single cycle of cell line development (with the initial and pivotal clinical trials using the same cell line), high throughput screening (where enabled) of cell lines for appropriate product quality and optimal titer, and, of course, typically the selection of the same host cell line as the innovator product.

Although there certainly will be pricing pressures in a competitive biosimilars market, is it likely that production technology will ever dictate the outcome in the market? This scenario is sometimes raised as a motivation to evaluate new process technologies (including fully continuous processing). However, if the sale prices of the biosimilars drop so low that production costs become a key differentiator in the marketplace, it is unlikely that those biosimilar products will offer a significant return on investment.

It is not yet clear whether the biosimilars market will have any lasting impact on bioprocess technology through the use of novel or innovative processes, or whether conventional processes will continue to be favored by biosimilar companies.

4.5 Harvest

As mentioned previously, the development and implementation of single-use bioreactor systems has been established in the biotechnology industry. For certain product volumes, single-use systems for clinical and commercial production may make sense given the reduced plant startup time that is possible, along with the potential to significantly reduce the requirement for support utilities such as SIP and CIP. One unit operation that has been slow to efficiently align with the vision of disposable systems is the harvest operation.

With single-use production bioreactor volumes in the 1–2 kL range and high cell densities utilized to drive multi-gram per liter titers, harvest unit operations have often continued to rely on centrifugation for efficient cell removal prior to final filtration. This approach presents challenges because single-use bioreactors cannot be pressurized to drive flow to the centrifuge, requiring either use of a feed pump or transfer of the bioreactor contents to a fixed vessel that can be pressurized as the centrifuge feed source. The former approach exposes cells to potentially high and disruptive energy dissipation rates, whereas the latter negates the cleaning benefit of the single-use bioreactor. Either approach based on centrifugation triggers the need for equipment (centrifuge, etc.) cleaning operations, which then prevent the

approach to a utility-lite facility that is the vision of single-use processing. Accordingly, development of single-use centrifugation systems/interfaces or other cell removal devices is an area where further innovation is appropriate. This type of technological approach is briefly described in [50].

In the interests of pursuing the utility-lite facility vision for single-use technologies, some companies have eliminated the centrifuge as the initial cell removal step and opted for a purely filtration harvest approach. The challenge with this approach is in identifying the initial filtration technology. Companies have pursued the use of depth filtration for cell removal and initial clarification. However, at these 1–2 kL scales of operation with high-density cultures, one must choose between an extremely large depth filtration area that has a large footprint and is costly or a more moderately sized depth filtration operation that can take as long as 24 h to harvest a batch. One potential approach to improving the filterability of pre-harvest cell culture fluid is that of flocculation of the cells and cell debris. Many flocculants tend to result in acidic conditions, which can cause product damage due to proteolysis. In addition, large amounts of polymeric flocculants and flocs may be a disposal issue, let alone a handling challenge. Accordingly, the non-centrifuge harvest approach is an area that would benefit from further innovation.

5 Conclusion

The current state of the art for industrial mammalian cell cultures has matured to a consensus platform of fed-batch operations at production scales up to 25 kL. The broad use of chemically-defined media and an improved understanding of media formulation and the influence of critical components have enabled more precise control of product quality and improved process consistency. With titers of 6–8 g/L or higher, 100-kg batches are possible with low COGs and very high production capacities. This combination of factors is a very attractive process design basis, with a long development history, a growing understanding of the causes of process variation, and experience with scale-up and facility transfer. Under what scenario is a more intensified process worth the additional investment and risk? There are several areas where further investment in the current process platform will likely provide significant returns. These focus areas will continue to improve this platform to ensure speed to clinic, efficient process development, streamlined process characterization, and validation of reliable and transportable commercial processes.

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