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Protein Production in Eukaryotic Cells

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Abstract The scientific and engineering aspects of design, development, scale-up, and manufacture of monoclonal antibodies are summarized in this chapter by outlining the key elements in the development of the expression cell line, cell culture, cell harvest, and protein purification process and exploring the effect of process technologies on production economics.

Keywords Bioreactor, Bioreactor sterility, Cell culture scale up, Cell harvest, Cell line screening, Chinese hamster ovary cell line, Continuous centrifugation, Cost modelling, Cost of goods, Depth filtration, Disc stack centrifuge, Economies of scale, Genetic stability, Host cell protein, Ion exchange, Medium optimization, Phenotypic stability, Process economics, Protein expression, Run rate, Sigma factor chromatography, Viral clearance

Contents

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1 Introduction

As the role of biologics in treating human diseases has evolved dramatically over the past decade, so has the technology to manufacture, test, and administer these products. This has been driven largely by improved understanding of the biological mechanism of action, the role of the structure of the molecules on the mechanism, and the role of the manufacturing process on the structure of the molecule. Among the various types of biologics, monoclonal antibodies now represent the largest segment of biopharmaceutical proteins in terms of sales. A 2015 study shows 8,182 kg of biopharmaceutical monoclonal antibody products were produced, representing nearly $60 billion in sales in 2013 [1]. Given this large demand for mAb products, along with increasing price pressures, and most of all an urgency to bring these biopharmaceuticals to patients, process scientists and engineers have developed “platform” processes with extremely high productivity; analytical scientists have developed sophisticated techniques to decipher attributes critical to quality; formulation scientists have developed formulations that allow storage at high concentrations for several years; and device engineers continue to develop modes of administration more convenient to the patient.

Depending on the commercial demand for the product and the available manufacturing facility, different manufacturing technologies are employed. The most prevalent among these is the fed-batch mode of bioreactor operation and batch mode of chromatography operation. However, the use of continuous manufacturing that relies on perfusion-based bioreactor operation and continuous flow chromatography operation is being developed to increase plant throughput [2]. This chapter will focus on batch manufacturing technology in order to anchor the reader in the most common process scheme used today. Also, this chapter will focus on manufacture of drug substance only. The reader is encouraged to refer to a review on recent progress in formulation development for protein therapeutics by Razinkov [3].

The scientific and engineering aspects of design, development, scale-up, and manufacture of monoclonal antibodies are summarized in this chapter by outlining the key elements in the development of the expression cell line, cell culture, cell harvest, and protein purification process and exploring the effect of process technologies on production economics.

2 Cell Line

2.1 Choice of a Host Cell Line

Protein therapeutics are generally produced from a recombinant production cell line, which is constructed by the introduction of the DNA encoding the therapeutic protein into a host cell line and harnessing the synthetic capacity of the cell to express the recombinant protein. The choice of host cell line determines critical aspects of therapeutic protein production, including yield, purity and quality of the protein, timelines, scale-up, and cost of goods.

Simple polypeptides, such as insulin, can be produced from bacterial systems, such as *Escherichia coli* (*E. coli*), which, owing to its short doubling time, has the advantages of rapid development of recombinant cell lines as well as low cost and short production bioreactor processes (reviewed by [4]). However, there are significant limitations to *E. coli* expression systems owing to the inability to perform disulfide bonding to assemble complex proteins and the lack of posttranslational modifications (PTMs) such as glycosylation, carboxylation, hydroxylation, sulfation, and amidation [5].

In contrast to bacterial cells, eukaryotic cells are equipped with the cellular machinery for the folding and assembly of complex proteins, as well as for performing PTMs. Yeast and insect cells can be used for production of therapeutic proteins [6] and, owing to their rapid cell division and scalability, have some of the same advantages as bacterial expression systems. However, they produce glycoproteins with carbohydrate structures that are different from human-type glycosylation and have the potential to impact both the in vivo activity and immunogenicity

of expressed proteins [5]. Systems for plant-made pharmaceuticals (PMPs) have been developed using transgenic plants [7], transgenic moss [8], or plant cell suspension cultures [9]. Again, some of the natural plant glycan structures are species specific, containing terminal beta (1,2) xylose and alpha (1,3) fucose residues that are not found in humans [10]. Efforts are ongoing to engineer PTMs in microbial [11], insect [12], and plant [13] cell systems in order to make these systems more suitable for protein therapeutic production.

Immortalized mammalian cells are most commonly used for recombinant therapeutic protein production [14]. Rodent host cell lines provide more humanlike glycosylation; they include mouse myeloma (NS0 and Sp2/0), baby hamster kidney, and Chinese hamster ovary (CHO) cells. However, these rodent cell lines also produce nonhuman alpha-gal and *N*-glycolylneuraminic acid (NGNA) glycoform structures that can affect in vivo clearance and immunogenicity, thereby impacting the pharmacokinetics of the therapeutic protein [15, 16]. Avoiding this issue, there are human host cell lines available for the production of fully human PTMs (reviewed by [17, 18]). Cell lines derived from the human embryonic kidney cell line (HEK-293) are used for the production of a number of approved therapeutic proteins, including recombinant clotting factors and fusion proteins, where additional PTMs such as gamma-carboxylation and sulfation are important for bioactivity [19]. The human HT-1080 cell line, derived from a fibrosarcoma, is used for the production of approved enzyme therapies, iduronate-2-sulfatase, agalsidase alfa, and velaglucerase alfa. Other human cell lines, PER.C6 (derived from human embryonic retinal cells; [20]) and CAP-T (derived from human amniocytes; [21]), are also being used to produce therapeutic proteins that are currently in preclinical and clinical development. Although human host cell lines offer advantages in terms of PTMs and associated bioactivity, there are theoretical concerns about the potentially increased risk of contamination of therapeutic proteins with adventitious viruses capable of infecting human patients. However, this has been addressed by incorporating the appropriate mitigation strategies during manufacturing, combining viral inactivation and clearance steps with suitable testing strategies [17, 19].

The most commonly used mammalian expression host is that of CHO cells and accounts for the production of >70% of approved therapeutic proteins [22], building a strong regulatory track record for this expression host cell line. As a rodent cell line, there is a species barrier to the production of viruses that can infect humans, and studies have confirmed that CHO cells are resistant to infection with many viruses that can infect humans [23]. Furthermore, the development of CHO-based expression platforms has enabled the removal of animal-derived components, such as serum, from the cell culture medium for cell line development and bioreactor processes, both reducing costs and concerns about adventitious virus contamination. In addition, not only are CHO cells amenable to the introduction of therapeutic protein genes, additional genetic engineering can be performed to modify the growth and metabolism of the cells and product quality attributes (reviewed by [24]). These engineering approaches have been enabled by the development of "omics" technologies and the accruing reference data for CHO

cells (reviewed by [25]) and by the availability of new genome editing tools for genetic engineering such as zinc finger nucleases, meganucleases, Talens, and the CRISPR-Cas9 system (review by [25, 26]). Tangible examples of these engineering approaches are the knockout of FUT-8 genes in CHO to enable the production of fucosyl antibodies conferring improved antibody-dependent cytotoxicity [27] and expression of single [28] or multiple heterologous genes [29] to improve glycan sialylation, which impacts glycoprotein half-life in vivo [28, 29].

As the CHO host cell is a current industry-standard platform, the remainder of this section will focus on CHO production systems.

There are in fact a plethora of different CHO host cell lines, as described by Wurm [30] and Lewis [31]. The original CHO cell line was derived from the ovary of an adult Chinese hamster by Puck [32] and later underwent cloning and other manipulations to derive a number of different CHO cell lines, including those commonly used for the production of therapeutic proteins. These include CHO-K1, CHO DG44, CHO-S, and CHO DUXB11, and these were originally cultured in medium containing serum. However, with the concerns about the cost, variation in the performance of different batches of serum, and the risks of contamination with adventitious viruses, these cell lines have been adapted to grow in animal-component-free media. The choice of individual CHO host is partly driven by the expression 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 expression system that complements this deficiency, as described below. The different CHO host cell lines can show differences in productivity phenotype, for example, recombinant cell lines from CHOK1 showed higher productivities for two difficult-to-express antibodies compared with cell lines created using DUXB11 [33]. However, the host cell lines are themselves heterogeneous, containing cells that show a diversity of characteristics, such as growth and ability to perform posttranslational modifications [34, 35]. These heterogeneous phenotypes result from genetic diversity, which can be observed as the varied karyology profiles of individual cells in a host population [30, 36] and from epigenetic variation. This genetic plasticity enables the adaptation of CHO cells to different culture media or culture conditions and contributes to the versatility of CHO cells for therapeutic protein production.

2.2 Gene Transfection

2.2.1 Stable Transfection and Selectable Markers

Typically, stable recombinant CHO cell lines are created by integrating linearized plasmid DNA encoding the therapeutic protein into the host genome so that, as the cell divides, the transgenes are transmitted to each daughter cell as the chromosomal DNA is replicated and segregated. Transfection methods to enable the transfer of the plasmid DNA to transfer across the cell membrane include

electroporation, calcium phosphate, cationic polymers, and lipid-based reagents. The process of integration of the plasmid DNA into the host genome is highly inefficient, and therefore, screening for recombinants is usually facilitated by including a selectable marker on the plasmid. This can be an antibiotic resistance gene, which can be selected by the addition of antibiotic to the cell culture medium or a metabolic gene such as DHFR or glutamine synthetase (GS), which can be selected by supplementing the cell culture medium with the specific enzyme inhibitors methotrexate and methionine sulfoximine, respectively (reviewed by [37]). Typically, transfectants that have incorporated the selectable marker and are resistant to the selection reagent also usually express the protein therapeutic as the genes are physically linked. Strong viral or housekeeping gene promoters are used to drive high levels of transcription of the therapeutic protein genes to maximize expression [38].

A number of strategies to improve the efficiency of the generation of high-producing transfectants have been developed and have been reviewed elsewhere [39]. These include the use of higher-stringency selection by using CHO host cells that have a knockout in the GS or DHFR endogenous metabolic marker that is used for selection [40] and/or attenuation of the selectable marker carried by the expression plasmid [41]. Other approaches incorporate chromosomal architectural sequences, such as ubiquitous chromatin opening elements (UCOE) or matrix attachment regions (MARS) into the expression plasmid to help promote expression of the therapeutic transgenes by enhancing or maintaining transcription once the plasmid is integrated into the host genome [38, 42]. Transposon-based expression systems can increase the frequency of high-yielding cell lines by increasing gene copy number and insertion at transcriptionally favorable sites [43]. Alternatively, expression plasmid DNA can be targeted by site-specific recombination to particular regions in the CHO genome that are known to be favorable for expression [44]. Similarly, expression plasmids can be targeted by site-specific recombination to regions of artificial chromosomes that have been developed to be maintained in CHO cells [45].

2.2.2 Transient Expression

Stable cell line development is time-consuming and resource intensive, and, at early stages of development, more-rapid methods with higher throughput, based on transient expression, are generally used to make therapeutic protein candidates for early characterization studies. Historically, transient expression systems based on the human HEK-293 cell line have been used for transient expression owing to its propensity for high transfection efficiencies and correspondingly high yields of recombinant protein (reviewed by [46]). Although transient expression in HEK-293 cells can be indicative of the expression levels of recombinant proteins seen in stable CHO cells [47], there are differences in product quality such as the glycosylation profiles [48, 49]. The desire to produce early-stage material that is more representative of the final production cell line has driven the development of

transient CHO systems that are capable of high yields. A number of CHO-based transient systems have now been developed involving engineering of the host cell line [50–53] and/or optimizing the transfection and production processes (reviewed by [54]). In these CHO transient systems, plasmid DNA is introduced into cells using either electroporation [55] or a range of reagents including polyethylenimine (PEI), calcium phosphate, or lipid-based systems [54, 56, 57]. The DNA that reaches the nucleus is transcribed and the mRNA processed and then transported to the cytoplasm where it is translated. No selection is applied, and the cells are allowed to grow and express over a period of a few days to a few weeks, with levels of the plasmid per cell reducing over time as the cells divide. Although CHO transient systems were initially developed for rapid expression of multiple candidates, the technology is being advanced for production at scale. With the achievement of titers exceeding 2 g/L at the 6-L scale [51], it is now becoming feasible to rapidly produce material for pharmacology, formulation, and toxicology studies without having to establish a stable cell line.

2.3 Clone Selection and Single-Cell Progeny

Stable transfection generates heterogeneous cell lines in terms of their productivity, growth, bioreactor performance, and product quality. This heterogeneity between individual transfectants arises from two sources. First is from the integration of the expression vector into different sites in the host genome and the variation in the number of copies of the expression vector, which impact the transcription of the genes encoding the therapeutic protein. Second, as described above, individual cells in the host population have heterogeneous phenotypes that determine characteristics such as doubling time, maximal cell density, metabolism, and product quality [30, 35]. This variation means that it is important to screen recombinant cell lines to ensure that candidate production cell lines express therapeutic protein with suitable product characteristics and that the growth and productivity parameters are suitable for scale-up in a production bioreactor. Finally, to ensure consistency of product quality and process performance, it is important to ensure that any cell line is clonally derived from a single originator transfectant, so that the cells in the cell line population are genotypically and phenotypically homogeneous. The processes for single-cell cloning are described below.

2.3.1 Cloning Processes

The regulatory guidelines direct that recombinant production cell lines should be "cloned from a single progenitor cell" (ICH Q5D).There are a number of different strategies and technologies to derive clonal cell lines (reviewed by [58]). Limiting dilution methods, where low concentrations of cells in suspension are distributed into multi-well plates, can be applied such that a cell line is recovered from a single

cell in an individual well. Traditionally, a statistical analysis of data on the recovery of colonies in wells has been used to support clonality of the derived cell lines [59]. However, with the advent of high-content imaging systems, limiting dilution statistics can be supplemented with detailed images that resolve and identify the originating single cell in a well at the time of plating. The capillary-aided cell-cloning method uses capillaries to transfer individual cells in a small droplet of culture medium from a dilute cell suspension into the well of a plate that is then visually assessed to confirm the presence of a single cell before addition of further medium [60]. Another approach uses the ClonePix robot system and involves dispensing cell suspensions into semisolid 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 [61]. The use of suitable fluorescent detection reagents in the semisolid medium enables enrichment of the colonies picked for those secreting recombinant product. The fluorescence-activated cell sorter (FACS) can be used for sorting of a cell suspension and single-cell deposition into individual wells of a multi-well plate [62]. The sorting capability of the FACS instrument can be leveraged by using fluorescently labeled reagents to detect either the product or a surrogate that is present on the cell surface and then sorting on the basis of the fluorescence signal [63]. Microfluidics is a developing technology that can also be applied to single-cell cloning [64]. Cells can be captured in picodroplets of culture medium in an oil emulsion, the resulting droplets can then be imaged on microfluidic chips, and those containing a single cell can be sorted and subsequently dispensed into plates. Additionally, as the recombinant protein is secreted by the cell into the culture medium in the droplet, this offers the potential to couple isolation of single cells with performing assays on the picodroplet for secreted product to assess yield or product quality [65].

2.3.2 Screening Strategies

Central to the cell line development strategy is that cell lines are screened in an established "platform" bioreactor process using production medium and feed, so that cell lines are selected as "fit to process" and minimize the requirement to perform further upstream process development before scale-up and manufacture of product for the clinic. Following cloning, the individual cell lines are assessed to find those that have suitable growth and productivity characteristics. In order to manage the assessment of the large numbers of cell lines, this process involves a screening cascade with a series of cell line evaluation steps where the numbers of cell lines reduce at each stage (Fig. 1). The first step generally simply identifies those cell lines expressing the therapeutic product, usually by detecting or measuring product secreted into the culture medium. Those cell lines expressing product are advanced to the next evaluation step that involves evaluating cell lines in fed-batch culture to assess both growth and productivity. Traditionally, this was performed using shake flask cultures, but manual handling restricts the number of

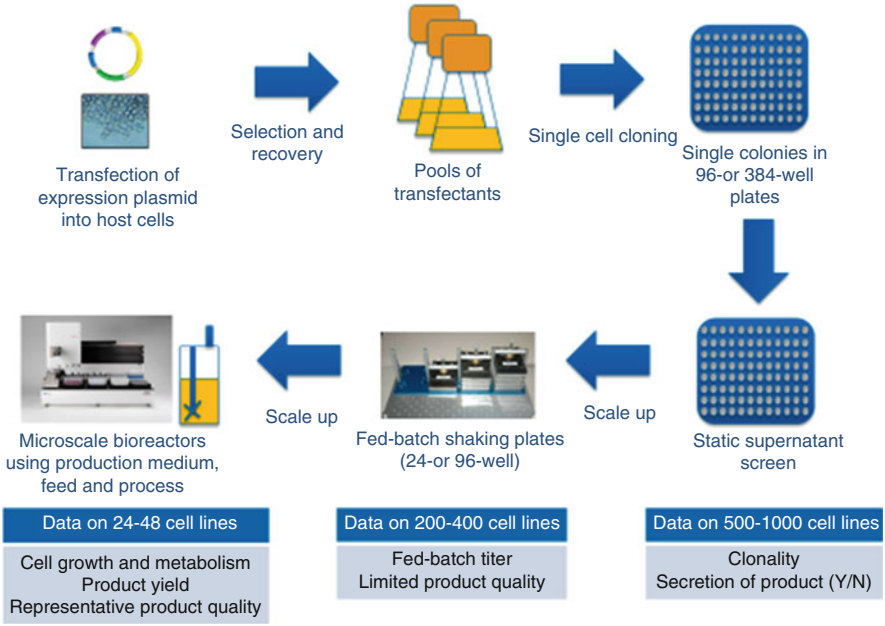

Fig. 1 A generic cell line development screening cascade. As the number of cell lines decreases at each screening step, more detailed data can be collected on each cell line in culture processes that are increasingly more predictive of the final production bioreactor production process. Example numbers of cell lines screened and the collected data at each stage are summarized in the *text boxes*

cell lines that can be evaluated in parallel to a few tens. However, the development of high-throughput, small-scale, fed-batch culture processes using multi-well plates allows hundreds of cell lines to be assessed in parallel [66]. Subsequently, further performance data can then be obtained on multiple (24–48) cell lines in parallel by using scaled-down bioreactor systems, which control pH and dissolved oxygen, generating data that are predictive of larger-scale bioreactors in terms of cell growth, productivity, and metabolism [67]. An additional output from the micro-scale reactor systems is the cell culture samples containing representative product for analysis of product quality attributes. The resulting cell line bioreactor performance and product quality data are assessed to select candidate production cell lines for further in-depth characterization before selecting the final clone for the creation of a master cell bank (MCB).

2.3.3 Product Characterization During Development of Stable Cell Lines

The cell line and the upstream process both contribute to determining the product quality attributes of protein therapeutics. Therefore, it is important to integrate product quality analysis into the cell line screening process. The generation of

analytical data is facilitated by high-throughput analysis of product within the cell culture medium [68] or by integration with high-throughput purification and analytical assays. The exact assays are determined by the properties of the product itself, but typically include evaluation of glycosylation, aggregation, fragmentation, and amino acid sequence integrity. Amino acid sequence integrity is assessed by mass spectrometry methods and peptide mapping [69] and confirms that the expected sequence is being produced. Product sequence variants containing one or more amino acid substitutions can occur as a result of mutations in the encoding DNA or misincorporation of amino acids during translation in the production cell line [69–72]. As these sequence variants are cell line specific, it is feasible to screen them out during clone selection if the causal genetic modification has occurred prior to cell cloning.

2.4 Phenotypic and Genotypic Stability

A critical requirement for the upstream production process is that it is robust and generates product of consistent quality across different batches and scales in order to satisfy both regulatory and commercial requirements. To achieve this, the production cell line must demonstrate suitable growth, productivity, and product quality over the generations required to run the manufacturing process (Fig. 2). Therefore, candidate production cell lines are typically evaluated for phenotypic and genotypic stability at different time points over approximately 70 generations before final clone selection to mitigate the business risk of selecting a cell line that is not sufficiently stable. Regulatory requirements for demonstrating cell line stability are described in the ICH Q5D guidelines.

A number of underlying genetic mechanisms of instability of product expression have been characterized, including gene loss, gene rearrangements, and gene silencing [73]. In addition, as described above, product-related variants owing to genome mutations can occur during cell line development and subsequent subculture. Initially, cells with these genetic changes can be at a very low level within a cell population, so that they are difficult to detect by standard product or genetic characterization methods. However, if these genetic changes confer a benefit to the cell in terms of reduced biosynthetic and metabolic burden, this can lead to faster cell growth, and in turn these aberrant cells can begin to increase in proportion in relation to other cells in the population and consequently impact product expression levels and/or product quality.

Regulatory approval requires that the production cell line is genetically characterized in accordance with the regulatory guidelines (ICH Q5B). This includes analysis of the integrated expression construct for copy number, typically by QPCR, and assessment of insertions or deletions of the transgenes and the number of plasmid integration sites, generally using Southern blotting. In addition to protein analytical characterization, confirmation of the protein sequence for the therapeutic protein is performed by sequencing genomic copies of the encoding

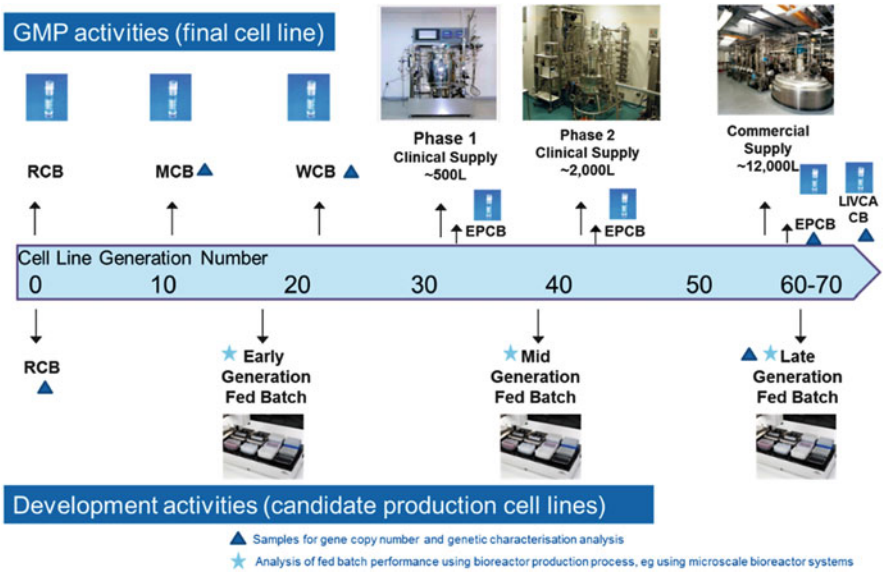

Fig. 2 Phenotypic and genotypic stability assessment in relation to manufacturing time scales. *Top panel*: seventy generations exceed the number of cell doublings required to expand a research cell bank (RCB; generation 0) to create master and working cell banks (MCB and WCB) and then to run the production bioreactors up to commercial supply scale (12,000 L). Cell banks are generated from cells from the production bioreactors – the end of production cell bank (EPCB) and also the limit of in vitro cell age cell bank (LIVCACB) that defines the upper limit of the number of cell generations used for the manufacturing bioreactor process. *Bottom panel*: candidate production cell lines are cryopreserved as RCBs and then *characterized* over a further 70 generations to demonstrate a suitably stable phenotype and genotype in cell culture processes that simulate the production process

genes or by sequencing cDNA produced from RNA transcripts. Next-generation sequencing is also beginning to be applied as a characterization tool for assessing copy number, structure, and sequences of therapeutic protein genes as well as transcript sequence [74].

2.5 Cell Banking

Production cell lines are cryopreserved to enable long-term storage and to sustain consistent manufacture of a therapeutic protein through the clinical development and commercial phases of the drug life cycle. Methods and strategies for the cryopreservation of production cell lines have been reviewed elsewhere [75]. Typically, a tiered system of frozen cell banks is used as outlined in the ICH Q5D guidelines. At the end of cell line development, candidate production cell lines are cryopreserved as a research cell bank (RCB) or pre-master cell bank

(pre-MCB).A single vial of the RCB of the final clone is then used to create an MCB containing several hundred identical vials. Cells from a high-viability culture are centrifuged, and the cell pellet is resuspended in culture medium containing a cryopreservant, typically 5–15% dimethyl sulfoxide, to create a homogeneous suspension that is then dispensed into individual vials before being frozen and stored in the vapor phase of liquid nitrogen at temperatures below –135°C. The process of preparing the vials is performed aseptically under controlled conditions to minimize the introduction of any environmental contaminants. The MCB is the starting point for manufacture of a therapeutic protein product, and so it is vital to safeguard the MCB to maintain drug supply. Hence, MCB vials are stored in at least two independent liquid nitrogen Dewars to mitigate risk of a catastrophic Dewar failure and to maintain supply of the therapeutic product. Also, in order to conserve the use of MCB vials, working cell banks (WCBs) are cryopreserved in a similar way to the MCB, each WCB consisting of a several hundred vials, being derived from a single vial of the MCB. A single vial of the WCB is then revived and expanded to provide culture to inoculate a production reactor. At early stages of clinical development or if only a few vials of the MCB are required each year for product supply, MCB vials can be used for manufacture, avoiding the need to generate a WCB. Historically, cryopreserved MCBs are very stable if stored appropriately, with cells reviving in a consistent way even after many years. The recovery data of the cell banks is tracked over time so that, if necessary, a new MCB can be generated to maintain drug supply.

Typically, cell banks are cryopreserved in small (1–2 mL) cryovials, and cells from each vial are revived into 50–100 mL of cell culture medium and then expanded into larger culture volumes over sequential passages to generate sufficient cells to inoculate the production reactor. At the commercial supply stage, there is a desire to shorten the timelines, and the duration of this expansion phase can be shortened by reviving a larger number of cryopreserved cells at the start of the process. This can be achieved by cryopreserving cells in larger cryopreservation bags (150 mL). In turn, this process requires the availability of high-density and high-viability cell cultures, which can be produced from a bioreactor process in a fed-batch or perfusion mode [75].

Cells used in the manufacturing process are also cryopreserved for the purposes of safety testing, which is described in the next section. Cell culture is removed from the production bioreactor to create the cell banks. If the cells are at high viability, the cells can be cryopreserved directly, whereas lower viability cells are serially subcultured until they reach high viability before being cryopreserved. Freezing high-viability cells ensures that the cells can later be successfully revived. These banks are termed end of production cell banks (EPCB) and limit of in vitro cell age cell banks (LIVCACB) when the cells have reached the maximal number of cell generations that will be used for the manufacturing bioreactor process.

2.6 Safety Testing

Safety testing of the cell banks and the culture harvest at the end of bioreactor production process is performed to ensure patient safety, as outlined in ICH guidelines Q5A and Q5D. Identity testing of the cell banks is performed to confirm the species of origin of the cell line and is important as the cell lines can become contaminated with other cell lines if not appropriately segregated [76]. Tests for microbial and viral contamination are performed on the cell banks and the bioreactor harvest or unprocessed bulk (UPB) according to ICH guidelines Q5A and using methods summarized by Mclean and Harbour [77]. The testing of the UPB, end of production, and limit of cell age banks (EPCB and LIVCACB; Fig. 2) confirms that microbes or adventitious viruses were not introduced during the production process, for example, through the use of contaminated raw materials in the culture process [78] or from the environment. In addition, the risk of introducing contamination with adventitious viruses is mitigated by the use of animal-component-free culture medium, screening raw materials, and heat treatment of culture medium to inactivate viruses. The development of next-generation nucleic acid sequencing technologies provides an additional method for testing and investigating potential incidences of contamination [79]. As next-generation sequencing technology gains regulatory acceptance, it has the potential to reduce the need for the in vivo testing that forms part of the traditional program of virus testing. It is well documented that CHO cell lines possess type A and type C endogenous retrovirus that express retroviral particles [80]. However, these particles have been found to be defective and noninfectious for cells from other animals, including human, and are not considered to present a safety risk [81].

3 Cell Culture Process

3.1 Inoculum Train

Manufacturing of a protein therapeutic starts with the thaw of a vial of a frozen cell bank. Cells are diluted in nutrient medium and placed in shake flasks. Cultures are progressively scaled up in larger shake flasks, by passaging to the next stage when they have grown to a predefined cell density range. Cells are then transferred to disposable bags (20 or 50 L) and subsequently to larger inoculum bioreactors, often known as seed bioreactors. For the earlier stages in shake flasks and disposable bags where pH is not actively controlled, CO_2-enriched air is used to buffer the growth media. For seed bioreactors, pH is controlled within a range by way of sparging CO_2 gas (acid) or dilute sodium hydroxide (base) as needed. Temperature is controlled throughout the inoculum train, and for seed bioreactors, dissolved oxygen (pO_2 measured in mmHg) is also controlled at a predefined set point. The

primary objective of inoculum train is to generate sufficient quantity of cells at high viability to inoculate the production bioreactor where protein production occurs.

3.2 Production Bioreactor

Nutrient and environmental conditions within the production bioreactor are optimized to ensure growth and maintenance of cells at high viability to enable maximum expression of protein of acceptable quality. Environmental conditions that need to be controlled include temperature, pH, oxygen (pO_2), carbon dioxide (pCO_2), and hydrodynamic shear. Agitation and aeration in the bioreactor are controlled such that the oxygen demand of cells is met and dissolved CO_2 is maintained within a predefined value. Agitation also ensures uniformity in concentration of cells and nutrients inside the vessel. Since many product attributes including glycosylation are primarily determined at the cell culture stage, control of bioreactor process parameters is very important. Also, culture viability before harvest is maintained sufficiently high so that the cell harvest process runs smoothly and product variants that may be formed due to released enzymes (e.g., sialidases) [82] are minimized. Low viability also results in higher levels of host cell DNA and proteins, which makes their clearance through the purification steps more challenging.

While the scale of production depends on the product demand, production stage bioreactors as large as 25,000 L are being used in the industry for suspension cell culture [83]. Production bioreactors are often stainless steel reactors although disposable reactors up to 2,500 L are also available [84] and are being increasingly used owing to their ease of implementation and routine operation, reduced change over time between batches, and flexibility in plant capacity as demand changes.

3.3 Modes of Operation

The most typical mode of operation, termed fed-batch mode, involves adding concentrated nutrient-rich feeds periodically during the course of the bioreactor run to supply nutrients such as glucose and amino acid consumed during growth and product expression. This mode is preferred for its balance of simplicity and ability to satisfy nutrient demands to very high cell densities and hence is the mode most used in the industry. It should be noted, however, that this configuration does not work for all molecular formats. If the protein molecule is unstable at the temperature and pH conditions of the bioreactor or because of enzymatic action, it becomes necessary to minimize the residence time of the product in the bioreactor, making a perfusion mode of operation preferable. For example, commercial production of recombinant human Factor VIII is performed using a perfusion system [85]. Also, if the production cell line is anchorage dependent, cells are often grown

on microcarriers. A seasonal influenza vaccine is produced in Vero cells that grow on microcarriers [86]. In addition to providing anchors for cell growth, microcarriers also protect cells from excessive shear.

An emerging trend in the industry is the interest in continuous bioprocessing [2]. The continuous system makes an attractive option to increase the throughput of the plant allowing manufacture of large amounts of product in a facility with smaller-scale bioreactors. Many cell retention devices can be used for perfusion process [87], but alternating tangential filtration (ATF) devices are particularly popular [88]. Also, a perfusion process may be used to increase the cell density in the last inoculum reactor, which in turn results in a higher seeding cell density in the production bioreactor. It should be noted that perfusion, which involves continuous addition of medium and removal of product with return of cells back to the bioreactor, does increase the volume of medium consumed as well as the complexity of operation.

3.4 Process and Media Optimization

During the design of the process, the nutrient concentrations in the basal medium as well as in the nutrient feeds are carefully optimized considering the shifting metabolic needs of the cells during the growth stage and during protein expression. Due to risks associated with transmissible spongiform encephalopathy (TSE) and other transmissible diseases of animal origin, industry has shifted away from the use of animal sera or media containing serum components. However, some serum or serum components are occasionally used during cell line development. Although some companies use only chemically defined media, many rely on animal protein-free formulations that do not have any components of animal origin but may contain complex raw materials such as hydrolysates. Industrial cell culture media contain a number of components and commonly include glucose, amino acids, vitamins, salts, trace metals, buffering agents, and antioxidants. Components such as growth hormones may also be included. Additionally, growth media contain shear protectants in the form of surface active polymers such as pluronic polyols [89]. Media optimization is usually achieved via rational understanding of cellular needs as well as through empirical experimental studies. An important criterion for medium design is to stay within a narrow range of osmolality (approximately 300–500 mOsm) where mammalian cells thrive. Equally important is the design of nutrient feeds, which often have higher nutrient concentrations compared to those in growth media. Since the growth media and nutrient feeds often contain multiple components that interact with each other, scientists rely on statistical experiments to improve process productivity and optimize product quality. Such experiments are carried out in benchtop bioreactors (2–5-L working volume) or in miniaturized bioreactors with working volumes as low as tens of milliliters [90]. Industry has been quite successful in continually increasing the titers for mammalian cell culture processes. A recent example of a significant titer increase,

accomplished by optimizing nutrient concentrations and process conditions without the need for a cell line change, was reported by Ahuja [91].

In addition to approaches mentioned above, advances on multiple fronts continue to enhance the understanding of mammalian cell metabolism. These advances relate to different omics technologies [92, 93] and flux analysis techniques [94]. These techniques help scientists generate hypotheses for further developing growth media and nutrient feeds.

3.5 Equipment Design and Maintaining Sterility

The primary goals of the mechanical design of the bioreactor are to prevent ingress of microbial and adventitious viral agents, to supply oxygen needed for cell growth and product expression, and to provide adequate mixing to ensure homogeneity. Sterility is achieved by ensuring that the bioreactors provide a closed environment with the sterile boundary and that the media, feed, and base used in the manufacturing process are sterilized through membrane filters as they enter the bioreactor. Bioreactors are commonly run at positive head space pressure (generally $\leq$5 psig) to minimize the risk of bacterial contamination. Requirement of the sterile boundary necessitates the design of a robust sterile-in-place (SIP) method and other operational procedures for bioreactor operation and maintenance, the details of which can be found elsewhere [95]. Sterilization cycles for bioreactors and autoclaves are designed to provide sterility assurance level (SAL) in excess of 10^{-6}, where SAL of 10^{-6} indicates the probability of one out of 10^6 sterilization cycles to be unsuccessful [96].

Adventitious agents of concern include bacteria, yeast, mycoplasmas, and viruses. Use of 0.1-μm filtration is generally effective in removing bacteria, yeast, and mycoplasma. For higher assurance of inactivation of mycoplasmas and viruses, heat treatment (high-temperature short time (HTST)) could be employed. HTST treatment of media and feeds involves exposure to temperature about 102°C for approximately 10 s [97]. This treatment has been successfully shown to inactivate multiple viruses including minute virus of mice (MVM), which has been implicated in facility contaminations as recent as 2011 [98]. In addition to heat treatment, another possible technology for inactivating viruses is the use of UV-C (ultraviolet light in the C spectrum) irradiation [99], which so far has found limited use in the industry. Yet another approach is gamma irradiation, which is frequently used for inactivating viruses in sera used for biologics manufacturing [98]. Gamma irradiation is also used to sterilize disposable bags and bioreactors [100].

The bioreactor usually has an aspect ratio (diameter to height ratio) of approximately 1:1.5, which is higher compared to that of a microbial fermenter (approximately 1:3) [101]. A shorter reactor would be better for maintaining homogeneity even though it might be less efficient in oxygen transfer for a given amount of power input [102]. Bioreactors are continuously mixed to ensure homogeneity of cells, nutrients, and environmental parameters such as pH, temperature, dissolved

oxygen, and pCO_2, as well as to improve gas–liquid mass transfer by reducing the size of the gas bubble as well as that of the liquid boundary layer. Many impeller types including Rushton, hydrofoil, elephant ear, marine, and pitched blade can be seen in the industrial bioreactors [103, 104]. Compared to Rushton impellers, more efficient impellers such as axial hydrofoil impellers make an attractive choice, as the latter can be operated with lower power input to provide the desired level of mixing and mass transfer, and they also provide better axial (vertical) mixing [105]. In large-scale bioreactors, it is common to have two impellers with the bottom impeller located above the sparger, which supplies gases (air, oxygen, and CO_2) to the bioreactor. Bioreactors may also contain baffles to aid adequate mixing. Also, if possible, nutrient feeds and base used for pH control are added through subsurface addition; this has been shown to significantly reduce pH fluctuations during base addition in the bioreactors [106].

Foaming, the extent of which depends upon the medium type and sparge conditions, is usually controlled using products containing simethicone. Foaming is not desirable as it can act as a trap for cells, which in turn can be damaged by bubbles bursting on the liquid surface [107]. Moreover, excessive foaming can compromise bioreactor sterility if the foam wets the exhaust filter.

3.6 Process Scale-Up

One must be aware of different physical and chemical environments that may exist as the process is scaled up from a small lab scale to a larger production-scale bioreactor, as maintaining similar environmental conditions is the key to successful scale-up. Parameters that are routinely controlled in a bioreactor are temperature, pH, agitation, and dissolved oxygen (pO_2). Temperature and pH set points remain the same as the process is scaled up. The pO_2 set point may require changing to account for the increased pressure in a larger bioreactor in order to maintain the oxygen concentration across scales.

Agitation rate changes with scale and is commonly estimated using the scale-up criterion based on same power input per unit bioreactor volume (P/V). P/V is a common scale-up criterion since it is a broad indicator of mixing and mass transfer characteristics. Mixing time is inversely related to agitation rate up to a point, and it generally increases with scale and is usually in the order of minutes in large-scale (10,000–15,000 L) reactors [108]. As long as they are significantly lower than characteristic times of gas–liquid oxygen transfer and cellular oxygen uptake [104], differences in mixing times across scales do not pose a problem as they have been found to be satisfactory for dispersing nutrient feeds and base, which are intermittently added in the process. Shear stress experienced by cells in a bioreactor can be estimated by the knowledge of agitation speed [109] or more precisely, by using computational fluid dynamics [110]. Excessive shear, which may generate turbulent eddies (known as Kolmogoroff eddies) with sizes similar to that of cells, can damage cells directly. However, it has been shown that the lethal level of shear

needed to directly damage cells is significantly higher than what is normally experienced in the bioreactors [111]. This offers flexibility in scale-up as agitation set point calculated based on P/V can be adjusted as necessary to optimize other parameters.

Compared to agitation, a more critical factor is the effect of gas sparging on bioreactor performance. Sparge rate and/or composition (relative proportion of air and oxygen) is continuously varied to compensate for the change in the rate of oxygen consumption during the course of the culture in order to maintain the pO_2 within a narrow range. It has been well established that major damage to cells in a bioreactor occurs when bubbles burst at the top of the surface [107]. High rate of sparging, in addition to damaging cells, can also cause excessive foaming. The other important effect of gas sparge rate and composition is the level of CO_2 in culture. Cellular metabolism results in evolution of CO_2 and has to be removed at the same rate to prevent accumulation, as high levels of pCO_2 have been shown to negatively affect the product yield and product quality [112]. High levels of pCO_2 in the bioreactor reduce the pH, which in turn results in higher base addition. This increases the osmolality, which may negatively affect the culture performance. Higher levels of pCO_2 can also affect the intracellular pH, which can result in altered process performance. Appropriate control of pCO_2 in the bioreactor is therefore critical to successful scale-up.

The gases (air and oxygen) sparged to meet cellular oxygen demand in a bioreactor also serve to remove CO_2. In large-scale bioreactors, removal of CO_2 is more challenging than in smaller vessels. As indicated earlier, it is typical for process engineers to maintain a similar P/V as the criterion for scale-up of agitation. This approach ensures adequate mixing, comparable shear imparted to the cells by the impeller, and comparable mass transfer efficiencies to satisfy cellular oxygen demand. However, because the larger-scale vessel is considerably taller, the residence time of gas bubbles is significantly longer, and they reach near-saturation levels of CO_2 concentration [113], thereby losing their ability to strip out CO_2. This in turn leads to an accumulation of CO_2 in the bioreactor. To ensure similar levels of CO_2 between scales, careful consideration of equipment design and sparging strategy is important. Equipment design could include impeller design, sparger design, number of spargers, and their placement. Design of sparging strategy, that is, the profile of sparge rate and composition throughout the culture, should consider cellular CO_2 metabolism/stripping in addition to oxygen metabolism/supply.

While designing the agitation rate and sparging strategy, the process engineer must take into consideration the constraints of mixing, shear stress, damage due to sparging, oxygen supply, CO_2 accumulation, and foaming. For example, although a high gas flow rate would be preferred for keeping pCO_2 levels low, it might result in excessive foaming. Figure 3 depicts the interplay between different constraints and how they all must be looked into for optimizing operating conditions in a bioreactor.

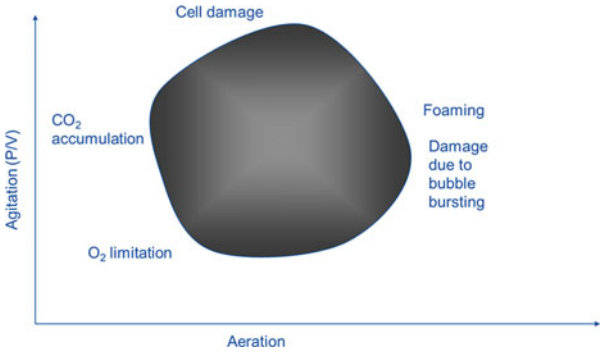

Fig. 3 Interplay of different constraints as function of agitation and aeration rates

The concerns listed above apply to fed-batch as well perfusion cultures with the latter having increased demands for oxygen transfer, CO_2 removal, and foaming due to much higher cell densities.

4 Harvest

Cell harvesting is the separation or retention of cells and cellular debris from the supernatant containing the target molecule. Selection of a harvest method depends greatly 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 in order 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 up to several weeks. Further details on cell retention techniques for perfusion can be found in the review by Voisard et al. [114]. 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 are typically followed by depth and sterile filtration for removal of small debris from the centrate. The ability to predictably scale the selected harvest method helps ensure successful production and is critical for conducting small-scale characterization studies for confirming parameter targets and ranges.

4.1 Methods for Cell Harvest

Batch harvest methods include tangential flow filtration (TFF), depth filtration, and centrifugation. Tangential flow filtration has traditionally been used for lower solid containing <2% and higher viability cell cultures.TFF retains cells using a membrane while the clarified filtrate flows through. The flow along the membrane surface helps keep the membrane from fouling. This method is linearly scalable and, depending on the membrane pore size, can generate clarified cell culture fluid with minimal downstream filtration. Since cells are concentrated during harvest, this method is limited by the concentration factor and product yield that can be achieved. While single-use options for TFF are available, reuse is generally economically more favorable.

Depth filters separate cells based on size exclusion by forcing the liquid through a porous medium in normal flow mode. The medium traps cells and cellular debris while the liquid flows through to a collection vessel. Depth filters of different nominal pore sizes are often used in series to allow the larger cells to be trapped in the first stage and cellular debris to be trapped in the second stage. As harvest volumes increase, the amount of depth filtration area also increases, leading to a larger footprint and less economic advantages when compared to TFF and centrifugation.

Centrifugation is the most widely implemented harvest method for large-scale manufacturing. Centrifuges separate cells from the product-containing liquid by exploiting density differences between the solid cells and the liquid. In a continuous disk stack centrifuge, the feed is accelerated via a rotating bowl, and the solids are thrown toward the outer radius where they contact a series of closely placed, angled disks. The solids travel down the sides of the disks, collect at the bowl periphery, and are discharged intermittently by opening and closing the bowl. The clarified liquid travels upward and out of the centrifuge. The clarified liquid typically is filtered downstream through a combination of depth and membrane filters to ensure complete solids removal. Centrifugation provides significant advantages over filtration including high process yields, effective and consistent clarification performance, and lower resources for process development. However, among the three most commonly used harvest methods, centrifugation is operationally more complex and more challenging to scale up.

4.2 Scale-Up of Centrifugation

Centrifugation takes advantage of density differences between cellular solid particles and cell culture fluid in order to achieve separation. Particles that differ in density will settle at different rates in response to an applied gravitational or

centrifugal force. Assuming laminar flow and approximating cellular particles as spheres enable application of Stoke's law, which defines particle settling velocity as:

$$V_{\mathrm{t}} = \frac{\omega^2 r\left(\rho_p - \rho l\right) d^2}{18\mu}, \tag{1}$$

where V_{t} is the settling velocity, ω^2 is the angular velocity, r is the distance of the axis of rotation, ρ_{p} is the density of the particle, ρl is the density of the liquid, and μ is the viscosity of the liquid.

Ambler related the particle settling velocity to the flow rate and settling area of a centrifuge using:

$$V_{\mathrm{t}} = \frac{Q}{\Sigma}, \tag{2}$$

where Q is fluid flow rate and Σ is a relationship Ambler derived for various types of centrifuges [115]. For a disk stack centrifuge, Ambler defined Σ as:

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

where N is the number of disks, ω^2 is the angular velocity, r^2 is the maximum disk radius, r^1 is the minimum disk radius, and α is the 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. Since sigma factors are known for each centrifuge, the scientist can solve for feed flow rate in Ambler's equation. 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 fully predict separation performance. Prediction of performance using the sigma factor relationship is more reliable when comparing Q/Σ from similarly-designed centrifuges. For example, a laboratory-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:

$$\left(\frac{Q}{\Sigma}\right)_{\mathrm{lab}} = \left(\frac{Q}{\Sigma}\right)_{\mathrm{production}}. \tag{4}$$

Even when using a scaled-down version of the production centrifuge, 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 solid volumes from batch to batch.

Several researchers have successfully developed small-scale models to mimic the shear created within the feed zone of a large-scale centrifuge [116–118]. When used with a laboratory-scale centrifuge, the shear models have had success predicting centrate clarity from a pilot-scale centrifuge [117].

4.3 Filter Sizing

Filter sizing for harvest operations is best determined empirically with scaled-down devices and representative feedstocks. In theory, Darcy's relationship between flow rate and the pressure drop resulting from the flow through a porous material can be used to describe filtration performance:

$$\frac{dV}{dt} = \frac{k\Delta PA}{\mu L} \tag{5}$$

where dV/dt is flow rate, k is bed permeability, μ is liquid viscosity, ΔP is pressure drop, L is bed thickness, and A is filtration area.

However, applying Darcy's law to biological filtrations is challenging because of the difficulty in calculating the bed permeability and the resistance (k/L) for these fluids. Though permeability and resistance could be estimated from the Kozeny–Carman expression [119], experimental measurements are usually a better option. Experiments can be performed either at constant pressure ($V_{\max}$) or at constant flux ($P_{\max}$) [120]. The $V_{\max}$ method applies the linear form of the pore-plugging model to predict the maximum volume that can be filtered:

$$\frac{t}{V} = \frac{1}{Q_{\mathrm{i}}} + \frac{t}{V_{\max}}, \tag{6}$$

where t is time, V is volume, Q_{i} is initial filtrate flow rate, and $V_{\max}$ is the maximum volume that can be filtered at the test pressure before the membrane fouls.

The experiment is performed by filtering at a constant pressure and measuring the filtered volume as a function of time. If the plot of t/v versus t is linear, then the gradual pore-plugging model applies and $V_{\max}$ is calculated from the inverse of the slope. If the plot of t/v versus t is nonlinear, then the constant flux method should be used. Generally, constant pressure experiments are best for screening depth filtration options and constant flux experiments are best for estimating filtration performance. Unless numerous studies are performed with multiple feedstocks, the experimentally determined filtration area is increased by 50–100% to provide a margin of safety for large-scale depth filtration harvest operations [121]. Depth filtration sizing experiments are relatively easy to perform, and generally similar harvest areas and filter types can be used across pilot and commercial scales.

Harvest operations for cell culture are typically filtration or centrifugation or a combination of both methods. Harvest performance is still heavily determined by

experimental data, although improved scale-down models and high-throughput systems continue to be developed.

5 Purification

5.1 Purification Process Targets

Purification of biopharmaceuticals must strike a balance between several competing goals to achieve an optimal result. While each drug is unique, these competing goals often encompass product quality, manufacturing facility fit, adherence to platform process principles, robustness, scalability, productivity, yield, cost of goods for manufacturing (COGM), employee safety, environmental impact, freedom to operate (intellectual property), and validation. While it is beyond the scope of this work to discuss all of these considerations in detail, a few definitions and general guidelines are provided below.

Product quality is defined as the suitability of either a drug substance or drug product for its intended use. This term includes such attributes as identity, strength, and purity (ICH Q8(R2)).Typical limits for process-related impurities and bacterial endotoxins in biopharmaceuticals are provided in Table 1. There are additional considerations for product-related substances and impurities that are specific to each biopharmaceutical. These species may need to be controlled and, therefore, must also be taken into account during design of a purification process.

Good manufacturing facility fit can be thought of as the ability to run a process optimally with few if any changes to the existing facility or equipment. Common purification process bottlenecks, such as buffer requirements that exceed storage capacity, are avoided. For monoclonal antibodies in particular, adherence to platform process principles, where most aspects of process design are fixed, can usually avoid many facility fit issues.

Process robustness is defined as the ability of a process to tolerate variability of materials, process parameters, and equipment performance without negative impact

Table 1 Typical limits for process-related impurities in biopharmaceuticals

Impurity	Limit	References
Host cell protein (HCP)	Varies based on risk assessment; often ng HCP/mg drug	[122], author experience
Small and synthetic macromolecule impurities	Varies based on risk assessment; often μg impurity/mg drug	[123], author experience
DNA	≤10 ng/dose	[124]
Endotoxin	≤5 EU/kg body weight per h[a]	USP <85>[b]

[a]For parenteral administration other than intrathecal

[b]The United States Pharmacopeia Convention <85> Bacterial Endotoxins Test. www.usp.org/usp-nf/harmonization/stage-6/bacterial-endotoxins-test

on quality (ICH Q8(R2)).As process deviations and upsets are possible occurrences during routine manufacturing, good process robustness is a critical goal of purification process development.

Process productivity (e.g., kg/year) and yield (e.g., kg/batch) are related but not identical. These parameters determine how much drug a given facility can produce per unit time and at what cost. Cost of goods for manufacturing (COGM) is impacted by productivity and yield, as well as other parameters such as raw material costs and labor costs. Process economics is discussed in greater detail later in this chapter.

5.2 *Purification of Monoclonal Antibodies*

Given the importance of the biopharmaceutical market and scale of production as indicated earlier in this chapter, tremendous effort has been devoted to mAb purification process development. Numerous comprehensive reference texts on the subject are available (e.g., [125]). Here, we will briefly describe the culmination of decades of biopharmaceutical development, resulting in the modern mAb platform purification process.

Figure 4 shows a process flow diagram for a common variant of the mAb platform purification process. This process begins with removal of cells and clarification by continuous centrifugation and depth filtration. The clarified culture broth containing the mAb is captured by Protein A affinity chromatography. This is followed by a dedicated viral clearance step – low-pH inactivation. The anion exchange flow through chromatography step is principally a viral clearance step, although it can also clear process-related impurities such as DNA. The cation exchange step serves to remove process and product-related impurities. Much of

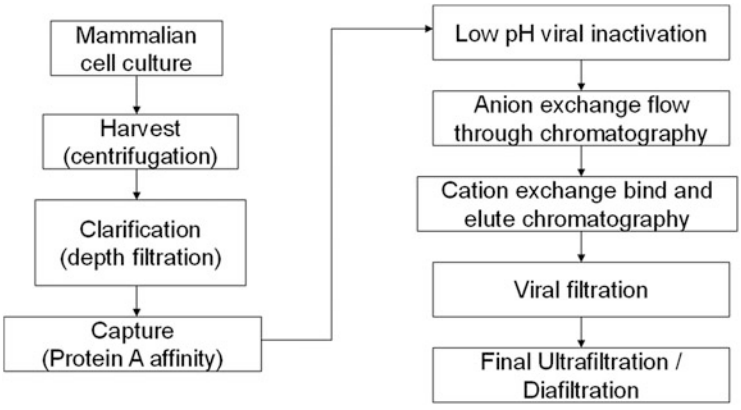

Fig. 4 Process flow diagram for a common variant of the modern mAb platform purification process

the process development effort is dedicated to optimization of this step for removal of product aggregates. Another dedicated viral clearance step, virus filtration, is performed after cation exchange. Lastly, the mAb is concentrated and buffer exchanged into formulation buffer by ultrafiltration/diafiltration.

In most instances, the Protein A column is placed first in the purification train to capture product from clarified cell culture broth [125–127]. This configuration provides an optimal balance of process similarity for different molecules, favorable COGM for routine commercial manufacturing, and process robustness. However, process models developed to predict cost of goods and facility capacity tend to be sensitive to Protein A capture column dynamic binding capacity (DBC) [128, 129]. As a result, the last decade has seen introduction of multiple generations of Protein A stationary phases designed to achieve higher DBCs.

5.3 Affinity Chromatography

Owing to the demand for mAbs, the industrial-scale use of Protein A chromatography with column diameters in excess of one meter is now routine. Staphylococcal Protein A (SpA) is a 42 kDa single-chain protein localized to the outer surface of *Staphylococcus aureus* [130–133]. Native SpA is composed of five Fc-binding domain-designated E, D, A, B, and C [134–136].

The potential of SpA to be used as an affinity ligand for protein purification was recognized early on. Initial SpA affinity resins consisted of native Protein A coupled to a base matrix usually through covalent bonding to amines. Since then, extraordinary improvements have been made in Protein A chromatography resins. Among the most innovative is the Z domain, which represents an analogue of the native B domain developed for purification of Fc-fusion proteins [137, 138]. A derivative of the Z domain engineered for greater alkaline stability, incorporated in mAb Select Sure from GE Healthcare, has gained widespread use for capture of mAbs and Fc-fusion proteins.

Protein A chromatography is most often used for capture of biopharmaceuticals. It serves to remove process-related impurities, including cell culture media components, host cell protein, and DNA. Fc-containing proteins are typically bound to Protein A at neutral or near neutral pH and eluted under acidic conditions below pH 4.0.

Other affinity matrices are used in mammalian cell culture processes. Notably, recombinant Factor VIII preparations have historically utilized custom-designed immunoaffinity resins [139]. However, as non-mAb recombinant proteins manufactured in 2013 represent less than 2% of the total mammalian cell culture production, these applications comprise a tiny fraction of the bioprocessing capacity represented by Protein A chromatography [1].

5.4 Ion Exchange Chromatography

Due to its gentle processing conditions and relatively low cost, ion exchange is among the most widely used separation modalities for purification of biopharmaceuticals. Most modern ion-exchange media are composed of a porous agarose, methacrylate, or polystyrene base matrix containing either strong or weak ionogenic groups. Ion-exchange membrane absorbers are also used for purification of cell culture-derived products, but to a lesser extent.

In most mAb platform processes, ion-exchange steps serve primarily a viral clearance function, in the case of anion exchange, and a polishing function for aggregate removal, in the case of cation exchange. For non-mAb processes, ion exchange serves a much wider variety of functions ranging from capture to polishing. Proteins are usually bound under low ionic strength conditions and eluted at higher ionic strength, although some proteins (mAbs in particular) have demonstrated higher binding capacity at intermediate ionic strength owing to electrostatic exclusion effects [140]. Binding and elution by varying pH can also be employed although this approach is not as widely used and generally requires more time and effort for process development.

Contributions from both thermodynamic and rate factors govern the performance of ion-exchange chromatography. The thermodynamic component is described by the equilibrium adsorption isotherm, while the rate is usually governed by mass transfer resistance. Early generations of ion-exchange media had relatively poor equilibrium capacity and mass transfer characteristics. By comparison, more recent iterations strike a good balance of equilibrium binding capacity and mass transfer rates for many protein biopharmaceuticals.

Mechanistic models of ion-exchange adsorption usually require a description of the equilibrium isotherm and in many instances require a numerical solution of the general diffusional transport model [141]. However, constant pattern solutions for column breakthrough are available for the external film model and the linear driving force model. For instances of strong binding, which can be approximated by a rectangular isotherm and which is frequently encountered with ion-exchange media at low ionic strength, exact analytical solutions are available [141].

5.5 Hydrophobic Interaction and Multimodal Chromatography

Hydrophobic interaction chromatography (HIC) and multimodal chromatography are widely used for purification of cell culture products. Because of the need for salts composed of kosmotropic anions and chaotropic cations (e.g., ammonium sulfate), HIC does tend to have greater drawbacks compared to other modalities. The high concentrations of salt required may necessitate special waste treatment prior to discharge from the manufacturing facility. Moreover, HIC can be quite

sensitive to a number of operating parameters and, therefore, requires more time and effort to develop a robust and scalable unit operation. Nonetheless, HIC unique selectivity often proves indispensable in biopharmaceutical purification, requiring the investment be made to overcome these challenges.

Proteins are commonly bound to HIC ligands at high ionic strength using ammonium sulfate, sodium sulfate, or sodium citrate and eluted by lowing the ionic strength. HIC is used extensively for demanding separations such as removal of product aggregates and fragments. It is most often used in a polishing configuration later in the process. While HIC can be used for product capture, this is relatively rare due to complications that can arise when adding large amounts of salt to cell culture harvest broths and the tendency of HIC resins to become fouled due to the higher proportion of impurities in these feed streams.

Multimodal chromatography utilizes ligands typically composed of both ion-exchange and HIC modalities. In general, these matrices mitigate some of the manufacturing drawbacks of HIC. For example, the use of concentrated salt solutions is often not required for multimodal chromatography. From a process development perspective, the time and expense to develop a multimodal purification step are intermediate between that of HIC and ion exchange. Therefore, it is usually evaluated before HIC for challenging separations that cannot be achieved by less selective methods.

Binding and elution of proteins on multimodal resins can occur under conditions similar to either HIC or ion exchange, depending on the specific nature of the protein. Some proteins show a strong tendency toward one mechanism, while others may be able to utilize either depending on other parameters such as solution pH. Multimodal chromatography is utilized in a wide variety of roles for removal of both process- and product-related impurities.

5.6 Scale-Up of Chromatography Steps

A number of factors must be considered prior to scale-up of process chromatography unit operations. These usually encompass equipment and hardware, column packing and pressure-flow relationships, dynamic binding capacity, yield, selectivity, and resolution [142]. Due to loss of wall support, higher backpressure is encountered in larger diameter columns as scale is increased for the same bed height. This effect is now relatively well understood and predictable using mathematical relationships for compressible media [143]. The quality of column packing is usually evaluated using relatively simple inert tracer pulse response experiments to measure height equivalent to a theoretical plate (HETP) and peak asymmetry. A practical guide to qualification of chromatographic columns and setting limits for these parameters is provided by Rathore et al. [144].

Capacity, selectivity, and resolution are usually optimized using laboratory-scale models. Dynamic binding capacity (DBC) is best measured using representative feedstocks with >1 cm diameter columns at the target bed height and mobile

phase velocity to ensure that both selectivity and resolution are maintained at scale; the process developer needs to have a good understanding of potential sources of variability and the normal operating ranges for all relevant process parameters. For example, aggregate removal by stepwise elution from ion-exchange resins tends to be sensitive to both pH and ionic strength [145]. If the process requires tighter operating ranges than can be achieved in manufacturing, process performance or product quality may suffer. Scale-up of gradient elution and prediction of optimal stepwise elution conditions using relatively simple mathematical relationships has been established by Yamamoto and coworkers [146].

5.7 Viral Safety Considerations

Viral safety of biopharmaceutical preparations is of paramount consideration. For products made using well-characterized cell lines, at least two unit operations with orthogonal clearance mechanisms have to be tested for clearance capability using model viruses [147]. Cell lines used for biopharmaceutical production often contain endogenous retroviruses. Moreover, adventitious viral contamination may occur through ingress from raw materials, cell banks, or process operations. Viral safety must be assured for both sources of viral contamination. In the authors' experience, a large proportion of questions from regulatory authorities concern viral safety matters. Therefore, attention to detail in this area during process development and execution of viral clearance studies is particularly important.

For monoclonal antibody processes, the majority of clearance is usually derived from low-pH virus inactivation (i.e., exposure to low pH, typically 3.6 or lower, sufficient to inactivate enveloped viruses), anion exchange flow through chromatography, and virus filtration (a dedicated membrane filter with a pore size, usually 20–40 nm, smaller than the size of the virus desired to be removed).The cation exchange step can also serve to clear viruses if needed [148]. Low pH, anion exchange, and virus filtration can often provide a combined log reduction value (LRV) of greater than 15 if implemented correctly for xenotropic murine leukemia virus, which is a commonly used model virus for clearance studies.

5.8 Ultrafiltration/Diafiltration (UF/DF)

In many cell culture processes, UF/DF is used as a process step to perform a simple buffer exchange and concentration function. They may also serve to remove small impurities with a high sieving coefficient. In practice, removal of many small-molecule impurities by UF/DF may not follow ideal behavior and can be described using mathematical models developed by Zydney and coworkers [149].

For monoclonal antibodies, the final UF/DF step serves to achieve the final drug substance protein concentration and exchange the product into formulation buffer.

As a result of the industry trend toward high-concentration formulations in order to reduce the volume of product dosed, this step can pose unique challenges. In particular, concentration polarization, high shear rates, and high viscosity can lead to scale-up challenges for this unit operation [150]. Furthermore, the requirement for a large amount of product can make this step challenging to develop even in a laboratory-scale setting. To avoid commonly encountered problems associated with slip-induced shear from peristaltic pumps, it is recommended that high-quality diaphragm pumps be used for both development and manufacturing.

6 Process Economics

Biotechnology is increasingly providing new and potentially effective protein-based treatments against many of humankind's most serious diseases. However, treatment in therapy areas such as oncology, neuroscience, and metabolic disease, all with significant unmet medical need, is not viable unless these novel products can be made in sufficient quantity and sold at a price that payers can afford. In addition, the market is becoming increasingly competitive with efficacy of the new drug being of primary importance but time to market and cost becoming key differentiators. Therefore, as this trend in rapid expansion of next-generation biologic portfolios continues to grow, getting to grips with process economics is very important.

There are a number of fundamental components that affect process economics including titer, scale, purification yield, time in plant, facility costs (depreciation, labor, and utilities), raw materials, and process configuration.

6.1 Titer and Economies of Scale

Recombinant protein titers vary widely depending on expression system and protein class. A well-established expression platform such as Chinese hamster ovary (CHO) expression of therapeutic antibodies can reach 10–15 g/L [126]. Other protein classes (e.g., blood factors) may be expressed at significantly lower levels. The impact on cost of goods manufactured per gram (COGM/g) by titer is very significant. As titer continues to increase, the impact of manufacturing costs become more apparent (Fig. 5). Beyond a certain titer, say above 4 g/L, the effect of COGM/g is less dramatic; however, the plant output continues to increase as titer increases, in the absence of purification bottlenecks. With a finite amount of time that can be devoted to process optimization before the program needs to move into clinical evaluation, the allocation of development effort to increasing titer versus other process improvements, downstream, needs to be carefully considered.

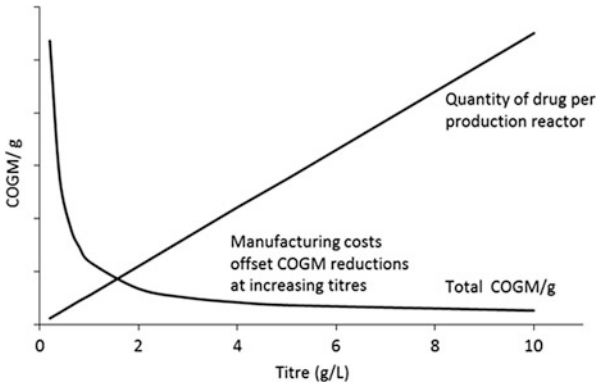

Fig. 5 Economies of scale. Typical cost trend as titer increases

6.2 Purification Yield

The overall process yield is a function of the performance of each individual purification step. Improvements in step yield through process optimization and in some cases removal of non-value added intermediate steps can increase the quantity of product per batch and thus reduce COGM. Typically the more complex the process and the more steps required to produce appropriate quality product, the lower the yield and the more expensive the final drug. For an established product type such as antibodies, process yields have risen in recent years from <50% to typically over 70% [151].

6.3 Batch Duration: Run Rate

A biologic manufacturing facility costs money whether it is used or not. Fixed costs, such as depreciation, labor, and power, are constantly accumulating and will be attributed to the COGM in that facility. Therefore, it is essential to maximize the potential number of batches that the plant can handle. Fermentation duration, number of bioreactors, number of purification suites, and the purification time are some of the key components that dictate run rate. Clearly the number of bioreactors and purification suites may not be easily changed; however, the way that the fermentation and purification process are optimized and run to maximize run rate is of paramount importance.

6.4 Material Costs and Process Configuration

Production of biologics requires the use of raw materials (chromatography resins, filters, buffer constituents, cell culture media, and water). The cost impact of raw

material used is dependent on scale and in certain cases (resins and filters) whether or not components are reused and, if reused, how many times. The decision to incorporate disposable or reuse is complex. While the disposable path may lead to higher raw material costs, this is potentially offset to some extent by reduction of operating costs and other indirect costs such as cleaning validation [152, 153]. The process configuration is ultimately determined through careful consideration and modeling of potential manufacturing scale, campaign size, and other manufacturing considerations, such as whether or not the process will move into a dedicated or a multiuse facility.

6.5 Cost Modeling

The choice of operating conditions, process performance, and scale of manufacture will all impact COGM. Cost models are typically used to help rationally determine the optimum solution for process configuration and potential process development strategies [154]. Developing a protein therapeutic has high uncertainty and is costly and subject to strict regulatory requirements. The old paradigm of "fail fast-fail cheap" in which early proof of principle was demonstrated before significant investments were made is no longer always the case. With some disease areas becoming increasingly well understood, the requirement to rapidly accelerate through the clinical phases leaves little time to make significant process modifications. Development of processes that are economically viable from the beginning of the process life cycle is becoming increasingly necessary. One way it has been possible to satisfy this contradictory expectation of high productivity, and accelerated timelines is by development of "platform processes" that work especially well for a class of molecules (e.g., mAbs). The use of cost models and other in silico process tools to evaluate and prioritize impact of cost-related factors (e.g., titer, scale, and process choice) with clinical data (potential dose, patient population) and manufacturing facilities is becoming fundamental to strategic and rational selection of process requirements and optimization goals [155].

7 Summary

The progress in bioprocess development and scale-up continues to keep up with the changing nature of treatment paradigms and economic pressures. On the one hand, a fairly solid platform process (described in this chapter) consisting of fed-batch bioreactors, three-column chromatography, and two viral clearance steps has been developed and routinely practiced at large scale for the manufacture of monoclonal antibody products to supply markets with high product demand. At the same time, the industry is also retooling itself to respond to personalized medicine approaches calling for greater number of products with lesser demand by developing disposable

bioreactors and columns. Leveraging the experience gained in developing processes for mAbs, progress is also being made in other recombinant proteins, such as Fc-fusion proteins, multi-specific proteins, and even other product modalities such as gene therapy and cell therapies.

Because of the need to evolve with the changing patient needs, bioprocess development and manufacture will never become a mature field; however, thanks to the rapid progress made over the past decade, it is well past its infancy.

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