Advances in Biochemical Engineering/Biotechnology 139
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# Mammalian Cell Cultures for Biologics Manufacturing

Springer

# 139
# Advances in Biochemical Engineering/Biotechnology



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Weichang Zhou · Anne Kantardjieff
Editors

# Mammalian Cell Cultures for Biologics Manufacturing

With contributions by

Anne Kantardjieff · Weichang Zhou · Scott Estes
Mark Melville · Susan Abu-Absi · Sen Xu
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ISSN 0724-6145 ISSN 1616-8542 (electronic)
ISBN 978-3-642-54049-3 ISBN 978-3-642-54050-9 (eBook)
DOI 10.1007/978-3-642-54050-9
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2013958240



Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

# Preface

Mammalian cells have now become common cell hosts for the production of biologics, and more than 50 % of approved recombinant protein therapeutics currently on the market are being manufactured in mammalian cell lines. Since the approval of Orthoclone OKT3 (the first biologic drug to be produced in mammalian cells) in 1986, routine titers, cell-specific productivities, and cell densities have increased dramatically. These improvements have been the result of decades of developmental work in multiple areas, including cell line development, process operations, and equipment.

This volume was put together to highlight the progress in using mammalian cell cultures for the manufacture of therapeutic biologics. It consists of ten chapters which provide an overview of biologics development and manufacturing in mammalian cell cultures. The first chapter entitled "Mammalian Cell Cultures for Biologics Manufacturing" provides an overview of licensed therapeutic biologics currently on the market, including details on market size, as well as commonly used production cell lines and cell culture operation modes. The second chapter entitled "Mammalian Cell Line Developments in Speed and Efficiency," provides an overview of the cell line development process, including host cell line selection, available expression systems, and commonly used selection strategies. In the third chapter "Cell Culture Process Operations for Recombinant Protein Production," an overview of current operation models for mammalian cell culture is presented, including batch, fed-batch, and perfusion processes. Details regarding process monitoring and control, including data analysis, are also included. The fourth chapter, entitled "Equipment for Large-Scale Mammalian Cell Culture," provides an overview of the commonly used equipment in industrial mammalian cell culture, with an emphasis on bioreactors. This chapter also provides insight into the use of disposables during seed train and production.

The next chapter, entitled "Development and Characterization of a Cell Culture Manufacturing Process Using Quality by Design (QbD) Principles" provides a case study of the application of quality by design principles during late stage process development. In the sixth chapter, "Product Quality Considerations for Mammalian Cell Culture Process Development and Manufacturing," a review of common product quality consideration in mammalian cell culture is provided. This chapter also includes a summary of the impact of cell culture conditions on product quality, and current strategies to control product quality profiles.

An overview of testing of adventitious agents is provided in the next chapter, entitled "Safety Assurance for Biologics Manufactured in Mammalian Cell Cultures: A Multitiered Strategy." In this chapter, a general overview of the tiered safety strategy commonly employed by the biopharmaceutical industry to mitigate adventitious agent contamination is presented. The eighth chapter, entitled " Mammalian Cell Culture Capacity for Biopharmaceutical Manufacturing," provides an overview of the current global manufacturing capacity and an analysis of market trends that will impact the future manufacturing expansions and utilization. The ninth chapter, entitled "Transcriptomics as a Tool for Assessing the Scalability of Mammalian Cell Perfusion Systems," provides a case study of the use of transcriptome analysis in mammalian cell culture process development. The final chapter, "Lifecycle Management for Recombinant Protein Production Using Mammalian Cell Culture Technology," provides a case study for product lifecycle management.

In summary, this volume represents a comprehensive overview of biologics manufacturing in mammalian cell lines, and includes a number of relevant industrial case studies. While it is inevitable that certain topics or areas were omitted from this volume, the authors have sought to provide extensive references to additional sources of information. We hope this volume will provide readers with a concise summary of state-of-the-art practices in the industry and an overview of the current challenges faced by biologics manufacturers. The editors would like to thank all of the contributors to this volume, the series editor and the publisher, who have made this volume possible.

Anne Kantardjieff
Weichang Zhou

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Adv Biochem Eng Biotechnol (2014) 139: 1–9
DOI: 10.1007/10_2013_255

Published Online: 21 November 2013

# Mammalian Cell Cultures for Biologics Manufacturing

**Anne Kantardjieff and Weichang Zhou**

**Abstract** Biopharmaceuticals represent a growing sector of the pharmaceutical industry, and are used for a wide range of indications, including oncology and rheumatology. Cultured mammalian cells have become the predominant expression system for their production, partly due to their ability to complete the post-translational modifications required for drug safety and efficacy. Over the past decade, the productivity of mammalian cell culture production processes has growth dramatically through improvements in both volumetric and specific productivities. This article presents an overview of the biologics market, including analysis of sales and approvals; as well as a review of industrial production cell lines and cell culture operations.

**Keywords** Biopharmaceuticals · Cell culture operations · Mammalian cell culture · Production cell lines

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## 1 Introduction

Biologically derived drugs represent a growing sector of the pharmaceutical market. This class of compounds, known as biologics or biopharmaceuticals, is derived from the genetic manipulation of living organisms. Biologics include recombinant DNA-derived proteins and monoclonal antibodies, along with gene therapies and bioengineered animals and plants. Biopharmaceuticals are used to treat a broad range of diseases, especially in the fields of rheumatology and oncology, as well as cardiology, dermatology, gastroenterology, and neurology. In many medical fields, biologics represent the sole therapeutic option available to patients.

## 2 Licensed Therapeutic Biologics

There are currently 230 approved biologics on the market [5, 7, 8]. Figure 1 shows the distribution of therapies among eight major classes of compounds: recombinant blood factors, including Factor VIII; recombinant thrombolytics and anticoagulants, including tissue plasminogen activator and hirudin; recombinant hormones, including insulin, human growth hormone, and follicle-stimulating hormone; recombinant growth factors, including erythropoietin and granulocyte colony-stimulating factor; recombinant interferons and interleukins, including interferon-$\alpha$ and interferon-$\beta$; recombinant vaccines, including hepatitis B; monoclonal antibodies (mAbs) and monoclonal antibody-based products; and other recombinant products, including bone morphogenic proteins, recombinant enzymes, and nucleic acid-based products. As can be seen, recombinant hormones are the most represented class of compounds (51 therapies, 22 % of approved biologics), and mAbs are the second most predominant class with 49 therapies on the market (21 % of approved biologics).

Of the 230 approved biologics on the market, 93 have been approved since 2006, which represents more than 40 % of commercially available biopharmaceuticals. Figure 2 shows the number of drugs approved by the US Food and Drug Administration (FDA) since 1998 [7]. This includes both small molecule drugs, filed as New Molecular Entities (NMEs) and biologics, filed under biologics license applications (BLAs). The pace of approval for new biologics has been growing over the past years. A total of 37 drugs was approved by the FDA in 2012. Of these, 14 were biologics, as filed under BLAs. This represents the largest number of approved biologics license applications in the past decade. Among new approvals, 32 % were for orphan disease indications. Although these rare diseases have small patient populations, they have also been shown to have a higher success rate in the clinic and require smaller clinical trials. As a consequence, many pharmaceutical and biopharmaceutical companies have added orphan drug programs to their product pipelines.

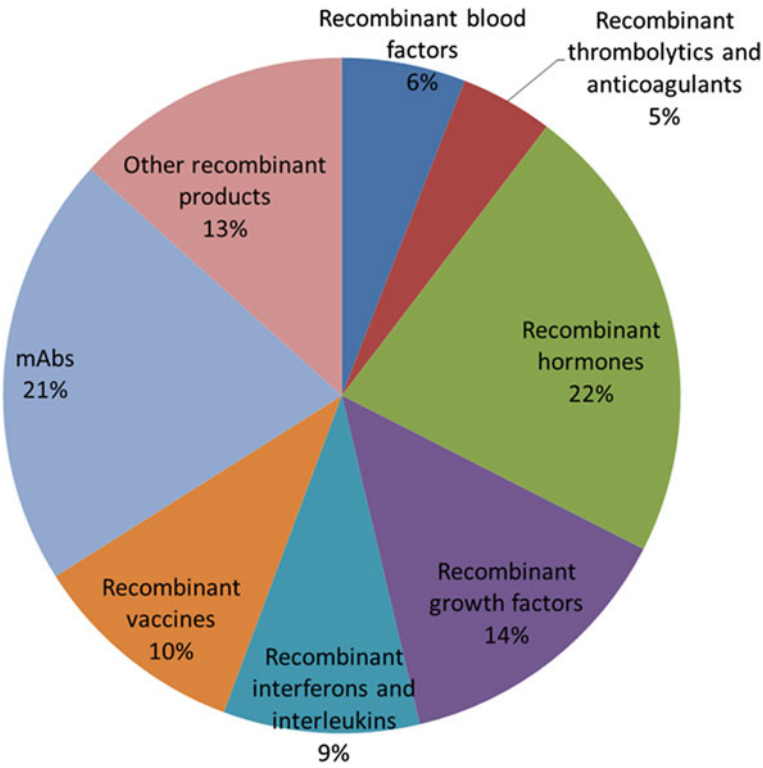


**Fig. 1** Distribution of the number of approved biologics by compound class

## 3 Market Size

Global sales of biologics reached US $124.9 billion dollars in 2012, a 10.4 % increase over 2011. Among them, sales of monoclonal antibodies and antibody fusion proteins were US $65 billion dollars, over 50 % of the total biologics sales. The growth of the US biologics market has mirrored that of the global biologics market. Growth data for the US market since 2002 is shown in Figure 3. As can be seen, the market has been growing steadily since 2002, albeit at a reduced pace in more recent years [2]. In 2011, total sales of biologics in the US market were US $53.8 billion, which represents a 4.9 % increase over 2010 sales [1].

Monoclonal antibodies are the best-selling class of biologics, with US sales in 2011 of ~$20.3 billion. In fact, sales of monoclonal antibodies have almost doubled since 2006, when US sales were $11.4 billion. This dramatic increase can be attributed to two factors: the first is significant sales of new entries into the market. Four monoclonal antibodies approved in 2011 had combined sales of $2 billion. The second is the growth in sales of the three top-selling monoclonal antibodies (Humira, Remicade and Rituxan), which each sold more than $3 billion in the US alone.

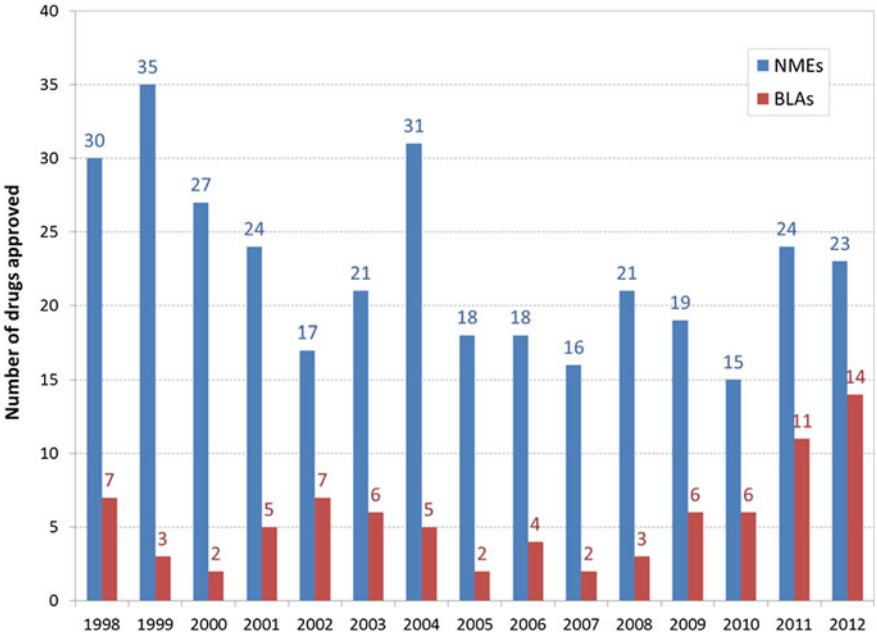


**Fig. 2** Number of drugs approved by the US food and drug administration (FDA) since 1998


**Fig. 3** Growth trends in the US biologics market

A second class of compounds that has been growing significantly in recent years is recombinant hormones, where insulin analogues account for three-quarters of reported sales. Sales of recombinant hormones in 2011 topped US $12.2 billion, up from $5.39 billion in 2006, an increase of more than 126 %. One insulin analogue, Lantus (Sanofi Aventis), was the second–best selling biologic drug in the United States in 2011, with total sales of $3.5 billion, only slightly behind AbbVie's Humira, with total sales of north of $3.5 billion [1].

There were more than 30 blockbuster antibodies and proteins in 2012, as defined by global sales in excess of US $1 billion dollars [6]. The top-ten highest-grossing biologics are shown in Table 1. Nearly all of the monoclonal antibodies and antibody fusion proteins, along with many blockbuster recombinant proteins such as erythropoietins, granulocyte colony-stimulating factor, coagulation factors, and replacement enzymes are manufactured by mammalian cell cultures.

## 4 Industrial Production Cell Lines

An analysis of industrial production cell lines used for the manufacture of biologics shows that 51 % of currently approved biologics are produced in mammalian cells (Fig. 4). Mammalian cell lines are especially predominant in the production of certain classes of biologics. Notably, 83 % of recombinant blood factors are produced in mammalian cell lines, 95 % of monoclonal antibodies, and 74 % of other recombinant products. Biologics of increased complexity, including products with extensive post-translational modifications, must be produced in mammalian cell lines in order to obtain the desired product quality profile including humanlike glycan profile [5, 7, 8].

Mammalian cell lines used for biologics production include Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and mouse myeloma cells, including NS0 and SP2/0, as well as human cell lines (HEK293, HT-1080). Of these, CHO cells are the most widely used, accounting for the production of more than 60 % of mammalian cell culture derived biologics currently on the market (Fig. 5). Chinese hamster ovary cells were first used for an approved biologics by Amgen in 1989, for production of Epogen, a recombinant human erythropoietin used for the treatment of anemia. Since then, CHO cells have become the most widely used mammalian cell line for industrial biologics production. Seven of the eleven monoclonal antibodies approved since 2010 are produced in CHO cells.

The productivity of mammalian cells for production of biopharmaceuticals has increased more than 20-fold in the past two decades [3]. Titers in the range of 1–5 g/L are now commonplace, especially for antibody production processes, with productivities as high as 10–15 g/L being reported in a fed-batch culture of 2–3 weeks [4]. This increase has been driven by two factors: the first is a dramatic

**Table 1** Blockbuster biologics in 2012

| Ranking | Product name | Target/mechanism of action | Class of compound | Company | 2012 global sales (vs. 2011) |
|---|---|---|---|---|---|
| 1 | Adalimumab (Humira Pen) | TNF alpha6 antagonist | mAb | Abbott; Eisai | US $9.534 billion |
| 2 | Etanercept (Enbrel) | TNF antagonist | Recombinant protein | Amgen; Pfizer; Takeda Pharmaceutical | US $8.406 billion |
| 3 | Infliximab (Remicade) | TNF alpha antagonist | mAb | 7 Centocor; Merck; Mitsubishi Tanabe Pharma | US $7.468 billion |
| 4 | Rituximab (Rituxan/MabThera) | CD20 | mAb | Roche (Genentech, Chugai); Biogen-IDEC | US $7.143 billion |
| 5 | Trastuzumab (Herceptin) | HER2 | mAb | Roche (Genentech, Chugai) | US $6.272 billion |
| 5 | Bevacizumab (Avastin) | VEGF | mAb | Roche (Genentech, Chugai) | US $6.139 billion |
| 6 | Insulin glargine (Lantus) | Insulin receptor | Protein | Sanofi | US $6.510 billion |
| 7 | Pegfilgrastim (Neulasta) | G-CSF receptor | Protein | Amgen | US $4.092 billion |
| 8 | Ranibizumab (Lucentis) | VEGF | mAb | Roche (Genentech); Novartis | US $3.975 billion |
| 9 | Epoetin alfa (Epogen/ESPO/ Procrit/Eprex) | Erythropoietin receptor | Recombinant protein | Amgen; Ortho Biotech; Janssen-Cilag; Kyowa Hakko Kirin Pharma | US $3.442 billion |
| 10 | Insulin aspart (NovoLog NovoRapid) | Insulin receptor | Recombinant protein | Novo Nordisk | US $2.940 billion |

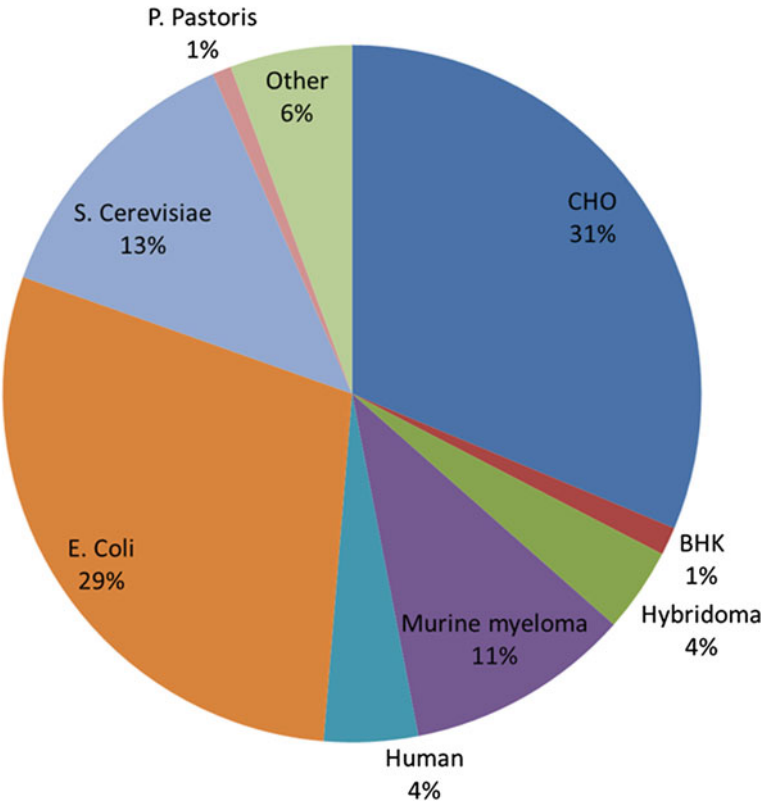


**Fig. 4** Distribution of cell lines used for industrial biologics manufacturing by number of licensed biologics until 2012

increase in the maximum achievable viable cell concentration and longer sustained cell viabilities in a typical fed-batch culture process. As a result, the total cell mass has significantly increased in fed-batch cultures. This is often expressed in terms of the integral of viable cell concentration (IVCC), which is determined by integrating the viable cell concentration over culture duration. The second factor attributable for increased titers is the development of cell lines with higher specific productivities ($q_p$). Specific productivities on the order of 10 pg/cell/day were commonplace a decade ago, and have now been replaced by high-producing cell lines with specific productivities of more than 50 pg/cell/day [3]. These rival or exceed the specific productivities of human plasma cells, the body's antibody production cells. These two factors combined have contributed to the higher titers now commonplace in biologics production in mammalian cells.

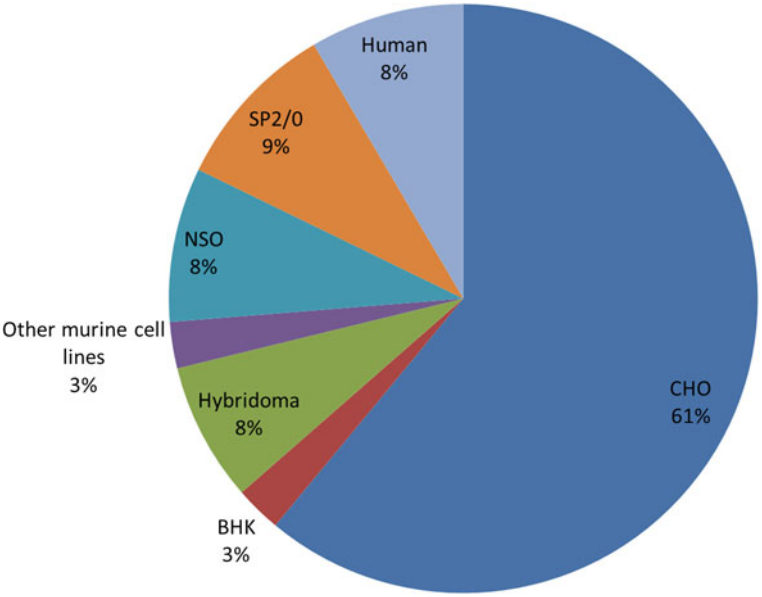


**Fig. 5** Distribution of mammalian cell lines used for industrial biologics manufacturing by number of licensed biologics until 2012

## 5 Industrial Cell Culture Operations

There are three major cell culture operations used in the industry, batch, fed-batch, and perfusion. Both batch and fed-batch cultures are used to produce stable molecules such as monoclonal antibodies due to longer residence time in culture, whereas perfusion cultures are required to produce labile molecules such as recombinant enzymes and coagulation factors. Several antibodies are also produced by perfusion cultures.

In a batch culture, all nutrients are added in the beginning. The nutrient concentrations are generally low to limit osmolality. These low nutrient supplies limit the maximum cell concentration, the culture duration, and the product concentration achieved. In a fed-batch culture, additional nutrients are added during the culture to prevent nutrient depletion, thus prolonging the cell growth phase and culture duration. This results in a much higher maximum cell concentration and longer culture lifetime. As a result, a much higher product concentration is achieved.

A perfusion culture is a continuous cell culture process with cell retention. Cells are retained in the reactor, while new culture media is continuously added in and culture supernatant is removed at the same rate to keep the reactor volume constant. A number of licensed therapeutic biologics products are produced in perfusion cultures to prevent product degradation and ensure better product quality for labile molecules.

## 6 Conclusion

Biologically derived drugs have continued to drive growth in the pharmaceutical industry. This is due partly to a sustained increase in the number of approved drugs in recent years. Furthermore, improved sales, especially for monoclonal antibodies, have contributed to the observed growth. Mammalian cells are the predominant expression system used for biologics production, partly due to their ability to generate humanlike posttranslational modifications essential for drug safety and efficacy. Improvements in process development over the past decades have resulted in higher titers and specific productivities, making higly robust and productive mammalian cell culture processes common place in the industry.

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Adv Biochem Eng Biotechnol (2014) 139: 11–33
DOI: 10.1007/10_2013_260

Published Online: 7 November 2013

# Mammalian Cell Line Developments in Speed and Efficiency

**Scott Estes and Mark Melville**

**Abstract** Mammalian cell expression systems are the dominant tool today for producing complex biotherapeutic proteins. In this chapter, we discuss the basis for this dominance, and further explore why the Chinese hamster ovary (CHO) cell line has become the prevalent choice of hosts to produce most recombinant biologics. Furthermore, we explore some of the innovations that are currently in development to improve the CHO cell platform, from cell line specific technologies to overarching technologies that are designed to improve the overall workflow of bioprocess development.

**Keywords** Automation · Bioprocess development · Cell engineering · Chinese Hamster Ovary cells · Protein expression

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## 1 Introduction

Twenty-five years ago, Genentech (now a part of Roche) received commercial approval for Activase®, a recombinant form of tissue plasminogen activator expressed in Chinese hamster ovary (CHO) cells. This accomplishment ushered in the era of utilizing mammalian expression systems to produce complex glycosylated therapeutics. Other established rodent cell lines such as NS0, Sp2/0, and BHK cells have been, and to a certain extent, continue to be used to develop biologics with Remicade®, Erbitux®, and Synagis® being the notable commercial successes. However, of the top-ten selling biologics which amassed over US $57 billion in sales in 2011, eight are produced from mammalian expression systems and of those eight, only one, Remicade®, is not produced from engineered CHO cells [33]. This dominance is further illustrated by a review of recently approved recombinant biologics derived from mammalian cell lines. In the last two years, five out of six utilize a CHO host for expression. Benlysta®, a therapeutic mAb to treat lupus patients, is produced from recombinant murine NS0 cells and represents the sole outlier in the group.

In contrast, the use of human cell lines to express recombinant therapeutics has been somewhat limited, despite the diversity of established human cell lines available. Over the years, regulatory agencies have approved recombinant biologics from two human cell lines, HEK 293 cells (Xigris®) and HT-1080 cells (Dynepo®, Elaprase®, Replagal®, and Vpriv®). Unfortunately, Xigris and Dynepo have been withdrawn from the market due to product-driven safety concerns or market challenges leaving only the enzyme replacement therapies marketed by Shire. With Biogen Idec's recent BLA submissions for extended half-life Factor VIII and Factor IX produced in HEK 293 cells, there is the possibility that the portfolio of approved biologics produced from human cell lines is poised to expand. Although these established human cell lines have been successfully utilized for the production of biologics, efforts have also been applied to the de novo derivation of human cell lines specifically for the expression of recombinant therapeutics and vaccines. This has most notably been achieved through the immortalization of primary cells with the adenovirus oncogene E1A. The most mature of these efforts is the PER.C6 cell line originally developed by IntroGene from fetal retinal cells. This cell line has been used widely for the production of vaccines and more recently for the production of therapeutic proteins with several clinical programs underway [35]. The PER.C6 cell line gained attention when the DSM and Crucell joint venture Percivia announced in 2008 that they had achieved mAb titers exceeding 25 g/L using a modified perfusion system in which the mAb was retained and concentrated in the bioreactor. Newer to the scene is a human amniocyte-derived cell line called CAP, marketed by CEVEC, that was also immortalized with adenoviral genes [21]. However, at this time there is little publicly available information to assess the robustness and reliability of this cell line.

Beyond cultured cells, a discussion of mammalian hosts would not be complete without acknowledging the success, albeit limited, of expressing recombinant proteins in transgenically modified animals. Patients have access to Atryn®, a recombinant antithrombin medication expressed in the milk of transgenic goats first approved in 2006 by the EMA and then by the FDA in 2009. Although this pioneering work established the proof of principle that a human therapeutic produced from a transgenic animal could gain regulatory acceptance, the biopharma industry has been reluctant to adopt this platform. To date, the only other approved product on the market derived from a transgenic animal is Ruconest®, a recombinant C1 esterase inhibitor approved by the EMA in 2010. The failure of this novel expression system to gain more traction in the industry likely reflects the substantial productivity improvements made in the last five years using the traditional cell line based manufacturing that significantly deflated a major impetus to consider hitching commercial development of new therapeutics to an emerging technology.

The modest interest in developing therapeutic proteins in hosts other than CHO reflects the overall attractiveness of the entire CHO expression package. There is extensive media and process expertise at the industrial scale, making their use virtually "plug and play". Moreover, it has a well-established safety profile and is a known entity with regulatory agencies. Arguments have been made that producing a therapeutic glycoprotein in a human cell line would be advantageous from the perspective that the therapeutic would have "human" carbohydrates and therefore be potentially more efficacious and/or less immunogenic. However, in the instances where there have been direct comparisons, analytical methods detected differences in the protein expressed from the recombinant human cell line relative to the endogenous human form of the protein. Furthermore, there was no evidence that the recombinant human form of the protein was safer or more efficacious than the recombinant CHO-derived counterpart. In the case of recombinant erythropoietin (EPO), comparisons of CHO and HT-1080 expressed product showed that there are detectable differences in the sialic acid content of the molecule produced from the different hosts [62]. However, the recombinant EPO produced in the human cell line was also distinguished by isoelectric focusing from endogenous erythropoietin isolated from plasma and urine [50]. In the same vein, there was also no compelling data to suggest an advantage for a human host cell line when CHO-derived Fabrazyme® was compared to HT-1080-derived Replagal®. Although the sialic acid and mannose-6-phosphate content differed between the recombinant alpha galactosidase produced from the two host cell lines, biodistribution in a mouse model and antigenicity studies found the two molecules to be comparable [45].

This is not to say that the CHO host options are not without potential issues. It has been well established that CHO and other rodent cell lines are capable of generating glycan structures not seen in humans. A naturally occurring mutation in the CMAH gene prevents the formation of Neu5Gc, a hydroxylated form of sialic acid in humans, yet this glycan moiety has been detected on several biologics produced from murine cell lines, as well as CHO [56]. There is no compelling evidence to date that suggests the presence of Neu5Gc adversely affects the safety

or efficacy of therapeutics. Nonetheless, the presence of circulating antibodies in humans directed to this sugar raises potential concerns that there is an elevated risk of altered clearance and antidrug antibodies response to Neu5Gc-bearing therapeutics [26]. Similarly, the α-1,3-galactose linkage is also absent in humans but is known to be expressed in CHO and murine cell lines [5]. The presence of the xenoantigenic gal-α-gal linkage is of greater concern, as there is credible evidence that the α-gal linkage can have an adverse impact on the safety profile of a biologic therapeutic. For example, SP2/0 (murine)-derived Erbitux® has been shown to trigger anaphylaxis in a subset of patients due to pre-existing IgE antibodies directed against galactose-α-1,3-galactose sugar residue [13]. As a result of these findings, product quality screening of clones needs to be directed specifically to these glycan structures and will typically result in clones with acceptable productivity being discarded due to concerns around elevated levels of either one of these glycans. Given the well-known metabolic pathways responsible for generating these glycan moieties and the development of some of the new genome modifying technologies mentioned later in this chapter, this shortcoming of the CHO host can be readily addressed to create a modified host cell line that does not suffer from the potential limitation of producing protein compromised by detectable levels of Neu5Gc or α-1,3-galactose.

## 2 Which CHO is the "Right" CHO?

Historically, there have been three CHO hosts routinely used to develop biologics. Two of these, DUXB11 and DG44, were isolated in the Chasin laboratory at Columbia University, New York [69]. These cells had undergone extensive mutagenesis to generate lines that were deficient in dihydrofolate reductase (DHFR) activity and hence dependent upon an exogenous source of nucleotide precursors for growth. This represented a readily manipulated phenotype suitable to select for genome integration and stable expression of exogenous DNA. This is accomplished by transfecting the cells with expression cassettes for the gene of interest and a DHFR gene. Posttransfection, cells are placed in selection media lacking nucleotide precursors. Given the ease and effectiveness of this approach, these cell lines found widespread acceptance in the industry as the starting host to generate production cell lines. Their suitability for this role was further enhanced due to the ability to select for a high copy number of the introduced expression vector by adding methotrexate (MTX) to the cultures. As MTX is a competitive inhibitor of the DHFR enzyme, applying this additional selection pressure on top of the absence of nucleotide precursors enables the selection and isolation of the minor population of cells that have undergone a spontaneous amplification of the integrated expression vector containing the DHFR selectable marker and, in most cases, the gene of interest. The presence of multiple gene copies helps to ensure maximum productivity for any given molecule by driving an excess of recombinant mRNA for the therapeutic protein of interest.

The third CHO option that has been extensively used is the wild-type CHOK1 cell line, and its derivative CHOK1SV (developed by Lonza). These hosts are usually paired with the other prevalent selection system used in the industry. This method, known as glutamine synthetase (GS) selection, capitalizes on the fact that, absent an exogenous source of glutamine, cell survival is dependent on the GS enzyme to produce glutamine [2]. With host cell lines such as murine myeloma-derived NS/0 cells, which have low endogenous GS enzymatic activity, this affords a simple selection scheme when using a GS selectable marker in the expression vector and glutamine-free selection media. On the other hand, CHO cells tend to have higher endogenous GS activity, making glutamine-free selection less efficient. However, similar to the DHFR/MTX system, the GS competitive inhibitor methionine sulphoximine (MSX) can be added to the media to apply additional pressure and select for CHO cells that are driving high levels of expression from the integrated vector.

The confluence of two unrelated factors has altered the landscape as it relates to the relative attractiveness of the GS and DHFR selection systems. Until recently, the technology associated with the GS, but not the DHFR expression system, has been encumbered by intellectual property. However, much of that protection has now expired, opening up the GS selection system for use without the burden of royalties on commercial sales. The second salient factor is the advances made in efficient genome engineering tools such as zinc finger endonucleases [49], meganucleases [8], TALENs [12] and CRISPR [7]. With these tools readily available, it is now relatively straightforward to create targeted mutations. This capability has been exploited by Eli Lilly and Lonza to create GS-deficient CHO cell lines which enhances the stringency of selection, in turn resulting in a greater proportion of high-expressing clones [22]. With the GS targeting zinc finger endonuclease utilized by Eli Lilly now commercially available from SAFC and the past IP issues around the GS system no longer an impediment, this selection system may be poised to become the dominant tool in the industry. It has the distinct advantage over the DHFR system of not requiring gene amplification to achieve suitable expression which can shave weeks off the development timeline. In an industry facing ever-increasing pressures to get candidates to the clinic faster, a switch from a DHFR to a GS based expression system represents low hanging fruit to achieve this end.

The CHO GS knockout represents one highlight in over 20 years of engineering CHO cells to imbue them with new phenotypes that would not have been readily achievable through classical methods of media and process manipulations. Some of the early pioneering work in this area included improving expression from a heterologous CMV promoter through overexpression of the adenovirus E1A gene [15] and altering glycan structure of recombinantly expressed proteins by over-expressing the alpha 2,6 sialyltransferase gene [44]. More recently, fucosylation modulation has been a subject of intense interest. It has been established that an afucosyl glycan is desirable on a subset of mAbs (i.e., for those intended for some oncology indications) due to the enhancement of ADCC activity [63]. Unfortunately, CHO cells invariantly produce fucosylated glycans on recombinant mAbs

making it highly unlikely that, even with exhaustive screening, a recombinant CHO cell line producing a predominately afucosyl mAb could be isolated. This obstacle was initially overcome through the laborious task of classical gene targeting via homologous recombination in CHO cells to create a host in which both alleles of the FUT8 gene (the transferase responsible for adding fucose) were knocked out [76]. Since that time, a variety of other strategies has been employed to establish engineered CHO hosts capable of producing hypo or a fucosylated glycans. These include knocking out or knocking down the genes for other key enzymes in the fucosylation pathway [36, 68, 80] to overexpression of native or chimeric GnT-III glycosyltransferase to drive formation of glycan structures that are not suitable substrates for the FUT8 transferase [23] and overexpression of the prokaryotic enzyme GDP-6-deoxy-d-lyxo-4-hexulose reductase to divert a key intermediate in the de novo pathway for fucose biosynthesis [71].

The engineering strategies described above represent but the tip of the iceberg. There have been dozens of publications detailing other host cell engineering strategies in which antiapoptotic genes, chaperones, and components of the unfolded protein response or the secretory apparatus have been manipulated to achieve a desired phenotype (reviewed in [52]). Although many of these publications hint at potentially interesting avenues of intervention to develop superior hosts for expression of recombinant protein therapeutics, the vast majority of them fail to demonstrate utility in a cell culture system that is industrially relevant, instead relying on models that incorporate transient expression, serum-dependent cell lines and/or a scale no larger than a T-flask. If the minimum criteria to demonstrate industrial utility are considered to be stable cell lines grown in a benchtop bioreactor with serum-free media, only a small number of published studies cross this success threshold (Table 1). What can't be ruled out is the possibility that some of these engineering targets have been successfully implemented in a commercial setting without being published.

With the currently available tools for precision genome modifications, together with the advancing understanding of CHO metabolism and the long-awaited publication of the CHO genome [30, 75], the ability to engineer CHO cells is greater than ever. This should accelerate the trajectory of successful engineering outcomes. Even challenging metabolic pathways that are under multitiered levels of feedback regulation could become amenable to successful manipulation by exploiting miRNAs, a class of regulatory molecules that can simultaneously influence multiple cellular targets within a metabolic network. [34]. There is currently a significant amount of interest in attempting to leverage these regulatory RNAs to improve CHO-based expression systems. Over the next few years, it will be interesting to see if the hope and promise of this application are realized.

**Table 1** A compilation of published reports in which cellular engineering strategies were successfully applied in an industrially relevant setting to improve productivity or product quality

| Cellular target | Engineering approach | Outcome | Reference |
|---|---|---|---|
| HSP 27 and HSP 70 | Overexpression | IFN-$\gamma$ titers increased 150 % as a result of reduced apoptosis and extended culture duration | [46] |
| FAIM | Overexpression | IFN-$\gamma$ titers increased 75 % along with higher sialylation as a result of reduced apoptosis and extended culture duration | [74] |
| FADD | Overexpression (dominant negative) | IFN-$\gamma$ titers increased 25 % along with higher sialylation as a result of reduced apoptosis and extended culture duration | [74] |
| ALG-2 | siRNA | IFN-$\gamma$ titers increased 110 % along with higher sialylation as a result of reduced apoptosis and extended culture duration | [74] |
| Requiem | siRNA | IFN-$\gamma$ titers increased 150 % along with higher sialylation as a result of reduced apoptosis and extended culture duration | [74] |
| CIRP | Overexpression | IFN-$\gamma$ titers increased 40 % as a result of improved SPR | [67] |
| Neu3 | shRNA | IFN-$\gamma$ sialylation levels increased 33 % due to lower sialidase activity | [79] |
| LDH and PDHK | siRNA | mAb titer increased 125 % due to increased SPR | [81] |
| Aven and E1B-19 K | Overexpression | mAb titer increased 66 % due to increased IVC | [24] |
| TAUT and ALT1 | Overexpression | mAb titer increased 50 % due to increased SPR | [66] |
| FUT8 | Knock-out (homologous recombination) | mAb 100 % afucosyl, ADCC activity increased 100-fold | [37] |
| FUT8 and GMDS | siRNA (delivered in bioreactor) | mAb afucosyl levels increased from 0 to 63 % and ADCC increased 30 % | [68] |

## 3 Who Knows Best: The Cell or the Engineer?

When it comes to the basic engineering of the host cell to overexpress the protein of interest, the industry has traditionally relied upon random integration of transgenes into the host genome posttransfection. This is an inherently inefficient process whereby the majority of transfected cells yield unsatisfactory production levels. As such, finding the rare high-producing cell lines has been a considerable challenge for many years. Several groups have independently discovered different genetic elements capable of influencing the chromatin environment to promote a transcriptionally permissive state, and employed them as flanking DNA elements in vectors as a tool to achieve higher productivity [3, 43, 78]. Although somewhat

counterintuitive, another strategy to manage the low frequency of high-expression challenge is to cripple the resistance gene present in the expression cassette. This increases the stringency of the selection, and enriches for cells that are able to overcome the defective resistance gene, either through the integration of many copies of the vector or by integration of the transgene into a transcriptional hot spot. To achieve this end, different strategies have been employed to compromise the efficiency of translating the selectable marker, such as engineering the DHFR open reading frame to employ predominately low-abundance codons [73], and attenuating the start codon for the zeocin resistance gene by replacing the native ATG start codon with an alternative start codon such as TTG [70].

Another approach that is particularly useful for the expression of multigenic molecules such as monoclonal antibodies is splitting the DHFR coding sequence into two pieces, with the two DHFR gene fragments genetically linked to the heavy and light chain genes through an internal ribosome entry site (IRES). To reconstitute a functional DHFR enzyme, each fragment of the DHFR protein is fused to a leucine zipper dimerization motif [4]. This strategy ensures that only those cells effectively expressing both the heavy and light chains of the antibody survive selection. These tools have helped maintain an impressive trajectory of continuous improvement with regard to cell line productivity. Despite this success, there has been a parallel effort to try to revolutionize the gene integration process. This avenue uses a controlled gene integration process that seeks to minimize the randomness of gene insertion, and thereby predestine daughter clones for predictably high transgene expression. Establishing this type of system comes with its own set of challenges, most notably achieving productivity levels that can match or exceed those currently being obtained with traditional, random integration methods. However, the appeal of a cell line development process that affords more control and predictability than random integration is quite strong. There are two basic systems that have been described that can accomplish the goal of having greater control over the gene integration event. One is through the use of artificial chromosome expression (ACE) technology, which allows one to build the gene expression cassette outside the production cell line, yet within an autonomously replicating genomic structure [48].

ACE technology has been available for several years as a means for introducing exogenous genes into mammalian cells [48]. These large genetic elements are similar to bacterial plasmids in the sense that they serve as autonomous genetic elements capable of replication and faithful segregation within the cell. There is also the added advantage that the minigenome can be exquisitely tailored with specific elements, such as promoters, enhancers, insulators, and the like. Published work allowed us to compare the performance of cell lines generated using ACE versus cell lines generated using a standard random integration approach. A collaboration between the Canadian Institutes of Health Research, Centre for Drug Research and Development (Canada), and Pfizer showed that ACE technology was effective in generating CHO cell lines expressing a model monoclonal antibody [38]. The studies demonstrated that cell lines could be generated quickly and achieve respectable titers. Several cell lines were subsequently examined for their

performance in fed-batch production, as well as assessed for gene expression stability [17]. The authors concluded that the ACE cell lines were similar in productivity and stability to the platform standard being used (random integration). This demonstrated that the approach is certainly a viable method for generating cell lines. However, the amount of work required to set up and utilize the ACE system in-house is considerable. Multiple vectors are required in order to build the expression chromosome, which is done in a stepwise manner. A flow cytometer and skilled operator are required to isolate the chromosome, which adds to the cost of supporting this system. As such, it would seem difficult to justify this additional complexity for an expression system that is comparable to the current industry standard. However, the ACE system is being offered to clients by at least one vendor as an available option.

The second method is to engineer the host cell line with an acceptor site within the host genome that is a site for gene integration using site-specific recombinases [54, 61]. The advantages of site-specific integration are primarily the predictability such a system might afford and the potential to engineer a preoptimized integration site. The obvious utility here is to create cell lines that have predictably high levels of gene expression from the very start, eliminating the need for brute force cell line screening. There are two common tools that utilize essentially the same mechanism: Cre/Lox, based upon the Cre-recombinase, and Flp/FRT, based upon the eponymous "flippase." Both of these systems were adapted for use in mammalian systems not long after their discovery and initial characterization in microbial systems, and were subsequently adapted for use in bioprocess development [39, 53]. The basic approach is the same, regardless of the specific recombinase being used. The first step is to introduce, by random integration, a reporter gene preloaded into the acceptor site cassette. The resulting clones generated are screened for expression of the reporter (commonly a fluorescent protein), and the highest-producing clones are identified. Typically the desire is to have a single integration site, so the clonal cell lines are often screened for copy number. The end result is typically a small number of single-integrant cell lines that are theoretically capable of supporting high levels of transgene expression. The biotherapeutic protein of interest is then swapped into the acceptor site by the appropriate recombinase, and the reporter gene is excised. These systems have been explored numerous times through the years in an attempt to generate improved host cell lines [10, 32, 40, 54], and one such system is also commercially available from Life Technologies (Flp-In™).

A more direct approach has been enabled by recent advances in genomics and elegant new methods for gene manipulation. That is, similar to the approach described above, the starting point of this new method is to identify a hotspot for the landing pad integration site. Instead of relying on random integration events and clone screening for the reporter gene signal, the cells themselves provide information regarding the location of transcriptional hotspots through evaluation of the transcription profiles of CHO cells using gene expression microarrays [18], [77]. Even more comprehensive is the newer technique of simply sequencing every mRNA in the cell (RNA-Seq) as a means to characterize the transcriptome

[72]. Regardless of the method, the outcome is that the most highly expressed transcripts are identified. This information, coupled with the recent release of the CHO genome [30], (www.CHOgenome.org) could be used to pinpoint chromosomal locations that are naturally occurring transcriptional hotspots. One can introduce a gene acceptor cassette into one of these regions, with minimal disruption to the naturally encoded genes by the host cell, and thus create an engineered host cell line that utilizes pathways the cell is already using to maximize gene expression. Targeting specific regions in the genome of mammalian cells has been relatively commonplace in stem cell research [55]. Moreover, the same molecular techniques have been used in CHO cells for the purposes of gene knockout or mutation for many years (reviewed in [42]). However, no one has yet demonstrated the convergence of these approaches with the specific application for bioprocess development.

## 4 There is Many a Slip Twixt the Cup and the Lip

The promise of site-specific integration was to achieve an optimized host cell line that would be predestined for high transgene expression. However, there are mechanisms of gene expression control beyond transcription that affect the ultimate production and secretion of the protein from the cell. Translational control is known to occur at all levels of protein synthesis: initiation, elongation, and termination. The posttranslational modification and secretion of proteins is also a controlled process that can influence the productivity and quality of proteins being produced. For example, changes in the translational machinery could alter the productivity of a cell line, whereas alterations in the secretory pathway could affect both the quantity and quality of the protein produced. Similarly, epigenetics, which are heritable changes in gene expression that are not caused by changes in DNA sequence, is another mechanism by which cell lines may control their gene expression. Such changes are most commonly understood to be caused by methylation of the genomic DNA [57]. The result of DNA methylation is a localized suppression of transcription, and therefore silencing of gene expression. This is a heritable, though dynamic process, and can be influenced positively and negatively over time. Finally, recent studies have pointed increasingly to the role of microRNAs in gene expression regulation [14, 25]. The implication here is that even if an "optimal site" were identified, there are posttranscriptional and epigenetic effects that can affect the expression of an exogenous gene from this site, and these effects can change over time. There are some tools that can be employed to counteract some of these effects. For example, so-called "insulating elements," such as matrix attachment regions (MARs), that protect chromatin from being methylated can protect against some of the gene-silencing effects [27]. Indeed, Selexis has developed a method to exploit the mechanisms of MARs to maintain chromatin in an open state that appears to permit rapid successive transfections, and thereby gene integration, into the initial integration site [28].

In addition to these aforementioned challenges, there are several other reasons that likely contribute to the failure of targeted integration systems to outperform the standard approach of random integration. It could be that, despite all that we know about these systems, there is still much left to be discovered and it is simply not yet possible to design an optimal expression system from the ground up. It may be that the approaches taken thus far are somehow flawed or incompatible with cell culture platforms that have been optimized for random integration and the expression of specific proteins. For example, the reporter genes used may be the best way to identify hot spots for fluorescent protein expression, but a different site may be optimal for heavy-chain and light-chain expression. It may also be possible that site-specific integration host cell lines that are superior to the industry standard have been developed, but that have not been revealed to the public domain as of yet. Finally, it may be simply that the standard platform of random integration coupled with a sufficiently powerful screening program is, taken altogether, inherently better than site-specific integration for the expression of protein biotherapeutics.

Despite these limitations, there remain distinct advantages that site-specific integration can offer over the random integration platforms used today. Site-specific integration provides predictability of expression. For a well-characterized site-specific host cell line, one can assume that the productivity of the heterologous gene will be within a comparably very narrow range. That is to say that the host cell line will have been predetermined to contain an integration site that is stable, and therefore not prone to transcriptional silencing. As such, by design there should not be nonexpressing or very low expressing cell lines. This has the potential advantage of greatly simplifying the cell line selection process. That is, given the assumption that all clonal cell lines derived from the transfection event are genetically identical at the site of integration, there is no need for an extensive cell line screening program because there is no "needle in the haystack" to find. This has the added benefit of saving the time that would normally be devoted to multiple rounds of clone screening. This lack of genetic diversity could have unintended consequences, however, as there are situations where a needle in a haystack is precisely what is needed (such as proteins that have significant product quality challenges, for example). Finally, there is utility for the initial nonclonal pool itself following the initial transfection. If the selection system is set up such that only host cells that integrate the transgene grow up out of the population, this population, like the clonal cell lines, should have a predictable level of expression. In this scenario, relatively large amounts of the recombinant protein can be generated in a very short time, with a low risk of failure (which would exist for a pool that does not express well, for example). This approach could be used for making material to supply development work, toxicology studies, or, potentially, material for Phase I clinical studies. Indeed, this approach has been used by Regeneron, utilizing their EEYSR (Enhanced Expression and Stability Region) system [1, 60]. The time savings of this approach, compared to establishing clonal cell lines, is considerable. However, it is important to note that although Regeneron has embraced this approach to accelerate speed to clinic, they opt for developing cell lines via traditional methods to produce material for pivotal studies and ultimate commercial launch.

## 5 Finding the Needle

Until the day arrives when high-efficiency, high-productivity cell line development methods are widely adopted, effective productivity screening technologies will still be required to facilitate finding the needle in the haystack. One system that has gained widespread adoption throughout the industry due to the relatively modest up-front capital cost, ease of use, and effectiveness is the ClonePixFL instrument developed by Genetix [11]. This technology combines the growth of colonies in methylcellulose embedded with a fluorescently labeled antibody directed to the product being expressed. As the antibody/antigen complexes precipitate around the secreting colony, fluorescent halos are formed, with the size of the halo presumably representative of the productivity of the colony. To enhance the throughput of the screening, the instrument includes imaging capabilities, software, and robotics capable of screening tens of thousands of colonies and transferring the most promising colonies to 96-well plates. As with any technology platform, there is the need for some initial optimization; in this case, it involves optimizing media composition to enable existing media platforms typically focused on supporting high-density suspension growth to meet the new demand of enabling robust growth of colonies at low densities in semi-solid media. There is also the challenge of understanding the most effective way to utilize the data that are generated from the ClonePixFL platform. For example, the early protein expression data, as measured by the fluorescent halo around a colony, must be correlated with subsequent expression once the clonal cell lines have been adapted to suspension growth and scaled up into a more "manufacturing relevant" production platform, in order for this approach to be truly effective.

The breadth of clone screening can be enhanced severalfold relative to the ClonePixFL by capitalizing on the throughput of flow cytometry, or fluorescence activated cell sorting (FACS). This platform has been utilized for many years as an effective tool that several groups have utilized either as an alternative to the ClonePixFL technology or as an enrichment tool prior to employing the ClonePixFL. The challenge for flow-cytometry based methods has been the means of detecting the secreted product. One method used frequently in the development of cell lines relies on the transient association of the secreted product with the extracellular matrix as a means to measure how much each cell is producing [6]. Although effective in many instances, this approach does have some limitations. Proteins that are intrinsically "sticky" limit the effectiveness of the screen as they have the potential to remain bound to cells after being secreted. Furthermore, similar to the ClonePixFL method, detection of the product requires an antibody to the recombinant protein being expressed. There are many commercially available options available for detecting antibodies or Fc-fusion proteins, however, early-stage cell line development projects for other (non-Fc-containing) recombinant proteins could be hampered by the absence of available reagents that recognize the protein being expressed.

Other flow-cytometry-based methods have been developed that eliminate some of the drawbacks of the Brezinsky method. These rely on a surrogate reporter to serve as the readout for expression levels of the gene of interest. The reporter molecules are typically fluorescent proteins or cell surface proteins that can be readily detected with fluorescently labeled antibodies. In order for the reporter to be a meaningful barometer of therapeutic protein expression, its open reading frame typically needs to be genetically linked to the expression cassette used to express the protein of interest. The use of an internal ribosome entry site (IRES) is a common strategy to bridge therapeutic and reporter genes, ensuring that both are translated from the same mRNA [19]. More recently, a reporter system that places the small open reading frame (ORF) for the cell surface protein CD52 in the 5′ UTR of the genes encoding therapeutic proteins has been described [9]. As with the IRES system, both reporter and therapeutic genes are expressed from the same mRNA ensuring that reporter levels correlate with therapeutic expression. In this case though, rather than relying on a viral element to direct translation of the second ORF containing the reporter, the 5′ UTR embedded reporter ORF is the first to be encountered by the ribosome scanning the bicistronic mRNA. By engineering the reporter ORF to utilize an inefficiently translated alternate start codon, the system ensures that only a small percentage of ribosomes initiate translation of the reporter, with the majority of ribosomes continuing to scan until the optimal Kozak initiation sequence of the therapeutic is encountered.

One factor to bear in mind with the antibodies used in both the ClonePixFL and FACS-based screening methods is the potential TSE and virus exposure risk these reagents pose. For the ClonePixFL, there is a fully recombinant monoclonal detection reagent produced in CHO using no animal-derived media components which mitigates this risk. For those who feel that the original polyclonal detection reagent produces more robust halos, this reagent at least goes through in vitro viral testing and is certified to be produced from sheep herds that are monitored for disease. At the other end of the risk spectrum are the commercially available antibodies typically used in FACS-based methods which tend to be polyclonal in nature and have been developed with research applications in mind, rather than development. As such, these lack the basic testing and precautions applied to the polyclonal ClonePix reagent. In addition, the purification of these reagents, typically by affinity purification, likely entails exposure to nonrecombinant human and, in some cases, bovine and equine proteins. The potential for commercially available antibodies to be formulated in storage buffers containing BSA should also not be overlooked, although some vendors may provide custom formulations that are free of animal-derived components when specifically requested to do so.

A simple solution to avoid the potential TSE exposure while still capitalizing on the throughput of flow cytometry is to use fluorescent protein reporters that abrogate the need for a detection antibody [51, 65]. When expressing other proteins in addition to mAbs, this also represents an effective alternative if antibody reagents have not yet been developed at the time cell line generation is initiated. An interesting twist on this approach has recently been published by scientists at Cellca Gmbh. Although the method uses GFP as a reporter, it differs from other

approaches in that the reporter is not incorporated in the expression vector. Instead, this novel clone screening methodology capitalizes on the ER stress induced by the metabolic burden associated with high-level expression of a recombinant mAb [41]. By engineering a host cell line to express a GFP reporter under the control of a truncated promoter for the ER stress inducible gene GRP78, they have shown good correlation between reporter expression and cell line productivity. The success of these platforms, which enables the screening of thousands, if not millions, of clonal cell lines, creates another problem. How does one exploit the seeming advantages that these technologies bring when it can mean maintaining and analyzing a very large number of cell lines?

## 6 Necessity is the Mother of Invention

One answer to this question is the development of automated platforms for managing cells, and arguably more important, for analyzing the products these lines produce. Automation in bioprocess development is a relatively small and defined niche in the larger world of automation technologies and platforms. Automation has been incorporated in many areas of the pharmaceutical industry, from the beginning to the end of the process. In drug discovery, for example, many companies have developed large automated platforms for compound library screening. These systems feature vast compound libraries that are integrated into robotics systems for sample handling and computer-controlled inventories. These in turn are coupled with high-throughput analytical platforms that house relevant screening assays. These systems have revolutionized chemical compound screening for drug discovery. The system developed at Bristol-Myers Squibb, for example, increased the numbers of compounds that could be screened by 24-fold, while at the same time streamlining the process in order to realize a fivefold reduction in cycle times [31]. Within the area of biotechnology, automation has long been part of the manufacturing setting. A key example is automated feedback control for the production process, such as pH and dissolved $O_2$ control in bioreactors and fermenters. Beyond this, however, automation and automation platforms have been relatively slow to be incorporated into bioprocess development.

The major factors of integrating a successful automated platform technology in bioprocess development include affordability, flexibility, utility, and adaptability. First, budgets for bioprocess development tend to be included in the much larger budgets of either research or manufacturing, and therefore may not be considered a top priority. Second, systems need to be flexible enough that they can be used for more than one narrow purpose. If the automated platform overspecialized it may stifle platform improvements and be vulnerable to quick obsolescence. Third, the automation must be fit for purpose. There are many examples of high-quality, well-engineered automation that, rather than fitting into a platform or process flow, would require that the platform be significantly altered simply to make use of the automation instrument. Lastly, an automated platform needs to be adaptable. This

is captured in some of the above points, but it is worth calling out separately that an automated platform which can be adapted to a variety of uses by different fields stands a good chance of being widely utilized.

Many automated technologies and approaches have been tried in bioprocess development and met with very limited success, or failed outright. More commonly seen are those technologies that were developed for another target audience, but the developers saw a potential application in bioprocess development. An example of this is the suite of large-scale automated platforms for cell culture passaging and maintenance from TAP Biosystems (formerly The Automation Partnership, and recently acquired by Sartorius Stedim Biotech). These systems have been designed specifically for passaging cells in different types of cell culture vessels, such as T-flasks, shake flasks, or roller bottles. They have been successfully implemented in research organizations and some manufacturing settings, but they have not seen wide acceptance in bioprocess development platforms. These are well-engineered, but ultimately expensive and inflexible automated systems, thus falling short of the affordability and flexibility criteria. The more rare case is that of a technology that was specifically designed for bioprocess development, yet still failed to be successfully incorporated. The SimCell from Bioprocessors (now Seahorse) is an example of such an instrument that could be utilized for both clone screening and process development. The core technology of the SimCell device was a microbioreactor (0.6 mL) that was printed into cassettes of six bioreactors per card. Each "vessel" could be automatically controlled for dissolved $O_2$ and pH, while also affording online feeding and sampling. A collaboration between Seahorse and Pfizer demonstrated the potential utility of the system in a very large-scale (180 microbioreactors) DOE for process optimization [47]. In this experiment, a subset of conditions was compared to similar conditions run on benchtop bioreactors, and the performance was very comparable between the SimCell and the benchtop systems. Despite the success of the technology, the SimCell was not able to penetrate the bioprocess development market sufficiently to make it a viable long-term technology, and is now no longer available. The shortcomings of the SimCell system were both its expense and its limited ability to integrate the SimCell into an established bioprocess development platform. Rather, the platform would need to be built around it.

One of the most successful approaches in using automated platforms in bioprocess development is the liquid handling system. These systems meet all the criteria for success mentioned above. For one, they are relatively inexpensive. Second, they are flexible in that many of the platforms have a variety of functionality from variety in volumes they can handle to vacuum attachments for filter work to decks that can manage different temperatures and even shaking platforms for specialized incubations. They also are practical in that they can improve workplace efficiency through high-capacity sample processing and 24 h operations, as well as improved accuracy as compared to manual operations. Finally, they are adaptable in that they can be used across all aspects of bioprocess development, from cell culture to assay setup to resin screening. As an example, scientists at Biogen Idec have developed a high-throughput screening platform that

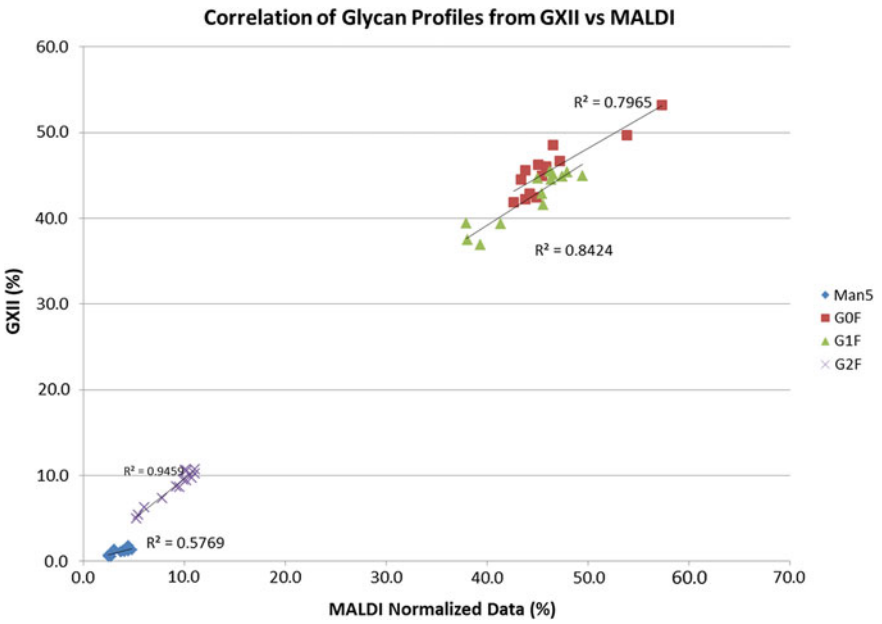


**Fig. 1** Correlation of glycan data. Several different clones were used to generate material for the same IgG1 monoclonal antibody. Purified antibody was analyzed using a high-throughput method developed for the GXII from Caliper and compared to the same samples analyzed using MALDI. The predominant species (Man5, G0F, G1F, and G2F) are shown for each cell line. Data and figure courtesy of Biogen Idec

can feed a variety of assays to support process development. The core of the system is a robotic liquid-handling platform that performs the initial Protein A purification of recombinant mAb from the culture supernatant, sets up a variety of assays in a 96-well plate format, and performs the incubations for various steps. Some assays required adaptation to the platform, but many were readily transferrable to that format. By using this platform for assay setup and execution, results for a large sample set for titer, sialic acid content, monomer/aggregate content by size exclusion chromatography, and glycan analysis were quickly generated [58]. Traditionally, most of these types of analyses (excluding titer) would not be considered for early clone screening campaigns because they are low throughput and time consuming. However, the assays were adapted to be accurate enough so as to be very comparable to the industry standards. For example, the glycan data that were generated in this high-throughput format correlate very well with data generated using MALDI-TOF mass spectrometry (Fig. 1). The data are not as high a resolution for the high-throughput method as for MALDI, but the correlation is more than adequate to identify large differences between clones, and the utility of being able to screen this many cell lines simultaneously more than offsets the reduction in data resolution. Similarly, automated liquid-handling systems have added a dimension to purification development that has dramatically

altered the scope and speed of establishing appropriate buffer conditions for purification steps. In a series of publications from the downstream purification group at Wyeth (now Pfizer), scientists described a revolution in conditional screening of resins that was completely enabled by scaled-down systems and automated liquid handling. As with the assay development work described above, the authors utilized a standard 96-well plate format coupled with a liquid handling system for plate and sample setup and incubation [16]. Using this platform, they developed a matrix screen for a variety of resins that was able to probe column conditions under an array of buffer conditions, varying in pH, ion, and ionic strength. This represented a tremendous improvement in terms of efficiency as compared to the traditional (at the time) bench-scale model column development.

In an elegant convergence of small-scale production and automated liquid handling, TAP Biosystems has created an extremely successful automated platform for bioprocess development in the ambr$^{TM}$ system. The ambr system utilizes a minibioreactor ($\sim$15 mL) coupled with a robust liquid-handling platform for adding or removing material to or from the culture vessels. The system is compact enough that it is meant to be operated in a biosafety cabinet and versatile enough that multiple experiments can be carried out simultaneously. One practical limitation is that the temperature control is managed by controlling the reactors in blocks of 12, rather than as individual units. The software controls are simple and do not require an experienced engineer to program the system, making the system accessible to a broader range of cell culture scientists. As such, the system has found use for production conditions, as a clone screening tool for evaluating multiple cell lines under production conditions, and as a means to passage a limited number of cell lines in an automated fashion. The system can also be coupled with analytical platforms to provide real-time cell culture performance data, such as cell density measurements. Attempting to capitalize on the success of the first-generation ambr, TAP Biosystems has released a larger-scale version, the ambr250, which has many of the same features of the ambr system, but is designed to address some of the major limitations of the original system. One is that the configuration of the ambr250 is designed to mirror a stirred-tank bioreactor more closely. The other addresses the volume limitation encountered with the original unit, which prevented sampling throughout the run to generate temporal assessments of product quality. It is a larger and more expensive system, and it remains to be seen if it will be as successful as its predecessor.

The incorporation of automation into process development has truly followed the familiar adage of "necessity is the mother of invention." Over the past decade process engineers have been faced with the demands of higher productivity and shorter timelines, while the industry as a whole has seen economic pressures that have forced them to find new efficiencies and streamline activities wherever possible. Therefore the industry has looked for solutions that are affordable, fill gaps and/or expand existing capability, can be broadly utilized, and have the potential to be modified as platforms continue to evolve. Many large, expensive niche items have not been broadly adopted, likely for these reasons. Rather, the adaptation and evolution of existing technologies, such as liquid-handling systems

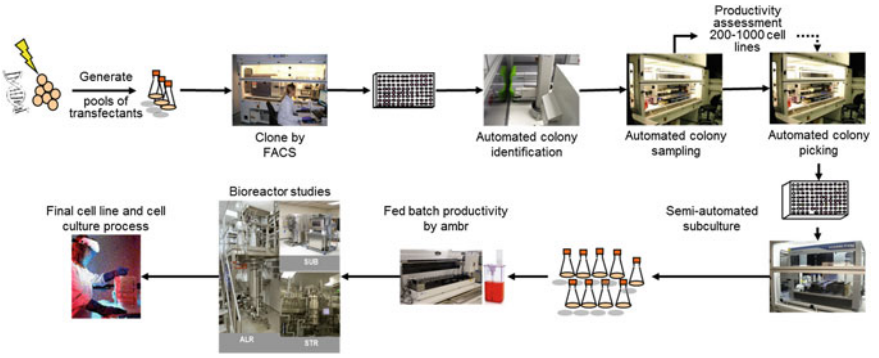


**Fig. 2** Islands of Automation. A process flow of cell culture development from transfection through to bioreactor assessment that incorporates discreet automated platforms linked together to maximize efficiency. Figure courtesy of Lonza Biologics plc

and simple robotics, have been the preferred strategy for incorporating new technologies into bioprocess development. A good example of this approach is the "Islands of Automation" developed by bioprocess engineers at Lonza (Fig. 2; [29]. The essence of the approach is to utilize automation specifically and discretely to where it can provide the most value, rather than trying to construct a single automated platform that does everything. The power of this approach is that it can be adapted to a variety of process development platforms with little difficulty and little disruption to an existing platform.

## 7 The Benefits of a Steady Platform

It is really this platform itself, in all its varied definitions, that has had the greatest impact on bioprocess development over this past decade. Successful platforms work by streamlining and simplifying development to a single process flow that is executed in the same way from program to program and product to product. Many aspects of "platformization" have been universally applied, such as serum-free processes (driven primarily by safety concerns), as well as adapting host cell lines to media that is consistent or compatible with the ultimate manufacturing process [64]. Some have taken this further, such as the "bioreactor evolved" CHO DG44 host cell line developed specifically to preselect cell lines already conditioned to the bioreactor environment [59]. The integration of a platform host and media expression system, coupled with effective scale-down models and analytical methods empowered by automation is the most effective approach to ensuring a satisfactory cell line development outcome. This type of integrated process can often result in the selection of lead clones that require little, if any, process development effort prior to the initiation of manufacturing runs to produce material for toxicology and clinical studies.

Bioprocess innovation, or platform improvements, has developed along two parallel paths. One path has been a consistent drive towards yield improvements, and has been followed virtually since the field of biotechnology began. This course of improvement has been essential to the success of biotechnology because it reduces the requirements for ever-increasing manufacturing capacity. Indeed, this so-called triumph of "biology over steel" helped to avert a capacity shortfall in the first decade of this century [20]. It does carry with it its own consequences, such as problems with an excess of capacity, but from a patient supply perspective, this is preferable to a market shortage. The leading technologies contributing to yield improvements these days can be found in the areas of cell line engineering and continued advancements in expression technology improvements. These technologies focus not only on improving transgene expression, but also on expressing more of the desired version of the molecule, for example, through manipulation of posttranslational machinery. The other path of bioprocess development, which has emerged more recently, is the path of greater efficiency. Some of the cell line engineering approaches, such as single-site integration, fall into this category, as do the expansion of automated platforms in recent years into all aspects of bioprocess development from cell culture to purification to analytics. Incorporation of these technologies into the bioprocessing workflow is a result of the combined pressures of budget limitations and expanding pipelines. This is the very definition of efficiency: do more with less. However, true to the innovative scientific nature of the people doing the work, not only were efficiencies realized and implemented, but improvements to the quality of the work were embedded into the processes. Process scientists have not been content simply to make processes more efficient, but rather have also strived to make processes better.

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Adv Biochem Eng Biotechnol (2014) 139: 35–68
DOI: 10.1007/10_2013_252

Published Online: 24 October 2013

# Cell Culture Process Operations for Recombinant Protein Production

**Susan Abu-Absi, Sen Xu, Hugh Graham, Nimish Dalal, Marcus Boyer and Kedar Dave**

**Abstract** The market for protein therapeutics has grown significantly over the past two decades and the pace of development continues to increase. It is a challenge to the industry to maintain the desired quality attributes while accelerating delivery to patients, reducing the cost of goods, and providing production flexibility. Efficient manufacturing scale production of protein therapeutics is required to continue to meet the needs of the patients and stockholders. This chapter describes batch, fed-batch, and perfusion processes and their utilization in the production of monoclonal antibodies and other therapeutic proteins. In addition, we have provided detailed discussions of the ongoing challenges of lactate metabolism and the future prospects of process monitoring and control.

**Keywords** Batch · Cell culture · Continuous processing · Data analysis · Fed-batch · Lactate metabolism · Perfusion

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## 1 Introduction

A typical cell culture manufacturing process is shown in Fig. 1. Manufacturing is initiated by the revival of cryopreserved cells. Cells are cryopreserved in small-volume vials (~2 mL) and, more recently, larger-volume vials (~5 mL) and high-volume cell bags [1, 2]. In traditional processes, the cells are thawed into small T-flasks, shake flasks, or spinner flasks and expanded in increasing numbers and sizes of flasks to achieve inoculation of a seed bioreactor. Large-volume cryovials and cryobags enable inoculation directly into small seed bioreactors (2–15 L) and eliminate the need for multiple passages in flasks. In addition to flasks, single-use technologies such as Wave™ bioreactors are routinely used during the inoculum expansion stages of cell culture processes [3]. Following the inoculum expansion and seed bioreactor steps, the cells are inoculated into the production bioreactor. During the production bioreactor step, the therapeutic protein is expressed by the cells. The primary recovery steps function to remove cells and cell debris and prepare the unprocessed bulk for purification.

Many types of production bioreactor formats have been developed and investigated over the years, particularly in academia. However, batch, fed-batch, and perfusion culture are currently the dominant modes of operation for commercial mammalian cell culture based processes. The goal of this chapter is to introduce the concepts of the most common forms of process operations. In addition, we have provided detailed discussions of the ongoing challenge of lactate metabolism and the future prospects of process monitoring and control.

## 2 Batch Culture

The batch mode of operation is a closed culture system in which a fixed amount of nutrients is added at the beginning of the culture. No additional nutrients are added, or fed, during the production phase. The growth curve of a batch process includes four distinct phases: lag phase, exponential growth phase, stationary phase, and death phase (Fig. 2). The growth rate varies during the culture cycle. The lag phase is a period of cell adaptation to a new environment, characterized by

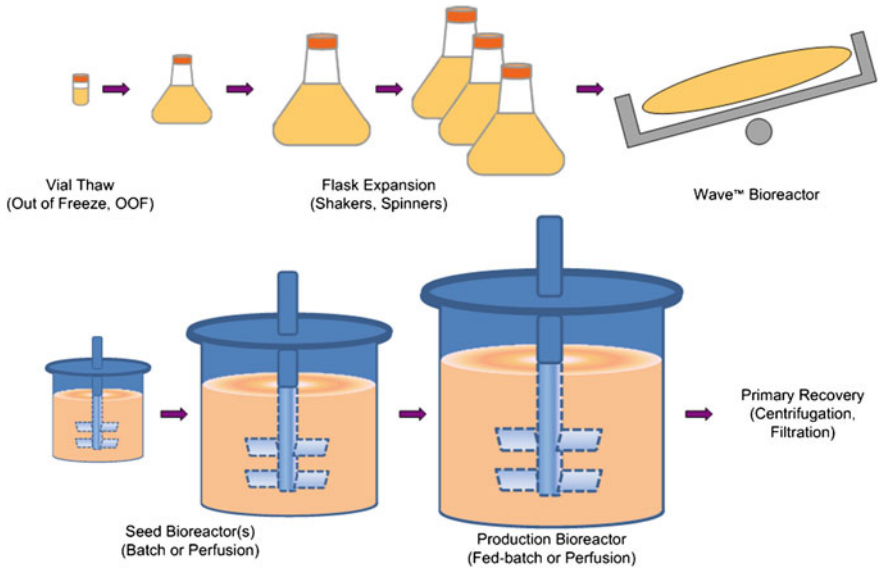


**Fig. 1** Stages of a cell culture manufacturing process. Cells are thawed from a cryovial (or cell bag) and expanded in increasing sizes of flasks. Inoculum expansion often also includes disposable bioreactors, such as Wave™. Cells are then inoculated into seed bioreactors to generate volume for the production bioreactor step. Following production of the therapeutic protein, the contents of the bioreactor are harvested via, for example, centrifugation and filtration. Differences between batch, fed-batch, and perfusion culture modes are described in subsequent sections

minimal increase in cell density. The duration of the lag phase is dependent on the seed culture used to initiate the batch process. During the exponential growth phase, the cells have adapted to their new environment and divide at a constant rate, resulting in a rapid increase in cell density. The growth rate during the exponential phase is often limited by nutrient availability. The third major phase is the stationary phase, where cells divide and die at a constant rate. Nutrient limitation, accumulation of metabolic by-products, and stress caused by high concentration of the recombinant protein are key characteristics. The final phase is the death phase, where the rate of death is greater than the rate of cell division.

Batch operation can be described by the following equations for cell accumulation, substrate (nutrient) depletion, and product accumulation:

$$\frac{dX}{dt} = (\mu - k_d)X$$

$$\frac{dS}{dt} = -q_s X$$

$$\frac{dP}{dt} = q_P X$$

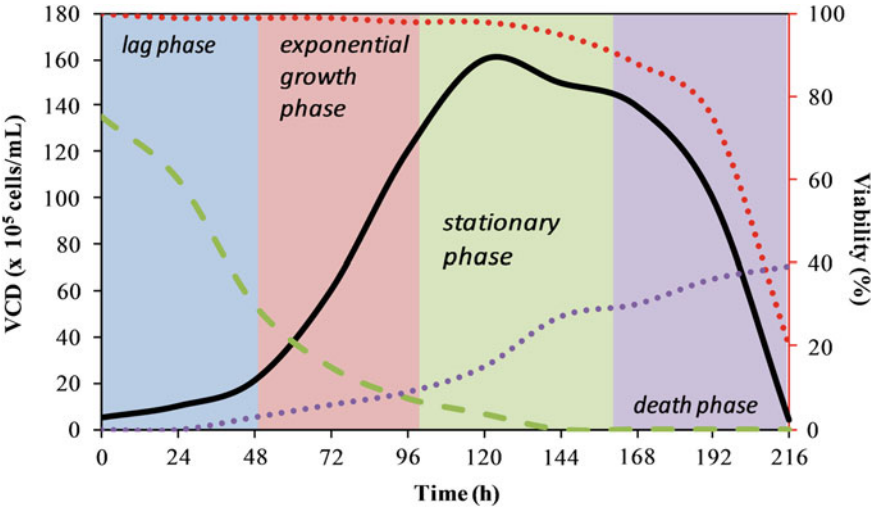


**Fig. 2** Mammalian cell growth in batch culture. *Black solid line* represents viable cell density (VCD); *red dotted line* represents cell viability; *green dashed line* represents glucose concentration in the culture; *purple small dotted line* represents product accumulation

where:

$X$ is the viable cell concentration ($10^6$ cells/mL)
$S$ is the substrate concentration (e.g., glucose; g/mL)
$P$ is the product concentration (g/mL)
$\mu$ is the specific growth rate ($day^{-1}$)
$k_d$ is the specific death rate ($day^{-1}$)
$q_s$ is the specific substrate consumption rate ($g/10^6$ cells/day)
$q_P$ is the specific production rate ($g/10^6$ cells/day)
$t$ is time (day).

As discussed in Sect. 1, inoculum propagation in animal cell culture is typically accomplished using a series of batch cultures with increasing cultivation volume. When sufficient cell numbers are achieved, the production bioreactor is inoculated from the contents of the seed bioreactor. The maximum cell concentration achieved in these batch cultures limits the split ratio and may require the use of several bioreactors in the seed train. Batch cultivation is not prevalent for use as the production bioreactor due to the limitations in achievable cell numbers and product titer.

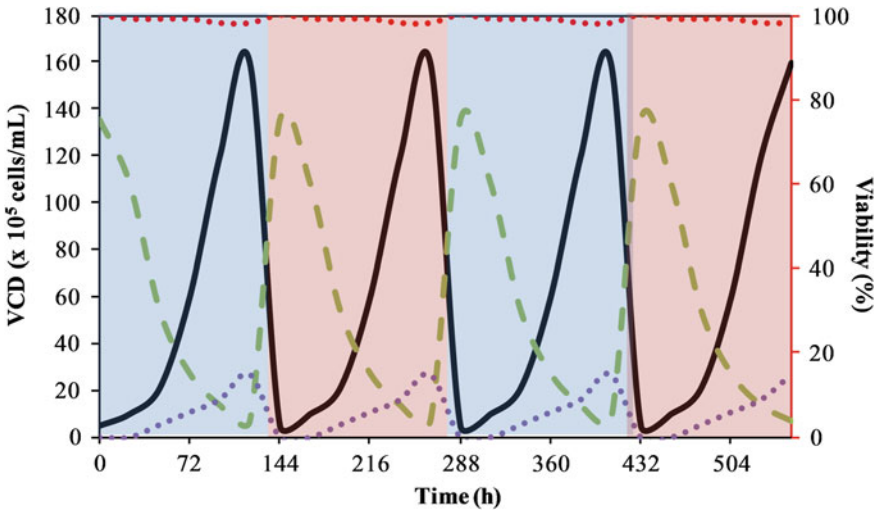


**Fig. 3** Mammalian cell growth in repeated batch mode. *Black solid line* represents viable cell density (VCD); *red dotted line* represents cell viability; *green dashed line* represents glucose concentration in the culture; *purple small dotted line* represents product accumulation

## 3 Repeated Batch Culture

Repeated batch, or intermittent harvest, processes are very similar to previously described batch processes. Cells grow in batch mode until nutrients approach levels that no longer support exponential growth and the culture is entering the stationary phase (Fig. 3). At that time, a portion of the cell culture is harvested along with the protein of interest, and the removed volume is replaced with new medium. This process can be repeated many times to generate product from each harvest [4].

Labile proteins, such as recombinant Factor VIII, that require short residence times at culture temperatures can be produced in repeated batch [5]. The product is harvested from the bioreactor after only 3–5 days instead of the weeks required for fed-batch. Repeated batch can also be utilized for microcarrier cultures. At the end of one batch culture period the medium is exchanged with fresh medium. In addition, cells can be detached and reattached to the microcarriers, if required.

For some cell types, especially stem cells, significant cell aggregation occurs in the stirred tank bioreactor culture. The cell aggregation may provide beneficial effects in terms of cell differentiation and productivity [6]. However, large aggregates can result in cell death and necrosis due to oxygen and nutrient limitations in the center of the aggregate [7]. Repeated batch operation can be used to control aggregate diameter for cell types that tend to aggregate [8–10]. The settling time and percentage of medium exchanged can be adjusted to select for the size range of aggregates that are optimal for the culture. Also, aggregate size may be

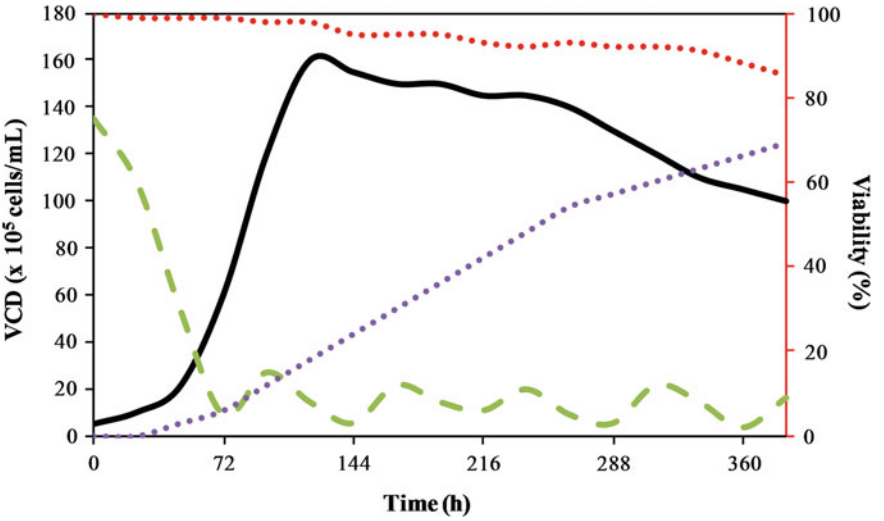


**Fig. 4** Fed-batch cell culture with substrate limitation. *Black solid line* represents viable cell density (VCD); *red dotted line* represents cell viability; *green dashed line* represents glucose concentration in the culture; *purple small dotted line* represents product accumulation. An initial batch phase leads to consumption of nutrients in the basal medium, after which a continuous feed (or multiple small bolus feeds) of concentrated medium is initiated

controlled by changing the liquid shear field (agitation rate) of the bioreactor in batch cultures [11] and repeated batch cultures [12].

Repeated batch is often used during the inoculum expansion steps of a manufacturing process allowing for multiple batches from a single thaw of cryopreserved cells. In the case of repeated batch during inoculum expansion, the product is not harvested. Cells may be transferred to another bioreactor or discarded prior to reinitiating the batch culture.

## 4 Fed-Batch Culture

For fed-batch/extended fed-batch cell culture operation, growth-supporting nutrients are added during the cell culture process to improve cell growth and productivity. This mode of operation has the same four distinct phases: lag phase, exponential growth phase, stationary phase, and death phase as a batch culture (Fig. 4). As nutrients are depleted, a feed solution is added to the cell culture. The feed solution is a concentrated solution of amino acids and vitamins with trace elements to support the cell culture while avoiding substantial dilution of the bioreactor contents. The addition rate of the feed can be used to modulate the growth rate of the culture and may help avoid or reduce unwanted glycolytic

pathway overflow metabolism effects [13, 14], such as lactic acid accumulation. The culture is ideally harvested prior to significant decline in culture health.

During fed-batch operation, the volume present in the bioreactor increases due to the addition of the feed medium. Fed-batch operation can be described by the following equations:

$$D = \frac{F}{V}$$
$$\frac{dX}{dt} = (\mu - k_d - D)X$$
$$\frac{dS}{dt} = D(S_M - S) - q_s X$$
$$\frac{dP}{dt} = q_P X - DP$$

where:

$D$ is the dilution rate (day$^{-1}$)
$F$ is the feed rate (liter/day)
$V$ is the culture volume (liter)
$X$ is the viable cell concentration ($10^6$ cells/mL)
$S$ is the substrate concentration (e.g., glucose; g/mL)
$S_M$ is the substrate concentration in the feed medium (g/mL)
$P$ is the product concentration (g/mL)
$\mu$ is the specific growth rate (day$^{-1}$)
$k_d$ is the specific death rate (day$^{-1}$)
$q_s$ is the specific substrate consumption rate (g/$10^6$ cells/day)
$q_P$ is the specific production rate (g/$10^6$ cells/day)
$t$ is time (day).

Fed-batch culture has been a common choice for large-scale production due to its operational simplicity and the preference to utilize established systems and facilities. However, in the absence of existing infrastructure, the fed-batch mode of operation may involve high start-up costs, resulting from the need for relatively large bioreactor plant capacity and associated large processing equipment [15]. As cell line and medium development technologies have led to increased productivity, the bioreactor capacity needed for commercial supply has been reduced [16]. This has led to an excess in production capacity, which was once expected to be in short supply. In some cases, high titers have resulted in purification bottlenecks, and manufacturers are decreasing the scale of the production bioreactors to compensate. The reduction in production bioreactor size coupled with the simplicity of the fed-batch approach is giving rise to the use of disposable bioreactors [17, 18]. More information on disposable bioreactors is included elsewhere in this volume.

Fed-batch culture equipment has lower direct costs than perfusion [15]. Process characterization and validation are relatively straightforward for fed-batch cultures.

In addition, cell expansion from working cell banks through the production bioreactor can be achieved with relatively fewer population doublings. As discussed in Sect. 5, perfusion cultures require the use of stable cell lines, often to 100 population doublings or more. The simplicity of fed-batch can result in a high degree of lot-to-lot consistency relative to perfusion culture, in which product quality attributes may shift as the cells age from the beginning of the cultivation period to the end. Downstream operations have been typically designed to accept batches of harvest from fed-batch bioreactors. In addition, due to the familiarity of fed-batch processes across the industry and in the health authorities, the approval time for fed-batch processes can be reduced compared to perfusion [19].

A simple feed medium is formulated by concentrating the basal medium (non-nutrient salts are not typically concentrated). However, optimization of a feed medium composition and associated feeding strategies requires consideration of nutrient consumption, by-product accumulation, timing and duration of feed, and the optimization of growth and productivity conditions [20–23]. Medium development can be labor-intensive and may require long timelines. Third-party vendors can be utilized for media development expertise with the addition of outsourcing cost. High-throughput, scaled-down cell culture systems or model processes in combination with a statistical design of experiment (DOE) approach can be used to optimize the development process [24]. More information on media development is included elsewhere in this volume.

Stepwise bolus addition of the feed solution to the production bioreactor is often selected for industrial processes as it is simple and scalable. A shortcoming to these types of feeding strategies is that they are not adjusted to compensate for variations in growth and nutrient requirements. Studies have shown that by-products such as lactate and ammonia can be minimized by maintaining low glucose and glutamine concentrations through frequent or continuous feeding [25]. Feeding strategies that take into consideration the real-time state of the cell culture through feeding algorithms have been demonstrated to reduce by-product accumulation [4, 26]. These types of feeding strategies have focused on reducing by-product accumulation through control of glucose and/or glutamine [27–31]. Feeding strategies whereby oxygen uptake rate (OUR) measurement is used to estimate the glucose consumption rate and determine the amount of feed medium required to maintain a low glucose level have also been demonstrated [23, 32]. Continuous feeding strategies are less common in manufacturing, mainly due to complexity of operations and validation [33, 34]. More information on feeding strategies is included elsewhere in this volume.

## *4.1 Lactate Metabolism in Fed-Batch Culture*

Although fed-batch operation is widely used in the industry, it is not without its challenges. Low lactate-producing cell culture processes are preferred for industrial applications because high lactate concentration can be detrimental to cell

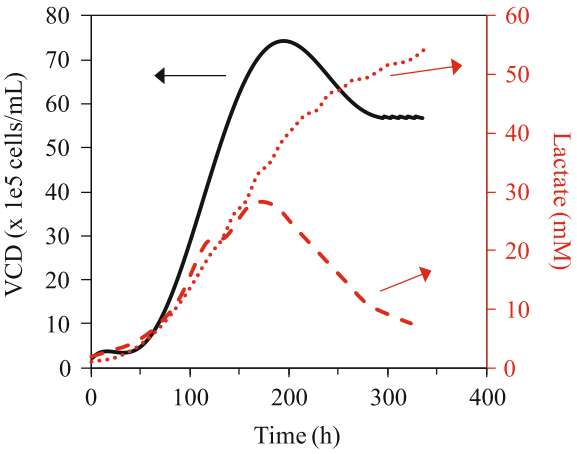


**Fig. 5** Mammalian cell growth and lactate metabolism in fed-batch mode. *Solid black line* represents viable cell density (VCD); *dotted red line* represents a scenario with only lactate production; *dashed red line* represents a scenario with lactate production in exponential growth phase and consumption in stationary phase

growth and productivity [35–37]. Prior to development of the manufacturing process, it is advisable to choose a low lactate-producing clone. Implementation of a process engineering solution to reduce lactate production at a later stage in process development can be more challenging.

Between 50 and 100 % of glucose is converted to lactate under aerobic conditions in most cases, which resembles the classical Warburg effect [38]. Lactate is rapidly produced and accumulated during the exponential growth phase due to the large energy requirement of the cells. Ideally, lactate is consumed to supply energy for cell maintenance and protein production in the stationary phase. Figure 5 shows two examples of lactate metabolism in fed-batch culture. Lactate metabolic shift is a significant feature in mammalian cell cultures and it is of practical benefit for extending cell longevity and improving protein production.

## *4.2 Media and Feeding Strategy Optimization*

Media optimization is one strategy to improve glucose utilization. By substituting glucose with slowly metabolized nutrients, such as galactose and mannose, one can significantly reduce lactate accumulation and induce lactate consumption by CHO cell cultures [39–41]. The ideal substitutes are those that enable sufficient cell growth yet prevent overflow metabolism to lactate, thus achieving the goal of more efficient media usage and less lactate accumulation. A few small-scale studies have shown a remarkable reduction in lactate production, and even induction of lactate consumption in CHO and NS0 cultures, by switching from hydrolysate-containing feed to chemically defined feed media [42, 43]. In addition, optimization of amino acids in basal and feed media using metabolic flux analysis led to a decreased lactate production rate in a small-scale CHO fed-batch process [44]. Similarly, basal and feed media optimization yielded a very high lactate consumption rate and low final lactate concentrations in five non-GS NS0 cell lines compared with a

previous process that used less-optimized media [45]. These results demonstrate that media optimization is an effective way to control lactate formation.

The addition of copper has been shown to promote cell growth and reduce lactate production in some cell lines. A 60 % reduction in lactate accumulation was demonstrated using a basal medium containing 5 μM $CuSO_4$ in fed-batch CHO culture [46]. Down-regulation of the transferrin receptor and LDH, and up-regulation of a cytochrome P450 family-1 polypeptide were identified and confirmed using CHO microarrays and Western blotting. Significant improvement in lactate metabolism was observed in large-scale manufacturing by adding a combination of metals to the basal medium. The same impact of copper on another CHO cell line at small-scale was recently reported [13]. A comparative metabolite analysis suggested that lactate-producing cultures have impaired mitochondrial and energy metabolism, and respiratory impairment was determined to be a key mechanism by which copper affects lactate metabolism. Phosphate was found to be important in GS-NS0 cell cultures. Phosphate feeding resulted in a twofold increase in viable cell density, a 1.7 times higher specific glucose consumption rate (qGlc), a 1.8 times higher specific lactate production rate (qLac) in the exponential growth phase, and a delayed cell metabolism shift from lactate production to consumption in a small-scale GS-NS0 fed-batch process [47].

Limiting the glucose level in cell culture by optimizing the feeding strategy can also be beneficial for controlling lactate production [23, 48]. Data show that cells grown under low glucose concentration produce less lactate than cells grown under high glucose concentration. A material balance study of hybridoma culture showed that 81 % of glucose was converted into lactate when glucose was in excess, and the conversion dropped to 52 % when glucose was limited [49]. Dynamic online feeding strategies for a CHO fed-batch culture using either glutamine or glucose as setpoints showed that maintaining glutamine (0.1 mM) or glucose (0.35 mM) at low levels could significantly reduce lactate production and trigger its consumption [20]. A glucose feeding strategy based on pH was developed to control lactate accumulation in a CHO fed-batch process at different scales [50]. The strategy was based on the premise that cells start consuming lactate when glucose is lower than 1 mM. A nutrient feeding was triggered by rising pH due to lactate consumption. Lactate thus could be suppressed at low levels (<34 mM) even for clones with high lactate production in a conventional fed-batch process (45–140 mM). The feeding strategy was successfully scaled up to 2,500 L and significant improvement in the production of multiple proteins was demonstrated. When complete lactate consumption is achieved, $pCO_2$ levels will rise, because $CO_2$ is sparged for pH control. For cell lines that have significant lactate consumption, feeding lactate for pH control can provide process benefits of ammonium and $pCO_2$ reduction [51].

Metabolic analysis is an effective tool to improve understanding of lactate metabolism with changing media components. In a recent study, it was shown that the metabolic flux distribution changes mostly in reactions involving pyruvate metabolism [52]. Correlation between reduced oxidative metabolism and high lactate production was also shown experimentally [53]. It has been suggested that inhibition of the flux through glycolysis in the stationary phase may be necessary

to trigger lactate consumption [34, 54]. The observed lactate consumption when glucose and galactose are used in combination as carbon sources could be explained by the glycolysis flux: cells are not able to provide enough pyruvate (glycolysis flux limited) to supply energy metabolism at the slow rate of galactose consumption, and lactate is used for pyruvate synthesis to feed the TCA cycle. In such cases, the influx of lactate is "forced" for sufficient energy metabolism. Recent findings show that the metabolic shift is not only limited to pyruvate–lactate conversion, but also involves a few other amino acids/intermediates, such as alanine, isocitrate, and succinate [13, 43].

## *4.3 Modulation of Cell Culture Parameters*

A few cell culture parameters (such as pH, temperature, and dissolved oxygen [DO]) greatly affect cell growth kinetics, antibody production rate, and cell metabolism. For example, a sharp decrease of lactate production rate in CHO and hybridoma cultures, along with decreases in other metabolites, was observed at a cultivation temperature of 32.5 °C compared to 37 °C [55]. When temperature is increased, more glucose is routed to lactate production. A wide range of pH was found to affect lactate formation greatly for different cell lines. Increases of qGlc and qLac were seen as the cultivation pH increased in the range of 6.85–7.8 in a CHO batch process [55]. Similar pH-dependent lactate metabolism was observed in a GS–CHO fed-batch process with a pH range of 6.6–7.2 and a CHO fed-batch process with a pH range of 6.8–7.4 [28, 56]. Because lactate formation is an anaerobic process, a very low DO level naturally promotes lactate production, and a high DO level is expected to reduce lactate formation. However, a very high lactate production rate in the exponential growth phase is commonly observed, even at high DO. An early study showed that lactate production significantly increased at low DO (0–10 %), and a change of DO within 10–100 % only produced a minor lactate production difference for a hybridoma cell line [57]. A separate study showed that DO must be maintained below 5 % for observable differences in lactate production rate in a CHO cell line producing t-PA [58]. The impact of DO levels typically used in industrial cell lines (30–60 %) is marginal. However, it is possible that the presence of DO gradients at large scale may result in localized regions of low DO, leading to an increase in lactate.

## *4.4 Cell Engineering-Based Approaches*

Genetic manipulations of the metabolic pathways have been heavily investigated for reducing lactate formation while increasing protein production [59]. Large-scale gene profiling of a hybridoma cell line showed that metabolic shift was affected by both biochemical reaction rate and gene expression level changes [33].

Specific targets that facilitate lactate production are frequently evaluated for this purpose, such as lactate dehydrogenase A (LDH-A), which catalyzes the conversion of pyruvate into lactate. Down-regulation of LDH-A has been a common strategy used for suppressing lactate production in CHO and hybridoma cultures, with a range of 21–79 % reduction of qLac reported in the literature [60–62]. Pyruvate is converted to acetyl-CoA by pyruvate dehydrogenases (PDHs) to enter the TCA cycle, and the activity of PDH is inhibited when phosphorylated by pyruvate dehydrogenase kinases (PDHKs). In a recent study, down-regulation of both LDH-A and PDHKs led to a 90 % reduction of lactate production in a fed-batch shake flask process and was further confirmed in small-scale bioreactors [63]. It was also reported that overexpressing multiple antiapoptotic genes, E1B-19 K and Aven (EA167), could alter the lactate metabolism of CHO cells [64].

Up-regulation of certain genes to channel more pyruvate into the TCA cycle has also been explored. Pyruvate carboxylase (PC) is a key enzyme that catalyzes pyruvate carboxylation to form oxaloacetate to enter the TCA cycle, and it typically has low activity. Expression of yeast PC in CHO cells was demonstrated to increase glucose flux into the TCA cycle and reduce lactate formation, as well as increase protein production [65], which is consistent with the observations in a BHK-21 cell line [66]. By expressing human PC, the same lactate reduction effect was observed in both adherent and suspension fed-batch CHO cultures [67]. Overexpression of antisense LDH-A and glycerol-3-phosphate dehydrogenase (GPDH) has also been found to be effective in reducing lactate production [68].

## *4.5 Scale-Up*

It is desirable to realize the lactate metabolic shift in mammalian cell culture manufacturing processes. The shift from lactate production to lactate consumption provides energy for cell maintenance/production, reduces toxic levels of waste products, and maintains lower osmolality. Small-scale bioreactor systems are used to develop fed-batch processes for the production of protein therapeutics. These small-scale systems ($\leq$20 L) do not exactly mimic the conditions of full-scale bioreactors ($\geq$1,000 L). Large-scale bioreactor systems are more challenging to mix, and they accumulate dissolved carbon dioxide to a greater extent than small-scale systems. In addition, lactate metabolism often becomes a challenge as a process is scaled up.

Large-scale data from the literature are relatively sparse compared with those of small scale. However, available data provide evidence that the lactate production rate often increases at large scale and the metabolic shift to lactate consumption is reduced or absent compared to the same process run at small scale. For example, much higher cell density was achieved in a 3-L bioreactor than that in a 2,500-L SP2/0 fed-batch process, with the same level of lactate produced at the two scales [69]. A recent report comparing an NS0 fed-batch process in a 15,000-L SS tank, a 200-L SS tank, and a 250-L SUB tank showed normalized peak lactate levels of 1:0.31:0.77, with the 15,000-L SS tank producing the highest lactate level, even

though cell density trends were quite similar between different scales [70]. In another NS0 fed-batch process, the final lactate level in a 10,000-L bioreactor was much higher than that in 2-L and 600-L bioreactors [71]. Notably, a divergence of lactate consumption rates was observed when scaling up from 2 L to 100 L in multiple non-GS NS0 cell lines [45]. An inhibitory lactate level (62.5 mM) was also reported in a 5,000-L CHO fed-batch process and a ~20 % lower lactate level was obtained in a 50-L bioreactor [37]. Large variation in process performance (including lactate) frequently exists in production runs, such as the trends observed in a 12,000-L CHO fed-batch process with over 200 runs [72].

It has been demonstrated that high osmolality leads to a high specific lactate production rate in CHO cell lines. A 50 % increase of qLac was observed when osmolality increased from 320 mOsm/kg to 440 mOsm/kg, and a 35 % increase of qLac was observed when osmolality increased from 350 mOsm/kg to 490 mOsm/kg in a serum-free CHO medium [73, 74]. Similarly, more glucose was channeled to lactate at higher osmolality (381 versus 276 mOsm/kg) in a GS-NS0 fed-batch process [75]. Even though the mechanism of osmolality impact on lactate metabolism is not fully understood, controlling osmolality at large scale may be important in reproducing the lactate metabolic shift observed at small scale.

Controlling $pCO_2$ is an important factor for controlling osmolality. High $pCO_2$ results in more base addition and elevated osmolality. It is well known that many large-scale bioreactor processes are plagued by high $pCO_2$ accumulation due to poor $CO_2$ stripping, either due to limitations in the engineering design of the bioreactor system, or from conservative operation due to concerns of the shear sensitivity of mammalian cells. High $pCO_2$ accumulation leads to more base addition for pH control (typically a few fold more than that at small scale), and partially inhibits glucose oxidation. In one particular case, increase of $pCO_2$ was shown to directly increase lactate production in a CHO perfusion culture [76]. The effect of high $pCO_2$ can thus contribute to both high lactate production and high osmolality in large scale. In return, high osmolality can also promote more lactate formation. The relationships among $pCO_2$, lactate, and osmolality are illustrated in Fig. 6. This cycle can be broken by reducing $pCO_2$. A higher air sparge rate can reduce $pCO_2$ by increasing $k_La$ and $CO_2$ stripping. A 50 % reduction of lactate concentration was observed when the air sparge rate was increased from ~1 LPM to ~2.5 LPM in a 2,000-L GS-NS0 fed-batch process [77].

Lactate accumulation is still a challenge for industrial fed-batch processes, even though many mitigation strategies have been investigated. The often-elusive lactate metabolic shift is not yet fully understood, and continues to be investigated.

## 5 Perfusion Cell Culture

In perfusion cell culture, a cell accumulation period is followed by a potentially long steady-state operation (Fig. 7). During these two phases, fresh growth medium is added to the bioreactor and spent medium or perfusate, typically containing

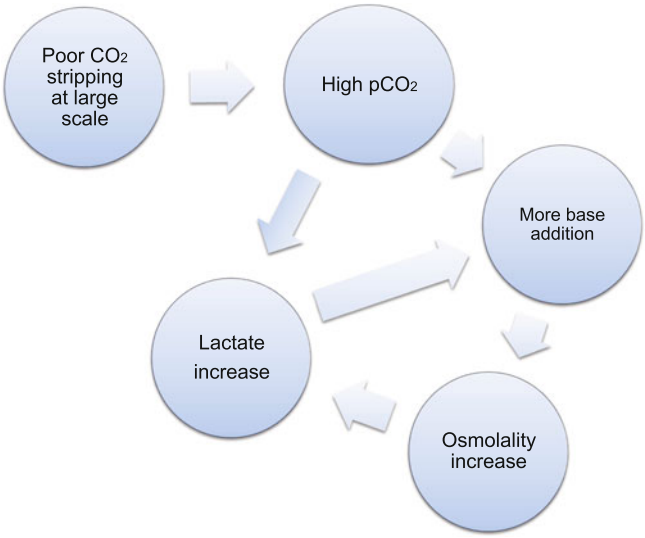


**Fig. 6** Relationships among $pCO_2$, lactate, and osmolality. Poor $CO_2$ stripping leads to high $pCO_2$ levels that result in base addition for pH control. Base addition results in an increase in osmolality. The high osmolality and $pCO_2$ levels result in lactate production, which also leads to additional base addition

the product and potentially cells, is removed (Fig. 8). A method for cell retention is needed to keep the cells in the bioreactor, and control systems have to be designed to maintain consistent flow rates, volumes, and cell densities as well as to control all typical growth conditions (such as temperature, pH, and dissolved oxygen). Perfusion cell culture has been reviewed in several recent articles [78, 79] and the advantages and challenges (compared with the more common fed-batch process) can be briefly summarized as shown in Table 1.

Higher cell densities are possible with perfusion when compared to fed-batch or batch processes because several limitations of batch processes are removed. Nutrients can be continuously provided by the replacement of the growth medium, and product and wastes can be continuously removed. This enables cell growth to continue until a second level of process limitation is reached, such as cell retention device capacity or bioreactor oxygen transfer rate. At this point cells must be discarded, either with the harvest stream (this may increase demands on harvest clarification systems) or in a separate stream of concentrated cells (which may result in small losses of product).

Perfusion has been implemented with all types of cell culture bioreactors, including stirred tanks, air-lift bioreactors, hollow fiber systems, rocking platforms, and reactors with packed beds. Conventional and single-use systems can be used, although options for cell-retention systems are currently limited with single-use systems.

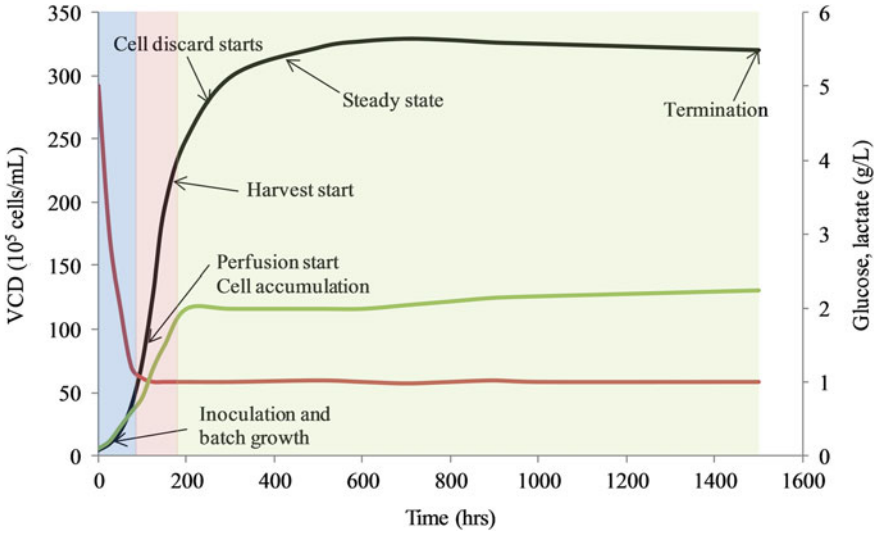


**Fig. 7** Mammalian cell growth in perfusion culture. *Black solid line* represents viable cell density (VCD); *green line* represents product concentration in the culture; *red line* represents glucose concentration. Initial cell growth is in batch mode until nutrient levels or other requirements trigger the start of perfusion. Cells are accumulated until cell discard is required to maintain bioreactor performance (e.g., at the limit of oxygen transfer or harvest production rate). Harvest can start when sufficient product is being collected for processing in a timely manner. During steady state, the objective is to maintain relatively constant conditions (e.g., within characterized limits) by controlling cell density (e.g., through cell discard), media addition, and harvest rates. The cell growth parameters should also be well controlled to ensure steady temperature, dissolved oxygen, and pH. The run is terminated when the cell age reaches a known limit, when growth conditions change, or when sufficient product has been harvested

Cell lines may be suspension-adapted or attachment-dependent and can be of any eukaryotic type including those derived from CHO, NS0, hybridoma, plant, and insect cells. Note that perfusion has also been applied to microbial fermentation systems [80, 81]. Any type of cell culture medium may be applicable, from chemically defined and peptide-free to those containing complex components and growth factors. Products produced can also vary from monoclonal antibodies to virus-like particles and labile proteins or peptides.

## *5.1 Cell Retention*

The cell retention system is crucial to a perfusion system. The complexity, throughput capability, efficiency, and reliability of operation of this device greatly affect the performance and usability of the process. The choice of device can affect cell growth or product quality and may have a significant impact on operational costs. Many types of cell retention devices have been developed for commercial

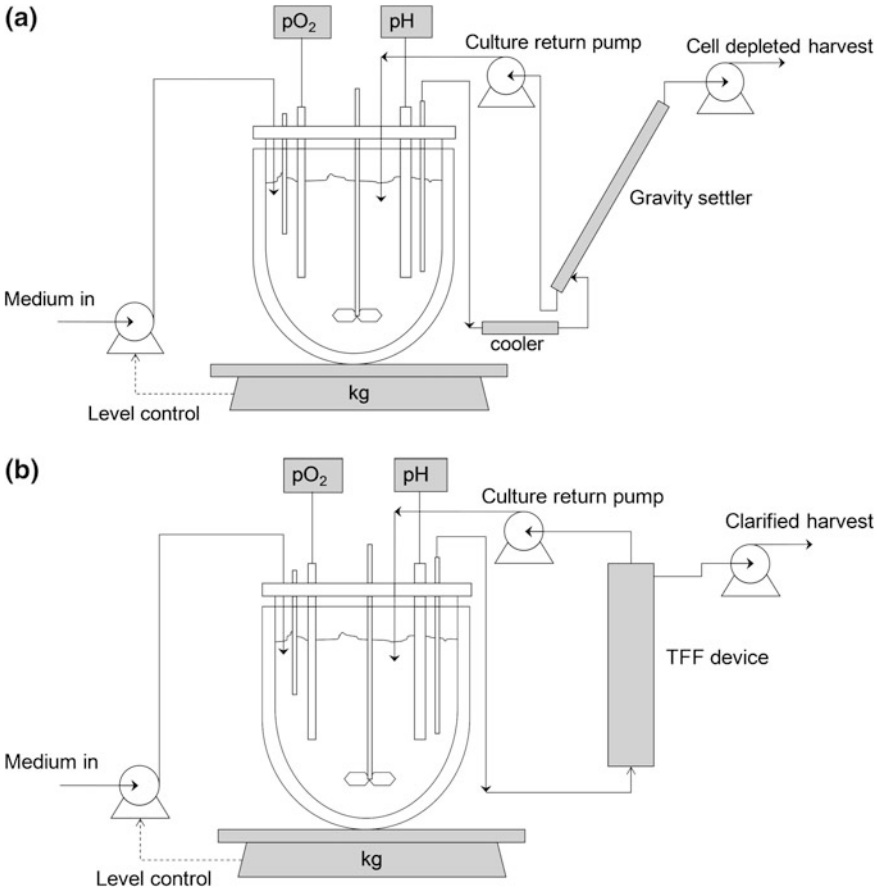


**Fig. 8** Two types of cell retention devices. Panel (**a**) shows a schematic of a gravity-based cell settler device and Panel (**b**) shows a schematic of a tangential flow filtration device

production and these can be based on several types of solid–liquid separation technology, including dead-end or tangential flow filtration, gravity settling, centrifugation, and cell immobilization. Figure 8 shows simple schematics of gravity settler and tangential flow filtration cell retention devices.

Filtration systems function by allowing spent medium to pass while retaining the cells for continued growth and production. Both tangential flow and dead-end filter-based systems may be subject to blocking and fouling with prolonged use unless preventive measures are used. These can include regular filter cleaning, back-flushing, or replacement. Tangential flow systems can be self-cleaning to some degree, although fouling due to macromolecular components (DNA, proteins, lipids) is hard to eliminate completely at shear levels suitable for mammalian cells.

**Table 1** Perfusion cell culture advantages and challenges

| Advantage | Challenge |
|---|---|
| Higher cell densities and increased bioreactor up-time can mean that smaller bioreactor volumes are required, reducing capital investment | Large media preparation and harvest fluid volumes require additional investment. Additional process equipment such as cell retention devices, pumps, harvest concentration systems, or continuous downstream processing systems can increase capital investment |
| High cell densities can be maintained for longer periods | Harvest product titers are typically lower |
| Steady-state operation can give consistent growth conditions with steady nutrient supply and no waste product build-up | Cell line must be stable and performance must be characterized over the length of extended runs |
| Product removal from the system after a short residence time can reduce degradation for labile products | Supplementary process equipment and control systems add complexity and must be well designed, integrated, and monitored |
| Batch definition and quality systems can allow process to continue despite issues with sublots | Process development and characterization, platform development, and scale-down modeling can be more complex |

Spin filters are cell screens mounted inside a bioreactor, typically on the motor shaft, and these can operate successfully for a few days. Wave bioreactors [82] can be obtained prefitted with a floating filter, through which perfusate can be withdrawn. The motion of the filter relative to the cell culture suspension in the bioreactor can help remove cells from the filter surface. Neither of these systems can be mechanically or chemically cleaned or defouled during use. Filter systems external to a stirred tank vessel can be temporarily taken offline or switched out for a back-up system for cleaning and a clogged single-use bioreactor system such as the Wave can be simply transferred to a new vessel for continued perfusion with a clean integral filter. External tangential flow filter systems [83] allow for back-flushing (ATF) [78] or automated cleaning cycles, and ultrafiltration filters [84] can be used to retain both cells and product, if the product is suitably stable, resulting in a harvest that is preconcentrated for downstream processing.

Filters and macro- or microcarriers can support the cell culture in a stationary manner or help in cell retention [85–87]. For hollow fibers, the cells are typically on the shell side of a tube bundle, whereas the tube side is used to perfuse nutrients and gases in, and product and spent media out (e.g., Maximizer, Xcellerator, Biovest). This system removes hydrodynamic stresses from the cells and can be used with suspension-adapted or attachment-dependent cells but can also result in very dense cell beds, potentially adding anoxia or nutrient gradients as another source of variable stress to the culture.

Macrocarriers and microcarriers have also been implemented in various formats to allow retention of attachment-dependent cells in a perfusive environment [88]. Microcarriers settle more rapidly than individual cells and may even be capable of magnetic separation. Macrocarriers and packed beds can be designed to seclude

the cells from external stresses, but in all these cases, renewal of the cell culture through continued cell growth and discard is more complex than with suspension-adapted cells. This may suit certain slow-growing cell lines or cell lines that produce best under slow-growth conditions and may be the only option for attachment-dependent cells.

Gravity-based systems such as conical or plate settlers and centrifuges are less prone to blockage but may not be as efficient for cell separation as filters. They may also add additional stresses to the cells, such as temperature changes or oxygen starvation if the cells are in the cell retention system for a prolonged period [89, 90].

Acoustic separation has also been used for perfusion cell culture (Applikon). This system is reported to work fairly efficiently but can cause cell aggregation [91] and is limited in scale. Hydrocylones are available for perfusion cell culture in single-use format (Sartorius) and these can be operated over a wide volumetric flow range by using intermittent flow through the device at the high flow rate needed for separation. They can be less efficient at cell separation than most other devices but they are not prone to blockage and are simple to maintain and operate. Scale-up could be achieved by multiplexing standard units.

The cell retention system choice must therefore take into account many factors in conjunction with the cell line and product needs as well as scalability, controllability, and capital cost [78].

## 5.2 Operational Considerations

The rate of perfusion of fresh growth medium can be calculated on a volumetric basis or on a cell-specific basis. The former can be in a flow rate expressed as reactor volumes per day and can vary over a wide range, depending on the nutritional content of the medium and the stability requirements of the product. A cell-specific perfusion rate [79] varies with the total cell number in the reactor system and is designed to provide a constant nutritional environment to each cell. This requires accurate cell counts, which can be problematical for nonsuspension cell cultures (e.g., hollow fiber systems or microcarrier systems). Perfusion rates can also be altered based on levels of nutrients, for example, to maintain a constant glucose level. This allows the cells to grow at constant low levels of nutrient, reducing, for example, lactate or ammonia production and allowing cell lines to grow in predictable or optimized conditions.

A volumetric cell-specific perfusion rate (CSPR) in units of nL/cell/day can be calculated using the following equation:

$$\mathrm{CSPR} = \frac{D}{X}$$

where: $D$ is the dilution rate or perfusion rate ($day^{-1}$) and $X$ is the viable cell concentration ($10^6$ cells/mL).

One approach to control of perfusion bioreactors is to discard cells from the bioreactor to maintain a more constant cell density [92]. By controlling the cell density in the bioreactor, there is less need to adjust the perfusion rate to maintain a constant CSPR throughout the run.

Perfusion operation can be described by the equations below. Due to the ability to maintain high cell viability by constant nutrient addition and waste removal, the death rate for cells in perfusion culture is often negligible in comparison to the growth rate. In addition, the contribution of the cell discard rate (CDR) to removal of product and substrate from the bioreactor is also very low. These equations assume that cells are present in the harvest stream ($X_H$), as in the case of a gravity-based cell settler device.

$$D = \frac{F}{V}$$

$$\frac{dX}{dt} = \left(\mu - k_d - \frac{\text{CDR}}{V}\right)X - DX_H$$

$$\frac{dS}{dt} = D(S_M - S) - q_s X - \frac{\text{CDR}}{V}S$$

$$\frac{dP}{dt} = q_P X - DP - \frac{\text{CDR}}{V}P$$

where:

| | |
|---|---|
| $D$ | is the dilution rate ($day^{-1}$) |
| $F$ | is the feed rate (liter/day) |
| $V$ | is the culture volume (liter) |
| $X$ | is the viable cell concentration ($10^6$ cells/mL) |
| $X_H$ | is the viable cell concentration in the harvest stream ($10^6$ cells/mL) |
| CDR | is the cell discard rate (liter/day) |
| $S$ | is the substrate concentration (e.g., glucose; g/mL) |
| $S_M$ | is the substrate concentration in the feed medium (g/mL) |
| $P$ | is the product concentration (g/mL) |
| $\mu$ | is the specific growth rate ($day^{-1}$) |
| $k_d$ | is the specific death rate ($day^{-1}$) |
| $q_s$ | is the specific substrate consumption rate (g/$10^6$ cells/day) |
| $q_P$ | is the specific production rate (g/$10^6$ cells/day) |
| $t$ | is time (day). |

The cell culture medium can be specifically designed for perfusion culture. Nutrient balance and buffering system needs may be somewhat different from those used for batch or fed-batch culture. For example, the perfusion rate and the cell growth rate can influence the nutrient density of the medium. For labile proteins, high perfusion rates may be used in order to remove the product from the

bioreactor rapidly. In this case, a rich medium with a high glucose or glutamine concentration would not be appropriate as cell growth or productivity could be inhibited. Conversely, a stable product may be retained in the growth chamber by a suitable ultrafiltration retention device and in this case a slow perfusion rate with a more concentrated growth medium may be more suitable. A properly balanced perfusion medium will, at steady state, supply the nutrients consumed by the cells and minimize excesses. Medium buffering level may also vary during the course of the culture. Less buffering capacity may be needed during steady state than that used during the cell accumulation phase. Variations in pH, temperature, osmolality, or even the use of induction agents [93] during steady state can be used to select for productivity over growth, but the utility of factors such as this will be cell-line dependent.

As described above, the perfusion rate may be controlled based on cell growth and cell count. Other factors may also be relevant, such as gas transfer capacity, heat transfer capacity (the ability of the bioreactor to maintain temperature when using a chilled medium supply and/or a chilled external cell retention loop), cell retention system capacity, downstream clarification and purification capacity, and product stability. A steady noncontrolled perfusion rate greatly simplifies the overall system and allows establishment of a steady state in many control loops, such as dissolved oxygen, pH, and temperature control as well as in vessel volume control and cell discard rate. However, this steady state may not be optimal for the cell culture if the system drifts and the control parameters require careful monitoring. An interruption of medium supply, for example, can perturb temperature, pH, and oxygen levels and vessel volume must also be controlled in such situations so that the removal of spent medium does not empty the vessel. These complexities can be overcome with a well-designed and integrated control system [94], but specific interlocks must be designed for perfusion culture systems.

Prolonged processing associated with perfusion culture adds additional stresses to a cell line. Genetic stability must be confirmed at the limit of cell culture for GMP production and cell phenotype drift may also be observed. Gravity separators tend to select for larger cells and aggregates, for example, so measures may need to be taken to compensate for or control the extent of these changes.

Because of the potential long duration of a perfusion cell culture process, process development and characterization studies can be very lengthy. The final production process format may be limited in overall culture time by cell line stability or the need to characterize the process fully over the entire process duration. In addition, scale-down of perfusion systems adds additional compromises to a model system [95]. Cell retention systems are not all ideally scalable, especially those that use proprietary devices with limited size options. Residence times in external loops should also be adjusted to match the full-scale system, but adjustment of the circulation rate may mean that external hydrodynamic stresses in pumped loops may not scale properly. A risk assessment approach can be used to identify which scaling parameters will be most closely targeted.

Strategies for batch definition in GMP perfusion culture are accepted by regulators if they are well defined and logical. Each separate batch should have a specific start and end event, lot number, and set of test data associated with it. Separate batches may be associated with separate clarification filters, distinct purification runs, or with timed harvest separations. Continuous purification processing (see below) must move batch separation and definition farther downstream, perhaps to a bulk-filling or even to a vial-filling operation. The system, however devised, should obviously allow for batch traceability.

## *5.3 Continuous Processing*

The future of perfusion cell culture operations includes more integrated capture steps. Recent publications from Bayer [96] and Genzyme [97] describe technologies for the semi-continuous or continuous capture of product from perfusion bioreactors. (Semi-) continuous capture has several advantages over more traditional batch operations for the primary recovery of the product. Traditional perfusion culture requires freezing of the clarified/concentrated harvest, particularly in the case of unstable complex glycoproteins, such as coagulation factors. Continuous capture by membrane adsorbers or resin chromatography shifts the inventory control point farther downstream and at potentially higher concentrations/lower volumes. Continuous processing can reduce processing time of the bioreactor harvest, which is important for maintaining the activity of unstable proteins [96]. The closed system operation also has advantages in terms of bioburden control [97]. Continuous processing can lead to faster development times because the same scale can be used in process development, clinical production, and commercial manufacturing. The footprint of the equipment needed for continuous processing is much smaller than traditional batch processes and can be easily scaled up by increasing the number of units. Continuous media and buffer preparation can further reduce the operational footprint.

Other industries have made a similar gradual conversion from batch to continuous processing [97]. In the case of bioprocessing, the advantage is that the production bioreactor step is the single synthesis step in the process (unlike small molecule production, which requires several synthesis steps). In order for continuous processing to be competitive with fed-batch processes for monoclonal antibodies, the media costs must be kept low. However, due to the high cell densities enabled by perfusion culture, volumetric productivity can be $\sim$ sevenfold higher than fed-batch. Therefore, perfusion titers $\geq$50 % of the fed-batch titers may be sufficient for cost-effective processes.

## 6 Monitoring and Control

Regardless of the culture mode employed, monitoring and control of bioreactors are paramount to ensuring robust performance in commercial manufacturing. Most prevalent monitoring instrumentation for bioreactors continuously report culture condition values for dissolved oxygen, pH, and temperature. In addition, the use of offline procedures provides data on the state of the cell culture as well as the physical environment of a reactor. New offline biochemical methods are becoming available as well as online, noninvasive, and quantitative tools and techniques to monitor new aspects of bioreactor systems. Noninvasive, real-time measurement of multiple biochemical species has been demonstrated using optical probes that use technology based on near-infrared [98] or Raman spectroscopy [99].

Very large datasets can be generated via the monitoring methods described above for each manufacturing run. Therefore, an important part of the process and facility design should be the selection and implementation of systems for data gathering and analysis. Once in production, the success of a batch will largely be defined by the data collected. The quality of the data systems is of paramount importance because these measurements will be used for real-time control of batches and for postbatch analysis, product release, and regulatory submissions. The collection and meaningful use of data is not trivial, even for the most experienced of companies. In designing a data system, solutions should be carefully selected for data acquisition, data aggregation, and data analysis.

### *6.1 Data Acquisition*

Modern facilities usually include a multitude of data-gathering instruments producing enormous amounts of data for every batch. Instruments contributing to the accumulation of data will usually include numerous temperature sensors, pressure sensors, pH probes, DO probes, off-gas analyzers, metabolic product analyzers, and cell counters, among others. However, in laboratories, older facilities, or improperly designed installations, a paucity of important data may still exist. The selection and placement of instruments is ideally the result of a collaboration between engineers and scientists who understand both facility and process design. The intent of the collaboration is to capture all the meaningful information in the process by recording data for variables that have the potential to affect process performance, equipment performance, environmental stability, raw material variability, and product quality.

Worse than a lack of data is inaccurate or misleading data. Temperature probes placed too close to neighboring steam lines or strain gauges susceptible to temperature fluctuations are examples of such implementations. Unresolved, these situations will at best result in unusable ignored data. More dangerous, however, is the possibility that inaccurate data may lead to unnecessary or even detrimental

manipulation of equipment or processes. To avoid this situation, selection of the appropriate instruments, design of their placement, and regular calibration, maintenance, and replacement are required for all manufacturing systems.

## 6.2 Date Aggregation

Because most production facilities will generate enormous amounts of data for a given batch, data archiving and aggregation must be considered. Data must be archived for retrospective analysis associated with lot release, process investigations, process performance monitoring, and regulatory inspection. Despite the proliferation of digital memory storage solutions, the abundance of data produced from online data streams may still warrant archiving using compression algorithms. Systems, such as the widely employed OSIsoft PI system, take digital data streams and selectively discard some data based on an algorithm such that the "forgotten" data can be reproduced by interpolation with a user-defined degree of accuracy [100].

Not all data of interest will be collected by online instruments. Many important data will reside outside the production batch control system and must be aggregated in order to become useful. Such data will include raw material information such as lot numbers, expiration dates, and release testing information. Other data may be collected after a batch is complete such as offline laboratory tests. Still other data may exist only in written or printed form such as handwritten batch records, or vendor documentation. The process of aggregating data from a variety of electronic and manual data archives is a serious challenge, especially for large and complicated data networks. Sophisticated software solutions, such as Discoverant from Aegis [101] or MII from SAP [102] can be customized to fit a company's needs. These systems are expensive, require customization to connect securely to a company's individual data systems, and must be validated and maintained to ensure that the data aggregation and analysis they perform are reliable. However, the potential benefit of rapid access to accurate data is of such magnitude that these large information technology projects are often justified by solid business arguments.

## 6.3 Data Analysis

Data that contain information about a process or batch may take several forms. Continuous numeric data are data that can assume any number within the limits of the measuring device. For example, temperature can be any value within the granularity of the temperature probe. Many other types of data may also be collected such as nonnumeric data (pass/fail, less than limit of detection, conforms, raw material lot descriptor), and ordinal data (low/medium/high). Data within a

particular stream may also be collected continuously (online probe), periodically (daily sample), or once per batch (release result). The result of this variety of datatypes is that each batch will have a complex multidimensional matrix of data that requires different statistical techniques for appropriate analysis.

## *6.4 Real-Time Data Analysis*

### 6.4.1 Process Control

Many production and laboratory systems are controlled through automated computer systems. In such a system, data produced during the process are used to make decisions about future process steps. Process control systems of this sort have well-developed science for control algorithms [103] and hardware and software solutions for any scale of production [104]. It is obvious that in such applications the accuracy, precision, and timeliness of data are critical in maintaining expected process performance and product quality.

### 6.4.2 Process Alarms

Beyond the use of process data for direct control of process functions, another important use is for the monitoring of process performance. The most basic application of process monitoring involves the establishment of limits for individual variables. If a variable exceeds the established limit, some action must be taken either by the process or by the operators (i.e., adjust the process controls, initiate an investigation, reject the batch). Limits may be derived from some knowledge of acceptability such as clinical data showing efficacy within a certain range, or development data showing process failure beyond a certain point. Ideally, the widest limits for any given process variable are dictated by an understanding of how that variable affects the needs of the product's customer (e.g., drug product critical quality attributes).

Robust processes do not operate near the limits that define acceptable product; they maintain control in a tighter range. This range is dictated by the variability inherent to the process, which may be introduced by measurement variability, equipment variability, raw material variability, and so on. Given adequate data, statistical limits may be calculated that represent the expected range for process operation based on inherent process variability, and assuming a level of confidence that is acceptable to the process owner. These statistically derived limits should fall within the product quality limits and are applied to variable monitoring to ensure that a process continues to perform as it has historically. Figure 9 illustrates the performance of a process variable over time with reference to its quality acceptance limits, or upper and lower specification limits (USL, LSL), and statistical control limits (UCL, LCL).

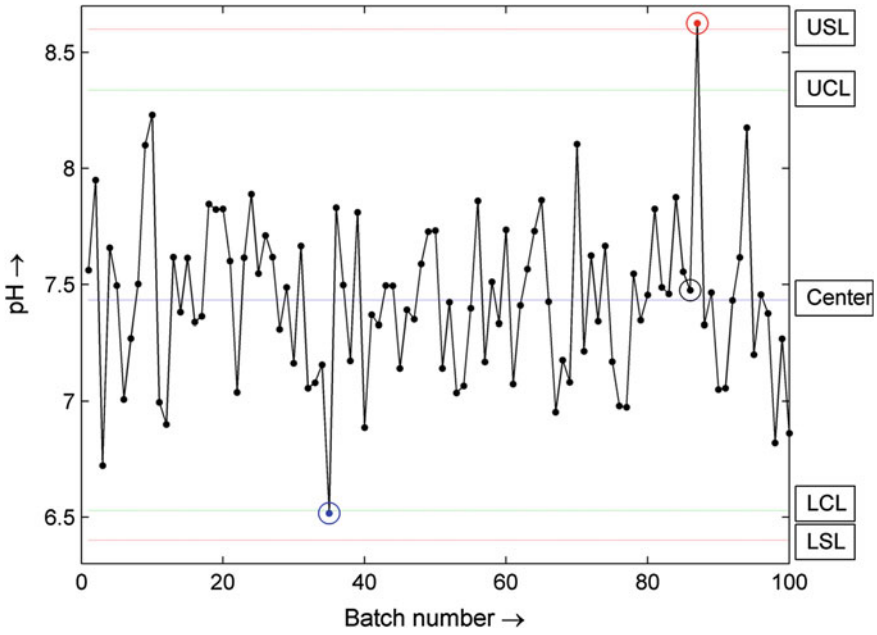


**Fig. 9** Univariate statistical process control (SPC) chart with violations. The *three circled points* in the figure represent different violations of statistical process control. The *circled blue point* violates the lower control limit, which means a value that low would rarely be expected based on inherent process variability. The batch should be investigated for the root cause of the low value. The black circled point represents the seventh in a series of values all above the mean. This could represent a shift in process performance that should be investigated for root cause. The *red circled point* is a batch that failed the specification limit. The root cause of the failure should be identified and corrected to prevent further batch failures and product discards

### 6.4.3 Online Multivariate Alarming

Online monitoring and alarm of individual variables is an expected form of process control, but it has important shortcomings. One example is that univariate monitoring is blind to interactions between variables. In real processes, many variables are dependent on each other and are expected to vary in a predictable pattern. An alteration in the variable relationship would suggest something is not behaving as expected and should be investigated. Advanced statistical techniques and software exist that allow for multiple variables and the relationships between them to be modeled based on historical data. This is termed multivariate data analysis (MVDA). The most common form of this modeling relies on the mathematical technique called principal components analysis (PCA) [105, 106]. PCA models can take large datasets and reduce them to a few important components that capture the most variable portions of the dataset. Each component is influenced by several individual variables and variable interactions, so monitoring a few components can be enough to ensure that all process variables continue to exhibit their

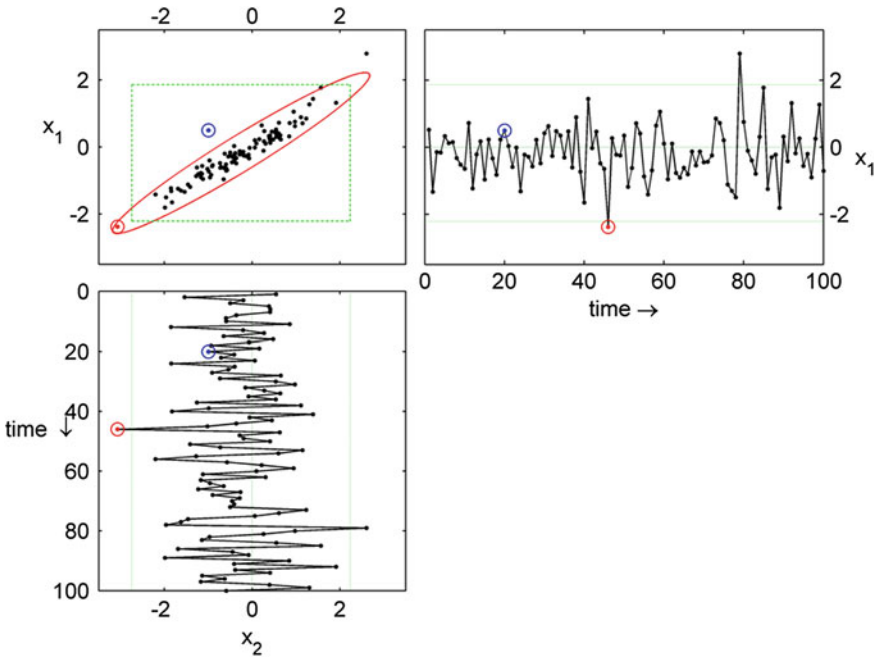


**Fig. 10** Multivariate monitoring of batch variables. Individual monitoring of variables that are related to each other results in both false-positive and false-negative errors

historical behavior. When a multivariate PCA model signals a pattern or excursion, principal components must be decomposed into the measurements that most strongly contribute to them in order to understand the physical meaning of the model data.

Figure 10 is an often-used example of the advantages of using multivariate monitoring over univariate methods. The example given is for retrospective monitoring of batches over time, but the same principle could be applied to two (or many) continuously monitored, related variables in a real-time monitoring scenario. In the example, two variables ($x_1$, $x_2$) are plotted against each other for many lots in the upper left corner. Clearly, the variables are correlated, and thus a specific relationship is expected between them. The ellipse surrounding the variables represents the area within which all points are expected to fall based on a multivariate model and given a predefined level of confidence. Based on the model, the circled blue point represents a break in the relationship between the variables that is unexpected. The circled red point, however, falls within the ellipse, and thus may be expected to occur based on the variability existing in the data. The variables are plotted individually in the lower left and upper right plots with corresponding confidence limits. Based on these univariate plots, the blue circled point is not at all unexpected and would not be flagged as unusual, whereas the red circled point is flagged in both cases as out of statistical trend.

Monitoring both variables independently essentially creates a rectangular acceptance region defined by the intersection of the univariate confidence limits, as pictured in the upper left plot. Because this ignores the important relationship between the two variables, application of this acceptance region produces both false-positive (rejecting a good batch) and false-negative (missing an out-of-trend batch) signals for data within the given example. The benefit provided by models that account for and simplify relationships between variables is amplified as the number of variables and the number of interactions increases.

## *6.5 Retrospective Monitoring*

In addition to real-time monitoring and control of processes, robust process monitoring systems include provisions for retrospective trending of batch data to detect changes in batch performance over time. This activity falls under the umbrella of statistical process control (SPC) and is an expectation of regulatory agencies. Classical SPC involves the charting of the same process variable (such as yield) over the course of many production lots. Statistical analysis can determine what the expected range of a variable is based on historical data, and any new lots outside the range can be identified as atypical. Additionally, data can be analyzed for shifts or trends that suggest that the process has begun to perform differently. A pattern of decreasing yield values for several lots may suggest that a trend is occurring that should be investigated. An increase in the variability of process results may suggest that new sources of variability have entered the process. Many statistical techniques have been developed to analyze process data of various types, and can be of significant value in ensuring process performance continuity.

As in the case of real-time monitoring, retrospective monitoring of process data can also be done using multivariate models. Advantages of using multivariate statistics again include the simplification of complex datasets into fewer, multivariate indicators, and the ability to monitor for parameter interactions. Multivariate statistics have been used in bioprocess manufacturing for many applications [107, 108], including fault detection and diagnosis [109], scale-up and process characterization [110], process comparability [111], root cause determination [112], and product attribute prediction [113]. Figure 11 illustrates one of the applications of multivariate data analysis, a scores plot of batches. Each point represents a single batch whose multivariate attributes are summarized by, in this case, just two scores, t1 and t2. This view shows a relative separation of two populations of batches. This type of separation could occur between two manufacturing sites, or two manufacturing scales, before and after a process or raw material change, or even as the result of process drift over time. Visualizing and quantifying differences between populations of batches enables the process owners to design more specific controls of process variability.

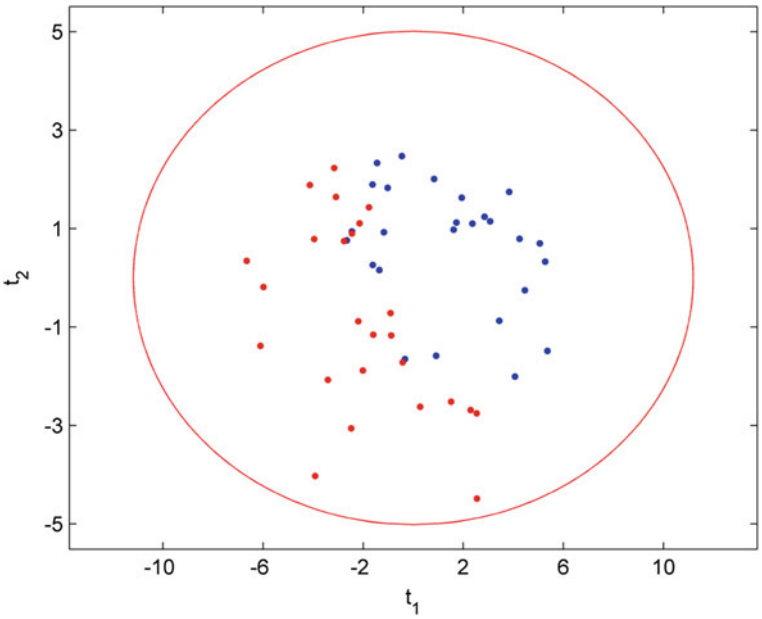


**Fig. 11** Principal component scores plot of historical multivariate batch data. The scores plot condenses the multiple batch attributes into a single point on a two-dimensional plot. Separation of groupings of batches within the *ellipse* (which represents the 95 % confidence limit) illustrates subpopulations within manufacturing history that performed differently. Investigation of the lot groupings can yield information about sources of process variation such as the effect of scale, manufacturing site, raw materials, and the like. Points outside the ellipse represent batches that were statistically inconsistent with the majority of the batches

## 7 Conclusion

The dominant culture method for the production of monoclonal antibodies is currently fed-batch, whereas perfusion processes are predominantly utilized for the production of labile therapeutic proteins. However, as cell line and media development technologies improve productivity, the need for facilities with six-packs (6 × 20,000-L bioreactors) has been reduced. The product demands can be met frequently with 2,000–10,000-L bioreactors. This trend towards smaller bioreactors has enabled the industry to embrace the use of disposable bioreactors. As the industry looks to the future of low-volume products, rapidly changing production demands, and personalized medicine, the concept of continuous processing is becoming more attractive.

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Adv Biochem Eng Biotechnol (2014) 139: 69–92
DOI: 10.1007/10_2013_259

Published Online: 5 September 2013

# Equipment for Large-Scale Mammalian Cell Culture

**Sadettin S. Ozturk**

**Abstract** This chapter provides information on commonly used equipment in industrial mammalian cell culture, with an emphasis on bioreactors. The actual equipment used in the cell culture process can vary from one company to another, but the main steps remain the same. The process involves expansion of cells in seed train and inoculation train processes followed by cultivation of cells in a production bioreactor. Process and equipment options for each stage of the cell culture process are introduced and examples are provided. Finally, the use of disposables during seed train and cell culture production is discussed.

**Keywords** Bioreactor · Cell culture · Cell retention · Disposable · Monoclonal antibody · Perfusion · Preculture · Scale-up · Seed train · Single use

**Abbreviations**

| | |
|---|---|
| CHO | Chinese Hamster Ovary |
| BHK | Baby Hamster Kidney |
| cGMP | Current Good Manufacturing Practices |
| DO | Dissolved Oxygen |
| WFI | Water for Injection |
| HVAC | Heating, Ventilation, and Air Conditioning |
| QC | Quality Control |
| RTD | Resistance Temperature Detector |

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## 1 Introduction

Mammalian cell culture is now an essential part of the pharmaceutical industry and used for the production of therapeutic proteins such as interferons, blood clotting factors, enzymes, vaccines, and monoclonal antibodies [1]. Genetically engineered host cells such as CHO, BHK, SP2/0, and NS0 myeloma cells are cultivated in large bioreactors to produce drugs for the treatment of life-threatening diseases including cancer, autoimmune diseases, and metabolic and blood disorders [1, 2]. The amount of protein to be made can be as high as 1,000 kg in some instances requiring bioreactors as large as 25,000 L in size (total volume). The bioreactor size and the number of bioreactors to be used are dependent on the yield and demand for the product [3–5]. A single 10,000 L (or five 2,000 L) bioreactor can produce 100 kg of product a year for a cell culture process with 1 g/L titer. This calculation assumes the bioreactor is run 20 times a year and the overall process yield from bioreactor stage to final product is 50 %. A process producing 5 g/L requires only one 2,000-L (or five 400-L) bioreactor to produce the same amount.

Large-scale manufacturing of cell culture products requires dedicated or multiuse facilities with full cGMP compliance [6, 7]. These facilities accommodate cell culture, purification, and fill/finish suites, QC laboratories, and all supporting utilities such as HVAC, clean steam, and WFI. The size of these facilities, construction, capital, and operating cost largely depend on the production capacity or the size of the bioreactors.

Table 1 presents the size of the bioreactors used by leading pharmaceutical companies [8]. As can be seen from the table, the volume of the bioreactors can be several thousands of liters. It should be noted that most of these facilities were built for running rather old processes with low yields (less than 1 g/L), and more compact facilities are expected to be built as yields go to as high as 5–10 g/L [2].

Aseptic processing is essential for cGMP manufacturing of biotherapeutics and cell culture areas must be designed, built, and operated to meet the necessary requirements [6]. Use of clean rooms, sterilization, and sanitization of the equipment, personnel training, use of clean steam and high-quality water (WFI), environmental monitoring, and so on are essential for cGMP operation. Even for a non-GMP area such as a process development and scale-up facility, aseptic

**Table 1** Bioreactor Size Used in Different Cell Culture Production Facilities (From [8])

| Company, location | Bioreactor size (L) | Total capacity (L) |
|---|---|---|
| Genentech, Vacaville | 25,000 | 200,000 |
| Amgen, West Greenwich | 20,000 | 180,000 |
| Biogen Idec, RTP | 15,000 | 90,000 |
| Lonza, Tuas, Singapore | 20,000 | 80,000 |

controls should be in place to prevent contamination of the cell culture. The equipment for cell culture needs to be sterile, used either as a presterilized component, or sterilized at the facility by autoclaving or by steaming in place (SIP). Raw materials such as medium, feeds, and base for pH control need to be sterile or sterile filtered using sterilizing grade (0.2 μm) filters. Use of biosafety cabinets or laminar flow hoods are required for open operations such as media and cell transfer. The gases used to aerate the cultures in bioreactors need to go through sterilizing grade filters.

In this chapter we outline a typical cell culture process, present individual steps involved, and discuss the equipment used in large-scale manufacturing of therapeutic proteins. The design, installation, and operation of this equipment are illustrated. Cell culture operations include vial thaw, seed train expansion, inoculum expansion, and production in the bioreactor [9, 10]. Harvesting of the bioreactor and purification of the product through downstream processing is beyond the scope of this chapter. Traditional stainless-steel-based, and disposable cell culture equipment are covered.

## 2 Cell Culture Process

Production of therapeutic proteins using mammalian cell culture starts with a frozen stock of recombinant cells, traditionally in the form of a cell bank vial. Depending on the scope of cell culture production a research cell bank, a master cell bank, or a working cell bank is used [9, 10]. All of these types of cell banks need to be sterile and free of mycoplasma. Master cell banks and working cell banks require preparation under cGMPs and are subject to full safety testing including adventitious agent testing. Typically, each vial of a cell bank contains 10–20 million cells in a 1–1.5 mL volume. A frozen stock of cells can also be prepared in a 50-mL cryobag to contain about 1–2 billion cells; this stock can start a culture at 5–10 L directly [11].

Figure 1 shows a typical cell culture process and outlines three different stages: seed train expansion, inoculation train, and production bioreactor.

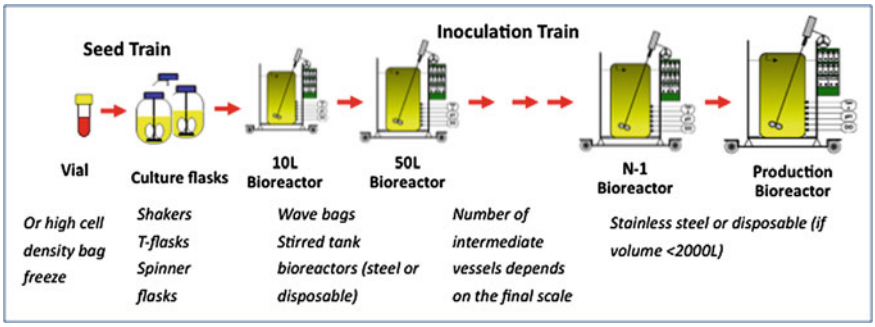


**Fig. 1** A typical upstream process used to produce biotherapeutics from cell culture

## *2.1 Seed Train Expansion*

Seed train expansion is used to increase the number of cells from a frozen vial (or bag) so there are a sufficient number of cells to seed the larger vessels. Examples of vessels are shown in Fig. 2. It starts with the thawing of cells, a centrifugation and resuspension step to remove freezing medium containing DMSO, and seeding a cell culture flask such as a T-flask or a shake flask containing fresh cell culture medium [12].

The size (volume) of the flasks used in the seed train expansion will depend on the seed cell density and split ratio. Split ratio is a measure of dilution of the cells of the seed during the inoculation to the new flask (or vessel). For instance, a 1:5 split ratio means one portion of the seed stock is added into four volumes of fresh medium, thus diluted by 1:5 after seeding. A higher split ratio is preferred because cells receive a higher amount of fresh medium (thus more nutrients) and inhibitory metabolic by-products are diluted as much as possible. However, there are limits to the allowable split ratios imposed by the growth kinetics of the cells in the medium used. For seed train expansion, cells need to be in the exponential phase of growth which sets a maximum cell density limit for the split. For some cells and media, there may also be minimum cell density requirements below which cell proliferation is affected. So between the minimum and the maximum cell densities, an optimal split ratio can be determined experimentally.

In a shaker-flask–based seed train expansion, the first shaker is typically a 125-mL shaker with 40 mL of culture (Table 2) at a seeding cell density of 0.4 million cells/mL. A lower volume and a lower cell density can be used if the vials have less than 16 million cells.

Cells are incubated in humidified $CO_2$ incubators for a period of 2–4 days before each passage depending on the cell growth and production schedule. At the end of the incubation period, the cell density is measured ($X$) and the volume of the inoculum for the next passage ($V_i$) is determined. Target seed density ($X_t$) and the target volume ($V_t$) for the next flask (or vessel) are used in this calculation. The required fresh medium volume ($V_f$) is then calculated as the target volume minus the seed volume.


**Fig. 2** Equipment used for seed train expansion: **a** T-flasks, **b** shaker flask, **c** roller bottles, **d** spinner flask, and **e** WAVE bioreactor on a rocking platform

$$V_i = (X_t / X).\, V_{\mathrm{t}} \tag{1}$$

$$V_f = V_t - V_i \tag{2}$$

Fresh medium and the seed inoculum are then added to the new flask at the calculated volumes and a new passage is initiated. The seed expansion in the example outlined in Table 2 utilizes 125 mL (40 mL working volume, wv), 250 mL (100 mL wv), 1 L (400 mL wv), and 3 L (1 L wv).

The incubators are typically operated at 36.5–37 °C and at a humidity of 50–80 %. The $CO_2$ is used to maintain the pH at the physiological range of 6.8–7.4 as close as possible. The percentage of $CO_2$ to be used in the incubator can vary between 5–10 % depending on the medium used and its bicarbonate level. Mixing, gas exchange for aeration, and $CO_2$ equilibration and heat transfer to the culture are accomplished by shaking the flasks on a shaker platform. The target speed of shaking depends on the size of the shaker platform and the size of the shake flask and can vary between 100–200 rpm. For the effective exchange of gas, the cap of

**Table 2** A typical shaker-flask–based seed train expansion from vial to seed a 10-L inoculum train bioreactor

| Stage | Culture volume (mL) | Flask size |
|---|---|---|
| 1 | 40 | 125 mL |
| 2 | 100 | 250 mL |
| 3 | 400 | 1 L |
| 4 | 2,000 | 2 × 3 L or 5 × 1 L |

the shakers should be kept loose unless it has a special design known as a vented-cap with gas-permeable film on it.

Seed trains can also utilize T-flasks, roller bottles, or spinner flasks instead of shaker flasks or WAVE bioreactors on a rocking platform. These systems are available at different sizes as reusable glass or disposable (single-use) units. The use of plastic or disposable systems is preferred because it eliminates the need for cleaning and sterilization of the flasks before and after use.

T-flasks are preferable for cultivating attachment-dependent cells but they can also be utilized for the initial stages of seed train expansion of suspension-adapted cells (Fig. 2a). T-flasks are sized based on the surface area; a T-25 T-flask has 25 $cm^2$ surface area and it can employ 5–10 mL of culture. As the volume of the culture goes up during the seed train expansion, the size of the T-flask becomes limiting. The largest T-flask has 150 $cm^2$ of surface area and can accommodate about 200 mL of culture. There is no mixing involved in T-flasks and the aeration is achieved by diffusion through the surface. Even though the T-flasks are kept in the incubator horizontally to maximize the gas exchange area, culture aeration is a major issue for this system.

The use of roller bottles (Fig. 2c) has limited application for suspension cultures even though some manufacturing processes use them for scale-up and even for production. Aeration and mixing in roller bottles are achieved by rotating the flasks horizontally. Cell suspension stays mostly at the bottom of the flask due to gravitation but a thin layer of culture is created on the surface of the flask during the rotation due to adhesion. This thin film allows gas exchange by diffusion to the cells and the movement of the cells to and from the thin layer provides continual mixing.

Spinner flasks (Fig. 2d) are designed to have a similar geometry and mixing system as bioreactors. A Teflon©-coated magnetic stir-bar and, in some systems, Teflon blades connected to a magnet provide mixing when spinner flasks are operated on a magnetic stirring platform. Spinner flasks are operated in humidified $CO_2$ incubators and they vary from 25 mL to 3 L in size. The top plate on spinner flasks can be modified to have a pH and DO probe, and in some cases a sparger to provide oxygen to the culture. These modified spinner flasks can, therefore, be operated as low-tech bioreactors.

The WAVE rocking platform (Fig. 2e) uses a presterilized and cell culture compatible bag to culture the cells [13]. Cells are mixed and aerated by the rocking motion that creates waves. The culture (medium, cells, or cell suspension) can be

transferred to the bag aseptically in a biosafety cabinet using a special inlet port. Culture can also be transferred to and from the bag using the tubing attachments and a sterile connecting device. Although WAVE bags can accommodate culture volumes from 1 L through 500 L, the use of WAVE bags for seed train expansion rarely needs volumes more than 50 L. Disposable (single-use) pH and DO probes can be used in WAVE systems for process monitoring and control. A special filtration unit can be integrated into the WAVE system to allow perfusion operation [13, 14].

Each system discussed above for seed train expansion has advantages and disadvantages. The actual choice of one system over the others depends on simplicity, reliability, user friendliness, and also personal preference. In some cases a combination of systems is preferred. Shake flasks followed by WAVE systems, for instance, can initiate a seed train process.

Thus far we considered expansion of cells from a vial up to a volume of 2 L. Above a 2-L volume of culture, the cells are traditionally seeded to a 10-L bioreactor, or to a 10-L WAVE bag as part of the inoculation train (see below). If a 10-L bioreactor or WAVE bioreactor does not exist, expanding the cells to multiple 3-L shake flasks and pooling them to seed the first-stage bioreactor is also an acceptable method to expand the cells.

The seed train expansion outlined above can be skipped partially or completely when 50-mL cryobags are used instead of frozen vials. These bags can contain 1–2 billion cells making it possible to start a culture at 5–10 L directly [11].

During the seed train expansion, cells are passaged and incubated for 2–4 days for each stage. Using the target cell density and the maximum allowable cell density for exponential growth, a split ratio target is determined. The culture duration is then set to allow the required expansion based on growth rate of the cells. If the split ratio target is 1:5, cells need to have at least 2.3 doublings before passage. If the doubling time is 20 h for the cells, a culture duration of 2 days is recommended.

## *2.2 Inoculation Train*

The inoculation train occurs at the completion of the seed train and further expands the number of cells to seed the production bioreactor [10, 11]. The number of vessels in the inoculation train depends on the size of the production reactor and the allowable split ratios for the expansion. For fed-batch operation, the volume expansion due to feeds should also be considered. A 20,000-L (final culture volume) production bioreactor requires a 4,000-L seed bioreactor (also called the N-1 bioreactor) with a split ratio of 1:5 for batch operation.[1] For a fed-batch operation,

[1] All the volumes indicated here are working volumes unless specified. The total bioreactor volume needs to be at least 20 % higher than the working volume; that is, a bioreactor with 20,000 L working volume has a 25,000 L total volume.

**Table 3** A typical inoculation train expansion for a 20,000-L fed-batch bioreactor with a starting volume of 15,000 L with a split ratio of 1:5

| | Working volume (L), min | Total volume (L) |
|---|---|---|
| N-5 | 5 | 7 |
| N-4 | 25 | 30 |
| N-3 | 120 | 150 |
| N-2 | 600 | 750 |
| Seed (N-1) bioreactor | 3,000 | 3,800 |
| Production bioreactor | 20,000 | 25,000 |

the production bioreactor can start at 10,000–15,000 L to accommodate the volume increase by feeding, and a lower volume of the seed is needed (2,000–3,000 L). A 3,000-L seed bioreactor will require a 600-L bioreactor for its seeding, and so on. Table 3 shows the sequence of the inoculum (inoculation) train process and the volumes required for each vessel with a split ratio of 1:5. It should be mentioned here that the values in Table 3 are the minimum volumes and it is possible (in some cases advisable) to use a larger bioreactor. For instance, the N-1 bioreactor is listed as having a total volume of 3,800 L, but a 5,000-L bioreactor would be appropriate as well.

If the cell culture medium used in the cell expansion is very rich, a split ratio higher than 1:5 is possible. This allows the inoculum train to use fewer bioreactors even though the total time for expansion will not change dramatically. A 1:10 split ratio, for instance, will require only 3–4 bioreactors instead of 5 for the inoculum train.

Use of a perfusion system in the seed (N-1) bioreactor allows expansion of cells to higher cell densities (30–80 million cells/ml), thus allowing a higher split ratio, as high as 1:100 [15]. In this case the inoculum train can be simplified further and smaller bioreactors can be used. A 200-L perfusion bioreactor will be sufficient to seed a 20,000-L fed-batch bioreactor with starting volume of 15,000 L, instead of a seed 3,000-L bioreactor. The inoculum train in this case will have three bioreactors with 8, 40, and 200 L minimum working volumes. Although there are clear advantages of running an N-1 bioreactor in perfusion mode, operational complexities in running such systems reliably need to be addressed.

Although bioreactors used for inoculum train are traditionally stainless steel stirred-tank bioreactors, disposable systems can also be utilized [13, 14]. WAVE systems can be used at 10, 50, 200, and 500 L scale. Disposable stirred-tank bioreactors are available at several scales as well (10, 50, 250, 500, 1,000, and 2,000 L) and they are being adopted gradually as part of the inoculation train or even as production bioreactors.

Culture of the cells for inoculum train expansion follows the same procedure discussed for seed train expansion. Cells are cultured for 2–4 days in each vessel depending on split ratio, target cell density, and maximum allowable cell density.

### 2.3 Production Bioreactor

The final vessel used for cell culture process is the production bioreactor where the cells are cultured to make the product. As mentioned before, the size of the production bioreactor is determined by the amount of product needed and the process yield. Due to extensive process optimization efforts in the last two decades, cell culture processes are now very efficient. Cell densities around 10–30 million cells/ml are achievable and the titers reach 5 g/L, in some cases even 10 g/L. With this efficiency of the process the required production bioreactor size can be as small as 1,000 L when used in a 6-pack configuration (six bioreactors per production suite) for fed-batch. For perfusion operation, even 200-L bioreactors will be sufficient to make products at as high as 500 kg/year [15, 16].

A production bioreactor is typically operated as a fed-batch culture even though other modes of operation such as batch, continuous chemostat, and perfusion are available. Fed-batch operations typically run for 11–18 days depending on the process [2, 10, 17]. The final yield, production kinetics, cell viability limits, and other operational considerations determine the length of the culture. At the end of the culture, the cells are separated out and the product is purified using an established downstream process.

For perfusion operation, bioreactors are typically equipped with a cell retention device and they can be operated for months [18]. Depending on the productivity of the culture, stability of the cells, and operational considerations, run lengths of 1–6 months are used in the biopharmaceutical industry.

## 3 Bioreactor Types

Several types of bioreactors are available for cell culture processes and they vary in terms of their design, geometry, mechanism of mixing and aeration, and material used in their construction [14]. The types of bioreactor systems can be categorized under homogeneous and heterogeneous systems as indicated in Table 4.

In homogeneous systems, cells are freely exposed to the medium and they are kept in suspension by the mixing mechanism used in the bioreactor. These bioreactors are easier to operate, provide a uniform environment to the cells, allow representative sampling, and can be mixed and aerated efficiently. In heterogeneous systems, cells are not directly exposed to the culture medium and they are maintained as stationary either attached to or trapped in a solid support. Fixed-bed bioreactors, fluidized-bed bioreactors, and microcarrier cultures use porous or nonporous microcarriers for cell attachment/trapment. In the fixed-bed bioreactors, microcarriers are kept stationary and medium is passed through the bed to supply nutrients and oxygen to the cells. Microcarriers are suspended by the upflow of medium in the fluidized bed whereas mechanical agitation is utilized in stirred-tank

**Table 4** Bioreactor types used for cell culture processes

| Homogeneous systems | Heterogeneous systems |
|---|---|
| Stirred tank | Fixed-bed bioreactors |
| Bubble column | Fluidized-bed |
| Airlift | Immobilized-bed |
| WAVE, rocking platform | Stirred-tank with microcarriers |
| | Hollow-fiber |

bioreactors with microcarriers. Cells in a hollow-fiber bioreactor grow in the space between the fibers and the fibers act as capillaries providing nutrients and oxygen to the cells [19].

In hetereogeneous systems, nutrients and oxygen are delivered to the cells mainly by diffusion. Diffusion through the cell layer in porous microcarriers or in a hollow-fiber system is not as effective for mass transfer as in homogeneous systems and can cause variations in cellular environment (concentration gradients) and cellular activities. Although these systems reach very high cell densities locally, formation of necrotic (nonviable) zones in the cell layer is a major problem for productivity of the cells [19].

## 4 Bioreactor Operation Modes

Bioreactors can be operated as batch, fed-batch, repeated-batch, semi-continuous, and continuous (chemostat or perfusion) depending on the process, product, and process economics. Batch and fed-batch processes are more typical for cell cultures although continuous cultures have been successfully implemented for commercial production of several products [1]. In a batch operation, the bioreactor is seeded using fresh medium and cell inoculum and the product is collected at the end of the cultivation, typically 7–10 days later. In fed-batch operation, cell density, viability, and productivity of the culture are enhanced by supplying the cells more nutrients in the form of concentrated feed [1, 14].

Continuous culture can be operated with and without cell retention in a mode where the bioreactor is constantly fed by fresh medium and harvested at the same rate [14]. If no cell retention is used, the cell density in the harvest is equal to the cell density in the bioreactor. In this operating mode the bioreactors, also called chemostats, have limits on how fast the medium can be exchanged before washing out all the cells from the bioreactor. High cell densities cannot be reached as cells are continuously removed. Low cell density and low throughput (volume exchange rate) limit the volumetric productivity in a chemostat operation.

Use of cell retention devices allows operating the bioreactors at higher volumetric exchange rates, thus allowing the cultures to reach higher cell densities [18, 19]. There is no appreciable washout of the cells and cell densities of 30–50 million cells/mL are achieved. As mentioned before, these bioreactors can

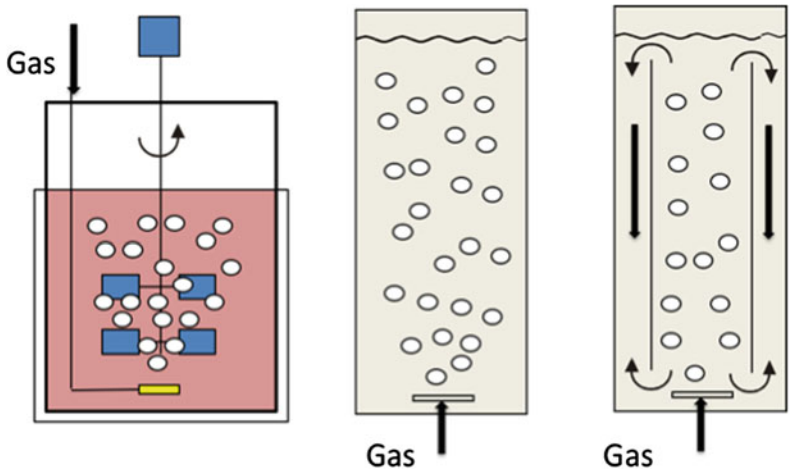


**Fig. 3** Mixing and aeration in stirred-tank (*left*), bubble-column (*middle*), and airlift bioreactor

be used as part of an inoculum train but they can also be the production bioreactors. Continuous perfusion bioreactors reach volumetric productivities much higher than fed-batch systems allowing the use of more compact bioreactors for the production. Cell retention systems are discussed later in Sect. 8.

## 5 Mixing and Aeration

In this section we consider only homogeneous systems, that is, stirred-tank, bubble-column, airlift, and WAVE bioreactors. Figure 3 illustrates the operation of these bioreactors and Table 5 summarizes the mixing and aeration mechanisms they utilize.

Stirred-tank bioreactors use mechanically or magnetically driven impellers to keep the cells in suspension, to homogenize the feed and base additions to the culture, and to disperse the bubbles for better aeration. The impellers are placed on a shaft, which can be top mounted, bottom mounted, centered, or angled. Depending on the size of the bioreactor, one or more impellers can be used [20].

Cell culture bioreactors can use several types of impellers and some of them are presented in Fig. 4. Depending on the design and orientation of the blades, the impellers can be classified as radial flow or axial flow impellers. The Rushton impeller, commonly used in microbial cultures, is a radial flow impeller indicating the mixing it provides acts mainly in the radial directions. In cell culture bioreactors, axial mixing (vertical) is as important as the radial one and axial flow impellers such as pitch-blade impellers are preferred. Use of baffles may be

**Table 5** Mixing and aeration systems used in homogeneous bioreactors

| Reactor type | Mixing mechanism | Aeration mechanism |
|---|---|---|
| Stirred tank | Impeller | Direct or indirect sparging |
| Bubble column | Bubbles | Sparging |
| Airlift | Bubbles, draft tube | Sparging |
| WAVE, rocking platform | Rocking | Surface aeration |

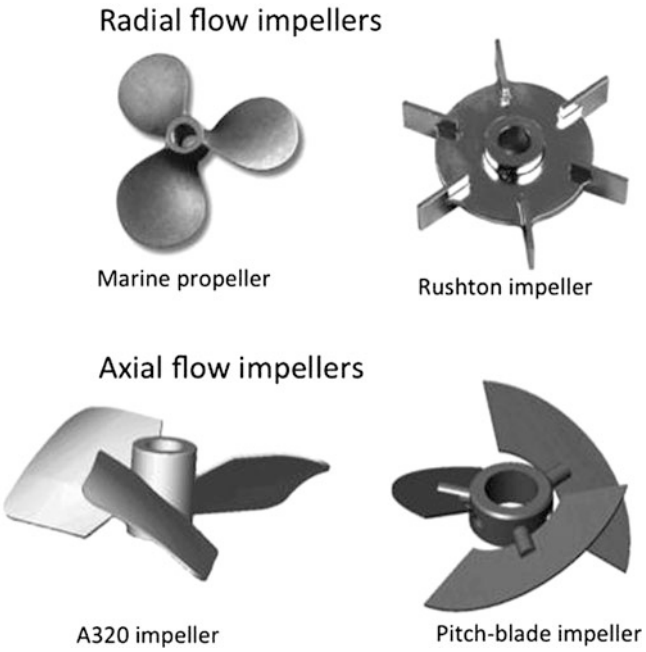


**Fig. 4** Types of impellers used in cell culture bioreactors

required in large-scale bioreactors to enhance axial mixing and to prevent vortex formation.

Bubble-column and airlift bioreactors do not use an impeller, but rely on moving bubbles for mixing. Rising gas bubbles generate turbulence and liquid circulation in the axial and radial directions. These bioreactors have a simple design with no moving parts. Placing a draft tube in a bubble column enhances liquid circulation horizontally and converts the bioreactor to an airlift bioreactor. Celltech (now Lonza) introduced the airlift bioreactors for large-scale cell culture production and successfully used them for many cell lines.

Aeration is an important aspect of the bioreactor design and it has to be optimized to increase the yield of the cell culture process [21, 22]. Both oxygen and $CO_2$ mass transfer need to be considered for aeration. Solubility of oxygen is very low (0.2 mM at air saturation) and the maximum number of cells that can be maintained in the bioreactor is directly proportional to the oxygen mass transfer.

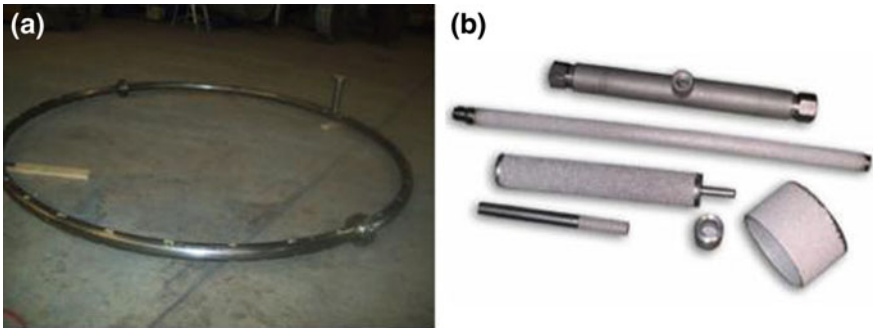


**Fig. 5** Types of spargers used in cell culture bioreactors: **a** macrosparger (arranged as a *ring* sparger), and **b** microspargers

The $CO_2$ is used to control the pH in the beginning of the culture. During the culture, the cells generate $CO_2$, and it has to be stripped out by mass transfer to maintain it below inhibitory levels.

Transfer of the oxygen to the cells and the removal of $CO_2$ can be achieved by several mechanisms [23]. Surface aeration and sparging are commonly used as aeration methods for cell culture. Although it has limited capacity for mass transfer, surface aeration can provide enough gas exchange for smaller vessels (<1 L) such as shake flasks or spinner flasks for cell densities up to 2–5 million cells/mL. As the volume goes higher the surface area to volume ratio in the vessel goes down, thus affecting the efficiency of mass transfer. Increased agitation rate for spinner or shake flasks, increasing rotation speed for roller bottles, and rocking speed for the WAVE system will enhance the efficiency of surface aeration. It should be pointed out that these rates cannot be increased indefinitely as mechanical or operational problems start affecting the cells. In sparged cultures, surface aeration contributes to the mass transfer but its contribution to overall mass transfer goes down as the scale goes up. Although the contribution of surface aeration to overall mass transfer is about 20–30 % in a 1-L stirred-tank bioreactor, it goes down to about 10 % in a 1,000 L, and it is negligible for bioreactors >5,000 L.

Direct sparging is very efficient for oxygen and $CO_2$ mass transfer and several types of spargers are used in the bioreactors (Fig. 5). Spargers are categorized as macro- or microspargers depending on the size of the sparger holes (or pores) they use. If not properly implemented, aeration using sparging can affect cell culture performance. Shear generated from the formation of the bubbles, their rupture, and the entrapment of cells in a foam layer were identified as the source of cell death in the bioreactors. Most of these issues were addressed in the last 20 years and sparging is now successfully used in cell culture to provide sufficient oxygen to more than 50 million cells/mL.

The use of Dow Corning® antifoam C emulsion to suppress the foam and the use of surface active agents such as Pluronic F-68 to minimize the attachment of

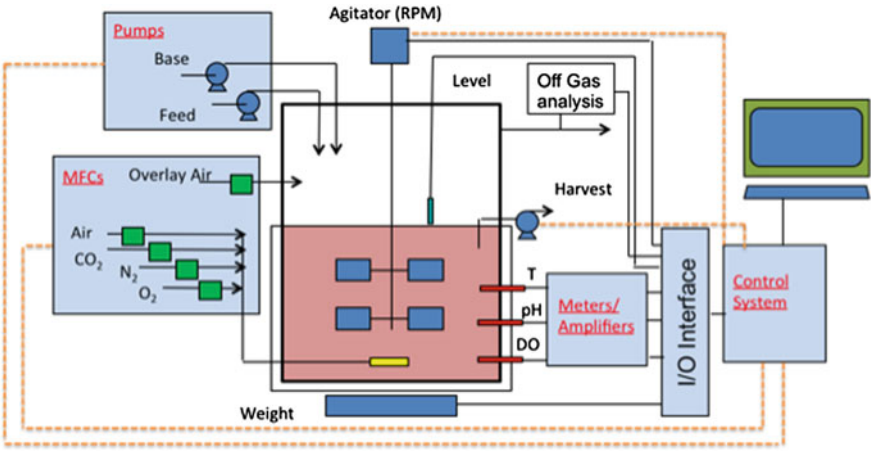


**Fig. 6** A bioreactor system with pumps, mass flow controllers (MFC), probes, meters and amplifiers, and process control units

the cells to the bubbles eliminated most of the problems related to foam and shear-induced cell damage [24].

Macrospargers have openings 0.5–2 mm in diameter and generate bubbles from 1 mm to several centimeters in size. The use of multiple holes on a sparger provides more bubbles to the culture and allows even gas distribution in the bioreactors. Although a ring configuration is more common, L-shaped spargers can also be used. Spargers are typically located below the impeller so that the bubbles generated are dispersed to the culture easily and uniformly.

Microspargers utilize micron-sized (2–30 um) pores for aeration and they come in different sizes and shapes. These spargers are made of sintered metal and sold as cylindrical elements to be connected to a main sparger line. Microsparger elements generate very fine bubbles (100–400 um diameter), which are extremely efficient for mass transfer of oxygen. To support the same cell density, a microsparger needs almost 10 times less gas flow compared to a macrosparger. A drawback of the microsparger is a reduction in $CO_2$ removal; the fine bubbles tend to saturate quickly with $CO_2$ and the tenfold reduction in bubble volume contributes to the loss of $CO_2$ mass transfer efficiency. A second concern with the microsparger system is the formation of a thick foam layer that requires more intervention for foam control and more antifoam use to manage.

The advantages of macro- and microspargers can be combined by fitting the bioreactor with both types of spargers. In this configuration, the microsparger is used mainly for oxygenation (main sparger) and $CO_2$ removal is accomplished primarily by the macrosparger (secondary sparger) [19].

## 6 Bioreactor Operation and Control

A bioreactor system, independent of size, consists of several components: (a) pumps for the addition of medium, feeds, base, and antifoam; (b) gas supply using mass flow controllers (MFC) (or solenoids); (c) probes (meters) and amplifiers to monitor and control cellular environment; and (d) a data acquisition and process control system. These components are presented in Fig. 6.

Peristaltic or diaphragm pumps can be used for addition of medium, feed, base, and antifoam, as well as cell suspension to the bioreactors. These pumps can be manually operated or remotely controlled by the automation software used. The sizing of the pumps with respect to their capacity in terms of L/min needs to be done properly so that they can run in the middle of their operating range.

Bioreactors use four gases, air, $O_2$, $N_2$, and $CO_2$ although some systems forgo the use of $N_2$ and/or air. The flow rates of these gases are typically controlled by the dissolved oxygen (DO; air, $O_2$, $N_2$ flow rate) and pH controller ($CO_2$ flow rate); the gas mixture is introduced to the bioreactor through the sparger. A certain gas flow is also needed in the overlay to sweep the headspace above the culture, mainly to remove $CO_2$ generated by the cells in culture.

Mass flow controllers (MFC) are special devices where the flow rate of the gas is measured and controlled. The size of the MFC (maximum capacity) has to be carefully chosen based on the bioreactor size and the gas sparge rate requirements of the culture. The accuracy and the sensitivity of MFCs at the very low end of their operation range can be an issue if MFCs are sized too big. Gas flow can also be regulated using a solenoid controller. Solenoids are much cheaper than MFCs as they rely on a pinch-valve to control the gas flow. In a solenoid-controlled gas delivery system, the opening of the valve is set to maximum flow rate. The pinch-valve controlled by the solenoid opens or closes at a frequency to match the gas flow desired.

The gases can also be delivered at a manually set flow rate. The overlay gas flow rate and, in some cases the flow rate to the secondary sparger used for $CO_2$ removal, are often controlled manually.

Bioreactors are equipped with probes to monitor the cellular environment such as temperature, pH, and DO. The probes are connected to the meters or amplifiers where the signal is converted to the values of temperature, pH, and DO. The data from these probes are also used to control bioreactor temperature and culture pH, aeration, and $CO_2$ removal by directing pump and gas flow controller function. The probes are calibrated using standards, special buffers for pH (pH 7.0, 10.0, or 4.0), and using $N_2$ (zero point) and air (100 % air saturation) for DO before use; they can be recalibrated (single-point) during use based on offline measurement of a bioreactor sample using a bench pH meter (pH only) or blood gas analyzer (pH and DO).

The probes have direct contact with the culture so they need to be sterilized before use. A glass probe for pH, a polarographic DO probe, and a resistance temperature detector (RTD) for temperature can be autoclaved or SIP for

sterilization. Optical probes based on fluorescent technology are gaining popularity for disposable systems as they can be applied as presterilized patches or probes as part of the disposable bag. These probes are not as robust as the conventional probes as their calibration can change during use because of a photo-bleaching phenomenon. Conventional probes can also be used in disposable systems using a special connecting device, as discussed later.

Bioreactors are operated at a positive pressure to minimize contamination risks and a pressure gauge is used to monitor the pressure. A pressure relief valve is utilized as a safety guard to prevent over pressurization. Bioreactor instrumentation may include offline gas analyzers that allow monitoring of $O_2$ and $CO_2$ in the gas phase. This allows continuous measurement of respiration rates and provides an estimation of viable cell density in the bioreactor. Other sensors such as cell density probes, $pCO_2$ sensors for dissolved $CO_2$, and so on, can be utilized in a bioreactor for control and/or information on the cell growth and cellular environment as well.

Level probes can measure volume in the bioreactor but reliability and robustness issues limit their application. Therefore, weight measurements are preferred, as they tend to be more precise and less cumbersome. The density of the culture is close to the density of the water and in most cases the weight can be treated as the volume. However, the actual density needs to be used in converting the weight to the volume for accuracy. Although bioreactors can be put on a scale, load cells are commonly used in the industry for weight measurements.

Agitation to the bioreactors is supplied by motors, each with a controlled gearbox to adjust and control the agitation rate. The connection of the impeller shaft to the motor needs a special coupling and a sanitary seal to prevent contamination. The design of mechanical or magnetic seals is very complex and it involves carefully selected gaskets, bearings, springs, and collars, to allow motion while sealing the system against leakage and contamination.

Bioreactors are integrated with a computer system for data acquisition and process monitoring and control. The computer system controls the pumps, gas flow rates, weight (or level), and process parameters such as temperature, pressure, pH, and DO. A computer system can also automate transfer of the medium and cells, cleaning and sterilization of the bioreactor, and harvesting operations. For cGMP operation, the computer system should be qualified/validated and comply with 21 CFR Part 11 regulations as well as the predicate cGMP regulations in 21 CFR part 211. A variety of computer systems that are in use includes Delta V (Emerson), Allen-Bradley (Rockwell), and FermWorks (Jova Solutions). These systems can trigger alarms when a parameter is outside the specified range; capture, store, and analyze data; and can control the process variables at user-defined setpoints. The data historian feature of the controller system can integrate online and offline data, compare several batches, and can generate campaign reports.

Bioreactor monitoring and control also relies on offline data generated from the samples withdrawn from the bioreactor. Cell density and viability determinations can be performed manually using a hemocytometer or automatically using particle counters such as a Cedex (Roche Diagnostics), Nova Bioprofile Flex (Nova

Biomedical), and ViCell (Beckman) analyzer. Metabolites (glucose, lactate, glutamine, ammonia), pH, DO, $pCO_2$, electrolytes ($Na^+$, $K^+$, $Ca^{+2}$), amino acids, and product can be analyzed using a variety of analyzers. Culture performance is monitored based on the data generated and manipulations such as feed additions can be made. Offline measurement of pH and DO can be used to recalibrate the online probes, when necessary.

## 7 Stainless Steel and Disposable Bioreactors

Quality, durability, and cleanability of the vessels used in cGMP production of pharmaceuticals are extremely important for the safety of the products. High-quality stainless steel (Type 316) is used extensively in biomanufacturing as the material of choice for tanks, pipes, and bioreactors. If the surface of the stainless steel contacts the product, extra precautions are in place that include electropolishing and passivation of the surfaces. A tight control of surface chemistry and roughness ensures complete cleanability of the surface so that the vessel can be reused.

A stainless steel bioreactor setup is presented in Fig. 7. This bioreactor has a working volume of 1,200 L and is housed in a one-floor facility. When the bioreactor size is bigger than 2,000 L, it is necessary to have more than one floor because of the height requirements. A 20,000-L bioreactor, for instance, requires a three-floor facility.

A stainless-steel–based bioreactor system requires supporting utilities to run and it uses extensive piping systems for fluid transfer, cleaning, sterilization, and harvesting. Clean steam and WFI are required for cleaning and sterilization of the bioreactors and supporting transfer lines. These systems are very expensive to build and validate, increasing the capital cost significantly. Cost of the stainless-steel bioreactors is also high and their validation takes a long time. In addition, the reuse of the bioreactor from one run to another can take a week for turnover (from one batch to another) or a couple of months for changeover (from one product to another). The methods used to clean bioreactors—both manual and automated clean-in-place—should be validated. Equipment surfaces and rinses should be assayed for residual product, medium, cleaning agents, and microorganisms. Specifications for residuals are calculated using assay data or limits of detection; product yields, purification methods, and clinical doses; and data from other cleaning validation studies.

Because they are hard-piped, stainless-steel–based bioreactor systems are difficult to modify. They are operated in a controlled environment by strictly following established procedures that were previously validated using expensive and time-consuming efforts.

Disposable bioreactors have been developed in the last decade and are now a viable option as bioreactors in clinical and commercial production facilities [13, 16, 25–27]. WAVE bioreactors have played an important role in cell culture


**Fig. 7** A stainless-steel bioreactor with instrumentation and piping for sterilization, gas flow, and fluid transfer

since their introduction in 1998. These bioreactors are basically biocompatible plastic bags that are agitated and aerated using a rocking mechanism on a platform. The WAVE systems are available in different sizes varying between 1 and 500 L and they are used mostly in the inoculum train expansion stage. A 500-L WAVE bioreactor can be used in small- to medium-size production but process scalability is an issue.

The stirred-tank design allows for the use of conventional design and scale-up principles for disposable bioreactors. Due to extensive efforts from many companies in the last decade there are several disposable bioreactor options on the market (Fig. 8). These bioreactors cover a volume range between 2 and 2,000 L. Most important, they have been implemented successfully in cGMP production. A 1,000 or 2,000-L disposable bioreactor can also meet the demands of a commercial-scale production when it runs a high yield process [16]. Disposable bioreactors do not need cleaning and sterilization and they can be installed and validated rather quickly. Facilities using disposable bioreactors can be easier to construct, validate, and they are cheaper to operate. Because there are no hard-pipes connecting the bioreactors to support utilities, disposable systems allow flexible, compact, and modular facility design. Disposable bioreactors are manufactured from materials that are compatible with culture medium and supplements, cells, and commonly used buffer and salt solutions. The materials of construction should meet compendia requirements such as those in the USP for class VI materials; there are similar requirements in the EP and JP [25, 26]. A biopharmaceutical producer may want to generate an in-house technical report that


**Fig. 8** Disposable bioreactors from different vendors. The bag used in the stirred-tank disposable bioreactors contains an impeller and a sparger. The probes are either integrated as part of the bag or they are inserted using an aseptic method after the bag is in place

confirms the compatibility of the disposable bioreactor with their product and process; data provided by the manufacturer of the bioreactor along with data from the literature can be used to support the conclusion that the bioreactor is compatible with the product.

A disposable stirred-tank bioreactor utilizes a presterilized (gamma irradiated) bag that contains all of the inlet and outlet ports necessary for liquids and gases. The bag also contains ports for medium, cell, and feed additions, probes, sampling, and inlet and outlet gas filters. The bag is held in place in a steel holder (support vessel) after being inflated and filled by the medium (and the cells). The support vessel can contain heating elements (electric heating blanket) or can be jacketed for temperature control.

The bag contains an integrated impeller and a sparger (Fig. 9) as part of the mixing and aeration system [16, 25, 26]. Mixing the bioreactor using an impeller in a bag is complicated and it needs a special design. Use of a superconductive levitation system is used in LevTech systems (Sartorius) and Xcellerex uses a magnetically driven impeller. The impeller is located at the bottom and its efficiency for mixing, therefore, is affected as the scale goes up (more liquid depth). The ThermoFisher (HyClone) system uses a pitched blade impeller attached to a piece of silicone tubing, which is connected to a plastic mixing gear at the top. A solid (steel) shaft is inserted through the silicone tubing and it latches onto the impeller allowing the impeller to be operated through the mixing gear. The shaft is angled in the ThermoFisher system and the impeller is located at the liquid level allowing for good mixing without the need for any baffles in the bioreactor. In the

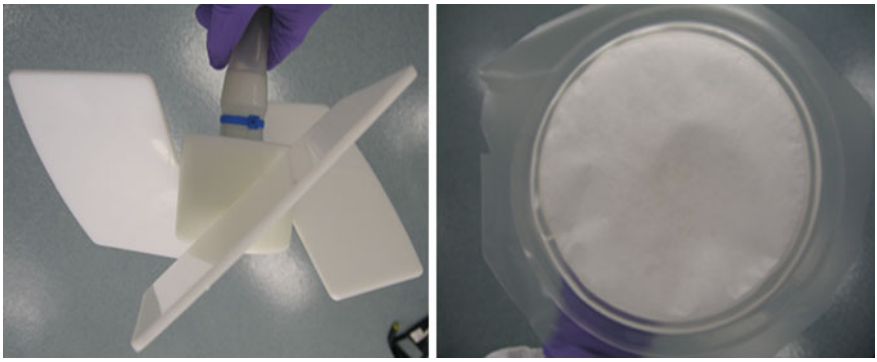

**Fig. 9** The impeller (*left*) and the Tyvec® sparger (*right*) used in ThermoFisher (HyClone) disposable bioreactors [25]

Sartorius system, two polycarbonate impellers are located on a shaft centered in the bioreactor.

Sparging in the bag is accomplished using a microsparger with 10–20 um diameter pores, although some designs contain a second sparger, a macrosparger, for $CO_2$ stripping. The microsparger can be designed as a sparging tip on a piece of tubing but it can also be designed as a Tyvec® patch on the bag as seen in Fig. 9 [16, 25]. Use of probes in the disposable bioreactor poses design and operational challenges. Fluorescent-based detection of DO and pH is possible and there are single-use probes that can be applied as sterilizable patches to the bags. These patches can be used with an external fluorescent light source to monitor the cell culture. Although they have the potential, these probes are not robust enough to be used reliably for cell culture. As mentioned before, the main issues are calibration drift and lifetime of the patch. Every time a reading is performed, fluorescent light is applied to the patch and a small quantity of the fluorescent dye in the patch is degraded. Thus, each patch has a lifetime, or a maximum number of measurements it can deliver. One strategy to deal with this issue is not to read the pH and DO values continuously but intermittently, every 1–2 s, for instance. Even though this strategy can extend the lifetime of the probe, it affects the pH and DO control.

Conventional pH and DO probes can be used in disposable bioreactors but they need to be sterilized and inserted into the bag separately [16, 25]. Insertion of a probe aseptically into the bioreactor is a risky and technically challenging process. One common way of performing this task is to use Pall KleenPak connectors with a bellow (Fig. 10). These connectors come as pairs, each sealed off by a removable film at each end. The end of each pair is pushed against the other until they latch each other. The film from each pair is peeled out together ensuring the sterility of the connection. Most of the bioreactor bags contain ports with sealed KleenPak connectors. The pH or DO probe is autoclaved in a special bellow with a KleenPak connector at the end. The sterile probe is then connected to the bioreactor by pairing the KleenPak connectors first. After the connection is made, the probe is

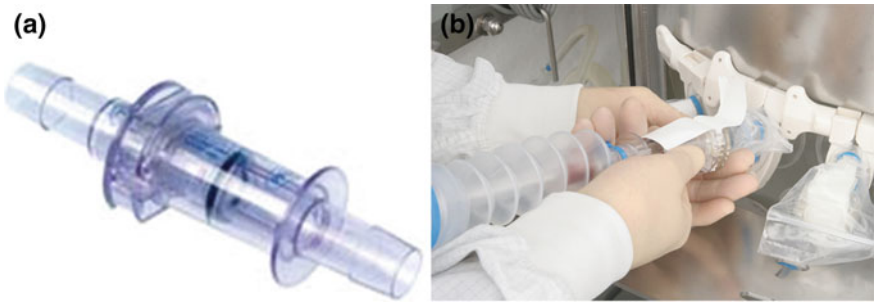


**Fig. 10** **a** Pall KleenPak connector and **b** its use to insert conventional probes to a disposable bioreactor [25]

pushed into the bioreactor, the bellow compresses, and the probe is ready to use (Fig. 10).

Many companies have used disposable bioreactors and their scalability has been demonstrated extensively [25–27]. Process and product comparability was studied for several products and disposable bioreactors were demonstrated to be comparable to stainless-steel–based counterparts.

## 8 Cell Retention Devices Used in Perfusion Bioreactors

As mentioned before, continuous perfusion is an essential part of cell culture and cell retention devices are key equipment for its operation. Many systems are available for cell retention and some of them are presented in Fig. 11 [18, 19]. These systems utilize filtration, sedimentation, and centrifugation for separating and retaining the cells in the bioreactor [18].

Filtration can be used as a cell retention method but filter clogging is an important issue for long-term performance. Using a tangential flow system instead of depth filtration can result in longer operation, as the cells and any cell debris are not directly pushed against the filter and the filters are kept "clean" by the tangential flow applied. The problem with traditional tangential filtration is the use of pumps and the shear induced to the cells during the operation. Alternative tangential flow (ATF) uses a fast-moving diaphragm instead of the pump (Fig. 12) and a hollow-fiber system to retain the cells [15, 18].

During operation of the ATF unit, the air pushes the diaphragm up in the pressurization cycle forcing the fluid in the fibers back to the bioreactor. Pressure from the bioreactor, or vacuum applied to the diaphragm allows the culture to move back to the fibers. During this cycle the fibers are flushed and the tangential flow generated "cleans" the fibers. The filtrate is constantly pulled from the system allowing a continuous perfusion. The ATF units can operate at high cell densities and for long time (2–4 months) without clogging [17, 18].

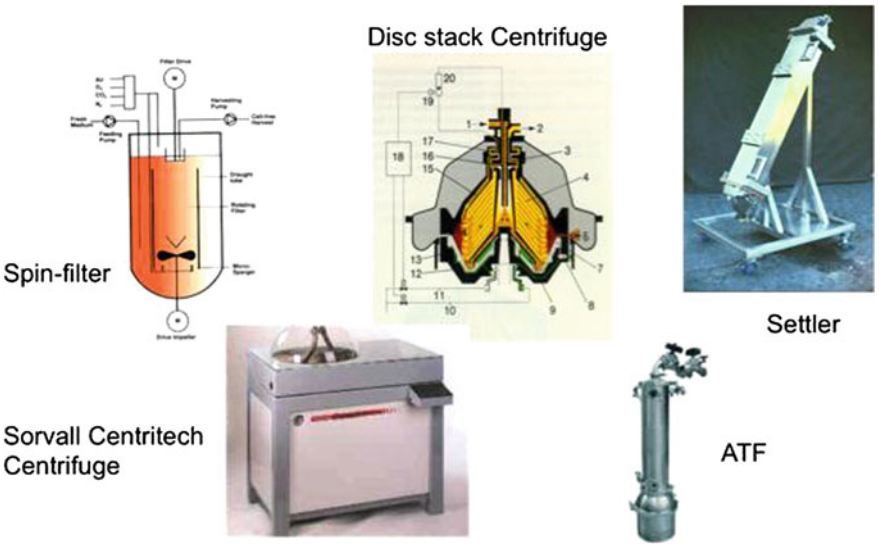


**Fig. 11** Cell retention devices used for perfusion bioreactors

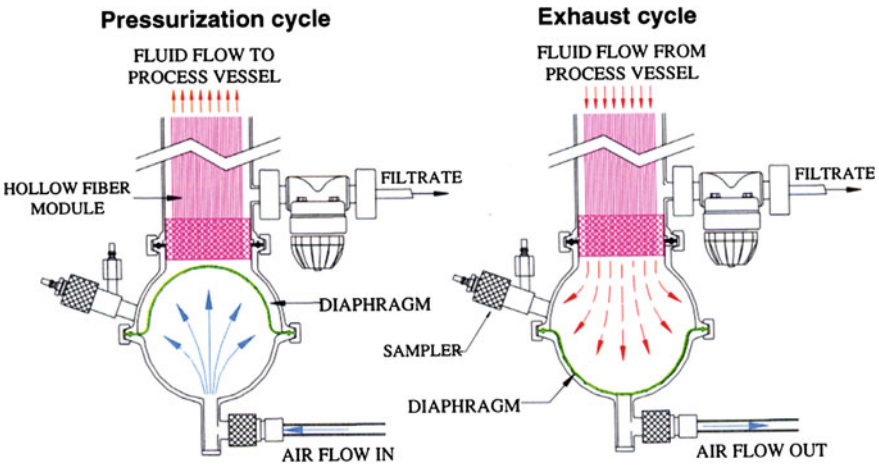


**Fig. 12** Operation of alternating tangential filtration (ATF) used for cell retention [15]

Another filtration-based cell retention device is the spin filter and it can be used internally and externally. The spin filter utilizes a fast spinning screen-based basket with a pore size of 10–100 um. Even though the pore size can be bigger than the cell size (10–20 um), the spinning motion prevents cells from passing through the screen by a lifting mechanism. Fouling of the screen over time and filter clogging are the main issues with spin filters. Screen size, rotation speed, structure of the weaving, screen material, flow rates, and so on can be optimized to operate the system for a longer time and for achieving higher cell densities.

Centrifugation offers cell retention without clogging but it has the disadvantages of low cell retention, operational complexity, and cleanability issues. Although batch centrifugation is an easy process for pelleting the solids and separating them out, continuous operation is rather complicated. Increasing the rotation speed can enhance centrifugation efficiency but this action results in compacting the cells, making them difficult to return to the bioreactor. Use of multiple stacks in a centrifuge helps in separating the cells from the medium significantly but it introduces cleaning issues. The spaces between the stacks are very difficult to clean after use. The Centritech centrifuge uses a disposable bag that separates the cells and periodically sends them back to the bioreactor [18].

Inclined settlers have the advantage of no moving parts and they are easy to build [18]. They contain multiple plates to increase the settling area, thus affording separation efficiency. The effectiveness of a settler for cell separation depends on the flow rate through the settler, cell density, cell size, and the degree of aggregation in the culture. Although a cell settler can be very effective for aggregated cells, it has very low separation efficiency for single-cell suspensions.

## 9 Conclusions

In this chapter we described the equipment used in typical large-scale cell culture processes. Seed train, inoculum train, and production bioreactor were covered in detail. Types of bioreactors and their operational modes were explained. Mixing and aeration in bioreactors and the types of impellers and spargers were outlined. Both conventional (stainless-steel–based) and disposable bioreactors were illustrated. Finally, cell retention systems were covered in the context of a continuous perfusion operation.

**Acknowledgments** I would like to thank Dr. Mark Leney and Dr. Roger Anderson for the review of the chapter and for their valuable comments.

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Adv Biochem Eng Biotechnol (2014) 139: 93–121
DOI: 10.1007/10_2013_217

Published Online: 5 July 2013

# Development and Characterization of a Cell Culture Manufacturing Process Using Quality by Design (QbD) Principles

**Daniel M. Marasco, Jinxin Gao, Kristi Griffiths, Christopher Froggatt, Tongtong Wang and Gan Wei**

**Abstract** The principles of quality by design (QbD) have been applied in cell culture manufacturing process development and characterization in the biotech industry. Here we share our approach and practice in developing and characterizing a cell culture manufacturing process using QbD principles for establishing a process control strategy. Process development and characterization start with critical quality attribute identification, followed by process parameter and incoming raw material risk assessment, design of experiment, and process parameter classification, and conclude with a design space construction. Finally, a rational process control strategy is established and documented.

**Keywords** Cell culture process characterization · Cell culture process development · Cell culture process scale-up · Control strategy · Critical quality attribute · Design space · Quality by design · Risk assessment

**Abbreviations**

| | |
|---|---|
| QbD | Quality by design |
| QTPP | Quality target product profile |
| CQA | Critical quality attributes |
| DOE | Design of experiment |
| CPM | Control point matrix |
| FMEA | Failure modes and effects analysis |

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## 1 Introduction

The quality by design (QbD) concepts embodied in the International Conference on Harmonization (ICH) guidelines Q8(R2), Q9, Q10, and Q11 have been applied to cell culture manufacturing process development and characterization [1–4]. The January 2011 revised FDA Guidance for Industry, Process Validation: General Principles and Practices, integrates QbD principles into process validation practices [5]. These guidance documents outline the application of QbD principles in the lifecycle of a product from process design, process definition, and process characterization to process validation and continued process verification. The expectation from regulatory agencies is that quality is designed or built into the product and its manufacturing process and quality cannot be adequately assured by testing [5]. The benefit of QbD is twofold: one is to provide a high level of assurance for product quality through lifecycle management of the product; the other is the potential for flexibility in the reporting responsibilities for movements within a registered design space [1].

The implementation of QbD principles means product characteristics are designed and fully understood and their linkage to patient safety and clinical efficacy is established, the interaction between critical product quality attributes and its manufacturing process are fully characterized, and control strategy

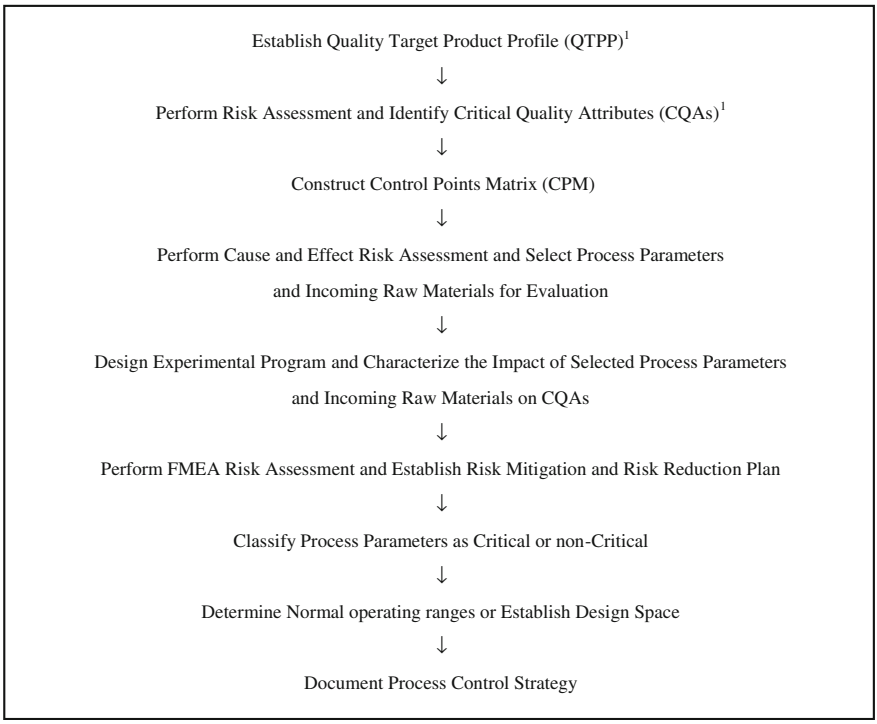



**Fig. 1** Overall QbD approach in developing and characterizing cell culture manufacturing process for establishing a process control strategy. The QTTP and CQA risk assessment are beyond the scope of this chapter

including design space is established to ensure that the manufacturing process is capable of consistently producing the product with the desired quality attributes [6, 7]. Figure 1 presents our approach in applying QbD principles to developing and characterizing a cell culture manufacturing process for establishing a process control strategy.

Development of a cell culture manufacturing process control strategy starts from identifying drug substance critical quality attributes based on the quality target product profile (QTTP). Critical quality attributes (CQAs) are identified through risk assessment that evaluates severity based on impact on patient safety and/or clinical efficacy [8]. The list of CQA(s) evolves during the development lifecycle. Then, a matrix is created to describe the interaction between critical quality attributes and process unit operations based on previous process development work, platform knowledge, literature information, and first principles. This control point matrix (CPM) visually indicates the origin, growth, reduction, or clearance of the quality attributes over the entire drug substance manufacturing process and demonstrates the process control points for each critical quality attribute.

Using the CPM as a guide, initial process parameter risk assessments are performed to evaluate the impact of process parameters and incoming raw materials systematically, within common cause variability, on critical product quality attributes. Process parameters are selected based on risk assessment for empirical evaluation using design of experiments (DOE) utilizing a qualified scale-down model. The purpose of the initial characterization study is to link process parameters to critical quality attributes. A resolution III or IV, fractional factional DOE is conducted depending on the number of parameters to be evaluated. Process parameters having statistically significant impact on CQA(s) are selected for further study using response surface DOE. The functional relationships between these process parameters and CQA(s) are fully characterized. A secondary risk assessment, failure mode and effects analysis (FMEA), is performed during technology transfer to the commercial manufacturing site. Risks identified during the FMEA are further reduced or mitigated through process excursion and/or process challenge studies.

Process parameters are classified as critical or noncritical postprocess characterization studies. The classification is performed based on risk assessment and experimental results from process characterization studies. Based on risk assessments conducted throughout the development lifecycle, those process parameters assessed as not likely to affect CQAs are classified as noncritical. For process parameters evaluated in characterization studies, if a parameter is both statistically significant and practically significant in affecting CQA(s), it is classified as critical. Otherwise, it is classified as noncritical.

A design space/operating space is constructed post parameter classification. Per ICH Q8, design space is the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality.

A cell culture process control strategy is established and documented based on information generated through risk assessments and process characterization studies during the development lifecycle. The establishment of analytical control strategy and microbiological control strategy is beyond the scope of this chapter.

In the next sections, we describe our practices for process parameter risk assessments, CQA-driven process characterization by design of experiment, process parameter classification, design space/operating space construction, and process control strategy establishment.

## 2 Development and Characterization of Cell Culture Manufacturing Process for Establishing a Process Control Strategy

The process development lifecycle consists of process design, process definition, process characterization, process validation, and continued process verification.

**Table 1** Control points matrix describing the probable quality attribute control points

| *Critical quality attribute* | *Analytical method* | *Unit operation influencing CQA(s)* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *1* | *2* | *3* | *4* | *5* | … | *N* |
| *CQA #1* | | | | O | | | | |
| *CQA #2* | | | | O | | | | |
| *CQA #3* | | O | | | ↑ | X | | |
| *CQA #4* | | | | O | ↕ | | X | ↓ |
| *CQA #5* | | O | | | X | ↓ | | |

O Origin of attribute at this unit operation
↑ Growth of attribute at this unit operation
↓ Reduction of attribute at this unit operation
↕ Potential for growth or reduction of attribute at this unit operation
X Significant reduction/clearance of attribute at this unit operation

After definition of an initial baseline process, characterization studies are initiated to understand fully the impact of process parameters and incoming raw material attributes, within common cause variability, on critical quality attributes. Process characterization starts with risk assessment. The intention of the initial risk assessment is systematically to evaluate the potential risk of process parameters and incoming raw material attributes from each unit operation, within common cause variability, on critical quality attributes. A cause and effect methodology is utilized in the initial risk assessment.

## *2.1 Construct CQA(s) Control Points Matrix*

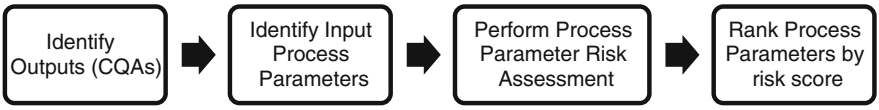
Prior to initializing process characterization, sufficient information should be available to describe, or reasonably estimate, the relationship between the unit operations and critical quality attributes. In order to facilitate the initial cause-and-effect risk assessment, a unit operation-based, control points matrix (CPM), is created to describe the probable control points (one or many) for each critical quality attribute. The matrix should include the most likely origin, growth, reduction, or clearance of the critical quality attributes across the entire drug substance manufacturing process.

An example of a unit operation-based control point matrix is displayed in Table 1. The control points matrix is used to guide the process parameter risk assessment by allowing unit operation characterization studies to focus only on the relevant critical quality attributes that are significantly influenced by the purpose or design intent of the unit operation. The control points matrix is updated as additional information becomes available.

## 2.2 Initial Process Parameter Risk Assessment

Initial process parameter risk assessments are based on process knowledge, that is, a combination of practical experience and theoretical understanding. The process parameter risk assessment is performed iteratively throughout the development lifecycle to prioritize development efforts. Depending upon an organization's experience and relative level of comfort conducting these risk assessments, they may be performed by a subject matter expert, or by a cross-functional team. Per ICH Q6, the degree of rigor and formality of quality risk management should reflect available knowledge and be commensurate with the complexity and/or criticality of the issue to be addressed.

The initial process parameter risk assessment is performed in four basic steps: (1) identify output, (2) identify input process parameters, (3) evaluate the probable risks, and (4) rank the process parameters by risk score.




The results from the risk assessment guide and prioritize the experimental program used to characterize each unit operation of the cell culture manufacturing process.

### 2.2.1 Identification of Outputs

Critical quality attributes are the main output analyzed in the initial process parameter risk assessment. Process performance indicators may also be considered.

### 2.2.2 Identification of Input Process Parameters

The inputs, or process parameters, are identified based on the operational knowledge and mechanistic understanding of each unit operation in the manufacturing process. A cause and effect diagram is a useful tool to organize and group process parameters systematically by function. The cause-and-effect diagram is constructed by placing the output (i.e., product and process attributes of interest) at the right side of the diagram, with the potential design factors (i.e., process parameters and incoming raw material attributes, e.g., concentration accuracy) on a series of branches and subbranches extending from the output axis. The process parameters can be grouped by function or process step to ensure no process parameters are overlooked.

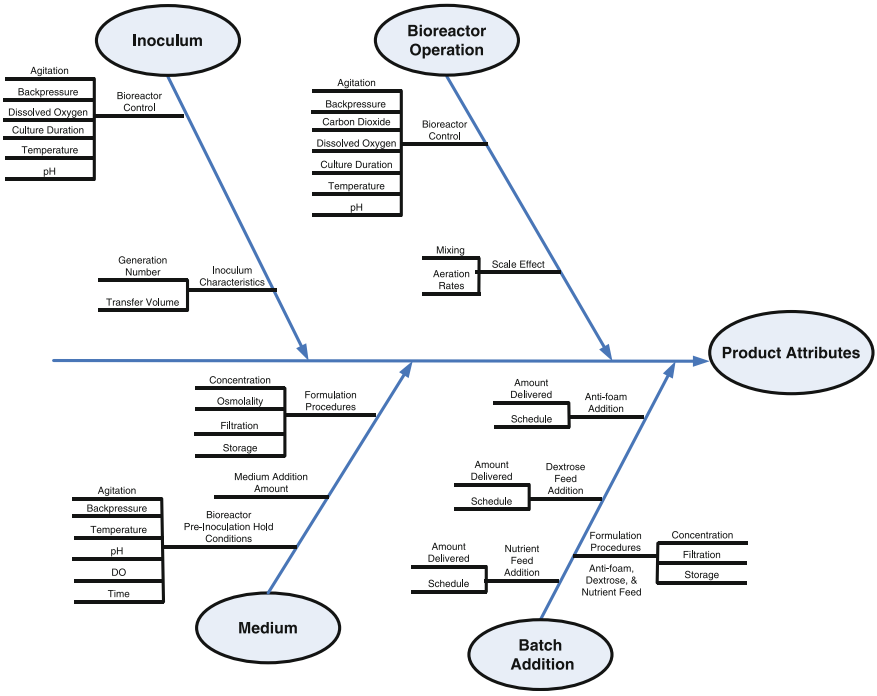



**Fig. 2** Cause-and-effect diagram indicating the process parameters analyzed in the process parameter risk assessment

The level of branching can be moderated to facilitate efficient communication to ensure the level of detail is appropriate. An example cause-and-effect diagram describing a typical production bioreactor process is given in Fig. 2 [9].

### 2.2.3 Risk Analysis

After identifying the relevant process outputs (CQAs) and process inputs (process parameters) for each unit operation, the risks of common cause variability in the input parameters that may affect the output parameters are assessed. The risk analysis is based on first principles, literature information, platform knowledge, manufacturing experience, scientific judgment of the subject matter experts, and molecule-specific empirical knowledge.

The process parameters can be classified into two groups: those that have the potential to affect critical quality attributes and those that do not. Process parameters that do not have the potential to affect critical quality attributes may be assigned a low risk score. Typically, low-risk process parameters are not formally studied in laboratory models or designed experiments and are classified as

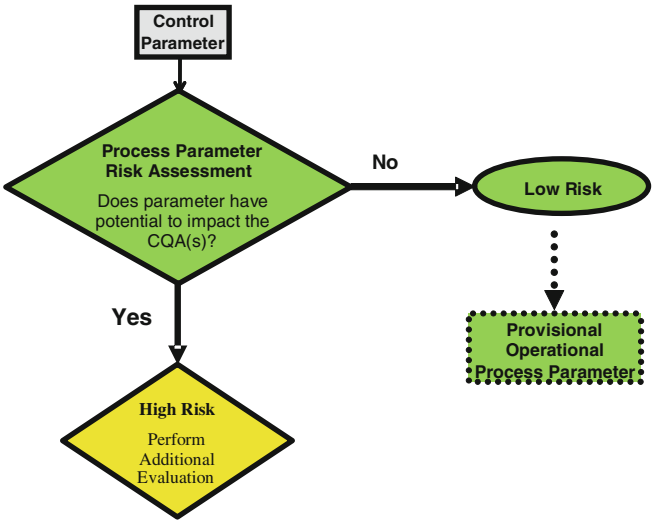



**Fig. 3** Logic diagram describing the initial process parameter risk assessment

noncritical with appropriate rationales. The remaining process parameters are classified as high risk, thus, they may have the potential to affect critical quality attributes and require additional evaluation to better understand, reduce, or mitigate risks. The process parameter risk assessment follows the logic diagram presented in Fig. 3. The initial process parameter risk assessment is an integral part of the development of a control strategy; therefore, this assessment should be adequately documented.

### 2.2.4 Raw Material Risk Assessment

The risks of variability inherent to the cell culture raw materials used to manufacture drug substances on CQA(s) are evaluated in the development lifecycle. The raw material components are analyzed to assess the intrinsic risk (use of the correct raw materials) and the extrinsic risk (lot-to-lot variability) on CQA(s) and other quality attributes. The assessment includes the risks introduced from a quality, technical, and procurement perspective. The initial risk assessment occurs prior to the manufacture of pivotal clinical materials, and is reassessed as the process evolves. For example, technology transfer and/or changes in the process or supply chain may initiate a reassessment.

The evaluation of raw material risk utilizes a series of weighted risk elements based on their criticality to the product or process, and the risk to the patient. Each raw material is assigned a three-tiered risk score (low = 1, medium = 3, or high = 5) for each risk element using a combination of platform knowledge, manufacturing experience, opinions of the subject matter experts, and molecule-

specific empirical knowledge. The summation of the individual risk scores multiplied by the risk element weight is calculated for each component. These values are used to rank the relative risks for each raw material component. As an example, the risk elements, and their respective weights, are described in table.

| Description of risk elements |
|---|
| Weight = 5 |
| • Variability has the potential to affect the drug substance quality attributes |
| • Ability of raw material to introduce bioburden, endotoxin, viral contaminates |
| • Known issues with raw material |
| Weight = 3 |
| • Molecular complexity |
| • Potential to affect process performance |
| Weight = 1 |
| • Experience with vendor |
| • Manufactured for pharmaceutical industry |

## *2.3 Risk Mitigation/Initial Process Characterization Experiments*

Following the identification of high-risk process parameters and raw materials, an experimental program is designed to characterize and mitigate the risks of identified process parameters on critical quality attributes within common cause variability.

### 2.3.1 Experimental Strategy

The experimental program is designed to characterize the manufacturing process to ensure consistent robust manufacturing capability. The high-risk process parameters are studied in a series of designed experiments intended to understand and mitigate potential risks further. Scale-independent process parameters are explored using a laboratory scale-down model. Scale-dependent parameters may be studied using intermediate or at-scale bioreactors.

The experimental program is typically initialized utilizing a highly leveraged design of experiments of a resolution sufficient to identify the main effects and some quadratic effects. Depending upon the number of relevant process parameters identified in the risk assessment process, a single or a series of screening experiments can be planned. Multivariate fractional factorial design of experiments of resolution III or IV run using one or several blocks are common. Based on the output from the screening experiment, additional studies may be performed to

characterize parameters further that have a statistically and practically significant effect on critical quality attributes.

Prior to designing experiments, the high-risk process parameters should be examined while acknowledging that not all process parameters are independent of each other (i.e., medium strength and medium osmolality). Potential correlations should be identified and taken into consideration.

### 2.3.2 Process Parameter Range of Interest

During cell culture manufacturing process characterization studies, the target setpoints of process parameters are determined based on process design and definition experimentation; process parameter ranges selected are intended to evaluate the impact of common cause variability in operations on critical quality attributes. Common cause variability is defined as the expected level of variability experienced during normal unit operations in a manufacturing environment when executed according to the batch record instructions.

The range of interest is determined from the current understanding of the at-scale control capability using a combination of operational variability, or the variance from target setpoints, and the measurement uncertainty of the device(s) that record the process measurement.

The operational variability is a measure of performance derived from sampling unit operations in the clinical manufacturing or commercial manufacturing facilities. The range encompassing common cause variability is chosen so that the probability of the parameter values being within the range of the target setpoints ± operational variability is at least 0.995 (or 99.5 %). Generally, six times the operational variability is selected to ensure that the values of a given process parameter will fall within this range irrespective of the underlying distribution [10].

The measurement uncertainty characterizes the dispersion of the values that could be reasonably attributed to the measurement. The measurement uncertainty is designed to reduce the false acceptance rate and is selected to ensure 95 % of the recorded measurements fall within the desired range. The measurement uncertainty is derived from either the measurement system design specification or historic calibration performance [11].

The summation of operational variability (containing 99.5 % of the observed values) and measurement uncertainty (containing 95 % of the recorded measurements) defines the recommended minimum range of interest used to characterize the process, as displayed in Fig. 4.

### 2.3.3 Laboratory Scale Models for Process Characterization

In most scenarios, performing process characterization studies at the manufacturing scale is not practically feasible due to the cost of operation, and limited availability of large-scale bioreactors. Therefore, laboratory scale models are used

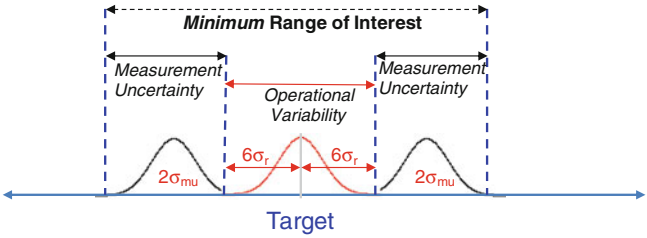



**Fig. 4** Determination of the range of interest for process characterization experiments

to perform process characterization experiments that define acceptable process ranges and establish predictive relationships between the scale-independent process parameters and critical product quality attributes. This approach is in alignment with ICH guidance [4]; small–scale models can be developed and used to support process development studies. The development of a model should account for scale effects and be representative of the proposed commercial process. A scientifically justified model can enable a prediction of product quality, and can be used to support the extrapolation of operating conditions across multiple scales and equipment.

The cell culture manufacturing process includes a series of shake flasks and conventional stirred-tank or disposable bioreactors to manufacture the unprocessed bulk drug substance. The culture expansion steps have a limited potential for impact on critical quality attributes due to negligible accumulation of product; therefore the focus of the scale-down model is typically on the production bioreactor unit operation.

The bioreactor configuration has five primary control loops intended to measure and control culture temperature, dissolved oxygen, culture pH, agitation rate, and vessel pressure by manipulating caustic and acidic pH control loops, air, oxygen, and carbon dioxide gas flow rates, vessel jacket heat exchanger, and the agitator drive. An example P&ID (piping and instrumentation diagram) is provided in Fig. 5.

The cell culture process parameters can be separated into two groups including scale-dependent and scale-independent parameters. The operating conditions for scale-independent parameters (i.e., temperature, pH, dissolved oxygen concentration) are conserved across different scales. The scale-dependent parameters (i.e., agitation rate, gas flow rates, nutrient addition volume) are adjusted to conform to the scaling strategy employed.

The scale-dependent parameters included in a bioreactor system are driven by gas–liquid and liquid–liquid mixing with the associated mass and heat transport phenomena. Mixing systems do not scale proportionally in all dimensions; therefore a basis for scaling up mixing unit operations must be chosen by balancing the characteristics that are important to the process under consideration. Scaling strategies are typically based on a combination of geometric similarity, kinematic similarity, dynamic similarity, and/or power per unit volume input.

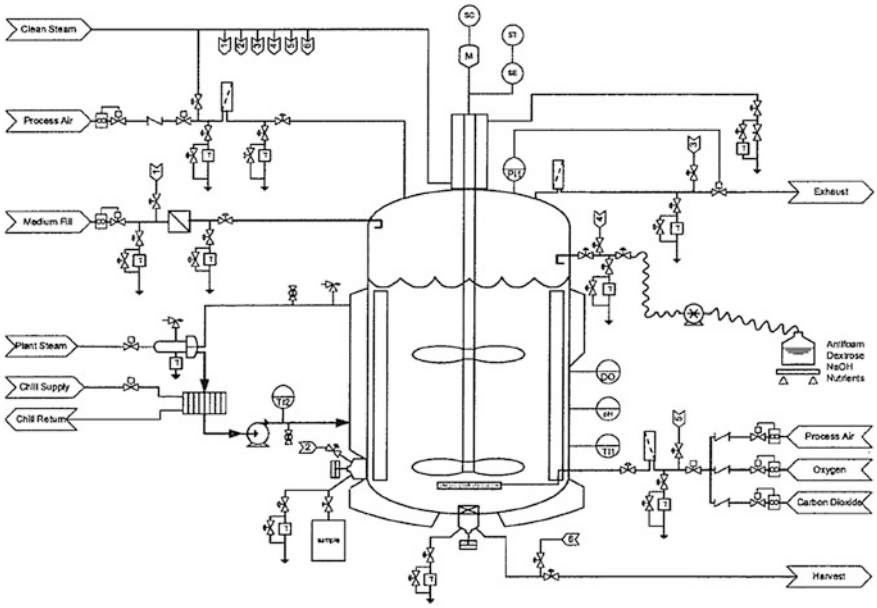



**Fig. 5** Example bioreactor piping and instrumentation diagram

Typically two of the four methods are selected, allowing the other characteristics to change. Bioreactor unit operations used for mammalian cell culture processes are usually scaled up by conserving the power per unit volume with geometrically similar vessels.

When scaling up on the basis of geometric similarity and constant power per unit volume, the relative agitator tip speed and the bulk mixing time increase. Increasing the agitator tip speed may increase the risk of shear damage to the cells; however, prior experiments have demonstrated that the risk of damage is minimal over the normal operating range of interest. Increasing the bulk mixing time will result in an increased risk of vessel heterogeneity which could affect the product's critical quality attributes and process performance. Equipment design and additional experiments should be considered if there is a high risk of vessel heterogeneity affecting culture performance or critical quality attributes.

In cell culture processes the proper scaling of gas flow rates to control dissolved carbon dioxide and dissolved oxygen levels is not trivial. As the process is scaled up, the mass transport of oxygen increases with vessel volume leading to a decreased volumetric flow rate of oxygen necessary to meet the culture demand. The resulting decrease in volumetric flow rate reduces the capability to remove carbon dioxide. An air balance is required in the sparger line to provide a sufficient volumetric flow for carbon dioxide removal. In addition, the medium chemistry and the profile of metabolic by-products (i.e., lactate concentration) may lead to a feedforward control strategy based on the interaction between dissolved oxygen

and pH control loops. In our system, the gas sparger configuration may be specified so that the amount of gas flow needed to maintain the dissolved oxygen control is the amount of gas needed for carbon dioxide removal. The carbon dioxide management in the at-scale and intermediate-scale bioreactors may be determined through process models that simultaneously solve the chemistry equilibrium and mass transfer equations through the course of the run assuming that the oxygen uptake rate and significant metabolic by-products are defined by the process conditions. The models are used to define a target air flow rate that allows for carbon dioxide off-gassing.

The interaction between multiple scale-dependent control loops presents additional challenges when scaling down cell culture processes to the laboratory bench scale. The power per unit volume is difficult to determine as the standard vessel geometry is modified to accommodate the reduced scale. In addition, the ratio between culture volume and surface area in contact with the head space increases, influencing the mass transfer rates for gases. As a result controlling the $pCO_2$ concentration at the laboratory scale is difficult to model. Additional experiments may be performed to understand the risks better that elevated carbon dioxide levels have on culture performance and/or product critical quality attributes.

The capabilities of the laboratory scale models are monitored throughout the development lifecycle and the risk, whether the scale-down models are representative of at-scale processes, is analyzed as sufficient large-scale information becomes available. The laboratory-scale models are analyzed by comparing results between the scale-down and at-scale processes for outcomes including critical quality attributes, other product quality attributes, and process performance indicators.

The scale comparison data for quality attributes are explored using statistical methods. The data from bioreactors run at process targets in the scale-down model (from process characterization and process design and definition studies) are compared to the data generated from at-scale clinical material manufacturing campaigns. An equivalence test (two-one-sided *t* test, TOST) with a predefined practical difference is used to test for equivalency between critical and other product quality attributes [12]. A practical difference threshold should be sufficient to support the claims, or intended use of the scale-down model. Based on these criteria, the suitability of the scale-down model relative to the at-scale process can be assessed.

The process performance indicators are also explored qualitatively by examining the process trends over time. Comparisons are made relative to the directionality and closeness of the time-series data.

If the performance of the scale-down model is not equivalent, additional analysis should be performed to determine if the process characterization results are sufficient to construct an adequate control strategy. If not, additional work should be performed to develop a better model, or generate additional data to mitigate risks.

### 2.3.4 Initial Process Characterization Experiments

The initial process characterization experiments are designed to evaluate the linkage between the scale-independent process parameters identified in the initial process parameter risk assessment and critical quality attributes. The process parameters are evaluated in the scale-down model exploring ranges that span the minimum range of interest. The process outcomes include the critical quality attributes influenced by the unit operation and any process consistency measurements used for routine process monitoring.

The experimental data are analyzed using a least-square regression analysis methodology to generate empirical models to estimate the relationship between process parameters and the critical quality attributes.

The signs and magnitude of the regression coefficients are examined to determine which process parameters have a statistically significant ($p < 0.05$) and meaningful impact on the process outputs. A meaningful effect is determined using the scientific judgment of subject matter experts when considering the potential sources of variability (analytical, operational, process, and model variability). The square-root sum of squares methodology is used to quantify a reasonable cutoff to identify a meaningful effect. For many quality attributes in the cell culture process an effect with a magnitude of >10 % is considered meaningful.

The outputs from the initial process characterization experiments typically reduce the number of high-risk process parameters to evaluate in higher-resolution DOEs.

## *2.4 Final Characterization Experiment*

The high-risk process parameters identified in the initial characterization experiments have a high probability of being classified as critical process parameters; therefore additional work is required to understand fully the functional relationship between the process parameters and critical quality attributes. Additional experiments may be designed to fill in the response surface to elucidate fully the design space by collecting the data necessary to resolve any interactions and/or quadratic effects that may be present within the proposed operating range. The experimentation required is dependent upon the level of information available. In addition, experimentation may also provide an opportunity to "re-center" the process targets to optimize process robustness prior to the manufacture of stability-indicating batches.

## *2.5 FMEA Process Parameter Risk Assessment*

After the process characterization experiments are complete, or during technology transfer, a second risk assessment is performed to ensure the control capability of the manufacturing facility meets the manufacturing process requirement. This risk

**Severity:**
Failure to control the process parameter results in a CQA outcome of the intermediate

- 9 = outside of
- 4 = near to
- 1 = well within

…the capability of the downstream unit operations

**Occurrence:**
Failure occurs:

- 9 = >10%
- 4 = 1-10%
- 1 = very unlikely

**Detectability:**
Failure is detected

- 6 = almost never, no capable measurement of process parameter or not enough ability to react
- 3 = sometimes/some capability to measure process parameter or partial ability to react
- 1 = almost always; capable measurement of process parameter and enough ability to react

**Fig. 6** Scores of severity, occurrence, and detectability in FMEA risk assessment

assessment examines the holistic control strategy encompassing all unit operations used to manufacture the drug substance to ensure that a comprehensive control strategy could be developed from the resulting data.

The secondary risk assessment uses a failure modes and effects analysis methodology to provide for an evaluation of potential failure modes for the process and their likely effect on outcomes and/or process performance. Once the failure modes are established, risk reduction can be used to eliminate, reduce, contain, or control the potential failures. The goal of the FMEA is to guide actions to eliminate or reduce potential future failures, starting with the highest-priority process parameters.

The FMEA risk assessment is performed by a cross-functional team of participants with an in-depth technical knowledge of the manufacturing process and control capability of the manufacturing facility. The assessment is led by a facilitator familiar with the quality risk management process who is independent of the process team. Stakeholders from process development and the commercial manufacturing site are involved in the review meetings.

Process characterization knowledge, which defines the link between process parameters and critical quality attributes, is required prior to performing the FMEA risk assessment. A basic knowledge of the magnitude of the impact of process parameters on CQA(s) and an understanding of the integration of the drug substance manufacturing process across multiple unit operations are necessary before performing the analysis. Each process parameter that can affect a CQA is considered a potential failure mode. Risk scores are assigned to each failure mode in the drug substance manufacturing process for severity, occurrence, and detectability.

To perform the analysis, risk scores are assigned to the severity of the harm, or severity of the negative effect on CQA(s), the occurrence of the cause of the harm, and how likely it is that the cause will be detected and corrected before the CQA(s) is (are) affected. Scores are assigned based on the impact on the CQA(s) of failing

to control process parameters (severity), likely frequency of failing to process parameters (occurrence), and the ability to detect process parameter failure in real-time (detectability).

The scoring of severity is based on the ability of the downstream unit operations to recover from a failure in the process parameter being scored. The risk scores for occurrence are specific for the occurrence rate for the cause of the failure and not the actual failure rate (as it's also dependent upon the ability to detect the failure). In most cases, only an approximate or qualitative estimate of the occurrence will be available. Detectability scores require that the cause of the failure be detected with enough lead time to react, leaving the CQA at an unperturbed level. Any knowledge gaps identified may lead to additional experimentation. Figure 6 describes the scoring rules for severity, occurrence, and detectability.

The risk scores for severity, occurrence, and detectability are multiplied together to arrive at a risk priority number (RPN). Failure modes with the highest RPN scores represent the greatest risk to the process. Judgment should be used to determine if risks are acceptable, or if risk reduction activities are required to control, mitigate, or reduce the potential failures. The outcome of the risk assessment, including any decisions made, should be documented appropriately.

## *2.6 Classification of Process Parameters*

The cell culture manufacturing process design, definition, and characterization studies conducted throughout the development lifecycle define the linkage to and the impact of process parameters on drug substance critical quality attributes. Based on data from these DOE studies and clinical material manufacturing experience, a decision tree, as presented in Fig. 7, is followed to classify process parameters as critical or noncritical [13, 14]. Per ICH Q8, a critical process parameter is defined as a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality.

As the figure demonstrates, based on risk assessments conducted throughout the development lifecycle, those process parameters assessed as not likely to affect CQAs are classified as noncritical and are not studied further. Process parameters that are identified during the risk assessments as having the potential to affect or that are known to affect one or more CQAs are selected for further study. From these studies the relationship between these selected process parameters and CQA(s) is characterized. If a parameter is both statistically and practically significant in affecting CQA(s), it is classified as critical. Otherwise, it is noncritical. In certain instances, select parameters that would have been classified as noncritical by the decision tree (e.g., parameters associated with viral clearance) can be further evaluated and classified as critical based on scientific judgment.

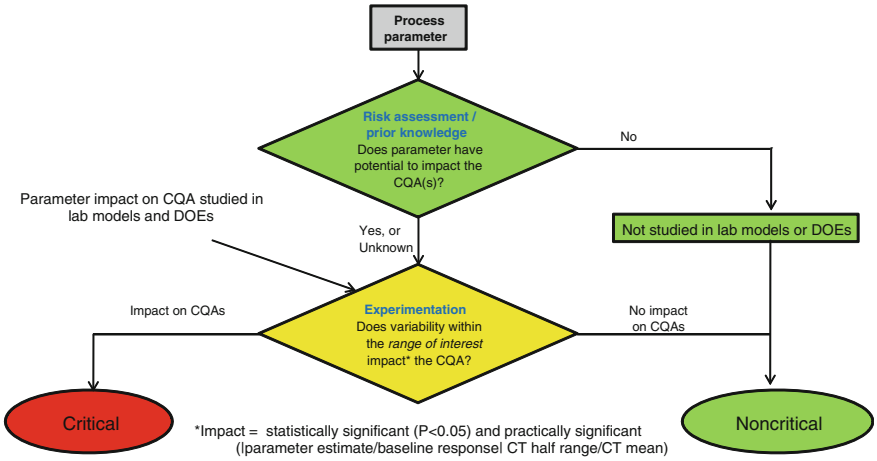



**Fig. 7** Logic diagram used to facilitate the classification of process parameters as critical or not critical

The statistical significance of the impact of a process parameter is determined by utilizing an appropriate statistical model of the experimental data from process characterization studies. If a process parameter is not significant at the 0.05 level (i.e., $p \geq 0.05$), then the impact of this process parameter on the CQA(s) is deemed to be within the variability of the process and the process parameter is classified as noncritical. If a parameter has a significant impact at the 0.05 level (i.e., $p < 0.05$), then the practical significance of the impact of this process parameter on CQA(s) is further evaluated.

The practical significance of the impact of a process parameter is determined by comparing the relative magnitude of the impact of the process parameter on CQA(s) in process characterization studies to the historically observed variability for the CQA(s) from the relevant step of the clinical material manufacturing (CT). The relative magnitude of the impact of a process parameter on CQA(s) is calculated by taking the parameter-scaled estimate from the statistical analysis and dividing by the baseline CQA(s) response, with the result expressed as a percentage. The historically observed variability is taken as half the range of the historical manufacturing data divided by the mean of the historical manufacturing data for a given CQA(s) (expressed as a percentage) for the relevant manufacturing step. If the relative magnitude of the impact of a process parameter is less than or equal to the clinical trial manufacturing process variability, then the impact of this process parameter would be deemed not to have practical significance, and therefore the process parameter would be classified as noncritical. However, if the relative magnitude of the impact of a process parameter is greater than the clinical trial manufacturing process variability, then the impact of this process parameter would be deemed to have practical significance on the CQA(s), therefore the process parameter is classified as critical.

$$\text{Practically significant when: } \left|\frac{\text{Parameter Estimate}}{\text{Baseline Response}}\right| > \frac{\text{CT Half Range of CQA(s)}}{\text{CT Mean CQA(s)}}$$

The variability of clinical material manufacturing is utilized as the comparator because it provides an estimate of the inherent variability of running the process as intended through multiple cycles of each unit operation with most process parameters conducted at their centerpoint condition. By contrast, the unit operation empirical studies intentionally varied process parameter conditions in order to estimate their respective effect on CQA(s). If the empirical studies demonstrated process parameter effects within the CT variability, then there is no practical impact on CQA(s) outside the CT manufacturing experience.

## *2.7 Process Excursion Studies*

Following the classification of process parameters, additional characterization experiments are performed for a better understanding of the impact of transitory excursions that exceed the normal operating range of the critical process parameters. The experiment explored probable failure modes that may temporarily affect a critical process parameter (i.e., pH out of range for between sample points). The experiment tests the robustness of the cell culture process, and the resulting data can be used to support investigations resulting from special cause events.

## *2.8 Construction of the Design Space/Operating Space*

A design space is constructed post parameter classification. The design space is constructed from the empirical knowledge gathered in experimental studies while considering sources of variability.

First, a knowledge space is constructed from the historical process development data representing the space evaluated by experimentation which may include areas of failures. The knowledge space is represented by a multidimensional region bounded by the critical process parameters for the relevant unit operation. Data contained in the knowledge space are analyzed using a least-square regression analysis methodology to generate empirical models to define the relationship between the critical process parameters and the critical quality attributes relevant for the unit operation under analysis. An initial space for each quality attribute is constructed within which the mean levels of critical product quality attributes are within the proposed specifications. This space is further reduced by performing a reliability analysis using Monte Carlo simulation to incorporate model uncertainty, analytical method variation, and process variation. The space is constructed to have a >90 % probability within which the critical quality attributes meet the proposed specifications [14].

The design space for the critical process parameters is determined from the common region of successful operation for the critical quality attributes.

### *2.9 Cell Culture Process Control Strategy*

The process development approach, described above, is used to construct a parametric control strategy for the cell culture unit operations. Starting with the molecule design and the identification of critical quality attributes, a series of risk assessments is performed to construct an experimental program used to characterize the unit operations. Using a systematic approach, a series of multivariate-designed experiments is performed with scale-down models to understand and mitigate risks and establish acceptable ranges for process parameters.

The logic diagram, presented in Fig. 7, is followed to classify process parameters as critical or noncritical based on their ability to influence critical quality attributes. A multivariate design space is established through statistically analyzing the historical development data, or the knowledge space. The operating ranges for the critical process parameters are determined from the common region of successful operation.

## 3 Case Study

An abbreviated case study is presented to demonstrate the methodology used for a protein currently under development.

### *3.1 Construct CQA(s) Control Points Matrix*

Process characterization activities for the upstream process focused on two product-related impurities that are generated in the cell culture bioreactor which are not significantly changed in downstream operations. The initial process parameter risk assessment for the cell culture process focused on these two impurities with the additional platform-specific process-related impurities introduced during cell culture operations.

### *3.2 Initial Process Parameter Risk Assessment*

An initial parameter risk assessment was performed to identify process parameters to examine experimentally.

### 3.2.1 Process Parameters

The identification of process inputs and process outputs was based on the control points matrix described in Sect. 2.1 and the respective fishbone diagram for the production bioreactor unit operation.

An assessment of risk was performed using the available information. Table 2 was constructed to document the rationale used during the risk assessment.

### 3.2.2 Raw Materials

An assessment of raw materials determined that custom-formulated complex raw materials consisting of multicomponent blends (i.e., medium and nutrient feed components) needed additional evaluation to analyze potential risk. The number of components in each formulation prevented the full characterization of each potential cause-and-effect relationship, therefore the potential risks were evaluated using a multifaceted approach. The robustness of the medium and nutrient feed formulations were evaluated through variation of the concentrations of high-risk raw materials in the scale-down model as part of the process definition and/or process characterization experiments. In addition, the lot-to-lot variability of the incoming raw materials was evaluated, first at small-scale by using multiple lots, and further confirmed at clinical material manufacturing by introducing multiple independent batches of high-risk raw materials in order to represent the diversity expected in commercial manufacturing. The raw material evaluation determined the process was robust and common cause variability in raw materials was not expected to have an impact on critical quality attributes.

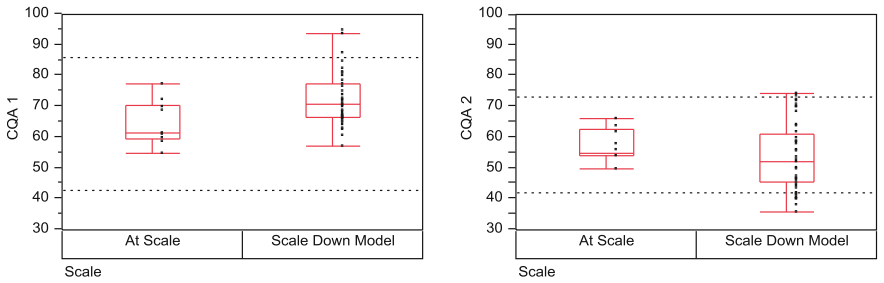
## *3.3 Scale-Down Model*

The scale-down model used for characterization experiments was based on a mechanistic understanding of the unit operation combined with historic platform knowledge acquired from the mammalian cell culture development group. The capabilities of the scale-down model were monitored throughout the development lifecycle by examining results from small-, intermediate-, and large-scale runs as information became available. A statistical analysis of the data, comparing the small-scale and large-scale results, was performed prior to the final classification of process parameters. An equivalence test (TOST) with a practical difference threshold of three times the standard deviation of the at-scale results was performed.

The performance of the scale-down model is displayed in Fig. 8. The analysis demonstrates that the scale-down laboratory models are fit for purpose and the data generated with the laboratory model are representative and predictive of at-scale manufacturing process performance; therefore, the scale-down models are

**Table 2** Process parameter risk assessment summary

| Process branch | Process subbranch | Process parameters | Risk to CQA | | Risk ranking | Rationale |
|---|---|---|---|---|---|---|
| | | | CQA #1 | CQA #2 | | |
| Bioreactor operations | Bioreactor control | Temperature, dissolved oxygen, dissolved carbon dioxide, pH, culture duration | Yes | Yes | Studied empirically | Potential for common cause variability in the bioreactor process parameters to influence product quality attributes and/or process performance in the production bioreactor |
| | | Agitation | No | No | Noncritical | The agitation rate is a scale-dependent parameter; therefore risk is minimal based upon historical performance of scale-down model |
| | | Vessel backpressure | No | No | Noncritical | The vessel backpressure is designed to minimize risk of foreign growth and has no direct link to the culture performance |
| | Bioreactor scale | Bioreactor scale | Yes | Yes | Studied empirically | Mixing time and gas transfer rates could affect process performance; therefore performance examined at an intermediate scale to reduce risk |




| Quality Attribute | CQA 1 | | | CQA 2 | | |
|---|---|---|---|---|---|---|
| Bioreactor Scale | N | Mean | Standard deviation | N | Mean | Standard deviation |
| Scale Down Model | 57 | 73 | 8 | 57 | 54 | 10 |
| At-Scale | 10 | 64 | 7 | 10 | 57 | 5 |
| Actual Difference | -8 | | | 4 | | |
| Practical Difference | 22 | | | 16 | | |
| p-value | <0.01 | | | <0.01 | | |

**Fig. 8** Scale comparison and equivalency test for normalized critical quality attributes. *Dashed lines* represent the practical significance levels

sufficient to evaluate the relationship between process parameters and product critical quality attributes.

In addition to analyzing quality attributes, trends for the benchtop bioreactors and at-scale manufacturing bioreactors were examined and found to be consistent, relative to each other for the process performance indicators examined (cell counts, culture viability, glucose, lactate, pH, and $pCO_2$). The data (not shown) largely remained within two standard deviations of each other and moved directionally in a similar pattern for the duration of the cell culture process.

Based on these data, the scale-down bioreactor model was determined to be fit for purpose to characterize the manufacturing process.

## *3.4 Initial Process Characterization Experiments*

Based upon the initial risk assessment, a two-block resolution III fractional factorial design augmented with replicate centerpoint observations was performed. In addition, process outcomes were measured at multiple timepoints to determine the impact of culture duration on process outcomes.

The minimum range of interest was determined for control parameters to facilitate selecting process ranges for the experimental program. Process ranges explored were intended to at minimum span the minimum range of interest. Table 3 provides an example for the process parameter bioreactor temperature.

**Table 3** Minimum range of interest for bioreactor temperature (°C)

| Expanded measurement uncertainty (MU) | 6$\sigma$ range for population standard deviation | 6$\sigma$ range with 95 % confidence limits | Minimum range of interest |
|---|---|---|---|
| ±0.26 | 0.31<br>–0.38 | 0.39<br>–0.49 | 0.65<br>–0.75 |

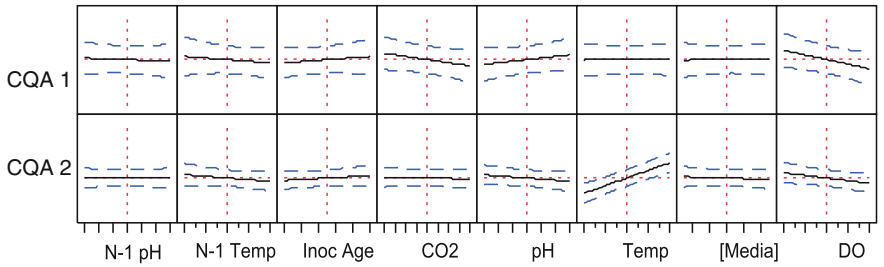



**Fig. 9** Prediction profile from initial process characterization experiment

The expanded measurement uncertainty value is based on the two sigma MU value provided by the manufacturer for the bioreactor RTD probe and transmitter measurement system; and the operational variability (six sigma range for the population standard deviation) is based on empirical data consisting of minimum and maximum deviation from temperature setpoint collected in the pilot plant facility. The minimum range of interest is derived from the summation of the expanded measurement uncertainty (2*sigma MU) and the operational variability accounting for a 95 % confidence interval.

An empirical model of the resulting data was constructed. The directionality and magnitude of the regression coefficient were analyzed. The prediction profile is displayed in Fig. 9.

Three process parameters, temperature, pH, and dissolved oxygen concentration in the production bioreactor were identified as having a statistically significant, meaningful, impact on output parameters.

## *3.5 Final Process Characterization Experiment*

The three process parameters identified as having a meaningful impact on critical quality attributes were further examined to elucidate the response surface. In addition, the data indicated it was advantageous to re-center the process targets to an operating region that increased process robustness; therefore a full-response surface study was performed.

A Box–Wilson circumscribed central composite design response surface experiment was selected in order to resolve any interaction that may be present between effective process parameters. Please see Fig. 10. The design provides factor interactions (multivariate response) with the cube points, as well as more

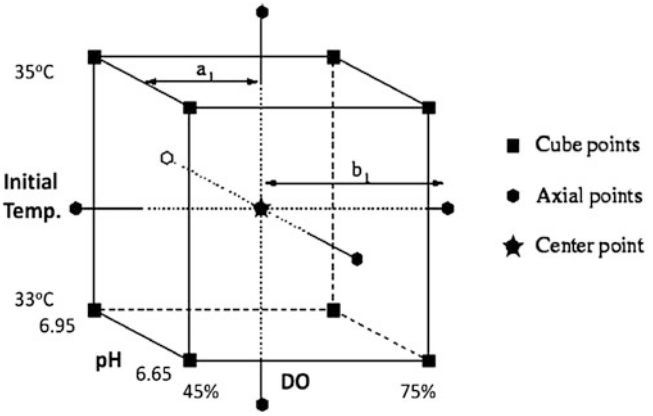



**Fig. 10** Central composite design

information on univariate (single factor) response and curvature with the axial points. The study was performed in multiple blocks in the laboratory used to support commercial manufacturing operations.

The experiment was designed to examine process ranges beyond the minimum range of interest while avoiding the edge of failure. Prior experimental data, from initial characterization studies, were analyzed to generate empirical models that predicted a response surface. The model outputs were used to predict probable regions of failure within the "potential" operating space. The process ranges explored in the response surface study were selected to avoid regions of failure conservatively [15].

The resulting data demonstrated that all points within the operating region, including the univariate axial points were within the proposed specification limits for each critical quality attribute analyzed. The data were further analyzed using an analysis of variance and an empirical model was constructed for the response surface. All three process parameters examined had a statistically significant impact on critical quality attributes over the range of interest. The prediction profilers from the response surface experiment are displayed in Fig. 11.

## *3.6 FMEA Process Parameter Risk Assessment*

A secondary risk assessment was performed to analyze the process prior to technical transfer to the potential manufacturing site. The FMEA risk assessment identified the effective process parameters from the initial process characterization study along with a continued examination of raw material variability as high-risk items. The process ranges examined in the initial characterization study were determined to be acceptable for the receiving site.

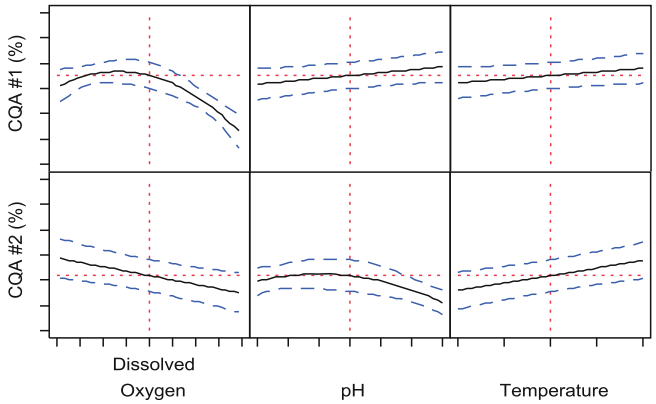



**Fig. 11** Prediction profiler from the response surface experiment

The FMEA analysis led to additional studies to examine transitory excursions caused by failure modes that have the potential to affect the high-risk process parameters.

## *3.7 Process Excursion Study*

A process excursion study was designed, in collaboration with the manufacturing site, to examine the impact of transitory excursions for the high-risk process parameters to simulate potential failure modes that may be encountered in a manufacturing setting. The process parameters explored included the production bioreactor culture pH, production bioreactor culture temperature, and production bioreactor agitation rate (a failure mode that may affect dissolved oxygen). The experiment explored each failure mode, or process parameter, independently.

The results enhanced confidence that the cell culture manufacturing process was robust, as the transient excursions tested did not have a meaningful impact on product quality results. The excursion data can be used to support investigations resulting from special cause events.

## *3.8 Classification of Process Parameters*

The classification of process parameters as critical or noncritical was performed following the methodology described in Sect. 2.6. The inherent critical quality attribute variability was calculated using data from the at-scale bioreactor runs that manufactured drug substance materials for human clinical trials. The data half-range divided by the mean value, expressed as a percentage, was calculated for each critical quality attribute of interest. These values were compared to the

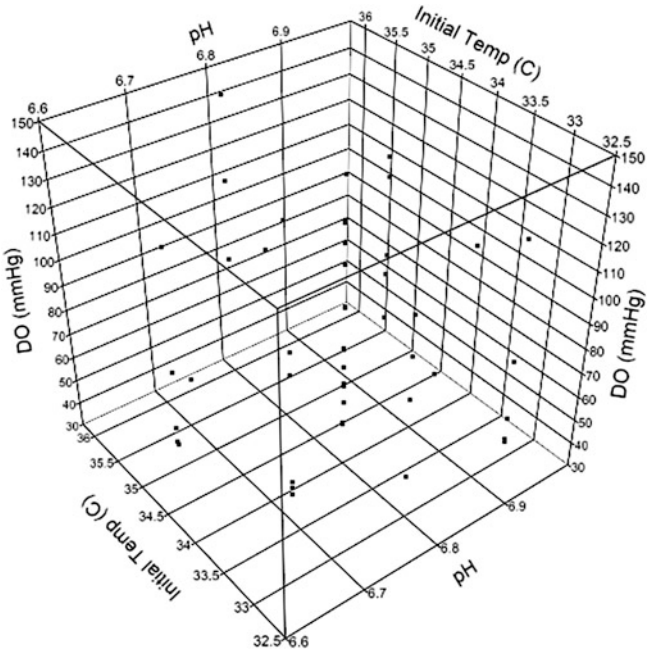



**Fig. 12** Graphical depiction of knowledge space for Unit Operation Three

parameter estimates divided by the baseline response from the process characterization studies. The resulting data indicated that the process parameters dissolved oxygen, temperature, and pH were critical.

## *3.9 Construction of Design Space*

A design space/operating space was constructed post parameter classification. A knowledge space was constructed from the historical process development data. The relevant knowledge space for the production bioreactor unit operation was represented by the three-dimensional cube bounded by the critical process parameters dissolved oxygen, pH, and temperature as indicated in Fig. 12.

Data contained in the knowledge space were analyzed using a least-square regression analysis methodology to generate empirical models to define the relationship between the critical process parameters (production bioreactor dissolved oxygen, temperature, and pH) and the critical quality attributes (CQA#1, and CQA#2).

The CQA#1 was primarily affected by the three main process parameters of dissolved oxygen, pH, and temperature. The resulting process model for CQA#1 is defined as:

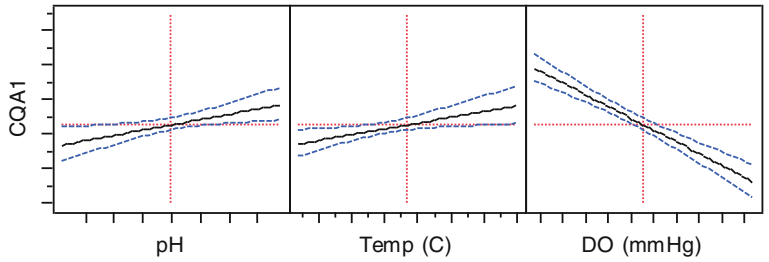
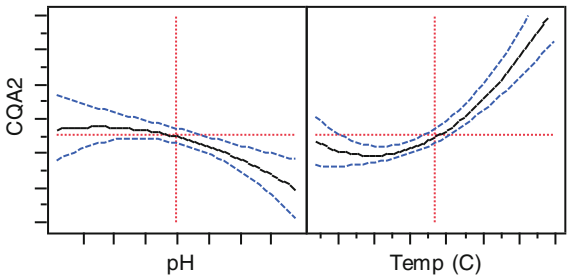



**Fig. 13** Prediction profiler describing the holistic process model for CQA 1

**Fig. 14** Prediction profiler describing the holistic process model for CQA 2




$$y_1 = 72.94 + 5.85x_1 + 5.53x_2 - 16.41x_3$$

where $y_1$ is the mean value of CQA#1, and $x_1$, $x_2$, $x_3$ are the normalized values of production bioreactor pH, temperature, and dissolved oxygen. Please see Fig. 13.

The CQA#2 was primarily affected by both pH and temperature. The resulting process model for CQA#2 is:

$$y_2 = 55.12x_1 + 20.00x_2 - 8.72x_1x_2 - 18.52x_2^2 + 6.54x_1^2$$

where $y_2$ is the mean value of CQA#2, and $x_1$, $x_2$ are the normalized values of production bioreactor pH and temperature. Please see Fig. 14.

The relationships of the two CQAs to three process parameters are shown in the contour plot displayed in Fig. 15. The blue dotted lines are the contours for the mean predicted value of CQA#2. The blue shaded region in these plots indicates the regions where the mean levels of CQA#2 will exceed the proposed specification limit. The red dotted lines are the contours for the mean predicted value of CQA#1. The red shaded region in these plots indicates the regions where the mean levels of CQA#1 will exceed the proposed specification limit. For each contour, the dots are in the direction of higher response values.

This space was further examined by performing Monte Carlo simulations incorporating process variability, analytical variability, and model variability. At each combination of pH, temperature, and dissolved oxygen, 5,000 values were simulated. In addition, the predicted value of a CQA was compared. If both

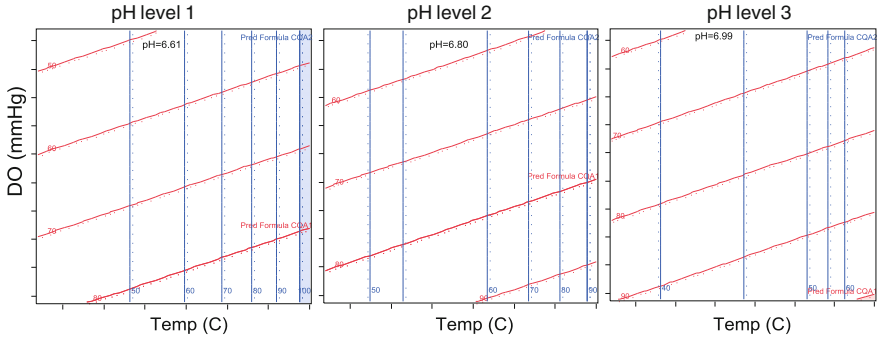



**Fig. 15** Contour profiler for CQA#1 and CQA#2 within the LY2189265 Unit Operation Three operating range

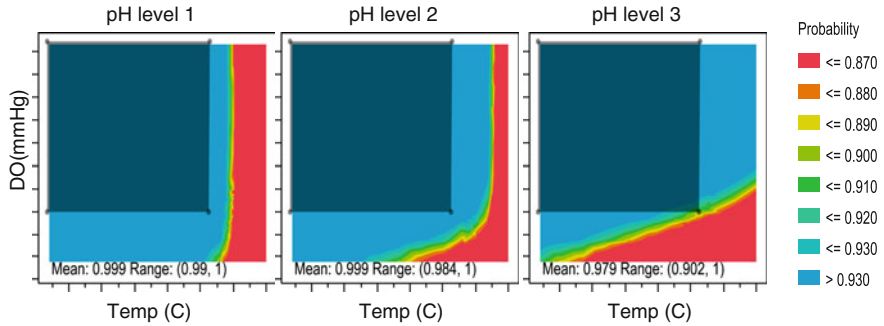



**Fig. 16** Unit Operation Three reliability analysis with resulting design space/operating space

simulated CQA values are simultaneously meeting their respective proposed specification limit, then this simulated run is defined as a success; if not, it is a failure. Reliability was defined as the number of successes over the number of simulations.

The reliability analysis is depicted in Fig. 16. There are eight different colors and each color represents a range of success probability. For instance, the red region has reliability of no more than 87 %, and the orange region has reliability between 87 and 88 %, and so on.

The reliability plot provides a graphic image describing the area that demonstrates an assurance of quality for both CQAs. Using a reliability cutoff of 90 % and reducing the space to a cube (to simplify operations), a series of mathematical equations was used to define the operating space.

**Acknowledgments** The authors acknowledge with thanks technical contributions from John Dobbins, Matthew Osborne, Sarah Demmon, Peter Lambooy, Kenneth Betz, Andrew Rusiniak, Matthew Hilton, Thomas Black, Steve Rose, Adam Rauk, Christopher Breen, Jeffrey Hofer, James Grimes, and Guillermo MiroQuesada. The authors also thank Anli Ouyang, Donald Olson, Matthew Osborne, and Michael DeFelippis for their technical review and comments.

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Adv Biochem Eng Biotechnol (2014) 139: 123–166
DOI: 10.1007/10_2013_214

Published Online: 9 June 2013

# Product Quality Considerations for Mammalian Cell Culture Process Development and Manufacturing

**Michael J. Gramer**

**Abstract** The manufacturing of a biologic drug from mammalian cells results in not a single substance, but an array of product isoforms, also known as variants. These isoforms arise due to intracellular or extracellular events as a result of biological or chemical modification. The most common examples related to biomanufacturing include amino acid modifications (glycosylation, isomerization, oxidation, adduct formation, pyroglutamate formation, phosphorylation, sulfation, amidation), amino acid sequence variants (genetic mutations, amino acid misincorporation, N- and C-terminal heterogeneity, clipping), and higher-order structure modifications (misfolding, aggregation, disulfide pairing). Process-related impurities (HCP, DNA, media components, viral particles) are also important quality attributes related to product safety. The observed ranges associated with each quality attribute define the product quality profile. A biologic drug must have a correct and consistent quality profile throughout clinical development and scale-up to commercial production to ensure product safety and efficacy. In general, the upstream process (cell culture) defines the quality of product-related substances, whereas the downstream process (purification) defines the residual level of process- and product-related impurities. The purpose of this chapter is to review the impact of the cell culture process on product quality. Emphasis is placed on studies with industrial significance and where the direct mechanism of product quality impact was determined. Where possible, recommendations for maintaining consistent or improved quality are provided.

**Keywords** Mammalian · Cell culture · Bioreactor · Process development · Product quality

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## 1 Introduction

The market for biopharmaceutical products is well established and is projected to increase at a rate of 10–20 % over the next decade [55, 233]. This increase can be attributed in part to the higher rate of approval for biopharmaceuticals compared to small molecule drugs, which is likely a result of enhanced efficacy and a better safety profile due to increased drug specificity and a stronger mechanistic understanding of the drug mode of action. A large majority of biological therapeutic products are manufactured via large-scale mammalian cell culture processes because mammalian cells are able to assemble and process complex protein molecules properly with suitable post-translational modifications.

Over the past three decades, product titers from mammalian cells have increased 100- to 1,000-fold such that the achievement of g/L titers, with a manufacturing cost on the order of several hundred dollars per gram, is now routine for many proteins; economic models suggest that further increases in product titers lead to diminishing returns [115]. As a result, there is reduced emphasis on titer and increased emphasis on reduction of development timelines, leading many companies to adopt a platform approach, whereby one process is applied to all related products for manufacture of materials for early-phase clinical trials. The use of a platform process is enabling from a timeline perspective, but for some programs, the process requires further optimization for product titer prior to commercial launch. As process parameters are modified to increase titers,

product quality may be negatively affected resulting in increased program risk. Any change in product quality could have an impact on product performance, and these changes must be justified through analytical comparability protocols [141]. In more extreme cases, justification may require additional pre-clinical or clinical evaluation prior to moving forward with the altered product [31]. As such, an understanding of the factors that affect product quality is essential for enhancing process performance while maintaining product quality.

Aside from the reduced focus on product titer, a number of factors have converged to create a growing interest in understanding and controlling product quality attributes of drugs manufactured by mammalian cell culture. Regulatory agencies and manufacturers have demonstrated increased attention to product quality as exemplified by the quality by design initiative [1, 37, 166, 172]. The initiative aims to build quality into the process early in development by rational choice of a target product quality profile through understanding of the critical quality attributes of the drug. These attributes are then met through consideration of protein design, host cell selection, and the development of a manufacturing process providing correct and consistent quality. The increased knowledge of how the process affects product quality may lead to further cost reductions by enabling more freedom for implementation of post-approval process changes and by minimization of batch rejection due to out-of-specification results.

Increased scientific knowledge regarding analytical product heterogeneity and the impact of product quality on clinically relevant outcomes [18, 24, 56, 70, 93, 116, 137, 140, 224] has led to further interest in rationally directing product quality. A prime example is the evolving understanding of how antibody glycosylation affects antibody drug properties including effector functions, leading to glycoengineering of antibodies, for example, for increased ADCC [12]. Better analytical tools continue to reveal new product quality issues such as amino acid misincorporation. Optimization of cell density for increased titers has led to new product quality issues related to process performance such as post-harvest product reduction. Further optimization and the continuing evolution of next-generation platforms (biobetters, ADCs, bispecifics) continue to push the need for understanding and control of product quality attributes.

Finally, understanding how to direct product quality rationally is of prime importance for developing a biosimilar product. In this case, exhaustive demonstration of analytical similarity using orthogonal methods will lead to reduced preclinical, clinical, and regulatory hurdles as well as increased marketplace acceptance [144, 183]. Typically, a lot surveillance program is in place to understand product quality variability over time from the originator material. As such, the quality profile is somewhat of a moving target until enough lots have been examined over a period of years. Even then, a step change in product quality may be observed from originator lots, presumably due to implementation of a process change by the originator company [178]. Having little knowledge of the originator process, the challenge for process development scientists is to create a modern manufacturing process that consistently results in a highly similar product from an

analytical and functional perspective (having no clinically meaningful difference) while achieving a commercially enabling product titer.

When targeting a specific product quality profile, the ideal approach is to match product quality coming out of the upstream process to that of the target quality profile; otherwise, the downstream process may be required to separate close isoforms resulting in substantial yield loss. Given these considerations, the goal of this chapter is to review the impact of the cell culture process on product quality for the manufacture of commercial biologic products.

## 2 Upstream Factors Affecting Quality

Product quality is affected by four main factors, including the transfected gene, the expressed protein, the cell expression system, and the bioprocess environment (Table 1). One approach to reviewing the impact of these parameters on product quality is to review each process parameter in a separate section. However, the impact of each process parameter is often unpredictable depending on the production system. Furthermore, it is often unclear whether a process parameter is directly affecting product quality or whether there is an indirect effect that is specific for that production system. Alternatively, a more directed, mechanistic approach was taken in this chapter by organizing the discussion by quality attribute. Each quality attribute section contains a short description of the nature and significance of the attribute. This is followed by a review of the impact of the upstream process on the attribute and conclusions and recommendations for controlling or directing the attribute in the cases where specific advice can be provided. Over 200 post-translational modifications have been described on proteins [161]. This review focuses on the most commonly observed modifications encountered in the production of biopharmaceuticals (Table 2). Analytical approaches for characterization are reviewed elsewhere [13].

## 3 Quality Attributes

### *3.1 Aggregation/Misfolding*

Aggregation is a concern in biological manufacturing inasmuch as aggregated protein in the final product may affect biological activity and has been linked to the development of adverse immunological responses [207]. In general, the downstream process is able to clear aggregates. However, a high level of protein aggregation from the bioreactor leads to a less efficient process with lower yield and increased burden downstream.

**Table 1** Upstream factors affecting product quality

| Primary factor | Description | Examples |
|---|---|---|
| Transfected gene | Integration site, copy number, codon usage, expression levels, selection marker | Random integration may increase clonal variability of primary transfectants; increased copy number increases the chance for mutation; codon usage can affect correct translation; increased expression may overwhelm biosynthetic machinery; MTX amplification may result in nucleotide limitation leading to genomic mutation; use of glutamine synthetase selection system will reduce ammonia accumulation which may affect glycosylation |
| Protein | Primary sequence and higher-order structure affects manufacturability including proper assembly, post-translational modifications required for efficacy, and susceptibility to chemical and enzymatic modification; engineering for enhanced manufacturability or product functionality | Oxidation of methionine and tryptophan; deamidation and formation of succinimide and isoaspartate; N- and C-terminal modifications including clipping, formation of pE, and amidation; glycation; glycosylation sites and heterogeneity; sulfation, phosphorylation, $\gamma$-carboxylation, and $\beta$-hydroxylation sites; sulfhydryl pairing, adduction, trisulfide formation, $\beta$-elimination; chemical and enzymatic clipping; folding and aggregation |
| Cell line | Choice of host cell species, tissue origin, clonal variation, cell stability vs. age; directing quality by clone screening or cell engineering for enhanced manufacturability or product functionality | Enzymatic repertoire and associated machinery affecting synthesis, folding and assembly, post-translational modifications, and degradation; process-related impurities including HCP, DNA, and viruses |
| Bioprocess parameters | Process design space leading to optimal and robust process performance and product quality | DO, pH, temperature, agitation, mix time, gas strategy, sparger design, runtime, cell density, cell viability, production mode, media formulation, feed rate, waste product build-up, base usage, osmolality, process related impurities, harvest process, scale |

**Table 2** Summary of protein heterogeneity observed on mammalian-cell-produced biopharmaceuticals and the impact of the cell culture process on each quality attribute

| Class | Quality attribute | Impact of the upstream process |
|---|---|---|
| Amino acid sequence | Genomic variants | Expression of product with a point mutation was observed to decrease from 27 to 1 % as the cell aged out to 140 days [90]; Point mutations observed, mechanism not investigated [164, 223, 227]; Increased gene copy number correlated with increased risk of expressed protein with a point mutation [47]; Gene amplification using MTX results in increased gene mutation potentially due to nucleotide limitation from MTX inhibition of DHFR [86]; Genetic mutation led to a cross-over event between antibody heavy and light chains [211]; An intron was translated into an antibody when expressed in CHO cells but not NS0 cells [9]; A mutation in the stop codon led to an antibody expressed with 17 extra amino acids [230] |
| | Amino acid misincorporation | ASN for SER variants observed in multiple systems due to mistranslation when using the ACG codon for SER [225]; mischarging was eliminated when alternate SER codons were used [86]; SER for ASN variants observed due to mischarging of tRNA when ASN was limiting [117, 216]; PHE to LEU/ILE or TYR variants observed when PHE was limiting [227]; PHE for TYR variant observed when TYR was limiting and HIS for TYR variant observed when both TYR and PHE were limiting [58] |
| | C-Terminal lysine removal | Material produced from ascites has a lower level of C-terminal lysine compared to in vitro produced material due to increased carboxypeptidase activity in ascites fluid [165]; Incubation of the product at low pH in culture supernatant or in ascites fluid results in reduced C-terminal lysine [145]; Increased C-terminal lysine was observed on the material produced in serum-free medium in a stirred tank reactor compared to the same material produced in serum-containing medium in a hollow fiber bioreactor [4]; Material from hybridoma cultures has increased C-terminal lysine compared to CHO-produced material; the level of C-terminal lysine was affected by feed strategy and temperature and by incubation in culture supernatant [46]; Increased copper concentration, decreased zinc concentration, and an increased ratio of copper to zinc correlated with increased levels of C-terminal lysine [142] |
| | Chemical clipping | No published data identified |
| | Proteolysis | Antibody fragmentation by host cell proteases has been observed for hybridomas [112, 147, 203], NSO cells [189], and CHO cells [65]. Degradation of other expressed proteins by host cell proteases has been observed, including interferon-$\gamma$ expressed in CHO cells [71, 148]; antibody fusion proteins expressed in CHO cells [48, 49, 169]; antithrombin III expressed in BHK cells [197]; factor VIII expressed in CHO cells [88, 174]; erythropoietin expressed in CHO cells [222]; and t-PA expressed in CHO cells [136]; Degradation glucagonlike-peptide-1-antibody fusion protein depended on the product sequence, host cell type used to express the product, clonal variation within a cell type, harvest time, culture temperature, batch versus perfusion culture, and use of the protease inhibitor benzamidine hydrochloride during cell culture to reduce product clipping [48, 49] |

(continued)

**Table 2** (continued)

| Class | Quality attribute | Impact of the upstream process |
|---|---|---|
| | N-Terminal leader sequence cleavage point | A point mutation in the leader sequence resulted in differential cleavage of the leader sequence [182]; Differential cleavage of the leader sequence observed [143]; A portion of the expressed product had the entire leader sequence remaining on protein [2]; Differential cleavage of the leader sequence was eliminated by modification of the leader sequence [123] |
| Amino acid modification | Glycation | Increasing concentrations of glucose in cell culture leads to increased glycation rate [163, 226]; Glycation is faster by galactose compared to glucose [163]; Glycation varies across different sites in a protein [146, 228]; Glycation is reversible; glycation of a susceptible lysine was eliminated by replacement with an arginine [146] |
| | Adduction to cysteine | Engineering an unpaired cysteine into an antibody resulted in adducted glutathione, adducted cysteine, and adducted free light chain; increasing the culture temperature from 33 to 35 °C reduced adduction of the free light chain; clonal variation was observed where a decreased cellular glutathione content or an increased ratio of light chain to heavy chain mRNA correlated with increased expression of the triple light chain species [30, 72, 73]; Expression of an antibody with an unpaired cysteine in the variable region resulted in cysteinylation [7, 63] |
| | C-Terminal amidation | The absence of C-terminal lysine created a C-terminal glycine, which was cleaved by the enzyme PAM followed by amidation of the newly exposed C-terminal proline [109, 113]; Increased amidation correlated with increased copper ion concentration [113] |
| | Deamidation | No published data identified |
| | Succinimide formation | No published data identified |
| | Isoaspartate isomerization | No published data identified |
| | Sulfation | No published data identified |
| | Phosphorylation | No published data identified |
| | N-Terminal pyroglutamate | N-terminal GLN was 90 % converted to pE by the end of the cell culture run (Dick et al. 2007) |
| | Glycosylation | Too many references to summarize. Refer to the sections describing glycosylation and degradation by glycosidases |
| | Methionine oxidation | No published data identified |
| | Tyrptophan oxidation | No published data identified |
| | $\gamma$-Carboxylation, $\beta$-Hydroxylation | Different cell lines vary in their ability to perform $\gamma$-carboxylation and $\beta$-hydroxylation [221]; Overexpressing the enzyme vitamin K epoxide reductase resulted in increased $\gamma$-carboxylation of Factor VII expressed in HEK293 cells [209], Factor X expressed in HEK293 cells [194], and Factor IX expressed in BHK cells [208] |

(continued)

**Table 2** (continued)

| Class | Quality attribute | Impact of the upstream process |
|---|---|---|
| Higher-order structure | Misfolding/Aggregation | Increased pH and osmolality reduce aggregation of an antibody which has a pI of 6.1–6.5 [61]; Reduced aggregation of interferon-$\beta$ observed at lower temperature, increased osmolality, by addition of glycerol, by encapsulation in microspheres, and by using perfusion culture [87, 170, 171, 188, 196]; Comp-Ang1 aggregation reduced by protein engineering [32, 99] and by production at lower temperature, increased NaCl concentration, increased pH, and addition of DMSO or proline [100, 101, 110]; Fusion protein aggregation was reduced by increasing the temperature from 32 to 34 °C and adding cystine [108]; Increased antibody aggregation observed when the ratio of light chain to heavy chain mRNA dropped below approximately 1.5 [131]; Increased temperature from 31 to 37 °C resulted in reduced aggregation by reducing product mRNA expression (Gomez et al. 2011) |
| | Incorrect disulfide pairing | The level of incomplete disulfide pairing was reduced by increasing copper ion concentration [25]<br>Lysis of viable cells during harvest led to post-harvest reduction of disulfide bonds; reduction can be inhibited by lower temperature, lower pH, adding EDTA to inhibit the enzyme, by increasing the oxidative environment through sparging or the addition of $Cu^{++}$ or cysteine, or by cell engineering [111, 122, 198] |
| | Trisulfide formation | The level of trisulfide observed on a CHO-produced antibody was found to vary across different bioreactor runs that varied runtime, feed strategy, and scale (Gu et al. 2009)<br>The level of trisulfide formation was directly linked to the formation of $H_2S$ due to the addition of cysteine to the bioreactor [127] |
| | Disulfide beta elimination | No published data identified |
| Process related impurities | HCP | The cell line (similar host) and culture process have less impact on the level and distribution of HCP compared to final culture viability [106, 126, 195] |
| | DNA | Increased runtime led to increased DNA [172] |
| | Viral particles | Different cell lines vary in viral particle production; pH variably affected viral particle production depending on the cell line; specific retroviral particle production rate increased due to addition of sodium butyrate or from reducing the temperature [21] |
| | Cell culture components | No published data identified |

Protein aggregation is a complex phenomenon that occurs through a number of distinct mechanisms [213, 214]. Unfolded/misfolded proteins have a propensity to aggregate due to the exposure of hydrophobic patches. Elevated temperatures increase aggregation via this mechanism both by increasing the fraction of protein in the unfolded state and by increasing the strength of hydrophobic interactions. Aggregation may also be affected by the exposure of the protein to various contact surfaces or air–water interfaces. Proteins can aggregate in the native state non-covalently via self-association through hydrophobic or electrostatic interactions or covalently through a number of mechanisms including the formation of intermolecular disulfide bridges by unpaired cysteines. Aggregates can arise during each manufacturing process step including cell culture, downstream processing, during a freeze/thaw process, during drug product manufacturing, and in the final drug product formulation [40, 207].

Proper intracellular protein assembly is required for high-quality protein expression. Elevated levels of product expression can overwhelm the intracellular machinery responsible for proper assembly, resulting in misfolded or aggregated protein, thus limiting productivity due to internal degradation of the improperly assembled protein [26, 180, 181, 186]. Alternatively, product overexpression can result in secretion of a fraction of the improperly assembled material. A number of cell line engineering approaches have been investigated to enhance the ability of the cell to secrete properly assembled product [105, 135].

A high level of extracellular aggregation (approximately 10 %) was observed in the production of a monoclonal antibody from a hybridoma [61]. Given that the pI of the protein was 6.1–6.5, the authors explored whether increased culture pH or osmolality could favorably affect protein aggregation levels. Indeed, increasing the osmolality at pH 7.1 from 300 to 395 mOs/kg $H_20$ resulted in aggregate levels falling below 3 %. Likewise, at 300 mOs/kg $H_20$, an increase in pH from 7.1 to 8.0 resulted in the aggregate level falling to 4 %. These factors were synergistic; optimal conditions, balancing cell growth, productivity, and product aggregation, were found at 350 mOs/kg $H_20$ and pH 7.5, which resulted in 4 % product aggregate.

A number of approaches have been explored for reduction of aggregation of $\beta$-interferon expressed in CHO cells. Reduced aggregation was observed at lower temperature, at increased osmolality, by addition of glycerol as a chemical chaperone, and by encapsulation of cells in microcarriers [87, 170, 188, 196]. Because aggregation continued to increase in cell-free supernatant, perfusion culture was also useful for reducing product aggregation [171].

A combination of protein engineering and bioprocess factors was used to reduce aggregation of Ang1 protein expressed in CHO cells. First, the molecule was engineered to reduce aggregate levels and to enable more efficient purification [32, 99]. Next, bioprocess factors were explored. Factors found to reduce aggregation include reduced temperature, increased osmolality via NaCl addition, increased pH, and addition of DMSO or proline as chemical chaperones [100, 101, 110].

The complexity of balancing aggregate reduction with cell growth, product titer, and other quality attributes is provided in a study to examine aggregate

formation of fusion proteins produced by CHO cells [108]. The authors explored the impact of culture DO, pH, copper ion, cysteine, cystine, and temperature. A combination of increasing temperature from 32 to 34 °C and the addition of an intermediate amount of cystine was found to provide the optimal balance.

The impact of the ratio of light chain to heavy chain mRNA on product aggregation was explored in the production of monoclonal antibodies from CHO cells [131]. The authors were developing a rapid method to screen for high-productivity clones and demonstrated a correlation between heavy chain mRNA expression and product expression levels. The authors also observed an increase in product aggregation when the ratio of light chain to heavy chain was below approximately 1.5. The study shows clearly that clones can vary widely in these attributes and in the secretion of aggregated product.

A high level of variability in protein aggregation from 1 to 20 % was observed in the development of a recombinant IgG from a CHO cell line [74]. The highest level of aggregation was observed when cells were cultured at 31 and 33 °C, with slightly lower levels at 35 °C and even lower levels (<5 %) were observed at 37 °C. Interestingly, the reduced temperature resulted in a 25–75 % increase in the ER volume and the level of ER chaperone proteins. However, the reduced temperature also resulted in a two- to fourfold increase in light chain and heavy chain mRNA expression, presumably overwhelming the ER machinery, resulting in a higher secretion rate of aggregated protein. When three other cell lines with high aggregate levels were investigated, an increase in culture temperature from 33 to 37 °C resulted in substantially less product aggregation and the same or higher product titer. These results suggest that reduced temperature may be effective at reducing product aggregation due to enhanced ER machinery unless the reduced temperature also results in a large increase in expression levels that may overwhelm the ER machinery, which then results in increased aggregate formation.

In summary, low aggregate levels are required for clinical application of the product. Minimizing the level of aggregated protein in the culture supernatant will lead to a higher yield and a reduced burden on the downstream process. The protein has a strong influence on the propensity to aggregate, therefore early screening in development is useful to eliminate problematic candidates, to re-engineer the protein if possible, or to prompt the initiation of studies to examine the impact of process conditions on product aggregation. These process factors include the cell line (clonal variability), expression levels, and culture conditions. Increased expression levels can lead to increased protein aggregation due to overwhelming of the protein synthetic machinery. Temperature plays a key role in protein aggregation both by affecting intracellular expression and assembly and extracellular protein–protein interactions. Extracellular aggregation can further be influenced by medium pH and ionic strength. Chemical chaperones may be useful to reduce aggregation rates. For proteins with a propensity to aggregate extracellulary, perfusion culture will minimize time of exposure in the culture supernatant to reduce aggregate formation.

## 3.2 Sequence Variants Due to Genetic Mutation and Amino Acid Misincorporation

Sequence variants arise from modifications of the amino acid sequence. Two mechanisms leading to unexpected sequence variants include genetic mutation and mistranslation either due to mischarging of the t-RNA or due to codon–anticodon mismatch, as described below.

A sequence variant observed during drug development using mammalian cells was first described some time ago [90]. In that study, a tyrosine to glutamine variant in the IgG1 heavy chain (Y376Q) was initially observed at 27 % in a HER2 antibody expressed by methotrexate amplified CHO cells. Interestingly, during sequential passage of the cell line to over 140 days, the sequence variant level dropped gradually to 1 %. The sequence variant was observed in 10 % of subclones from this amplified cell line, demonstrating a heterogeneous population of cells within this isolate. Examination of this and other transfected isolates prior to amplification revealed the presence or absence of the mutation, depending on the isolate. However, this mutation was not detectable in the original genetic material transfected into the cells. The conclusion was that the mutation occurred during the initial process of transfection and isolation, but prior to amplification.

Since that study, improved analytical methods have led to a number of more recent findings of sequence variants with the mechanism identified as a genetic mutation, translational misincorporation, or undetermined. Remarkably, in a recent presentation on antibody production by CHO cells, sequence variants were detected in 10 of 17 projects including 24 of 43 clones evaluated with misincorporation levels of 0.2–40 % [121]. Another presentation reported finding sequence variants in 10 of 75 cell lines including 9.5 % of nonamplified cell lines and 21 % of amplified cell lines [92].

A correlation between gene copy number and sequence variation was hypothesized in development of an Fc fusion protein using the Lonza CHOK1SV/GS expression system [47]. Peptide mapping data revealed that about 7–10 % of the protein from a subclone of the lead cell line had leucine in place of phenylalanine at the eleventh amino acid from the N-terminal end. Further investigation revealed that 12 % of the mRNA expressed by the subclone had a TTC → CTC mutation, leading to the observed variant. The variant originated as a genomic mutation in the parent clone of the lead cell line and was observed in subclones of this cell line, each of which had 20–25 copies of the expressed fusion protein gene. The sequence variant was not observed in another lead parental line, or clones derived from it, each of which had 6–8 copies of the expressed fusion protein gene.

Sequence variants were observed in lead clones from both early and late stage development projects [223]. In the early-stage project, separate variants were found in two of the four top clones. One variant was a methionine to arginine mutation (M83R) at 5 %, and the second was a proline to threonine variant (P274T) at 42 %. In the late-stage project, one of the four top clones had a leucine to glutamine variant (L413Q) at 0.3 %. The mechanisms leading to the observed

variants were not determined. In a separate study of IgG1 expression from four early-stage CHO clones [164], a serine to glycine variant (S52G) was observed in one clone at 0.2 % (mechanism also not indicated).

A model system (HGPRT/6-TG) was used to support a hypothesis that amplification of DHFR–CHO cells using methotrexate may cause mutations leading to sequence variants [86]. The authors proposed that inasmuch as the DHFR enzyme is involved in nucleotide synthesis needed for DNA replication, and MTX binds to DHFR to inhibit DHFR activity, addition of MTX for selection after transfection likely leads to the starvation of nucleotides during the initial MTX selection period. The nucleotide starvation could result in genetic mutation. To test their hypothesis, the authors evaluated the sensitivity of MTX amplified cell lines to 6-TG, which would indicate the mutation rate associated with the HGPRT gene; loss of sensitivity indicates a mutated gene. The authors show that selection and amplification in increasing concentrations of MTX make the cell lines less sensitive to 6-TG, thus supporting their hypothesis. The authors go on to demonstrate a 0.8 % serine to arginine (S167R) sequence variant in the light chain of a MAb due to a genomic nucleotide mutation.

A codon-specific variant of ASN for SER was observed in multiple cell types and for multiple proteins [225]. The systems investigated include: four CHO clones from one IgG1, a second IgG1 expressed in either CHO cells or E. coli, and an IgG4 expressed in either CHO or NSO cells. The ASN for SER variant occurred at multiple positions throughout each protein at levels varying from 0.01 to 0.77 %, depending on the protein, the expression system, the position within the protein, and the production process. However, although there are six possible codons for serine, the variation was restricted to SER coded with the ACG codon. Alternatively, not all ACG codons resulted in the observation of a variant. The variant frequency was not associated with cell age out to 100 days. From the sum of the data, the most likely mechanism is mistranslation due to codon–anticodon mismatch. In a related study of IgG expressed in CHO cells [86], a 0.1 % S63N mistranslation associated with the ACG codon was rendered undetectable (<0.01 %) when the codon was changed to TCT or TCC, whereas other AGC codons within the protein still contained the ASN variant; productivity was not negatively affected by the codon change. These results suggest that serine should be coded using the TCT or TCC codon in place of the ACG codon for recombinant protein expression.

The reverse variant, SER for ASN, was also observed [216]. However, in contrast to the codon-specific findings described above, the most likely mechanism is mischarging of the tRNA. In a first IgG expressed in CHO cells, SER for ASN variants were observed throughout both the heavy and light chains at 1.4–4 %. These variants were observed in multiple clones both before and after amplification as well as in two other CHO expressed proteins including a second IgG and a fusion protein. These variants correlated with ASN depletion and were not observed when the medium was sufficiently supplemented with additional ASN. These results suggest misincorporation due to charging of the ASN tRNA with SER when ASN was limiting. The mechanism was further explored, demonstrating

that high concentrations of serine did not cause misincorporation when ASN was present [117]. However, when ASN was limiting, increasing concentrations of SER resulted in increasing misincorporation of SER. Supplementation of glutamine during ASN depletion partially reduced misincorporation of SER, presumably due to increased intracellular biosynthesis of ASN from GLN.

Two variants arising from genetic mutation were identified in one IgG1 expressed in CHO cells, and two amino acid misincorporation variants were identified in a second IgG1 expressed in CHO cells [227]. The two genetic variants were found at separate sites on the protein. The first was a GLU to ASP variant at T14 in the light chain that was found at 0.4 % in a 2L lot and at 0.5 % in a 100L lot. The second was a THR to ASN variant at T24 of the heavy chain at 2.7 % in a 2L lot and at 4.0 % in a 100L lot. Further examination of the latter variant revealed a C to A substitution accounting for the observed THR to ASN variant in approximately 2 % of the DNA, which corresponded approximately with the amount of expressed variant. The two misincorporation variants were observed from multiple clones from multiple transfections when PHE was limiting. The first was a PHE to LEU/ILE variant found at 0.1 % in only one position within the protein. The second was a PHE to TYR variant found at up to 0.6 % at almost all PHE positions in the protein. With proper supplementation of PHE, neither variant was observed.

A separate group reported the reverse variant, a PHE for TYR misincorporation when TYR was limiting [58]. These variants were observed up to 3 % across several antibodies. When both TYR and PHE were limiting, HIS for TYR variants were observed. When TYR, PHE, and HIS were limiting, no further variants were observed. Tyrosine supplementation eliminated the observance of these variants.

A unique observation, a gene crossover mutation, was observed during development of an IgG specific for IgE [211]. One of the candidate cell lines (unspecified) produced a molecule with an additional minor heavy chain band of slightly lower molecular weight by SDS-PAGE, whereas a minor band of slightly higher molecular weight was observed for the light chain. More detailed studies confirmed a cross-over event between the V genes of the antibody between Arg108 of the light chain and Ala124 of the heavy chain. This cross-over resulted in a variant H chain that had 16 fewer amino acid residues than the expected sequence.

Another unique observation, expression of an intron, was observed in production of a monoclonal antibody anti-IGF-1 receptor from CHO cells [9]. The authors observed two unexpected variants on reverse-phase HPLC and identified these variants as containing one or both heavy chains with 24 additional amino acids from an intron sequence that is normally spliced out prior to translation. These variants were not observed on this antibody produced in a GS-NSO expression system.

A recently reported unique observation is identification of a mutation in the stop codon leading to light chain extension in an IgG1 produced from CHO cells [230]. The TAA stop codon mutated to a GAA codon was detected at a level near 15 %, leading to a corresponding similar level of expressed protein with glutamine at that

position continuing for another 17 extra amino acids until reaching the next stop codon.

An interesting related topic is the recent manipulation and exploitation of the translation process to incorporate unnatural amino acids in mammalian cells for introduction of unique, site-specific chemistries [139, 220]. These techniques have been used for a number of antibody engineering projects including introduction of site-specific linkers to create bispecific antibodies [119] or for attachment of antibody–drug conjugates [6].

The amino acid sequence defines the most fundamental aspect of identity for a biologic drug. Sequence variants arising from genetic mutation or misincorporation pose a high regulatory risk. As such, these studies highlight the importance of detecting and eliminating sequence variants throughout the development process. Codon optimization of the expressed protein is recommended. Clone selection should be based on sequence verification (genetic and expressed protein) at the time of identifying the lead cell clone as well as at the limit of in vitro age. The use of platform or off-the-shelf cell culture media and feeds may lead to amino acid limitation for some cell lines, which in turn could result in production of sequence variants. In order to prevent sequence variants in clinical material lots that may vary upon scale-up, a time-course study of amino acid profiles should be evaluated prior to locking down the clinical process. If any amino acid is found limiting, the medium should be supplemented to avoid limitation; increased productivity or process robustness could be an additional benefit of this study.

### *3.3 Differential N-Terminal Leader Sequence Processing*

Proteins targeted for secretion through the endoplasmic reticulum contain an N-terminal signal peptide [206]. The signal peptide leader sequence is generally between 15 and 30 amino acids, but can be over 50 amino acids long [193]. Following translocation into the endoplasmic reticulum, the signal peptide is cleaved by signal peptidase [157]. There is no consensus signal peptide sequence; rather signal peptides typically contain three distinct regions including a positively charged amino terminus, a central hydrophobic core, and a more polar carboxy terminal region [206]. The precise point of signal cleavage can only be predicted to an accuracy of 78 % [232]. The signal peptide can have a large impact on protein productivity [120, 193]. However, variants can arise as the result of differential cleavage of the signal peptide.

Differential cleavage of the signal peptide was observed on an antibody from a hybridoma cell line that contained a mutation in the leader sequence [182]. The parent cell line had a well-defined cleavage site with a 19 amino acid leader sequence. The mutant contained a proline instead of glutamine at the –2 position relative to the cleavage site. This mutation resulted in two variants: the first with 19 amino acids cleaved off, and a second with 21 amino acids cleaved off.

In production of an antibody from CHO cells, alternative signal peptide cleavage sites were observed for both heavy and light chains [123]. The heavy chain contained three variants including a main variant where 19 amino acids were cleaved, a second variant comprising 1 % where 21 amino acids were cleaved, and a third variant comprising 1.7 % where 22 amino acids were cleaved as the signal peptide. Altering the signal peptide sequence by two amino acids resulted in production of a homogeneous heavy chain length with 19 amino acids cleaved as the signal peptide. The light chain contained a main variant and a variant with an alternative signal peptide cleavage site resulting in 4 % of the light chain with two fewer amino acids. More extensive heterogeneity in the leader sequence of the heavy chain on a monoclonal antibody was observed by others [143].

In production of IgG1 from CHO cells, a heavy chain variant was observed that still had the entire leader sequence attached [2]. However, in any single antibody molecule, only one of each of the two heavy chains had the leader sequence remaining. Two other minor leader sequence variants were also noted for the heavy chain. The authors conclude that the particular leader sequence used might not be optimal.

In summary, the signal peptide leader sequence that directs the protein for secretion through the endoplasmic reticulum typically has a defined cleavage site, although cleavage site variability has been observed leading to N-terminal variants. These variants can be a few amino acids longer or a few amino acids shorter, or potentially the entire leader sequence could remain. This phenomenon is likely to be affected by the protein being expressed, the leader sequence used, and the ability of the cell line to cleave the leader sequence. The clinical significance of this variability will be product-specific. It is advisable to look for leader sequence variability early in development and, if possible, to choose an expression system (cell line/clone and leader sequence) that does not result in variability, or alternatively, design the downstream process to remove any undesired variants.

## *3.4 N-Terminal Pyroglutamate*

The N-terminus of a number of endogenous peptides and proteins (including antibodies) can variably contain a pyroglutamate [5, 128]. Proteins containing an N-terminal pyroglutamate (pE) are known as blocked proteins, because an enzyme is required to remove the pE to enable protein sequencing [5, 41]. Although pE is a cellular metabolite, it cannot be added directly to the protein [128]. Instead N-terminal pE is a post-translational modification of glutamine or glutamic acid, either of which can cyclize to form pE. Cyclization of glutamine to pE results in the loss of ammonia leading to a net reduction in mass of 17 Da and a net acidic shift of the protein, whereas glutamate conversion to pE results in the loss of water leading to an 18 Da reduction in mass and no net change in protein charge [140].

For some peptides and proteins, this variability affects biological function [10, 179], and the resistance to N-terminal peptidase action can result in increased

stability [202]. The aberrant formation of pE may be related to several pathological conditions, such as amyloidotic diseases, osteoporosis, rheumatoid arthritis, malignant pheochromocytoma, and melanoma [98]. However, for many proteins, including antibodies, no biological significance has been ascribed to the variability of pE formation.

N-terminal pE is commonly observed on recombinant cell culture produced proteins, including at the N-terminus of both heavy and light chains on antibodies, ranging from a few percent to essentially quantitative conversion [28, 62, 137, 156, 167, 173]. Injection of a recombinant antibody into humans or rats resulted in a steady increase in pE [140, 224].

The conversion of N-terminal glutamine or glutamate to pE can occur through enzymatic or chemical mechanisms. Enzymatic conversion is by glutaminyl cyclase, of which two forms exist including one form retained within the Golgi and a second secreted form [42, 179]. Chemical modification of glutamic acid to pyroglutamate is a relatively slow process, whereas glutamine readily converts to pyroglutamate through chemical modification [28, 45]. Given these mechanisms, pE can potentially result from intracellular or extracellular enzymatic action in the bioreactor or from chemical modification in any step of the production process.

Despite the wide observance of pE on recombinant cell culture produced proteins, limited data are available to assess the impact of the bioprocess environment on pE formation. In one study, CHO-produced IgG samples were analyzed from the end of a 15-day bioreactor run and at various points downstream [45]. The results demonstrated 90 % conversion of N-terminal glutamine to pE at the end of the bioreactor process and almost complete conversion by the end of the purification process. Based on data from a model peptide and the finding of further increases in pE downstream, the authors suggest that the primary mechanism of conversion is chemical degradation. However, their data do not rule out a contribution from enzymatic conversion.

In summary, although both enzymatic and chemical mechanisms can lead to the formation of pE, data investigating the actual mechanism of pE formation in cell culture are sparse and the clinical impact of varying levels of pE is largely unclear. However, the formation of pE from glutamine results in a charge difference and the formation of pE from glutamine or glutamate results in a mass difference. Both of these modifications can complicate product characterization and comparability work. As such, it is advisable to maintain a consistent level of pE. More work is needed to understand the impact of the cell culture environment on pE formation from N-terminal glutamate and glutamine.

## *3.5 C-Terminal Lysine/Arginine*

A number of proteins variably contain a C-terminal lysine or arginine residue [91, 184]. The presence of residual C-terminal lysine/arginine results in a net basic shift in charged-based assays and can also be detected through peptide mapping and

mass spectrometry [91]. For some proteins, this variability affects biological function [184, 185], although for many proteins, including antibodies, no biological significance has been ascribed to this variability except for the theoretical possibility to affect PK when injected subcutaneously [93].

The C-terminal lysine/arginine is added during protein synthesis and subsequently removed by a basic carboxypeptidase [184, 185]. The basic carboxypeptidases are a family of enzymes residing within various compartments in the cell, on the cell surface, and in secreted forms, that cleave C-terminal basic residues including lysine and arginine. The enzymes differ in tissue distribution, pH optimum, co-factor requirement, specificity, and susceptibility to inhibitors.

C-terminal lysine/arginine variants are commonly observed on recombinant cell culture produced proteins, ranging from partial to almost complete absence of the C-terminal basic residue [91, 118, 130, 137, 173]. Given the mechanism of removal and the location of carboxypeptidases, loss of the C-terminal basic residue can potentially result from intracellular or extracellular enzymatic action in the bioreactor. Furthermore, injection of a recombinant antibody into humans or rats results in rapid and complete removal of C-terminal lysine [24, 224].

C-terminal lysine variability was reported during production of OKT-3 antibody from a hybridoma [165]. When cultured in ascites, no C-terminal lysine was observed. However, when cultured in tissue culture, a variable level corresponding to approximately 8 % of the material contained a C-terminal lysine. The C-terminal lysine was completely removed from the antibody when the tissue culture produced material was incubated in ascites fluid for one hour. Heat inactivation of the ascites fluid at 50 °C for 45 min abolished the ability of the ascites fluid to remove the C-terminal lysine. The results suggested the presence of a basic carboxypeptidase affecting the levels of C-terminal lysine.

Several methods for reduction in C-terminal lysine variability were described in association with the production of an antibody by a hybridoma [145]. First cell culture supernatant was clarified and concentrated approximately 100-fold, at which point the sample contained approximately 50 % C-terminal lysine. In a first approach, incubation of the concentrated supernatant at pH 4 for 24 h at 25 °C resulted in complete removal of C-terminal lysine. In a second approach, dilution of the sample in a 1:1 ratio of ascites fluid for 16 h at 37 °C resulted in 20 % residual C-terminal lysine. In a third approach, incubation of the sample with a commercially available carboxypeptidase B resulted in 5 % residual C-terminal lysine. An alternative approach, expressing the antibody without C-terminal lysine, was also suggested but not demonstrated.

Variability in the level of C-terminal lysine was observed in the production of an antibody by SP2/0 cells [4]. Three batches were analyzed, including two produced in a serum-containing medium in a hollow fiber bioreactor, and a third in a serum-free medium from a stirred-tank bioreactor. The first lot from the hollow fiber bioreactor in serum had no detectable C-terminal lysine, whereas the second lot had approximately 5 % of the antibody with one C-terminal lysine. The lot from the stirred-tank bioreactor in serum-free medium had both one and two C-

terminal lysine forms, accounting for 15 % of the product. The observed variability in C-terminal lysine did not affect the biological activity of the molecule.

C-terminal lysine heterogeneity on a human antibody produced by a hybridoma and a CHO cell line was investigated [46]. The majority of material from the hybridoma cell line contained either one or two C-terminal lysines, whereas the majority of the CHO-produced material contained no C-terminal lysine. The authors further reported that antibodies produced by hybridomas typically contain 40–80 % of the product without C-terminal lysine, whereas 90–100 % of the product from CHO cells typically contains no C-terminal lysine. Altering the feed strategy and temperature in the CHO cell process resulted in a shift from 28.1 to 4.1 % C-terminal lysine. The authors further investigated the mechanism of C-terminal lysine variability. Overnight incubation of hybridoma-produced material at 37 °C in 50 % cell-free CHO cell culture supernatant resulted in essentially complete removal of the C-terminal lysine. Furthermore, when a carboxypeptidase inhibitor from potato tuber was added to this mixture, the lysine was not removed, and no removal was observed when the material was incubated in PBS. These results strongly suggest the presence of active extracellular basic carboxypeptidases that can efficiently remove C-terminal lysine.

C-terminal lysine levels varied from 4.9 to 30.9 % on an antibody from CHO cells [142]. To investigate this variability, three factors in the cell culture process were initially evaluated, including temperature, runtime, and copper ion concentration. Of these factors, culture time and temperature had a small impact, whereas the copper concentration had a strong impact on the level of C-terminal lysine. The authors hypothesized that the availability of zinc, a co-factor required by basic carboxypeptidases, could potentially be affected by the level of copper in the medium. In support of their hypothesis, increased copper concentration, decreased zinc concentration, and an increased ratio of copper to zinc correlated with increased levels of C-terminal lysine. Through time-course studies, the use of Western blotting for basic carboxypeptidases, demonstration of extracellular carboxypeptidase activity, and other arguments, the authors described a model to explain the variable levels of C-terminal lysine through both intracellular and extracellular enzymatic mechanisms.

In summary, C-terminal lysine/arginine variability is a natural process whereby the lysine/arginine is added to the C-terminus of a protein during translation and subsequently removed by action of a basic carboxypeptidase. The rate of removal will be affected by the suitability of the protein as a substrate, by the level and distribution of intracellular and extracellular basic carboxypeptidase enzymes, and by bioprocess factors expected to have an impact on enzymatic activity (pH, temperature, metal ion concentration, etc.) or enzyme distribution. Although variation in C-terminal lysine content is expected to have little impact on performance for most biologicals, this heterogeneity results in additional charge and mass variants. As such, minimization of this variability is beneficial from a product characterization perspective.

## 3.6 C-Terminal Amidation

A number of bioactive peptides require C-terminal amidation for activity [43, 159]. The amidation mechanism involves a substrate with a C-terminal glycine and action of the enzyme peptidylglycine alpha-amidating monooxygenase (PAM). The PAM enzyme cleaves the C-terminal glycine from the protein and amidates the exposed C-terminus, resulting in a net basic shift in the molecule and a net loss of 1 Da in mass (in addition to the loss of glycine); [109]. The catalytic center of the enzyme includes two copper ions, and co-factors for the enzyme include oxygen and ascorbate. A number of PAM isoforms exist to target the enzyme through various secretory compartments within the cell and externally [19].

C-terminal proline amidation was unexpectedly found on the heavy chain of an IgG1 produced in CHO cells [109]. The C-terminal sequence was PGK. Removal of the C-terminal lysine by a cellular carboxypeptidase resulted in exposure of a C-terminal glycine. The terminal glycine served as a substrate for PAM, resulting in removal of the glycine, and amidation of the adjacent proline residue.

Similar observations of C-terminal proline amidation were reported by a separate group in the production of an IgG1 expressed in CHO cells [113]. In this case, the authors observed increased amidation that correlated with increased copper ion concentration. At 2L scale under controlled conditions, an increased copper ion concentration from approximately 400 to 1,000 nM resulted in a linear increase in amidation rate from approximately 6 to 15 %. When pooling data from different processes and different scales, the correlation was still apparent, but was no longer linear. As a result, other process parameters were likely affecting the amidation level. The finding of increased copper concentration leading to increased amidation is consistent with the requirement of copper ion for PAM activity. The authors did not determine whether amidation was an intracellular or extracellular event.

In summary, a protein with a C-terminal glycine is potentially subject to action by PAM, resulting in removal of the glycine and amidation of the adjacent amino acid. Although prevalent in bioactive peptides, this quality attribute was only recently observed in the course of drug development, the level of which was increased by the addition of copper ion. Further work is necessary to understand this phenomenon including the clinical impact and whether this is an intracellular or extracellular event.

## 3.7 Glycosylation

Given the prevalence, natural heterogeneity, and significance of protein glycosylation, it is not surprising that the oligosaccharides of glycoproteins are widely studied. This section could be a chapter in its own right. However, a number of excellent reviews are available on the subject. As such, this section only

summarizes the most pertinent aspects and directs the reader to other more comprehensive discussions.

There are two main types of glycosylation including *N-linked* and *O-linked glycosylation* [14, 76, 104, 134, 149, 187, 192, 204]. N-linked glycosylation sites are well-defined by the consensus sequence ASN-X-SER/THR, where X is any amino acid except proline. The initial glycoform at each glycosylation site is also well defined, as a conserved $Glc_3Man_9GlcNAc_2$ structure is transferred to the protein en block while the protein is being translocated into the ER. As the protein travels through the ER and Golgi, a number of enzymes act on the N-linked oligosaccharide first to trim back the outer glucose and mannose residues to create various forms of high mannose structures, followed by the addition of N-acetylglucosamine, galactose, sialic acid, and fucose to form complex structures. Hybrid structures are also observed, where one portion is characterized as high mannose, whereas the other portion is characterized as complex. The most common type of O-linked glycosylation with respect to biopharmaceutical production is initiated in the Golgi by the addition of GalNAc to a SER or THR site; although each site is reproducibly glycosylated, there is no analogous consensus sequence for identification of O-linked sites. The structure is then elongated by the addition of sugars including Gal, GalNAc, GlcNAc, Fuc, and sialic acid. A number of other types of O-linked glycosylation have also been observed on biopharmaceuticals including O-fucosylation and O-mannosylation. Glycan heterogeneity arises due to variable processing of the N-linked or O-linked oligosaccharide as the glycoprotein travels through the biosynthetic compartments including variable addition of the oligosaccharide altogether, a phenomenon referred to as variable site occupancy. This resulting heterogeneity is important inasmuch as oligosaccharide structure is often critical to protein function and has been shown to affect glycoprotein folding, stability, conformation, routing, activity, potency, solubility, receptor binding, clearance rate, tissue clearance, susceptibility to protease digestion, antigenicity, immunogenicity, and isoelectric point.

One determining factor of glycosylation heterogeneity is the protein itself; even different sites on the same protein can have dramatically different structures. For example, antibody Fc glycosylation is typically comprised of a majority of biantennary complex structures lacking sialic acid [104], whereas CD4 primarily contains highly sialylated biantennary complex oligosaccharides [191], and EPO typically contains highly branched and highly sialylated complex structures [176]. The three glycosylation sites on t-PA include one site that exhibits variable site occupancy, a second site that has predominantly complex forms, and a third site that contains predominately high mannose forms [190]. Glycoprotein properties can be altered by engineering a protein to introduce or knock out a glycosylation site, for example, to remove a glycosylation site in the variable region of an IgG for enhanced binding [38] and manufacturability (reduced heterogeneity), or to introduce glycosylation sites in EPO to increase circulatory half-life [53].

The cell line also has a dramatic influence on protein glycosylation through differential expression of glycosyltransferase enzymes and other factors associated with the glycosylation process [11, 12, 20, 51, 76, 103]. Microbial, yeast, insect,

and plant cells are able to perform glycosylation, although differential processing and the display of nonmammalian structures and sugars has limited the usefulness of these expression systems for production of therapeutic glycoproteins. Alternatively, a number of efforts are underway to engineer both mammalian and nonmammalian cell types for improved glycosylation. Approved therapeutic glycoproteins have been expressed with good success in human cell lines including HEK293 and HT-1080 cells and in mammalian cell lines from rodent species including CHO, BHK, NS0, SP2/0, murine hybridoma, and C127 cells [68]. However, differences between the oligosaccharides from the human and nonhuman cell lines have been observed. Most notable is the presence of two nonhuman epitopes. The predominant form of sialic acid in all these expression systems, including human and nonhuman lines, is N-acetylneuraminic acid. A second form variably added by the rodent expression systems is N-glycolylneuraminic acid, a nonhuman sialic acid that may be undesirable from an immunologic viewpoint [67]. The rodent cell lines can also attach an immunogenic alpha-linked galactose residue, although the level is quite variable and often essentially undetectable in CHO cells [17, 201]. The presence of elevated levels of alpha-galactose in the variable region glycosylation of the antibody Erbitux expressed in SP2/0 cells has been linked to anaphylactic reaction in humans due to the presence of pre-existing antibodies against this epitope [34]. Aside from species and tissue-specific differences, clonal variation from a well-characterized cell line can be expected [199] and a number of mutants have been isolated with altered glycosylation [57].

The bioprocess environment also plays a key role in defining oligosaccharide heterogeneity. For example, glycosylation can be affected by pH, temperature, DO, the shear environment, the medium formulation and feed rate, cellular energy state, osmolality, cell growth state, runtime, expression level, metabolic waste build-up, and production method (batch, fed-batch, perfusion) [23, 44, 75, 96, 97]. However, in most cases, the impact on protein glycosylation, if any, is unpredictable. In the case of medium formulation, the feeding of glycosylation precursors, co-factors, or inhibitors has been useful for specifically directing the desired type of glycosylation [16, 83, 200, 219]. Ammonia build-up generally results in reduced glycosylation possibly by increasing the pH of intracellular compartments, by altering gene expression, or due to detoxification of the ammonia through sequestration of galactose [3, 29, 44, 66, 69, 95]. Last, the oligosaccharides can be remodeled in vitro after production and purification of the glycoprotein. This includes both digestion to trim the oligosaccharide and synthesis to add monosaccharides, with the aim of affecting glycoprotein function or creating more homogeneous products [50, 94, 212].

In summary, given the impact, it is important to design processes with correct and consistent glycosylation. The protein, cell, and bioprocess environment all play a role in defining the array of oligosaccharide structures found on the protein of interest. Cell line and protein engineering can be used to affect protein glycosylation and clonal variation can be expected. Given a protein and cell line, bioprocess factors can be varied to understand how these parameters affect glycosylation heterogeneity, although the approach is largely empirical. A more

direct approach to affect glycosylation specifically includes supplementation of the medium with precursors, co-factors, or inhibitors associated with oligosaccharide synthesis. Control of ammonia concentration may be required for consistent oligosaccharide biosynthesis.

### *3.8 Glycation*

Glycation is the attachment of a reducing sugar to lysine or an N-terminal amino acid by what is known as the Maillard reaction; the attachment initiates as a reversible Shiff's base and can further react to form a more stable Amadori product and other more advanced glycation end products [163]. The Amadori product can potentially be removed from the protein by a deglycating enzyme [158]. Circulating proteins normally contain a low level of glycation, for example, 14 % for human IgG [70], which is increased in diabetic patients due to elevated blood sugar levels [152]. The reaction rate for each monosaccharide varies; for example, galactose is more reactive than glucose [22]. Depending on the site of attachment, glycation has the potential to affect biological function of the protein. Furthermore, glycation results in a net acidic shift in protein isoelectric point due to loss of the positively charged functional group on lysine. Glycation of a recombinant protein drug can occur during cell culture with elevated sugar levels (reviewed below), from formulation with a reducing sugar [8, 64, 162], from administration in dextrose infusion bags [60], or naturally after injection into the patient [70].

The first report providing a direct link to the cell culture environment demonstrated glycation at 8 of 48 sites on a CHO-produced IgG1 [163]. The glycation rate at each site varied from 1 to 12 %. Overall, the molecule was 82 % non-glycated, 16 % monoglycated, and approximately 1 % each doubly and triply glycated. Glycation sites were observed on both the heavy and light chain. One site was within the CDR region, although the low level of glycation at that site did not translate into an observable effect on ligand binding. The most and least reactive lysines were separated by a single histidine, which was thought to play a role in glycation. Supporting this hypothesis, from molecular modeling the histidine side chain and the more reactive lysine were spatially aligned. The impact of glucose and galactose concentrations during culture was evaluated in combinations of 11.5–31 g/L total sugar in the medium. Increasing concentrations of sugar resulted in increasing glycation. There was an approximately 0.3 % glycation increase for each g/L glucose addition, whereas a 0.6–0.9 % glycation increase was observed for each g/L galactose addition.

A hot spot for glycation was discovered in an IgG1 expressed in CHO cells [228]. In a first lot of material, 58 % of the molecules were glycated. Although glycation was observed at five sites across the heavy and light chains, four sites were only 0.2–0.6 % glycated, whereas 57.8 % glycation was observed at K49 of the light chain, which was just outside the CDR region. A second lot demonstrated 13 % glycation, with 6.1 % glycation at the light chain K49 position. Incubation of

this second lot in 50 mM glucose for 24 h at 37 °C in sodium bicarbonate buffer pH 7.4 resulted in 53 % glycation across six sites, five of which ranged from 0.2 to 0.5 % glycation, whereas the K49 site had 45.5 % glycation. In an extreme case, incubation at similar conditions in 1.2 M glucose resulted in 100 % of the molecules with glycation across 13 sites. The K49 site was 90.1 % glycated, and 10 other lysine sites were 2.8–14.1 % glycated. Under these extreme conditions, glycation was also observed at low levels on the N-terminal heavy chain glutamate (1.5 %) and the N-terminal light chain aspartate (3.3 %). To evaluate whether free lysine can interfere with this reaction, the experiment for the second lot (13 % glycation) was repeated at 50 mM glucose with up to 500 mM lysine. This resulted in some suppression of glycation from 59 to 41 %. In contrast, incubation of a third lot of purified antibody (41 % initial glycation) in fresh cell culture medium with 50 mM glucose at 33–37 °C for 5–10 days resulted in no change in glycation. Perhaps increased glycation would have been observed had the second lot with lower initial glycation been used in this test. Alternatively, the cell culture medium may have been acting to inhibit the glycation process. Through comparison of 12 other closely related antibodies and molecular modeling, the authors propose that a nearby aspartate residue is acting to accelerate glycation at the light chain K49 position in this antibody.

The antibody described in the previous paragraph was further explored to understand the impact of the cell culture process in order to develop robust strategies for controlling glycation [226]. Glycation levels for the antibody were high (30–60 %) using standard processes for either transiently or stably transfected CHO cells, suggesting that the expression system or expression level was not the primary factor affecting glycation levels under these conditions. In 14 cultures spanning a range of titers and bioreactor scales, glucose levels remained comparably high for most of the culture duration: 9 g/L during the second half of the culture, and typically >5 g/L at the time of harvest. However, in 60-mm unfed dish cultures where the concentration of glucose started near 6 g/L and ended near 4 g/L, the glycation rates from nine different stable clones were relatively lower at 18–21 %. Several different feeding protocols were evaluated in bioreactors to provide progressively lower glucose concentration and tighter control, for example, by using continuous feeding. Lower glucose concentrations correlated to lower glycation levels down to 6 % glycation using 1 g/L glucose target with continuous feeding. Modeling suggested that the glycation reaction was first order with respect to both the glucose and nonglycated antibody concentrations.

Site-specific glycation was also observed in a different CHO-produced antibody, in this case at K98 near the CDR3 region of the heavy chain [146]. Overall, the molecule had 40 % glycation, the majority of which was at the K98 site. This site is typically an arginine for IgG1 and IgG2 molecules, although 20 % of molecules contain a lysine at this position. The overall glycation of 16 other IgG1 and IgG2 molecules including a mix with K98 and R98 demonstrated substantially less glycation. Through forced glycation studies (incubation in 50 g/L glucose pH 7 phosphate buffer 37 °C for 91 h), the increased susceptibility of this antibody to glycation was further demonstrated. Incubation of highly glycated material in

phosphate buffer for 100 h at pH 7 resulted in a reduction in glycation from 42 to 20 %; this reversibility demonstrated that a substantial portion of the glycated material was in the form of a reversible Shiff's base. Although the elevated level of glycation did not affect potency, lot-to-lot variability in glycation levels (20–55 %) resulted in variable charge isoform profiles, making product characterization more difficult. Engineering the K98 to R98 resulted in equal potency while eliminating the charge heterogeneity associated with glycation variability.

In summary, glycation is a natural chemical process resulting from attachment of a reducing sugar to lysines throughout the protein (and to a lesser extent to N-terminal amino acids). The level of glycation will be product- and site-dependent and also depend on the concentration and type of reducing sugar in the cell culture process. The impact of glycation will also vary, depending on the glycation site. Low glycation levels are normal and are an expected outcome of the cell culture process. However, in addition to having a potential impact on biological function, glycation affects the charge heterogeneity profile which can complicate characterization in routine assays used for product release. As such it is advisable to maintain consistent and low levels of glucose (<3 g/L) during the cell culture process. The glucose concentration over the first half of the culture has less impact because the glycation process is initially reversible and the initial product concentration is relatively low. For tighter control, a separate glucose feed is advisable to enable compensation for normal process variability in the glucose consumption rate.

## *3.9 Cysteine Variants*

With the ability to form disulfide bonds, cysteine is a unique and relatively reactive amino acid [27]. Undesirable cysteine variants can arise through a number of mechanisms [138]. These include: improper intracellular assembly resulting in the absence of an expected disulfide bridge or in an incorrect paring; beta elimination of a disulfide pair resulting in an unreducible thioether linkage from the loss of a sulfur atom; the addition of an extra sulfur atom in a cysteine bridge resulting in a trisulfide bond; the extracellular enzymatic reduction of a disulfide bond; and adduct formation on a free cysteine resulting in the addition of a small molecule with a sulfhydryl group or in the cross-linking of the protein with another peptide or protein that contains an unpaired cysteine.

Incomplete disulfide bridging is undesirable, although low levels of free sulfhydryls are often observed [231]. For one antibody produced from CHO cells, decreased potency was linked to incomplete disulfide pairing [25]. The process from an early-stage cell line led to 16 % of the product with an incomplete disulfide bridge, whereas the same product from a late-stage cell line demonstrated twice that level. The authors found that adding increasing concentrations of copper ion, a known catalyst for disulfide bond formation, resulted in lower levels of incomplete disulfide bridging down to 3 % at 100 μM $CuSO_4$ with minimal impact on culture performance.

A naturally unpaired cysteine can be problematic inasmuch as it will have a tendency to react with other sulfhydryls within the cell or the cell culture medium. In one case, a series of papers examined an unpaired cysteine that was engineered into an antibody to provide chemistry for attachment of a drug conjugate [30, 72, 73]. The authors found that glutathione, cysteine, or a free light chain could be variably bound to the unpaired cysteine. The variant containing the extra free light chain could be removed downstream, but lower levels of this variant were desired to reduce the downstream burden. When examining clonal variation, the authors found that a decreased cellular glutathione content or an increased ratio of light chain to heavy chain mRNA correlated with increased expression of the triple light chain species. Bioprocess factors including temperature, pH, DO, and type of hydrolysate were varied; of these, increased temperature (from 33 to 35 °C) was the most effective at reducing the formation of the triple light chain species. The presence of adducted cysteine or glutathione was not problematic for the conjugation chemistry, but variable levels of adduction by these two species complicated analytical characterization. By incubating the purified antibody in a combination of cysteine and cystine to remove the glutathione to replace it with cysteine, the authors were able to create a homogeneous molecule that was fully adducted with cysteine.

Variable levels of cysteinylation were also observed on an unpaired cysteine in the Fab region of antibody produced by CHO or hybridoma cells, which resulted in variable product structure and potency [7, 63]. In this case, the adducted cysteines could be removed from the purified protein by mild reduction with free cysteine. The authors speculate that the cysteinylation occurred intracellularly, because once the product was decysteinylated, the unpaired cysteine was no longer solvent exposed. However, as the authors pointed out, intracellular adduction of glutathione would also have been expected, but none was observed. Perhaps there was extracellular conversion of any adducted glutathione to cysteine based on the findings in the previous paragraph that cysteine can replace adducted glutathione in the presence of sufficient levels of cysteine and cystine.

Several studies have demonstrated and investigated post-harvest reduction of disulfide bonds, leading to product degradation [111, 122, 198]. Degradation occurs due to release of enzymes and enzyme co-factors from healthy cells that lyse during the harvest step. As a result, the potential for post-harvest reduction will increase at high cell viability and high cell density at the end of a bioreactor run coupled with sufficient cell lysis during the harvest step. Post-harvest product reduction can be inhibited using a number of approaches including lowering the pH or temperature, by adding various enzymatic inhibitors including ETDA, by engineering the cell for reduced expression of thioredoxin 1, or by increasing the oxidative environment of the harvest by adding $Cu^{++}$ or cysteine or by increasing the level of dissolved oxygen in the harvest tank.

Although trisulfide bond formation has been characterized as being a rare modification that occurs via a mechanism involving $H_2S$, [155], this modification was observed in all IgG subtypes from both recombinant and natural sources [85]. The trisulfide variant can be reverted to a disulfide form through mild reduction

[160]; this reversion also occurred after injection into rats [85]. The level of trisulfide observed on a CHO-produced antibody was found to vary from 2 to 26 % across different bioreactor runs that varied runtime, feed strategy, and scale [85]. In a more detailed follow-up study, the level of trisulfide formation was directly linked to the formation of $H_2S$ due to the addition of cysteine to the bioreactor [127]. The authors were able to minimize the formation of the trisulfide variant by lowering the level of cysteine supplementation.

In summary, free cysteines and those that form disulfide pairs are both prone to the formation of product variants, including adducted species, incorrect pairing, and the addition or elimination of a sulfur atom in the disulfide bridge. The redox environment, the presence of divalent transition metals, the level of sulfhydryl-containing molecules that can participate in redox reactions, and the presence of reducing enzymes and co-factors can all contribute to the formation of variants during the cell culture process or subsequent harvest step. Cysteine is an unstable molecule in the cell culture medium; when added to the culture medium in the presence of oxygen, cysteine will readily oxidize to cystine within a few hours, a process that is accelerated by the trace metals in the culture medium. Therefore, cystine may be a better choice for cell culture medium supplementation.

## *3.10 Enzymatic Degradation by Proteases and Glycosidases*

In addition to containing the necessary machinery for proper product synthesis, mammalian cells naturally harbor corresponding degradative enzymes [15, 39, 153, 175], raising the possibility for product degradation during cell culture or in downstream processing. These enzymes include proteases, glycosidases, phosphorylases, and the like, necessary for normal protein turnover or for a specific regulatory function. The enzymes are found within various cell compartments, on the cell surface, and are secreted extracellularly and can vary widely in specificity.

For degradation to be significant, the enzyme must be synthesized by the host cell (or be present in media additives) and come in contact with the product intracellularly or be released externally (by secretion or cell lysis), where the enzyme must be stable and active toward the product under bioreactor or downstream process conditions. As such, the rate of degradation, if any, is expected to be a function of: the product (whether is it susceptible); the enzymatic repertoire of the host cell (cell type, clonal variation); the upstream bioprocess conditions (such as pH, temperature, cell density, cell viability, the presence of co-factors or inhibitors, and runtime) that may affect the expression, activity, stability, and distribution of the enzyme; and the downstream process (co-purification of the enzyme and product, and activity and exposure time in each downstream step). Concentration of the product by ultrafiltration will also lead to concentration of the enzymatic activity, which will substantially increase the degradation rate. Low pH tends to increase the activity of many degradative enzymes, however, neutrally active enzymes are also prevalent. Reduced temperature will result in reduced

enzymatic activity. Specific inhibitors are available for some enzyme classes, although process compatibility, regulatory considerations, or cost may limit their usefulness. A number of studies have demonstrated the presence of proteases with varying properties in cell lines used for protein expression including hybridoma [125]; NSO [189]; BHK [125], and CHO cells [35, 36, 54, 177]; these studies characterized protease activity within cells, in the cell culture supernatant, or in downstream processing steps using model substrates.

Further studies have established a direct link between product degradation and host cell protease activity. Antibodies are considered relatively resistant to protease activity, an attribute that facilitates process robustness and the ability to manufacture under high density, long-term fed-batch conditions leading to high product titer. Nevertheless, antibody fragmentation by host cell proteases has been observed for hybridomas [112, 147, 203], NSO cells [189], and CHO cells [65]. Degradation of other expressed proteins by host cell proteases has been observed as well, including: interferon-$\gamma$ expressed in CHO cells [71, 148]; antibody fusion proteins expressed in CHO cells [48, 49, 169]; antithrombin III expressed in BHK cells [197]; factor VIII expressed in CHO cells [88, 174]; erythropoietin expressed in CHO cells [222]; and t-PA expressed in CHO cells [136]. In general, these studies characterize the activity and suggest approaches to minimize degradation. During cell culture, options are to harvest early, operate in perfusion mode to limit exposure of the enzyme, or add an inhibitor. Downstream options are to purify the enzyme from the product, avoid conditions that activate the enzyme, or add an inhibitor. The most comprehensive investigation demonstrated substantial differences in degradation of glucagonlike-peptide-1-antibody fusion protein depending on the product sequence, host cell type used to express the product, clonal variation within a cell type, harvest time, culture temperature, batch versus perfusion culture, and use of the protease inhibitor benzamidine hydrochloride during cell culture to reduce product clipping [48, 49].

The potential for extracellular enzymatic hydrolysis of sugars from recombinant glycoprotein oligosaccharides in cell culture was first recognized in 1991 [77]. In particular, sialidase, $\beta$-galactosidase, $\beta$-hexosaminidase, and fucosidase activities were demonstrated in cell lysates and culture supernatants from CHO, NSO, hybridoma, and 293 cells [78, 79]. However, these activities were measured using model substrates, and further work is necessary to demonstrate whether these activities are capable of altering the oligosaccharides on the product of interest [82].

For example, fucosidase purified from the supernatant of an industrial CHO cell culture process was able to release fucose from oligosaccharide substrates only after removal of the oligosaccharide from the protein [81]. As a result, despite finding a high level of this activity in the supernatant during cell culture, this enzyme is not expected to have an impact on the heterogeneity of cell culture produced glycoproteins.

In contrast, further characterization of the CHO cell sialidase activity demonstrated the presence of a uniquely elevated level of a neutrally active cytosolic sialidase that is released through cell lysis and is able to hydrolyze sialic acid from a glycoprotein product in the culture supernatant [80]. Results from this study

demonstrate or suggest a number of approaches to minimize degradation by extracellular CHO sialidase. Because the mechanism of release is cell lysis, degradation is minimized by maintaining high viability or by using perfusion culture to minimize contact time. Alternatively, metal ions can be used to inactivate the enzyme, provided it is compatible with the process. A specific sialidase inhibitor 2,3-dehyro-2-deoxy-N-acetyl neuraminic acid is an effective approach to suppressing degradation by sialidase during cell culture; however, due to the high cost, this method is more conveniently used as a diagnostic control to determine whether degradation by sialidase is occurring, and is not cost effective for large-scale cell culture. Interestingly, the CHO cell sialidase preferentially cleaves 2,3-linked sialic over the 2,6-linked isomer; inasmuch as CHO oligosaccharides have only the 2,3 form, cell line engineering to express 2,6-linked sialic acid would result in substantially lower degradation by cytosolic CHO cell sialidase. CHO variants can also be isolated or engineered to contain reduced sialidase activity. Last, low protein media formulations appear to destabilize the enzyme; it is possible that protein additives sequester metals in the cell culture medium that would normally inactivate the sialidase. Since then, a number of other studies have demonstrated a correlation between extracellular sialidase activity in CHO cell culture leading to lower sialic acid content [33, 84, 107, 129, 132, 133, 150, 151]. A number of genetic approaches have been used to knock down expression of the sialidase enzyme specifically [59, 154, 229]. In a recent study, four different CHO sialidase enzymes were investigated, including a lysosomal form Neu1, the cytosolic form Neu2, a plasma membrane bound form Neu3, and a second lysosomal form Neu4 that was not further investigated after concluding that is was a pseudogene in CHO cells [229]. The lysosomal forms of sialidase are known to be active only at low pH and are very unstable near pH 7 [79]. The cytosolic form was described above.

The most interesting aspects of this study are the results from the Neu3 form of sialidase. Because this enzyme is secreted to the cell surface, cell lysis is not required for exposure of the enzyme to the extracellular product. When the three enzymes were expressed in COS7 cells, the activity measured using an artificial substrate at pH 4.6 was highest for the Neu3 enzyme, and siRNA to knock out Neu3 resulted in a 30 % increase in the sialic acid content of CHO expressed interferon-$\gamma$. However, as stated above, the demonstration of activity toward an artificial substrate at a pH substantially different from that of cell culture does not directly demonstrate that interferon-$\gamma$ can be degraded by the Neu3 enzyme. Furthermore, the process of engineering the cell line could result in other changes, including intracellular biosynthesis, which was not investigated. Therefore, further work is needed to provide a direct link between the Neu3 sialidase enzyme and glycoprotein sialylation.

In summary, product degradation by host cell enzymes can result in reduced yields, undesired product variants, and reduced process robustness. The potential for enzymatic degradation must be assessed on a case-by-case basis and will depend on the product, the cell line, and the bioprocess conditions. Conditions that tend to minimize enzymatic degradation include low cell density, high cell

viability, low temperature, high pH, and short exposure time (e.g., perfusion may be preferable to fed-batch), although exceptions are to be expected. Depending on the downstream process, a degradative enzyme could potentially be co-purified and activated under downstream conditions that differ from those in the bioreactor. Specific inhibitors could potentially be used based on identification of the enzyme causing the issue, although process compatibility, cost, and regulatory considerations will need to be assessed.

## 3.11 γ-Carboxylation and β-Hydroxylation

The post-translation modifications of $\gamma$-carboxylation and $\beta$-hydroxylation are important for the function of a number of proteins including those involved in blood coagulation [89, 114]. Both modifications occur through enzymatic action in the ER. $\gamma$-Carboxylation is performed by the vitamin-K-dependent enzyme $\gamma$-glutamyl carboxylase, which carboxylates glutamic acid residues. $\beta$-Hydroxylation is performed by the enzyme ASP/ASN hydroxylase, which hydroxylates aspartate or asparagine residues. Very little has been published regarding the impact of the bioreactor process on these modifications. However, the ability of the host cell to perform these modifications clearly has a large impact on the extent of modification [210, 221]. Overexpression of $\gamma$-glutamyl carboxylase in CHO cells did not increase the ability of the cell line to perform $\gamma$-carboxylation [168]. Alternatively, overexpressing the enzyme vitamin K epoxide reductase resulted in increased $\gamma$-carboxylation of Factor VII expressed in HEK293 cells [209], Factor X expressed in HEK293 cells [194], and Factor IX expressed in BHK cells [208]. More work is necessary to understand the bioprocess factors affecting these modifications.

## 3.12 Process-Related Impurities

Process-related impurities associated with cell culture production primarily arise from either the medium or the cell line. These components need to be identified and controlled to a low and consistent level to ensure product safety. In a chemically defined protein-free medium, cell culture components are typically not a concern inasmuch as the components have a low molecular weight and are readily removable. Typically, impurity clearance of these media components is based on theoretical arguments or based on demonstration of clearance of a few components that are either toxic such as methotrexate or represent a certain class of components such as antifoam as a hydrophobic additive or pluronic as a surfactant. More work may be required if using a protein additive, a hydrolysate, serum, or other less-defined components. Process-related impurities from the cell line are typically characterized as DNA, host cell proteins (HCP), and viruses (or viral-like particles, retrovirus, etc.). The WHO defines an acceptable residual level of DNA

as <10 ng/dose [217]. Although there is no rigorous regulatory guideline for HCP, commercial processes typically contain less than 10 ppm HCP [52]. Viral clearance validation is required prior to performing a clinical study [102].

The impact of the cell culture environment on the level of endogenous retrovirus particles was explored for CHO cells [21]. The particle level increased steadily over the course of cell culture. Of the parameters explored, the cell line had the largest impact. The specific rate of particle production varied widely across the five cell lines evaluated, resulting in up to a 3-log difference in final viral particle count. Culture conditions that did not have a strong influence on cell metabolic activity including scale, seed density, feeding, and cell bank used, also did not strongly influence the specific rate of retroviral particle production or the final particle count. Variation of DO above the standard setpoint of 30 % had little impact on particle production, whereas growth at 0 % DO resulted in reduced culture performance and reduced particle count. For one cell line, pH variation from 7.15 to 6.90 had little impact on retroviral particle count. For a second cell line, the retrovirus titer increased moderately (up to one log) as the pH was lowered from 7.4 to 6.7 due to an increase in the specific rate of retroviral particle production. An increase in the specific retroviral particle production rate was also observed upon addition of sodium butyrate up to 6 mM (up to 1-log increase) or from reducing the temperature from 37 to 33 °C (up to a 1.5-log increase), and applying both temperature shift and sodium butyrate resulted in up to a 2-log increase in the specific retroviral particle production rate.

Host cell proteins can potentially be released from the cell by secretion or cell lysis. Evaluation of the impact of the cell culture environment on HCP concentration is complicated by the fact that host cell proteins represent a collection of hundreds to thousands of species [126]. Quantification of HCP is typically by ELISA using polyclonal antibodies prepared from HCPs generated from a mock-transfected cell line [52]. The material used for a reference and for generating the antibodies is from cell lysate, cell culture supernatant, and/or from a downstream sample such as a post protein A flow-through pool [215]. The suitability of the antibody is determined by evaluating coverage on a 2-D gel [218]. Proteomic studies incorporating 2-D gels are also being increasingly used to understand the impact of the culture environment on the level and distribution of host cell proteins [106, 126, 195]. In general, these studies report that the cell line (similar host) and culture process have less impact on the level and distribution of HCP compared to final culture viability. However, large differences were reported only when the final culture viability was lower than that typically found in commercial cell culture processes.

Intuitively, it is likely that the amount of DNA that will need to be removed will vary directly with the concentration of lysed cells. Although no direct correlation could be found, one study demonstrated a correlation between increased runtime and increased DNA [172].

In summary, the cell culture process has the potential to affect process-related impurities. Although downstream processes are typically able to clear upstream process-related impurities, providing consistent and predictable levels of these

impurities enables the downstream group to assure removal. Factors affecting cell metabolism, cell density, and cell viability are likely to have the strongest influence on the level of process-related impurities originating from the host cell line.

### *3.13 Other Chemical and Biological Modifications*

Aside from those described in previous sections, a number of other chemical or biological modifications have been observed on commercial products manufactured by mammalian cell culture. The common chemical modifications include asparagine deamidation, isomerization of aspartate to succinimide or isoaspartate, oxidation of tryptophan or methionine residues, and peptide cleavage [137, 205]. It is likely that factors in the cell culture process will affect these modifications. However, no publication could be found to provide a direct link of any of these modifications to the cell culture process. Other biological modifications include sulfation and phosphorylation [210]. More work is needed to better understand the impact of the culture process on these other chemical and biological modifications.

## 4 Addressing Variability in Product Quality

When assessing product quality from upstream samples, the typical initial approach is to purify the product sufficiently to enable characterization by primary methods such as SDS-PAGE, IEF, HP-SEC, and glycan profiling along with a relevant functional assay. If product variability is observed, the best approach is to apply additional characterization methods to determine what is specifically causing the heterogeneity. The approach to addressing variability depends on the mechanism leading to variability including whether the impact is caused by chemical or biological factors and whether the modification is occurring intracellularly or extracellularly. A time-course study using in-process bioreactor samples and product held in cell-free supernatant is useful for understanding the mechanisms leading to product quality variability. The approach to addressing variability also depends on the stage of development, for example, whether changes in the product or cell line are viable options for addressing the issue. Approaches to affect product quality specifically can be investigated based on known mechanisms of occurrence as outlined in this chapter. Last, an empirical approach can be applied by exploring the design space of upstream process parameters. Alternatively, downstream approaches can be used to remove undesired variants.

## 5 Summary, Conclusions, Outlook

Mammalian cell culture produced proteins are subject to a number of modifications, each of which may affect the physical, chemical, or biological properties of the molecule. The combination of these modifications leads to a staggering number of possible protein isoforms, for example, estimated to approach $10^8$ for a recombinant antibody [124]. Empirical approaches are still required to investigate this complex issue, however, recent efforts have added substantially to our understanding of the types of modifications that can occur, where they occur in the molecule, the rate of occurrence, the specific mechanisms affecting each modification, and the criticality associated with each modification. These efforts in turn provide more directed approaches to maintaining consistent product quality or affecting product quality for enhanced comparability or product performance.

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Adv Biochem Eng Biotechnol (2014) 139: 167–183
DOI: 10.1007/10_2013_213

Published Online: 30 May 2013

# Safety Assurance for Biologics Manufactured in Mammalian Cell Cultures: A Multitiered Strategy

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**Abstract** Contamination by viral and microbial agents is a serious risk for biopharmaceuticals produced by mammalian cell culture processes. In order to effectively mitigate the risk and minimize the occurrence of such contamination events, a multi-tiered approach has been adopted to safeguard the manufacturing processes from A to Z. The multi-tiered approach consists of three separate, yet complementary, elements: (1) control and testing of raw materials in general, and animal sourced materials (ASM) in particular; (2) in-process and release testing for adventitious agents with emphasis on viruses based on risk assessment; and (3) demonstration of an adequate, robust, and consistent viral clearance capability by the downstream purification process. The implementations of these measures will be described in the context of regulatory compliance and GMP manufacturing.

**Keywords** Viral safety · Viral clearance · Viral contamination · Adventitious agents

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## 1 Introduction

Microbial (bacteria, fungi, mycoplasma) and viral contamination is a shared safety risk for all biologics produced in mammalian cell cultures (MCC). Microbial contamination is a legitimate concern for MCC-based production. For example, it has been reported that 15–30 % of the cell lines examined are contaminated by mycoplasma [1]. However, the combination of bioburden reduction measures, appropriate cGMP and environmental controls, and robust in-process monitoring/testing can often effectively prevent such contamination. On the other hand, contamination by viruses is more difficult to control, detect, and prevent in comparison to that caused by microbial agents for several reasons, and thus poses the most serious safety challenge for both regulatory agencies and the biopharmaceutical industry. First, commonly used production cell lines are of rodent origin such as Chinese Hamster Ovary (CHO) and mouse myeloma NS0 cells. These cells produce retrovirus-like particles (RVLP) encoded by their genomes [2–4] and hence the presence of RVLP in the MCC production is inevitable due to its endogenous nature. It has been reported that the level of RVLP expression in CHO can vary up to 3 $\log_{10}$ among clonal cell lines [5]. Second, viruses are much smaller than microbial agents and thus cannot be effectively removed by 0.2 or 0.1 μm conventional filters. Third, viruses are ubiquitous in the environment as other adventitious agents; they can be potentially introduced into cell cultures throughout the manufacturing process via contaminated cell substrate, raw materials, culture media, or even personnel. Fourth, there is no simple and sensitive method that can reliably detect a broad range of viruses. Finally, some viruses can infect cells without causing any apparent changes in cellular metabolism or viability such as sudden pH fluctuation, alteration of population doubling time, or cytopathic effects (CPE), making it difficult to detect the contamination in a timely manner [6]. Because of these challenges, a multitiered approach has been adopted to minimize the risk of viral contamination. The approach consists of three independent, yet complementary tiers in order to achieve safety assurance for biopharmaceutical products (biologics): (1) control and testing of raw materials in general, and animal-sourced materials (ASM) in particular; (2) in-process and release testing for adventitious agents with emphasis on viruses based on risk assessment; and (3) demonstration of an adequate, robust, and consistent viral clearance capability by the downstream purification process.

This chapter provides a general overview of the tiered safety strategy commonly employed by the biopharmaceutical industry to meet current regulatory requirements, thus ensuring maximal product and patient safety.

## 2 Control and Testing of Raw Materials

Since the 1980s, there have been several publicly known cases of viral contamination in MCC manufacturing processes involving murine minute virus (MMV),

reovirus (Reo), Cache Valley virus (CVV), epizootic hemorrhagic disease virus (EHDV), and vesivirus [7–11]. Despite significant efforts, the verifiable source and/or route of contamination has not been determined for any of these cases. However, raw materials and ASM in particular were incriminated in almost all these cases as supported by circumstantial yet compelling evidence. Commonly used ASM in MCC production include fetal bovine serum (FBS), bovine serum albumin (BSA), and porcine/bovine-derived enzymes such as trypsin. It has been well documented that many viruses have high prevalence in the cattle and swine populations. For example, bovine viral diarrhea virus (BVDV) can be readily detected or isolated in FBS [12, 13]. It is also well known that many important human pathogenic viruses such as influenza and hepatitis E have their reservoirs in animals. Therefore, ASM are considered the highest risk for introducing adventitious viral contaminants into MCC processes. Naturally, the use of ASM in any stage of MCC production will certainly draw the attention of the regulatory agencies and subject the process to much closer scrutiny. It is in the companies' best interest to make every possible effort to eliminate the use of ASM in the MCC processes as recommended by the harmonized guidelines [14, 15] in order to reduce the real as well as the perceived risks of viral contamination.

When the use of ASM cannot be completely avoided, it is important to have an effective quality control system in place to minimize the viral risk associated with the ASM. Potential risks for transmissible spongiform encephalopathy (TSE) and adventitious viruses need to be assessed when materials of ruminant origin are used in the production process. In this regard, MCC production processes involving the use of FBS and/or BSA are closely scrutinized by regulatory agencies worldwide because of the potential risk of bovine spongiform encephalopathy (BSE). Therefore, it is important to have all the documents for the bovine-sourced materials to demonstrate their suitability for the intended use. It is highly desirable to use FBS, BSA, or any other bovine-derived raw materials sourced from countries or regions with negligible BSE risk according to the World Organization for Animal Health (OIE), thus minimizing the BSE risk and reducing the associated regulatory concerns.

ASM should be screened for viruses using appropriate testing methods (cell-based infectivity assays or nucleic-acid-based assays) with adequate controls to ensure the validity of the testing results. For example, bovine-derived raw materials are tested using cell lines susceptible to various bovine viruses. For viruses that do not have a reliable cell-based detection method, an alternative method such as polymerase chain reaction (PCR) or an in vivo method can be used. Although testing is necessary and important, it is inherently hampered by factors such as assay sensitivity, representative sampling, testing volumes, matrix inhibition, and nonhomogeneous distribution of the contaminants. Therefore, it is recommended that negative/nonreactive testing results are interpreted and accepted with caution as they could potentially be false stemming from the limitations described above.

In addition to testing, a robust system needs to be in place to manage the vendor quality supply chain effectively. It is important to understand clearly the raw material manufacturing process and the robustness of the vendor supply chain

through a rigorous vendor qualification process and quality audits. Whenever possible, it is recommended that ASM used for biologic production are treated with efficacious inactivation/removal methods to reduce the risk of adventitious viruses. Virus reduction unit operations such as viral filtration, gamma irradiation, and thermal inactivation are effective methods commonly used for ASM treatment by suppliers in order to reduce the viral risks. The virus reduction capacity is estimated by the laboratory scale spike and recovery studies using appropriately selected model viruses. It is highly recommended to obtain such information from the ASM suppliers for review as part of the risk assessment. This review mainly focuses on these questions: (1) did the laboratory scale spike and recovery studies represent the production scale unit operation as closely as possible; (2) were the model viruses selected appropriate; and (3) were sufficient controls included in the studies to ensure the validity of the results. Together, the answers to these questions would determine whether the reported virus reduction capacity, often expressed as the log reduction factor (LRF) or log reduction value (LRV), was a valid and accurate estimation. If deemed necessary, an ASM production process can be customized by adding one or more virus reduction unit operation to mitigate the viral risks. To assess the effectiveness of virus reduction by such a customized ASM production process, laboratory scale spike and recovery studies are performed to provide evidence that the treatment is effective in eliminating viruses. The design and execution of these studies follow the principles below to ensure data validity and accurate estimation. (1) The laboratory scale studies resemble the production scale as much as possible, particularly for the known critical process parameters (CPP); (2) relevant or worst-case model viruses are used in the laboratory scale studies; and (3) sufficient controls including those for possible inhibitions by matrices must be included in the studies.

In summary, raw materials represent potential sources for viral contamination and ASM pose the highest viral risk among all the raw materials. To mitigate the viral risks associated with raw materials effectively, priority should be placed on elimination of all ASM by investing in research and new technologies such as the development of chemically defined media (CDM) for MCC production. When the use of ASM cannot be avoided, it is important to implement appropriate material sourcing controls and risk reduction measures to minimize the potential of introducing viral contaminants via ASM.

## 3 In-Process Testing

There are two types of MCC processes based on feed strategy: fed-batch processes and continuous processes (also known as perfusion processes). A fed-batch process takes multiple weeks of cell expansion to complete a bioreactor starting from a vial of master cell bank (MCB) or working cell bank (WCB). For continuous processes, a bioreactor is often maintained for months with routine scheduled harvesting and feeding. The lengthy cell culture time under optimal growth conditions

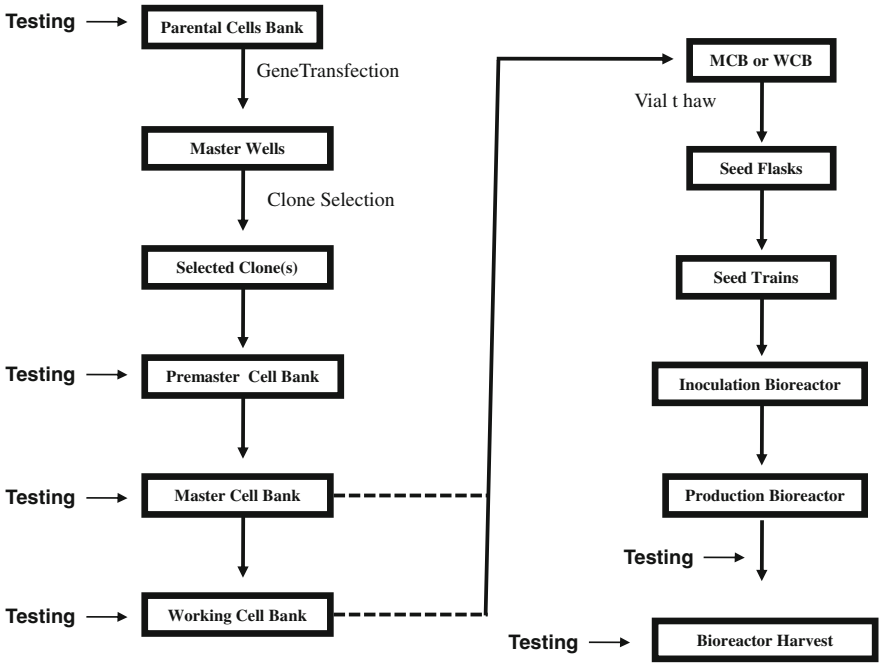


**Fig. 1** A representative schematic in-process testing throughout the MCC process

is an ideal environment for virus replication and propagation. In theory, a single infectious virus could potentially bring down the bioreactor and potentially contaminate the entire manufacturing facility if not detected in time. As a result, a comprehensive in-process testing strategy not only ensures product safety but also protects the manufacturing facility.

In-process testing is carried out throughout the MCC production process from parental cell line to bioreactor harvest. Figure 1 illustrates a general in-process testing scheme for a representative MCC production process. The scope, extent, and testing assays for the individual in-process testing should be based on the risk profile of the testing subject. The parental cell bank (PCB) is screened for adventitious agents based on risk assessment prior to transfection. The primary purpose of the PCB testing is to provide assurance that the starting cell line is free of detectable adventitious agents prior to MCB manufacturing. If the parental cell line is obtained from an external source, it is important to obtain all the information from the supplier as part of the risk assessment to ensure that the adventitious agent testing is adequate. The extent of the PCB testing may vary depending on known risks such as cell line history and ASM exposure or internal policies among different companies as there are no specific regulatory guidelines for the testing. Pre-master research cell banks (pmRCB) are derived from clonal cells containing the gene of interest. These banks are carefully characterized for product

**Table 1** Testing and characterization of MCB, WCB, and cells at or beyond the limit of in vitro cell age[a]

| Category | Test description | MCB | WCB | Cells at the limit of in vitro cell age |
|---|---|---|---|---|
| Adventitious viruses or nonendogenous viruses | In vitro (cell cultures) | + | +[b] | + |
| | In vivo (animals and eggs) | + | +[b] | + |
| | Antibody production[c] | + | – | – |
| | Other specific viruses[d] | + | – | – |
| Retroviruses and other endogenous viruses | Infectivity | + | – | + |
| | Electron microscopy | + | – | + |
| | Reverse transcriptase[e] | + | – | + |
| | Other specific viruses[f] | As appropriate | – | As appropriate |
| Others | Sterility | + | + | + |
| | Bacterial/fungal stasis | + | +[g] | + |
| | Mycoplasma | + | + | + |
| | Mycostasis | + | +[g] | + |
| | Identity test | + | + | – |

[a] Modified from Table 1 in Ref. 15
[b] For the first WCB, this test should be performed on cells at the limit of in vitro cell age
[c] Such as MAP, HAP, applicable for cell lines of rodent origin
[d] Tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate based on ASM exposure and/or cell line susceptibility to specific viruses, for example, mouse minute virus (MMV) should be tested for CHO cells and adenovirus should be tested for human embryo kidney (HEK) cells
[e] The test is not necessary if the retrovirus infectivity test is positive
[f] As appropriate for cell lines that are known to have been infected by such viruses
[g] These tests can be omitted if they have been performed during MCB testing, and no changes in media used for WCB production

titer, stability, and growth profile in order to select the best candidate for MCB preparation. The pmRCB testing is usually dictated by specific requirements of the GMP cell bank facility into which the selected pmRCB will be introduced for MCB production. There are no specific regulatory guidelines for the testing of the pmRCB and companies have the freedom to tailor the level of testing based on risk assessment and business needs. However, it is recommended that the selected pmRCB is tested for mycoplasma and sterility at the minimum to mitigate cross-contamination concerns. Additional viral testing can be omitted provided no ASM are used in the pmRCB production and the PCB has been extensively screened for viruses.

Regulatory guidelines provide specific testing requirements for MCB, WCB, and cells at the limit of in vitro cell age [14–17]. The required individual tests specified by the guidelines for these banks are summarized in Table 1. Depending on the species origin, the extent and emphasis of viral testing for these banks can differ significantly. In general, cell lines of human or primate origin require more extensive viral screening than rodent cell lines as human–human and primate–human transmissions are likely easier to occur and cause diseases than rodent–human ones.

Sterility and mycoplasma tests along with bacterial/fungal stasis and mycostasis are standard for any MCB or WCB regardless of the species origin.

Under normal circumstances, only a single MCB is made for any given molecule entity. The established MCB is expected to serve as the sole source of the starting cell substrate throughout the product lifetime. Consequently, the MCB is extensively and broadly screened for viruses. General screening tests for adventitious viruses include both in vitro (cell culture) and in vivo (animals and embryonated eggs) testing. In addition, tests for specific viruses are required for the MCB testing. For an MCB of rodent origin such as CHO, the tests for specific viruses include hamster antibody production (HAP) and mouse antibody production (MAP) tests. The MCB is also tested for bovine and/or porcine viruses depending on whether it has had prior exposure to these ASM. Retrovirus screening is also required for the MCB testing as retroviruses may have the potential to cause serious disease including cancers. Because the commonly used production cells such as CHO are of rodent origin and known to produce endogenous retroviruses, the retrovirus screening is of particular importance. Despite the fact that no infectious endogenous retroviruses have ever been isolated from CHO cells, retrovirus screening remains indispensable for the testing of CHO-derived MCB. The methods for retrovirus detection include reverse transcriptase (RT) assay, cell-based infectivity assay, and transmission electron microscopy (TEM). It is often unnecessary to complete all the tests before using the MCB to start the MCC manufacturing process. The specific requirements for bringing MCB cells into a GMP manufacturing facility vary among companies and can be influenced by origin of species, cell line history, and ASM exposure. However, the testing results must demonstrate that the MCB is free of detectable adventitious agents prior to the initiation of clinical trials.

Since the WCB is the direct descendant of the MCB that has been thoroughly tested with satisfactory results, the WCB testing does not need to be as extensive and vigorous as the MCB testing. As shown in Table 1, the WCB is only screened for general adventitious viruses by in vitro and in vivo methods.

Cells at or beyond the limit of in vitro cell age must be tested at least once prior to Biologics License Applications (BLA, United States) or Marketing Authorization Application (MAA, European Union) submission [15]. The term "end of production cells" (EOPC or EPC) has also been used to refer to cells at the limit of in vitro cell age [17, 18]. Cells at or beyond the limit of in vitro age are defined as "cells derived from a pilot or production scale bioreactor, which have reached a passage level or population doubling level at or beyond the maximum level anticipated in production." The testing of cells at or beyond the limit of in vitro cell age serves dual purposes: to provide assurance that cell passages/expansions under the manufacturing conditions do not induce new endogenous viruses that were not detected before, and to ensure that the manufacturing process is not prone to contamination by adventitious viruses. Consistent with the purposes above, the testing of cells at or beyond the limit of in vitro age usually will not take place until the upstream culture conditions have been finalized. The testing should be

performed using cells that have been expanded under pilot or production scale conditions [15]. It is desirable to add additional cell passages or population doublings for the bioreactor from which the cells will be harvested for testing. This can be readily achieved by extra passages at the flask or seed train stage of the cell expansion process following the vial thaw. By testing the cells that are several passages beyond the expected in vitro cell age, the possibility of having to repeat the test will be reduced in case there is a scale increase in the production bioreactors in the future. If the cell viability is sufficient to bank the cells at or beyond the limit of in vitro cell age, an extended cell bank (ECB) can be made and all the tests can then be performed using the established ECB. Testing of cells at or beyond the limit of in vitro age testing is similar to MCB testing except that the methods for detecting retroviruses are more sensitive. Retrovirus testing for cells at or beyond the limit of in vitro cell age typically includes a co-cultivation assay for retroviruses that cannot be readily detected by conventional infectivity assays. If there are significant changes in the cell culture process such as a new culture medium, the testing of cells at or beyond the in vitro cell age may have to be repeated.

Bioreactor harvests, commonly known as unprocessed bulk (UPB), are screened for viruses and other adventitious agents. For fed-batch processes, UPB testing is performed on each individual bioreactor because pooled harvests could potentially dilute the contaminants, thus reducing the detectability. For continuous processes, UPB testing is often performed on pooled harvests. However, special measures are needed in order to prevent contamination by adventitious agents during storage prior to the final pooling and testing. Predetermined acceptance criteria are required for UPB testing in order for the quality assurance (QA) function to release or reject batches appropriately. A representative UPB testing scheme is described in Table 2. It is worth emphasizing that the scope and extent of UPB testing should be determined by taking multiple factors into consideration, including the origin and history of the production cell line, the results and extent of virus tests performed on MCB and WCB, presence or absence of ASM in culture media, and the virus clearance capacity of the downstream purification process.

Viral contamination events reported recently [6, 19–21] have prompted a growing number of companies to implement fast turnaround PCR testing for specific viruses prior to bioreactor harvest as a containment measure. The in-process MMV testing by PCR was first implemented by Genentech in the early 1990s following a widespread MMV contamination event that affected the entire GMP manufacturing facility [7]. The implementation of MMV PCR testing allowed Genentech to detect the second MMV contamination prior to the bioreactor harvesting and effectively prevented the spread of MMV in the facility, thus significantly shortening the time for decontamination and cleaning. Although such in-process testing for specific viruses prior to harvesting is not yet required by regulatory guidelines, it has increasingly become common practice in the industry to provide an early warning system for specific virus contamination. The detection of virus contamination prior to bioreactor harvesting can significantly reduce the cost of cleaning, loss of production, and possibility of supply interruption as the

**Table 2** Unprocessed bulk testing for adventitious agents[a]

| Adventitious agents | Test | Description |
|---|---|---|
| Microbial | Bioburden or sterility | Acceptable limits are set if bioburden test is used |
| | Mycoplasma | Mycostasis is required to ensure data validity |
| Viruses | In vitro (cell cultures) | Three indicator cell lines are used, including the production cell line, a human cell line, and a nonhuman primate cell line |
| | Other specific viruses | Testing specific viruses known to infect the production cell line such as MMV for CHO cells |

[a] Modified from Table 2 in Ref. 17

result of the virus contamination. It is worth noting that the implementation of such an early warning system has largely been driven by devastating virus contamination events in the industry. Consequently, the in-process testing assays developed only provide early detection for a single virus such as MMV [6, 7] or vesivirus 2117 [20]. Various new nucleic-acid–based detection technologies can now readily detect multiple viruses simultaneously with rapid turnaround time [22–25]. It is conceivable to proactively design and develop a new generation of in-process testing assays that can provide early warning for multiple viruses based on the virus susceptibility of the production cell line. For example, CHO cells are susceptible to many different viruses belonging to a dozen or so families based on existing studies [26–28]. It is therefore possible to develop an assay that can provide effective early warning for all viruses known to infect CHO cells should the bioreactor be contaminated by any individual virus in the group.

Most of the above-mentioned in-process testing such as MCB, WCB, and cells at or beyond the limit of in vitro cell age are usually performed by specialized testing companies. These testing companies play a vital role in ensuring the safety of biologics produced in MCC. On the other hand, any missteps ranging from operational error to quality system gaps at these testing companies could have an enormous adverse impact on the sponsoring company and patients. Any out of specification (OOS) results immediately place the affected project or molecule on hold pending the outcome of the root cause investigation. The true positive results usually can be quickly confirmed by retesting or other orthogonal assays, thus allowing the sponsor to take appropriate actions to resolve the matter. However, false positive results can inflict unnecessary loss of time and/or materials. False positive or OOS results in general can have many different causes such as medium components, ASM used in the actual screen tests, cross-contamination, and operational errors, just to name a few. For example, insulin was identified as the root cause of an unusual OOS result observed in an in vivo viral assay for MCB testing [29]. In another case, it was demonstrated that the false positive result was caused by the contaminated horse serum used in the cell-based in vitro viral screen assay for bovine viruses [30]. Unfortunately, root causes for many OOS or putative false positive results cannot be determined despite tremendous efforts by all parties involved. When the initial positive results cannot be confirmed and there is no

compelling evidence indicating that it is a false one, then the positive result should be assumed to be a real one and immediate appropriate actions are needed in order to minimize the impact. It is in the best interests of both the sponsor and the testing company to minimize the occurrence of the false positive testing results by being proactive. One specific recommendation is that testing companies and sponsors work together to ensure ASM used for in-process testing such as FBS are sourced and controlled to the same rigors of quality and safety standards as those used for MCC manufacturing to minimize false positive results during testing. Regardless of whether the positive results are true or false, quick resolution is highly desirable for all parties involved to minimize the adverse impact of such events, which can only be achieved by working together, sharing all the relevant information, and devoting adequate resources in a timely manner.

The regulatory guidelines provide high-level requirements for MCB, WCB, and UPB testing as shown in Table 1 and 2, yet it is up to the individual company to determine the specific details, such as assay duration and number of cell lines in the case of in vitro virus testing based on the risk profile and intended use of the testing subject. For example, in vitro viral assays commonly used for screening of adventitious viruses are designed to detect a broad range of viruses by using multiple endpoints and can be carried out with either 14-day or 28-day duration. The 28-day assay includes an additional passage of culture media onto fresh indicator cells on day 14 post-inoculation with an intended purpose to increase the assay sensitivity. Both MCB and UPB are screened for adventitious viruses by the cell-based in vitro virus assay, yet these two testing subjects exhibit distinct risk profiles in terms of potential impact, cell culture processes, and likely contamination level. First, MCB is the starting cell substrate for the life cycle of the product and a contaminated MCB could potentially affect the entire product franchise. In contrast, each individual UPB represents only one particular batch of the bioreactor harvest, hence any contamination would be limited to the given batch or batches involved. Second, because MCB is the starting cell substrate, a low-level viral contaminant could turn into a full-blown contamination event through adaptation and propagation in subsequent extensive cell expansions. On the other hand, UPB is at the end of the cell culture process; low-level viral contamination will not have the opportunity to propagate further and likely will be removed by a downstream purification process. Third, MCB manufacturing involves multiple manipulations from vial thaw, cell passage, centrifugation, and re-suspension, to final vial aliquot, all taking place in an open environment. Viral contaminants could be inadvertently introduced into the MCB during any of these manipulations with similar probability via the culture medium, cryopreservation medium, or operators. Conversely, UPB is produced by a gradual bioreactor-to-bioreactor scale-up process in a closed system and a viral contaminant is most likely introduced into the UPB either at the time of inoculation of the production bioreactor or earlier, should it occur. Assuming a single infectious virus is introduced into UPB during the inoculation of the production bioreactor, the single virus would have sufficient time to adapt and produce an enormous amount of progeny viruses if it can initiate the infection, making it readily detectable by

14-day in vitro assay. This notion can be best illustrated by the vesivirus 2117 example experienced by Genzyme. It was found that vesivirus 2117 isolated from the contaminated bioreactor failed to cause noticeable CPE without passage in CHO cells when inoculated at a very low level. However, inoculation of CHO cells with the cell culture materials (UPB) from the vesivirus-2117-contaminated bioreactor consistently resulted in obvious CPE between 5 and 10 days post-inoculation [11, 31]. If the virus could not initiate the infection, it is then not a safety concern at all as the contaminant will certainly be removed by the downstream purification process. Finally, there is an inherent risk of viral contamination during the in vitro virus assay itself as discussed earlier, thus causing false positive results. The most vulnerable points for introducing viral contaminants are during the initial inoculation and subsequent medium change or culture passage. The additional passage in the 28-day assay inevitably increases the risk of false positive testing results. Based on the above assessment, it would make perfect sense to test MCB using the 28-day in vitro viral assay in order to maximize the possibility of detecting low levels of viral contaminants. The increased risk of a potential false positive associated with the 28-day assay is worth being taken in order to maximize the detection of low-level or slow-growing viruses. On the other hand, the assessment provides a scientific basis to justify the 14-day assay for UPB testing to maximize the benefit of adequate virus detection and meanwhile to minimize the chance of potential false positives.

## 4 Virus Clearance by Downstream Purification Process

In order to assure the safety of biologics produced in MCC, the downstream purification process is designed to provide sufficient and effective virus clearance. This is achieved by incorporating multiple unit operations dedicated to virus inactivation or removal as shown in Fig. 2. The sole purpose of the dedicated virus clearance unit operations is to remove or inactivate viruses. Therefore, it is important to ensure that these dedicated unit operations can do so consistently and effectively. In the meantime, these unit operations do not have any adverse impact on product quality or cause significant yield loss. If any chemicals are introduced in the dedicated virus clearance unit operations, they ought to be readily and effectively removed by the purification process. The spike and recovery viral clearance studies are required in order to demonstrate that the downstream purification process is able to clear known viral contaminants adequately (such as endogenous RVLP) as well as those inadvertently introduced into the process [15, 17]. These studies are carried out using scaled-down laboratory models that resemble the corresponding production scale unit operations as closely as possible. It is highly desirable to pull all the materials used in the virus clearance studies from the production-scale manufacturing to ensure their representativeness. Materials from alternative sources can also be used as long as their representativeness can be assured. The model viruses selected for the laboratory scale

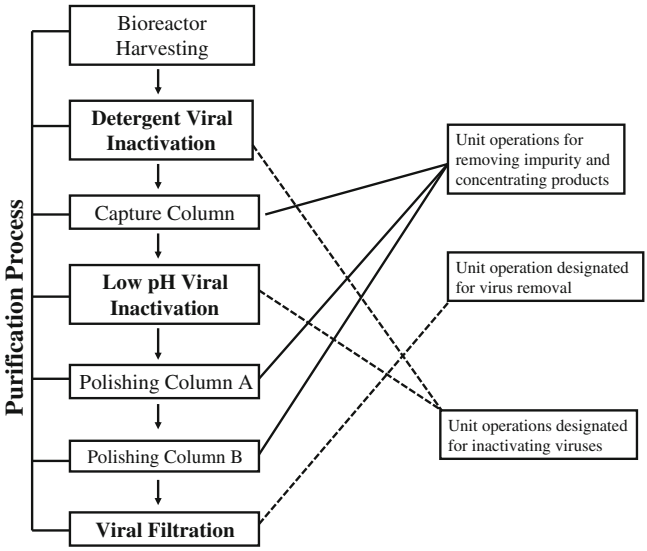


**Fig. 2** Diagram of a representative downstream purification process with multiple dedicated unit operations (in *boldface*) for viral clearance

clearance studies are relevant to the viral risks identified based on factors such as ASM exposure, endogenous viruses, and virus susceptibility of the production cell line. Multiple model viruses are often evaluated in the laboratory scale viral clearance studies to ensure that the purification process is capable of eliminating viruses with different biological, biochemical, and biophysical properties. It is generally expected that each model virus is assessed by two different unit operations with orthogonal mechanisms of action. The virus clearance is expressed as the logarithm reduction factor, which is defined as the $\log_{10}$ of the ratio of the total virus spiked into the starting material and the overall virus recovered in the forward process material for the next unit operation in the purification process. The overall LRF for a downstream purification process is the sum of LRF achieved by the individual unit operations as demonstrated using the laboratory scale models. The use of logarithm scale to measure the virus clearance capacity simplifies the calculation and quantification. More importantly, it indicates mathematically that the viral contaminants can only be greatly reduced, but will never be eliminated to zero by the downstream purification. If the bioreactor harvests contain known viral contaminants such as endogenous RVLP, then it is necessary for the downstream purification process to reduce the known viral contaminant to an acceptable level by achieving a specific LRF target as demonstrated by using a relevant or specific model virus. As outlined in Table 3, the LRF target is determined by the necessary safety factor and the calculated amount of the known viral contaminants in one single dose. Although not explicitly specified, the regulatory agencies worldwide often expect a minimum safety factor of $\geq$6.0 $\log_{10}$ for any viral contaminants known to exist in the bioreactor harvest such as the endogenous RVLP. There are

**Table 3** LRF target calculation for the viral contaminant known to exist in the bioreactor harvests

| Average product titer in the bioreactor harvest | 3.0 mg/mL |
|---|---|
| Dose[a] | 300 mg |
| Overall purification yield | 50 % |
| Volume of harvest needed to make one dose = 300 mg ÷ (3.0 mg/mL × 50 %) = 200 mL | |
| Average virus (e.g., endogenous RVLP) counts determined by TEM or other methods | $5.0 \times 10^6$/mL |
| Estimated virus (e.g., RVLP) in one dose = 200 mL × ($5.0 \times 10^6$/mL) = $1.0 \times 10^9$ or 9.0 $\log_{10}$ | |
| Safety factor[b] | ≥6.0 $\log_{10}$ |
| *Target LRF for the known virus (e.g., RVLP) = 9.0 $\log_{10}$ + (≥ 6.0 log) ≥ 15.0 $\log_{10}$* | |

[a] The highest dosage is used for the calculation

[b] A minimum safety factor of ≥6.0 $\log_{10}$ is expected for BLA/MAA approval

no specific LRF targets for nonspecific model viruses because as they simulate adventitious viruses that are not expected in the production cells or the bioreactor harvests.

## 5 Viral Safety for Clinical Trial and Marketing Applications

Regulatory safety requirements for commercial approval are more stringent than those for clinical trial authorization. Table 4 compares the regulatory expectations/requirements for clinical trial applications (IND in the United States; CTA in the European Union) and commercial applications (BLA in the United States; MAA in the European Union). The recent guideline by the European Medicines Agency (EMA) is particularly useful in ensuring an adequate viral safety data package to support clinical trial applications [32]. The difference in regulatory expectations/requirements outlined in Table 4 reflects the evolving nature of the process development and distinctive risks associated with commercial production upon the approval of BLA/MAA. In the early clinical trial phase, it is likely that no WCB has been made yet and both upstream and downstream processes are still being optimized. Therefore, it is impractical or impossible to fulfill all the expectations/requirements intended for the marketing authorization (BLA/MAA) in the IND/CTA. The sheer differences in the number of batches produced and patients exposed also contribute to the more stringent requirements for BLA/MAA. The more stringent requirements are intended to ensure that the commercial productions are operated within the proven acceptable ranges (PAR) for the parameters that are critical for virus clearance. The establishment of PAR requires a tremendous amount of time and resources. It is essential to start the systematic characterization of the unit operations involved in virus clearance as early as possible as the clinical development gradually proceeds towards commercialization so that all the expectations/requirements will be met at the time of BLA/MAA preparation.

**Table 4** Regulatory expectations for IND/CTA and BLA/MAA submissions[a]

| Expectations/requirements | IND/CTA | BLA/MAA |
|---|---|---|
| *In-process testing* | | |
| MCB testing | Yes | Yes |
| WCB testing | No | Yes |
| Testing of cells at or beyond the limit of in vitro cell age | No | Yes |
| Unprocessed bulk testing | Yes | Yes |
| Endogenous RVLP counts | RVLP determined from a single bioreactor is acceptable | RVLP are determined from a minimum of three bioreactors. |
| *Virus clearance studies* | | |
| Model viruses | 1 to 2 | 3 or more |
| Virus partitioning across all fractions[b] | No | Yes |
| Effects of resin life cycle on virus removal[b] | No | Yes |
| Effectiveness of virus elimination by column cleaning/regeneration procedure[c] | No | Yes |
| Use prior in-house data from similar products with well-characterized unit operations | Yes | No |
| Laboratory scale model studies | Carried out at representative set points are acceptable as long as the actual process is run at the same set points | Data are provided to demonstrate robust virus clearance within the proven acceptable ranges for the critical parameters |

[a] See references 15–18 and 32 for detailed requirements for IND/CTA and BLA/MAA
[b] Only when chromatography unit operations are contributing to overall virus clearance by the downstream purification process
[c] Only when there are known viruses such as RVLP in the bioreactor harvests

## 6 Summary

Processes for manufacturing biologics in MCC may vary from company to company or product to product in specific details such as cell substrate, medium formulation, feed strategy, bioreactor type, and purification scheme, yet they use similar technology to grow cells in large-volume cultures and thus are all prone to contamination by infectious agents such as viruses. The safety of biologics produced in MCC is of the highest priority for patients. Regulatory agencies and the biopharmaceutical industry have adopted the multitiered strategy to ensure the safety of life-saving modern medicines. Appropriate controls of raw materials by sourcing, vendor quality management, and testing reduce the risks of adventitious agent contamination. Extensive in-process testing of MCB, WCB, and unprocessed bulk provides

assurance that the cell substrate and starting materials for the production of biologics are free of detectable adventitious agents. Viral clearance studies using a panel of model viruses demonstrate that the purification process not only can adequately clear the known viral contaminants such as RVLP but also remove/inactivate viruses that may have inadvertently been introduced into the process. Together, these practices provide the maximal safety assurance and minimize the potential health risks to patients. There has been no single case of virus transmission by biologics produced in MCC since the first such product was introduced in the early 1980s, thus validating the effectiveness and robustness of the multitiered safety strategy.

Finally, it is important for readers to remember that regulatory guidelines only provide high-level principles instead of protocols. Specific practices and approaches used to achieve regulatory compliance and safety assurance could vary from company to company depending on in-house experience.

**Acknowledgments** The author wants to thank Ms. Michelle Quertinmont, Dr. Sean O'Donnell, and Dr. Marie Murphy for carefully reviewing the draft manuscript and providing helpful comments and suggestions. Drs. Qi Chen, Ray Nims, and Thomas Kreil provided their invaluable and expert critiques during the preparation of this chapter, for which the author is deeply in debt to each one of them. Finally, the author would like to express his gratitude to Drs. Morrey Atkinson, Uma Kuchibhotla, Catherine Srebalus Barnes, Jerome Kinzel, Victor Vinci, Jace Fogle, Allison Kennington, Tongtong Wang, Michael De Felippis, and Terri Vicenzi for their support and encouragement.

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Adv Biochem Eng Biotechnol (2014) 139: 185–225
DOI: 10.1007/10_2013_215

Published Online: 9 June 2013

# Mammalian Cell Culture Capacity for Biopharmaceutical Manufacturing

**Dawn M. Ecker and Thomas C. Ransohoff**

**Abstract** With worldwide sales of biopharmaceuticals increasing each year and continuing growth on the horizon, the manufacture of mammalian biopharmaceuticals has become a major global enterprise. We describe the current and future industrywide supply of manufacturing capacity with regard to capacity type, distribution, and geographic location. Bioreactor capacity and the use of single-use products for biomanufacturing are also profiled. An analysis of the use of this capacity is performed, including a discussion of current trends that will influence capacity growth, availability, and utilization in the coming years.

**Keywords** Biomanufacturing · Biopharmaceuticals · Capacity · Contract manufacturing · Excess capacity · Forecast · Mammalian cell culture · Single-use

**Abbreviations**

| | |
|---|---|
| BLA | Biologics License Application |
| BPSA | BioProcess Systems Alliance |
| CMO | Contract manufacturing organization |
| GMP | Good manufacturing practice |
| MAA | Marketing Authorization Application |
| ROW | Rest of world |
| WFI | Water for injection |

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## 1 Introduction

As outlined in our recent report analyzing the supply and demand for mammalian cell culture capacity for biopharmaceutical manufacturing [1], technological advances in manufacturing processes, improvements in expression levels, widespread acceptance of single-use bioreactors, the emergence of biosimilar development, and consolidation in the bio/pharmaceutical industry continue to result in significant changes in the supply and demand for mammalian cell culture capacity. This chapter provides an updated analysis of the supply for this capacity along with a discussion of current trends that will influence the growth, availability, and utilization of this capacity in the coming years. We define biopharmaceutical products as therapeutic recombinant proteins or monoclonal antibodies produced via the large-scale culture of recombinant microbial or mammalian cell lines. Biopharmaceuticals are a subset of the broader class of biologic products, which also includes hormones and enzymes isolated from natural sources, synthetic peptides and oligonucleotides, vaccines, gene and cell therapy products, blood and blood derivatives, and polyclonal antibodies.

In our analysis of the supply of manufacturing capacity for biopharmaceuticals, we also make a distinction between products produced in mammalian cell culture and microbial fermentation because there are significant differences in the manufacturing processes and in the bioreactor and facility designs required for each. Mammalian cell culture processes have different safety concerns compared to

microbial fermentation, such as potential contamination by adventitious viral agents, and different bioreactor requirements, so separate and different facilities and equipment are used for each type of production technology. In addition, products made in mammalian cell culture are more complex proteins containing significant post-translational modifications, such as glycosylation, that are less common in products made in microbial systems. Although it is possible to convert a mammalian cell culture bioreactor and facility into microbial fermentation capacity (or vice versa), this is a time-consuming and costly exercise and is therefore infrequently done by companies with either type of capacity. As a result, the microbial fermentation and mammalian cell culture markets are distinct, requiring separate analyses of the supply and demand for each type of manufacturing capacity.

In this chapter we present an analysis of the supply for mammalian cell culture capacity used to produce products approved for the US and European markets. Our analysis of manufacturing supply includes manufacturing facilities outside these areas (e.g., India, China, and Southeast Asia) that are used to produce biopharmaceuticals intended for sale in global markets but does not include those facilities used to produce product only for sale in non-US or European markets (e.g., "ROW" markets). We established this approach for our supply and demand databases because products intended for sale in global markets will need to be manufactured in facilities with the capacity to supply these markets. At present, this provides coverage of the vast majority of mammalian cell culture capacity; however, we do recognize that "local market" capacity is becoming more important and may eventually significantly influence the global marketplace. The products that create the demand for manufacturing capacity include recombinant proteins such as cytokines, enzymes, hormones, receptor agonists, and soluble receptors, as well as monoclonal antibody-related products, including full-length monoclonal antibodies, antibody drug conjugates (e.g., immunotoxins), antibody fragments, and $F_c$-fusion proteins. Many emerging vaccines are also produced in mammalian cell culture, however, these products are not included in our analysis of demand for capacity, and facilities dedicated solely to the production of vaccines in cell culture are not considered in our analysis of supply.

## *1.1 The Growing Market for Mammalian Cell Culture Capacity*

As shown in Fig. 1, worldwide sales of biopharmaceutical products approved in the United States or Europe have grown steadily over the past decade, reaching nearly $117 billion in 2011, or approximately 12 % of the total $956 billion pharmaceutical market. The growth of biopharmaceutical product sales has been driven largely by the success of several "blockbuster" products (defined as those products with sales over $1 billion) combined with the continued expansion of commercialization of monoclonal antibody products for a variety of indications.

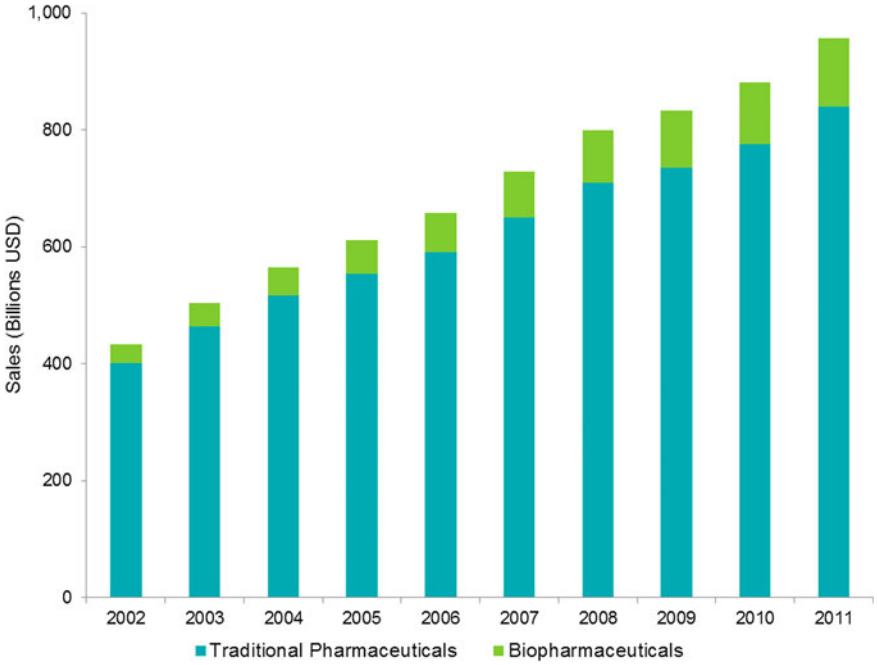


**Fig. 1** Biopharmaceutical product sales. Total sales of traditional pharmaceuticals (*blue*) and biopharmaceuticals approved in the United States or Europe (*green*) are shown by year for the past decade. Sales information was obtained from company annual reports and other publicly available sources. When specific sales data are not available, various methods are used to estimate these sales based on available data and reasonable assumptions

Led by these blockbusters, the growth in total sales of all biopharmaceuticals continues to outpace that of the overall pharmaceutical industry.

Since 2003, BioProcess Technology Consultants has maintained a database of biopharmaceutical products on the market or in development in the United States and/or Europe. As of December, 2012, our Biopharmaceutical Product database contained over 450 active products (phase II and beyond) being developed by over 170 companies. These active products are expressed in mammalian, microbial, and other systems (i.e., plant, insect). Of the 154 biopharmaceutical products approved and marketed in the United States and/or Europe, 89 (58 %) are produced in mammalian cell culture. These products include 51 recombinant protein products and 38 monoclonal antibody-related products.

As shown in Fig. 2, the sales of these biopharmaceutical products produced in mammalian cell culture have grown steadily over the past decade as many of these products entered the marketplace and experienced rapid sales growth, leading to a number of biopharmaceutical blockbuster products. Although overall sales of these products has increased, growth in sales of nonantibody biopharmaceuticals produced in mammalian cell culture has slowed in recent years due to a significant

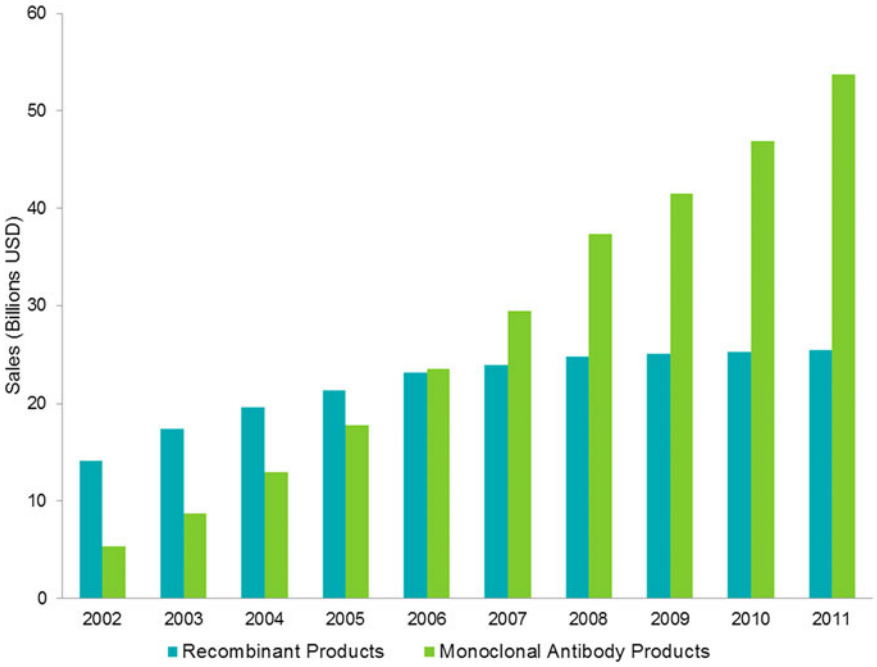


**Fig. 2** Sales of biopharmaceutical products produced in mammalian cell culture. Total sales of monoclonal antibody-related products (*green*) are compared to sales of all other biopharmaceutical products (*blue*). Sales information for products approved in the United States and/or Europe was obtained from company annual reports and other publicly available sources. When specific sales data are not available, various methods are used to estimate these sales based on available data and reasonable assumptions

decline in sales of erythropoiesis-stimulating agents (ESAs) and a decrease in the number of nonantibody products in development and reaching the market.

By contrast, sales of monoclonal antibodies and antibody-related products have grown steadily since 2001. These sales grew rapidly from 2002 to 2008 with the launch and market success of several monoclonal antibody products that eventually became blockbuster products. Consistent with the global financial crises and slowdown of the entire pharmaceutical industry, the sales growth of monoclonal antibody-related products slowed in 2009, but resumed in 2010 and appears poised to continue growing steadily through to 2017. The total US and European market for therapeutic monoclonal antibodies in 2011 was approximately $54 billion, an increase of nearly 15 % compared to approximately $47 billion in revenues from 2010. If the sales of monoclonal antibody-related products continue to grow as they have generally done over the past decade, the overall value of the monoclonal antibody market could exceed $70 billion in the next 5 years [2]. Supporting this forecast, a recent report by Visiongain predicts that overall revenues for monoclonal antibody therapies will reach $62.3 billion by 2015 [3].

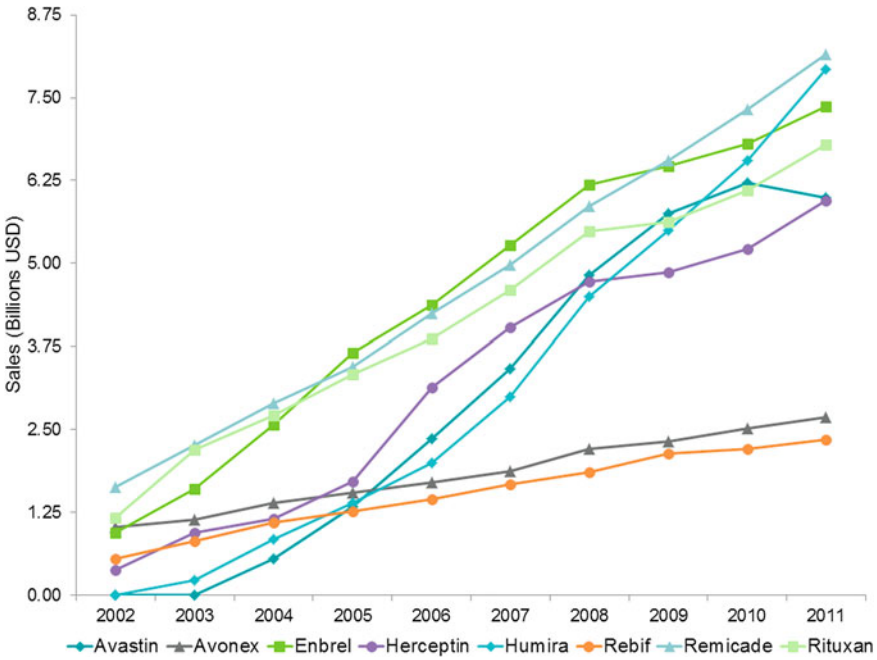


**Fig. 3** Sales growth of current commercial mammalian cell culture products. Annual sales of the top six monoclonal antibody-related products (Remicade, Enbrel, Humira, Avastin, Rituxan, and Herceptin) compared to two recombinant proteins (Rebif and Avonex) for the period 2002–2011. Sales information was obtained from company annual reports and other publicly available sources. See text for details

The sales data presented in Fig. 2 clearly show that monoclonal antibodies and monoclonal antibody-related products have driven the biopharmaceutical market growth over the past decade. Not unexpectedly, these products all contribute significantly to the current and future demand for mammalian cell culture capacity. The rapid growth in sales of these products over the past decade is clearly evident when the sales growth profiles of the six top-selling monoclonal antibody-related products (five monoclonal antibodies and one Fc-fusion protein) are compared to the sales growth profiles of the top two selling nonantibody products made in mammalian cell culture, Rebif and Avonex, both interferon-$\beta$ products. Each of the eight products shown in Fig. 3 had total sales in 2011 of $2 billion or more, with the six monoclonal antibody-related products having sales of greater than $5.5 billion each. As can be seen in Fig. 3, the average compound annual growth rate for the monoclonal antibody-related products over the 10-year period from 2002 to 2011 was nearly 30 % compared to the slower average sales growth (14 %) of Avonex and Rebif over the same period. One important aspect of this trend is that monoclonal antibody products generally act as antagonists, resulting in a higher product requirement per patient (or per dollar of revenue).

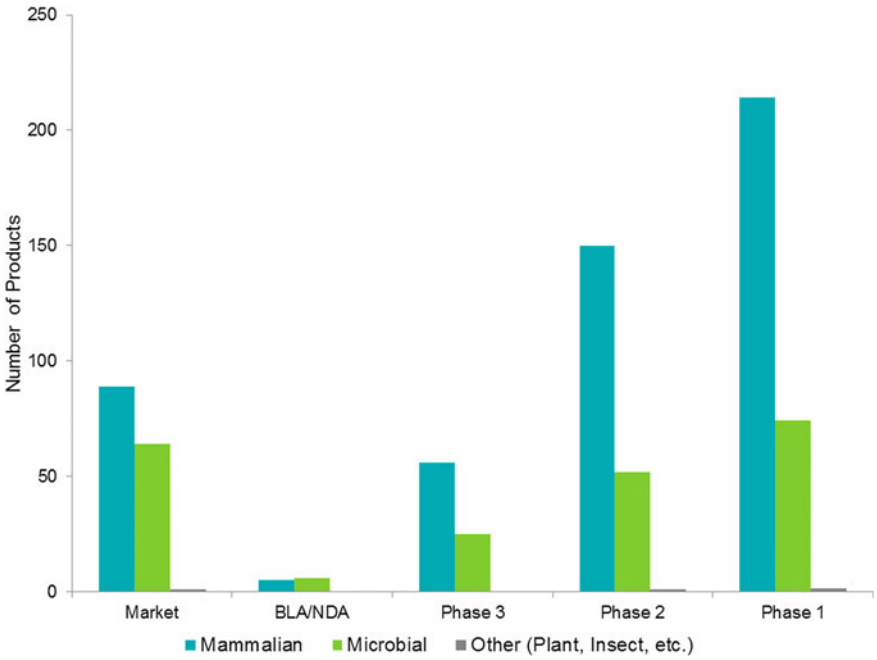


**Fig. 4** Distribution of products by production technology. The number of products produced in mammalian cell culture (*blue*), microbial fermentation (*green*), and other production hosts (*grey*) are shown for each stage of clinical development

As evident from the data shown in Fig. 3, although products currently in development will contribute to the growth in demand for mammalian cell culture capacity in the future, the sales growth of products on the market today will continue to drive the demand for this capacity in the coming 5–7 years. However, as with products such as Rebif and Avonex, when the current blockbuster monoclonal antibody-related products reach maturity, their rate of sales growth will eventually slow down or even decline. As a result, the volume of mammalian cell culture capacity required to produce them will also level off and be shifted to newer products, including biosimilars, with emerging demand for capacity.

By our current estimate, there are nearly 600 novel biopharmaceutical products currently in some stage of clinical development in the United States or Europe, with nearly 75 % of these products produced in mammalian cell culture (see Fig. 4), the majority of which are monoclonal antibodies or antibody-related products. Most of the remaining products are produced by microbial fermentation with a small percentage being produced using other production hosts, including insect cells, plant cells, or other emerging alternative systems. Not surprisingly, approximately half of the products currently in development are in phase I clinical trials indicating the robustness of the biopharmaceutical product pipeline and the high interest in developing these products. Another 35 % of the products in

**Table 1** Success rates for pipeline clinical products

| Stage of development | Next phase | Success rate to next phase (%) | Success rate to market (%) |
|---|---|---|---|
| BLA submission | Market | 90 | 90 |
| Phase III | BLA submission | 64 | 58 |
| Phase II | Phase III | 45 | 26 |
| Phase I | Phase II | 76 | 19 |

development are in phase II clinical trials with the remaining approximately 15 % either in phase III clinical trials or awaiting market approval.

Table 1 displays a critical parameter that influences the demand for future capacity for pipeline products: the probability of success. The probability of a product in development to advance successfully from one stage of development to the next in a given year highlights the inherent risk involved in developing biopharmaceuticals. These success rates are derived from published reports and data from our own Biopharmaceutical Product Database [1, 4–8].

The manufacture of biopharmaceuticals can be conducted by the company developing or marketing the product, or it may be contracted out to a third-party manufacturing organization. The biopharmaceutical contract manufacturing organization (CMO) market has grown in the past decade along with the overall growth of the biopharmaceutical and total pharmaceutical markets. Total biopharmaceutical CMO revenues were approximately $1 billion in 2001 and grew to approximately $2.5 billion in 2007 [9, 10], and approximately $2.6 billion in 2009 [11]. Based on Lonza's report of "strong performance driven, by biological manufacturing" [12] and Boehringer Ingelheim's "gratifying growth… based on the favourable development in the biopharmaceutical area" [13], we estimate that overall CMO revenues have increased significantly in the past several years [14]. We anticipate the long-term outlook for the CMO market as positive, with sales anticipated to grow modestly by 5–10 % in the coming years [14].

Assuming that total CMO sales increased to approximately $3.5B in 2011, CMO revenues would have remained fairly constant as a percentage of total biopharmaceutical industry sales (2.5–3.5 %) over the past decade. Despite the increasing trend towards outsourcing in the biopharmaceutical industry for early-stage biopharmaceutical companies, and the robust pipeline of products in development, the somewhat flat growth in CMO revenues as a percentage of the total biopharmaceutical revenues is the result of several factors. First, growth in the CMO market during this decade has been driven primarily by an increase in outsourcing manufacturing for products in clinical development, and the growth in the number of products in clinical development is not as significant as the growth in revenues generated from commercial products. A high percentage of CMO revenues is also derived from development and manufacturing services for these pipeline products, thus the growth in revenue from these services is also lower than the growth in commercial product sales. In addition, the majority of products on the market today were developed or in-licensed by biopharmaceutical

companies that have their own manufacturing capacity and manufacture their own products. As a result, a relatively small percentage of commercial products (on a volumetric basis) are manufactured by CMOs. As more and more products that were developed and manufactured for clinical trials by CMOs reach the market, we may see a higher growth rate in CMO revenues should companies choose to continue to utilize CMOs for commercial production after approval. Finally, companies that choose to outsource some of their manufacturing tend to outsource more mature products with slower revenue growth rates than newer products, as demonstrated by Genentech in choosing to outsource production of Rituxan rather than Avastin when additional capacity was temporarily needed to support the sales of both products.

## 2 Methods and Assumptions for Forecasting Supply

Over the past 10 years, BPTC has developed and refined a series of assumptions, algorithms, and methods for estimating, analyzing, and forecasting the supply and demand for mammalian cell culture manufacturing capacity. These methods and assumptions are continuously reviewed and updated based on publicly available information regarding mammalian cell culture manufacturing capacity, benchmarking, and selected interviews and discussions with key opinion leaders and experts in process development, manufacturing, quality control, and regulatory affairs for biopharmaceutical products.

Our bottom-up approach to the analysis of supply and demand for mammalian cell culture manufacturing capacity is based on estimates of total bioreactor capacity for individual facilities (supply) as well as annual product requirements for current commercial products and products currently in human clinical trials (demand). The analysis and forecasts provided in this chapter are based on BPTC's proprietary Manufacturing Facility database, which includes information on mammalian cell culture manufacturing facilities capable of servicing the global biopharmaceutical markets, and our Biopharmaceutical Product database, which includes information on product dosage requirements, patient populations, estimated process yields, and other important factors for biopharmaceutical products currently marketed in the United States and/or Europe, as well as for product candidates in clinical development for these geographical areas (phase II, III, or BLA/MAA).

Although no database will have complete coverage of either supply or demand, BPTC's proprietary databases are based on public-source information collected over the past 10 years and are continuously updated. These databases have a very high degree of coverage of commercial facilities and late-stage/commercial products. Our Biopharmaceutical Product database includes information on nearly 450 late-stage clinical (phase II or later) and commercial biologic products produced by mammalian cell culture, microbial fermentation, or other production technology. Our Manufacturing Facility database includes nearly 250

**Table 2** Detailed overview of mammalian manufacturing companies and facilities

| Capacity type | Number of companies | Number of facilities |
|---|---|---|
| CMO | 50 | 62 |
| Excess | 31 | 43 |
| Product | 43 | 87 |
| Total | 124 | 192 |

biopharmaceutical manufacturing facilities with either mammalian cell culture or microbial fermentation capacity capable of manufacturing bulk recombinant proteins and/or monoclonal antibody products for clinical or commercial use. A more detailed overview of our Manufacturing Facility Database for mammalian manufacturers is presented in Table 2. The capacity is distributed between product companies (i.e., biopharmaceutical or pharmaceutical companies who develop and/or market biopharmaceutical products), CMOs, companies that have both CMO offerings and biopharmaceutical products of their own (referred to as "excess capacity" companies in our analysis).

## *2.1 Estimating the Supply of Mammalian Cell Culture Capacity*

To estimate the current supply of mammalian cell culture capacity, we collect and track publicly available information on both pilot and commercial manufacturing facilities used for the GMP production of biopharmaceutical products for clinical trial and market. For consistency, bioreactor capacity information in our Manufacturing Facility database is listed as the working volume of each production bioreactor as we believe this to be the most appropriate indicator of available bioreactor capacity. For each company, the total volumetric capacity as well as the number and size of individual bioreactors is included in our database. For analysis, the overall manufacturing capacity at each facility, measured in liters, is converted to cell culture liter-equivalents per year using the operating and process assumptions described below.

In most cases the total installed mammalian cell culture capacity at biopharmaceutical companies worldwide is published. However, some companies do not publicize this information, especially for pilot facilities used to produce material for clinical trials. In these cases, relying on our industry experience, we have developed metric-based estimates for the installed manufacturing capacity based on publicly available information such as the reported square footage of the facility or the cost of constructing the facility, which are generally collected from company websites, annual reports, presentations, and other literature sources. This information, when compared to the industry average metrics, allows us to estimate the total installed capacity with reasonable accuracy.

Although tracked within our Manufacturing Facility database, non-cGMP facilities and GMP facilities using roller bottles, hollow-fiber bioreactors, and

other nonstandard production methods or facilities to manufacture cell or viral therapies are not included in the volumetric totals. However, single-use or disposable bioreactor capacity is included in our database and analyses as accurately as possible. Facilities designed for production of clinical supplies (particularly early-phase clinical supplies) are often quite flexible, but not likely to be able to support supply of a commercially licensed product. Facilities are considered "commercial" if they are currently used for production of commercial products or if they are designed for and/or capable of manufacturing commercial products in our estimation. We include all bioreactor-based manufacturing capacity that is used for the manufacture of either clinical or commercial supply.

Although the ever-changing dynamics of the biopharmaceutical industry make it virtually impossible to ensure that 100 % of all manufacturing facilities used for production of pre-clinical, clinical trial, and commercial material are included in our database, we believe that we have information on a sufficient number of these facilities to ensure accurate and complete analysis and forecasts of mammalian cell culture manufacturing capacity. Our information on manufacturing capacity covers essentially all major facilities in the United States and Europe as well as most of the major manufacturing facilities in Asia and other emerging markets to the extent that these facilities are designed to meet US and EU regulatory standards and provide products for the US and/or European market. Our current analysis of the supply of mammalian cell culture capacity is focused on facilities currently approved or in development for the US and European markets, however, we do include capacity in other geographies that is designed or capable of meeting the regulatory requirements for supply of material to these markets. We note that capacity in these emerging markets, although modest, is growing rapidly, especially with the emergence of biosimilars.

Our analysis of the supply of mammalian cell culture manufacturing capacity is based on the assumption that biopharmaceutical products produced using this capacity are manufactured using a relatively standard fed-batch process. For the vast majority of biopharmaceutical products produced in cell culture this is the case allowing direct comparisons from product to product and facility to facility. However, some biopharmaceutical products are manufactured using alternative cell culture approaches, especially perfusion culture. Because of the continuous nature of perfusion cultures, the volumetric productivity of a perfusion bioreactor is significantly higher than that of a similarly sized bioreactor operating in a fed-batch mode. To account for these differences in productivity, we use a "perfusion factor" of five times (5x) the bioreactor capacity to adjust perfusion bioreactor capacity to an "equivalent fed-batch capacity." This conversion allows us to compare capacity used for producing products in perfusion culture directly to that used for the more typical fed-batch culture and also to enable comparison of industrywide capacity supply with demand on a consistent basis. Because the number of bioreactors using perfusion culture is relatively small, any errors introduced by this conversion factor are relatively small.

Wherever possible, information on facility construction or expansion projects in progress or in late-stage planning is also included in our Manufacturing Facility

database as is information regarding facility closings. Information on facility expansion projects is collected from public sources, including press releases, company annual reports, analysts' reports, and other company literature and presentations. For these projects, we collect similar information as for existing facilities, including the number and size of the bioreactors, downstream processing capabilities, and overall facility size. A projected "year-online" for these projects is estimated from available information so that the supply of capacity can be forecast at the appropriate time. A plant is considered to be "online" when it is capable of cGMP manufacturing. If a manufacturing plant has been closed, we note whether the facility has been closed with no intent of future reopening (i.e., decommissioned or repurposed) or "mothballed" (i.e., closed but maintained in a manner that would allow for a rapid future start-up). The capacity of these closed or mothballed facilities is not included in our overall analysis of supply and demand for cell culture capacity.

In our analysis and estimates of future levels of mammalian cell culture capacity, this capacity is considered available for use by the company the year after the announced completion or online date of the facility. Due to the uncertainty in the timing of construction projects and the fact that not all are publicly announced, accurately estimating future capacity can be challenging, especially for projects that may be in the early planning stage, unannounced, or not yet started. To account for potential capacity from these unknown and upcoming projects, capacity projections from 2015 forward incorporate an increase over the previous year based on an assumed average year-over-year growth rate of 6.75 % in addition to the announced capacity for that year. The growth rate is estimated from the projected growth rate for capacity expansion from 2011 through 2014 based on information currently in our database. For comparison, the actual year-over-year percentage growth in mammalian cell culture capacity for the period 2003–2010 along with the projected growth rate for the period 2011–2013 is shown in Fig. 5. As seen in the Fig. 5 the growth rate of mammalian cell culture capacity has decreased dramatically in recent years. Although it is difficult to estimate the exact magnitude of capacity expansion more than 3 years in the future, we do not foresee a return to the large year-over-year increases in capacity seen in the early part of this decade.

Throughout our analysis, capacity estimates for the time period 2015–2017 based on the assumed 6.75 % linear growth rate of new capacity are referred to as "adjusted" capacity. For comparison to these growth-rate–adjusted analyses, some of our analyses were also performed using unadjusted capacity estimates in which we assumed no expansion of cell culture capacity in 2015 and beyond other than the completion of projects already announced. These estimates are referred to as "unadjusted" capacity.

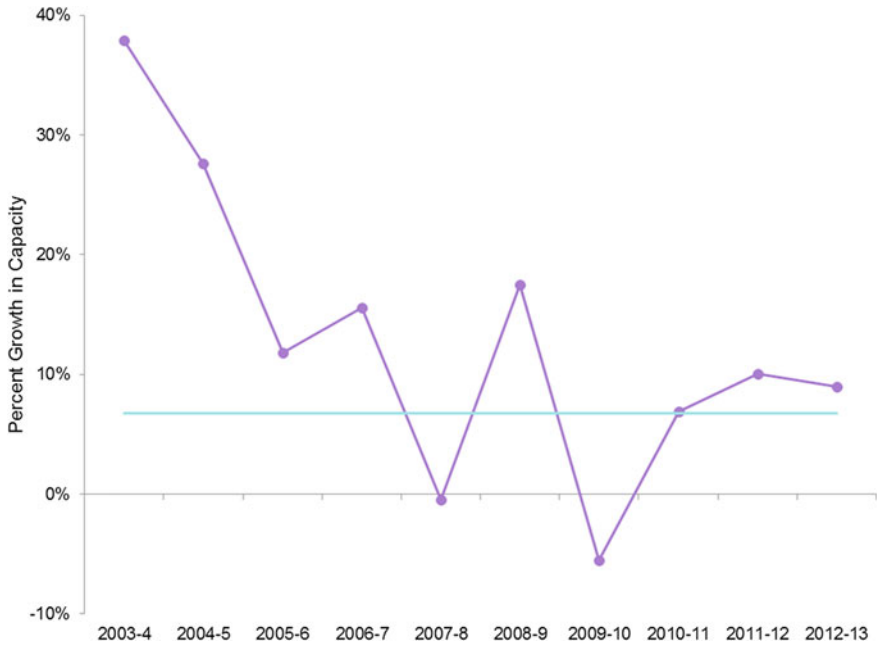


**Fig. 5** Year-over-year growth rates in cell culture capacity. Percentage growth in cell culture capacity from year to year is plotted for actual capacity growth in the period 2003–2011 and forecast growth in the period 2012–2014 based on publicly announced expansion projects. The *blue line* at 6.75 % shows the estimated percentage growth in capacity used in projections for the period 2015–2017

**Table 3** Distribution of product company manufacturing facilities

| Facility type | Number of facilities 2011 | Number of facilities 2017 |
|---|---|---|
| Commercial cell culture | 48 | 56 |
| Clinical cell culture | 25 | 31 |
| Total | 73 | 87 |

## 3 Segmentation of Capacity

Our analysis of the supply of mammalian cell culture capacity and forecasts for growth of this capacity include manufacturing capacity utilized by product companies, CMOs, and excess capacity. As shown in Table 3, as of December 2012, our database contained over 120 companies engaging in the manufacture of mammalian biopharmaceuticals comprising over 180 active facilities.

### 3.1 Product Company Facilities

BPTC's Manufacturing Facility database includes 43 product companies worldwide with bioreactor capacity for the production of biopharmaceutical products for clinical development and/or commercial sale in the United States and/or Europe. The current and projected future distribution of the 87 product facilities operated by these companies between those having commercial manufacturing capabilities and those with clinical supply capability is shown in Table 3. Nearly two-thirds (48 of 73) of the product company facilities in our database had mammalian cell culture capacity suitable for commercial production in 2011. Most of these facilities currently have installed capacity of greater than 5,000 L.

By 2017, we anticipate that the list of product companies with commercial manufacturing capabilities will expand to 56. It is possible that other product companies will establish commercial manufacturing capabilities by that time either through acquisition of existing facilities, construction of new facilities, or expansion or licensing of existing facilities. Along with those product companies having commercial capabilities, we have also identified 25 facilities for noncontract manufacture with capabilities and capacity to produce clinical trial material. We expect this number to increase slightly to 31 companies by 2017. As with the number of companies having commercial manufacturing capabilities, those with the capabilities to produce clinical trial material may increase even further as new projects are announced.

In the future, the number of product companies that decide to establish early-stage clinical manufacturing facilities may grow beyond our projections due to a number of factors including (1) the increasing availability of disposable process technologies that enable construction of pilot manufacturing facilities at considerably lower capital investments than in the past (2) the trend towards higher productivities and yields reducing the volume and/or number of bioreactors required to produce material for clinical trial, and (3) the FDA phase I GMP guidance [15], which clarifies the regulatory expectations and requirements in the United States for the manufacture of phase I clinical trial materials. These trends effectively reduce the capital investment and time required for establishing internal manufacturing capacity, particularly for early-stage clinical supplies, which may result in more companies making the decision to establish their own capacity.

It is interesting that the number of product companies with commercial cell culture manufacturing capabilities is significantly higher than the number of CMOs with this capability, and the number of product companies with clinical cell culture capacity is lower than the number of CMOs. Although clinical capacity for product companies may be slightly underreported because larger firms, in particular, do not always report pilot plant capacity, it is still a clear trend and is consistent with smaller biotech companies outsourcing manufacturing during early, high-risk clinical trials and bringing manufacturing back in-house after receiving product approval. Of course, once a company has invested in

**Table 4** Product companies with greater than 100,000 L of cell culture capacity

| Company[a] | Type | 2011 Volume (L)[b] | 2017 Volume (L) |
|---|---|---|---|
| Amgen | Commercial | 260,000 | 228,000 |
| Biogen Idec | Commercial | 106,000 | 196,000 |
| Bristol-Myers Squibb | Commercial | 11,900 | 131,900 |
| Celltrion | Commercial | 50,000 | 140,000 |
| *J&J* | *Commercial* | *230,000* | *230,000* |
| Lilly | Commercial | 137,000 | 147,000 |
| *Novartis*[c] | *Commercial* | *83,000* | *163,000* |
| Pfizer | Commercial | 144,500 | 149,000 |
| Roche | Commercial | 600,000 | 600,000 |
| *Sanofi* | *Commercial* | *153,000* | *263,000* |
| *Others (33) with <100,000 L*[c] | *Commercial* | *342,750* | *503,920* |

[a] Companies in italics have perfusion culture capabilities. For these companies, that portion of their actual capacity reported as available or used for perfusion culture has been converted to an equivalent fed-batch bioreactor volume by multiplying the actual bioreactor volume by a perfusion factor of five

[b] Includes active capacity only; mothballed or closed facilities not included

[c] Company also has facilities denoted as excess contract capacity, not included in this total

commercial capacity, maintaining clinical capacity is relatively straightforward and generally justified if the company continues to have biologics in their pipeline.

A list of product companies with greater than 100,000 L of total cell culture capacity is provided in Table 4. It is interesting to note that nearly half of the companies listed in Table 4 have multiple manufacturing sites which exemplify the increasingly sophisticated risk minimization strategies used by large companies to ensure redundant supply for large-volume or large-revenue products. It also reflects the increasingly global nature of our industry, the impact of mergers and acquisitions, and the increasing tendency for the larger companies to locate at least some of their manufacturing capacity in tax-advantaged areas.

## 3.2 Contract Manufacturing Organizations and Excess Capacity Companies

A similar breakdown of CMOs and excess capacity companies with manufacturing capacity for the production of clinical or commercial biopharmaceutical products is shown in Table 5. Among 80 companies offering manufacturing services, there are 104 CMO and excess capacity facilities, 21 of which have facilities and capabilities that are commercial. As described earlier, these facilities are either currently being used for production of commercial products or were designed and built to meet the regulatory requirements for commercial manufacturing in BPTC's estimation. We further assume that those CMOs capable of manufacturing commercial products also have the capabilities and capacity to manufacture product for clinical trials.

**Table 5** Distribution of CMO and excess capacity manufacturing facilities

| Facility type | Number of facilities 2011 | Number of facilities 2017 |
|---|---|---|
| Commercial cell culture | 21 | 27 |
| Clinical cell culture | 65 | 78 |
| Total | 86 | 105 |

Several companies (e.g., AbbVie) utilize their manufacturing assets for production of their own products as well as offer a certain percentage of this capacity or specific facilities, occasionally via subsidiaries, to others on a contract basis. To the extent possible, we analyze the facilities used as excess capacity as a separate group along with facilities dedicated for contract manufacturing and for product manufacturing. However, inasmuch as the number of companies in this category is relatively small, for some of our analyses the facilities are assigned to one category or the other based on our perception of their primary function. Throughout the period of performing these analyses, these assignments have changed and will continue to change as business drivers and focus areas for individual companies change.

The CMO market is rapidly changing and evolving with several CMOs gearing up to establish new commercial manufacturing capabilities. By 2017, we anticipate that several more CMOs will have large-scale commercial capacity. In addition, it is also possible that other companies—either existing CMOs with capabilities only to manufacture clinical trial material or companies seeking to enter the biologics CMO market—will have established commercial-scale capacity by that time. This could happen either through acquisition of existing facilities, construction of new facilities, or expansion or licensing of existing facilities.

In addition to the 21 CMOs and excess capacity facilities that currently have commercial manufacturing capabilities, we have also identified 65 CMOs and excess capacity facilities that currently have the capability to manufacture clinical trial material. We estimate that the number of CMOs and excess capacity facilities with capabilities to produce product for commercial sale will increase sixfold by 2017 and the number of CMOs and excess capacity facilities with manufacturing capabilities for production of clinical trial material will increase 13-fold by 2017. Note that some CMOs, such as CMC Biologics and Fujifilm Diosynth Biotechnologies are adding capacity using single-use or "disposable" bioreactor technology, which is accounted for in our models.

A full list of CMOs and excess capacity companies with greater than 50,000 L of total cell culture capacity based on information in our database is provided in Table 6.

## 4 Current Supply of Mammalian Cell Culture Capacity

On an unadjusted basis, total worldwide capacity for mammalian cell culture is forecast to increase from approximately 28,00,000 L in 2011 to nearly

**Table 6** CMOs and excess capacity companies with greater than 50,000 L of cell culture capacity

| Company[a] | Capacity type | Type | 2011 Volume (L)[b] | 2017 Volume (L) |
|---|---|---|---|---|
| AbbVie | Excess | Commercial | 62,000 | 75,000 |
| Boehringer Ingelheim | Excess | Commercial | 188,000 | 220,000 |
| *CMC Biologics* | CMO | *Commercial* | *31,750* | *60,000* |
| GlaxoSmithKline | Excess | Commercial | 73,200 | 98,200 |
| Innovent Biologics | Excess | Commercial | 0 | 75,200 |
| *Lonza Biologics* | CMO | *Commercial* | *155,700* | *244,900* |
| Samsung | CMO | Commercial | 0 | 120,000 |
| *Others (73) with <50,000 L*[c] | – | *Commercial* | *213,246* | *304,996* |

[a] Companies in italics have perfusion culture capabilities. For these companies, that portion of their actual capacity reported as available or used for perfusion culture has been converted to an equivalent fed-batch bioreactor volume by multiplying the actual bioreactor volume by a perfusion factor of five

[b] Includes active capacity only; mothballed or closed facilities not included

[c] Company also has facilities denoted as product company capacity, not included in this total

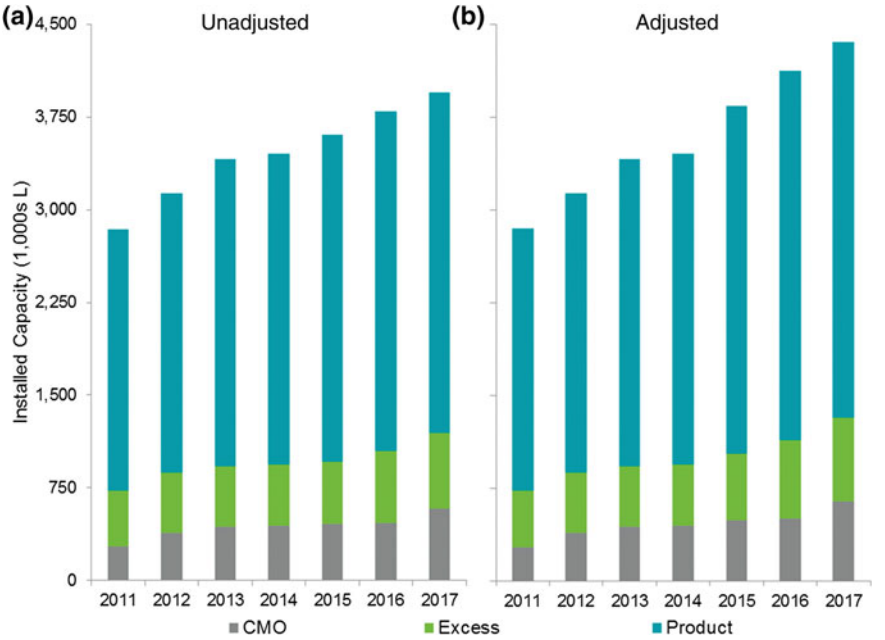


**Fig. 6** Distribution of mammalian cell culture capacity by company type. **a** Distribution of mammalian cell culture capacity (equivalent fed-batch basis) among CMOs, excess capacity companies, and product companies on an unadjusted basis (assumes no growth in capacity in 2015–2017 beyond already announced expansions). **b** Distribution of mammalian cell culture capacity among CMOs, excess capacity companies, and product companies on an adjusted basis (assumes linear growth in capacity in 2015–2017 in addition to already announced expansions)

40,00,000 L by 2017 (see Fig. 6a). This growth in capacity will come predominantly from expansion of capacity among product companies from approximately 21,00,000 L in 2011 to nearly 28,00,000 L in 2017. When adjusted for potential capacity expansion in the years 2015–2017, the total worldwide capacity for mammalian cell culture will increase to nearly 44,00,000 L in 2017 (see Fig. 6b), again driven primarily by capacity expansions among product companies. The total amount of industrywide capacity is increasing, but the rate of growth of this capacity over the past 3 years has slowed to less than 10 %. As previously reported, global cell culture capacity increased steadily from 2002 to 2008 [1, 16], but remained relatively flat in 2009–2010. Our forecast is for moderate growth in the coming years.

## 5 Growth in and Control of Mammalian Cell Culture Capacity

As discussed earlier and shown in Fig. 5, the average year-over-year growth rate of mammalian cell culture capacity for the period 2003–2011 and forecast growth in the period 2011–2014 based on publicly announced expansion projects is 6.75 %. However, the growth in total industrywide capacity has dramatically decreased in recent years, dropping from an average growth rate of almost 40 % in 2003–2004 to

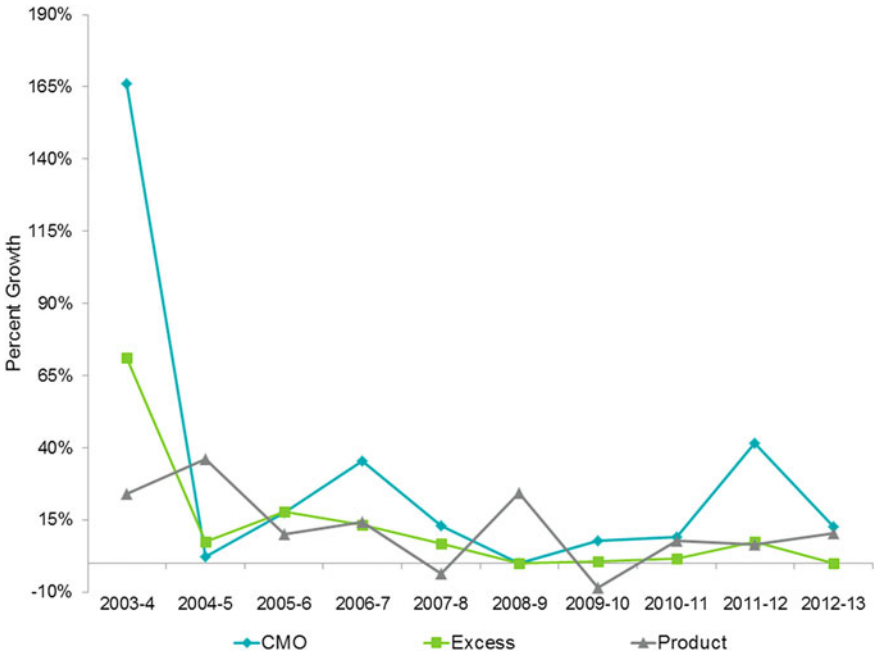


**Fig. 7** Year-over-year change in capacity as a function of company type

**Table 7** Explanation of spikes and troughs in Fig. 7

| Years | Capacity type | Company | Capacity event (L) | Net effect on total capacity (L) |
|---|---|---|---|---|
| 2003–2004 | CMO | Lonza | +60,000 | +244,000 |
| | Excess | Boehringer Ingelheim, Novartis | +103,000 | |
| | Product | Novartis | +81,000 | |
| 2004–2005 | Product | Amgen, J&J, Celltrion, Roche[a], Regeneron | +377,600 | +377,600 |
| 2007–2008 | Product | GenMAb, Lilly, Stada[b] | +33,250 | −30,750 |
| | | Amgen | −64,000 | |
| 2009–2010 | Product | J&J, Alexion | +120,000 | −180,000 |
| | | Roche, GenMab, Merck KGaA | −300,000 | |
| 2011–2012 | CMO | Gallus Pharmaceuticals, Lonza | +101,200 | +101,200 |

[a] Genentech at the time of the expansion
[b] Norbitech at the time of the expansion

7 % in 2010–2011. This significant decrease in the amount of new or expanded capacity is the result of several large construction projects being completed on top of a small capacity base in the early 2000s as well as the increase in expression levels and overall yields resulting in a lower volumetric demand to produce a given quantity of product. A breakdown of the expansion of capacity at CMOs, excess capacity facilities, and product company facilities is shown in Fig. 7.

Predictably, the year-to-year change in capacity for all three company types is not a smooth curve upward or downward but shows relatively high variability, especially in the early years of this decade. Table 7 shows the capacity events and the net effect on the total capacity for those notable spikes and troughs in Fig. 7. Although it is difficult to estimate exactly how much capacity expansion will occur in the coming years, we do not foresee a return to the large year-over-year increases in capacity seen in the early part of this decade.

A closer examination of the distribution of mammalian cell culture capacity among those companies controlling the largest amount of capacity relative to the rest of the industry shows nearly 75 % of the total worldwide mammalian cell culture capacity is owned or controlled by the 10 companies shown in Table 8. Half of the worldwide mammalian cell culture capacity is currently controlled by just five companies, Roche (including Genentech), Johnson & Johnson (including Centocor and all other subsidiaries), Boehringer Ingelheim, Amgen, and Lonza. Even more impressive, Roche controls nearly as much capacity as the 93 other companies not included in the top 10 companies (600,000 vs. 744,746 L). Of the 10 companies currently controlling the most cell culture capacity, one (Lonza) is a CMO, two (Boehringer Ingelheim and Novartis) have facilities that operate facilities as excess capacity companies, and the remaining seven are product companies.

By 2017, the total capacity controlled by the 10 companies with the largest amount of cell culture manufacturing capacity is forecast to decrease to approximately 63 % due mainly to expansions and construction of new capacity outside

**Table 8** Top 10 companies with mammalian cell culture capacity 2011 versus 2017

| 2011 Rank[a] | 2017 Rank[a] | Company[b] | 2011 Volume (1,000s L)[c] | 2017 Volume (1,000s L) |
|---|---|---|---|---|
| 1 | 1 | Roche | 600 | 600 |
| 2 | 5 | Amgen | 260 | 228 |
| *3* | *4* | *J&J*[b] | *230* | *230* |
| 4 | 6 | Boehringer Ingelheim | 188 | 220 |
| *5* | *3* | *Lonza*[b] | *156* | *245* |
| *6* | *2* | *Sanofi*[b] | *153* | *263* |
| 7 | 9 | Pfizer | 145 | 149 |
| 8 | 10 | Lilly | 137 | 147 |
| *9* | *7* | *Novartis*[b] | *125* | *205* |
| 10 | 8 | Biogen Idec | 106 | 196 |
| – | – | *All Others*[b, d] | *745* | *1,467* |

[a] Only companies within the top 10 in a given year are ranked for that year and have capacity listed in the table. Capacity for companies not ranked are included in the "All Others" category
[b] Companies in italics have perfusion culture capabilities. For these companies, some or all of their total capacity has been multiplied by a perfusion factor of five as described previously
[c] Includes active capacity only; mothballed or closed facilities not included
[d] In 2011 the "All Others" category includes 93 companies; in 2017 "All Others" includes 109 companies

the top 10 companies. This expansion of capacity by companies outside the top 10 capacity holders is driven by the 93 companies included in this group as well as 16 additional companies without any manufacturing capacity in 2011 who are currently adding capacity that will come online by 2017. The rank order of companies controlling most of the industry's capacity will also shift by 2017 as Sanofi-Aventis and Lonza bring new capacity online, each surpassing the capacity of J&J and Amgen. Amgen's decrease in capacity is reflective of the 32,000 L Freemont facility divestiture to Boehringer Ingelheim.

## 6 Geographic Distribution of Cell Culture Capacity

The geographic distribution of mammalian cell culture manufacturing capacity used for production of biopharmaceutical products for the US and European markets on an unadjusted and adjusted basis is shown in Fig. 8. As can be seen from these data, nearly 60 % of this cell culture manufacturing capacity is currently located in North America, predominantly in the United States. Approximately 33 % of the cell culture capacity is located in Europe and nearly 10 % of this capacity is located in Asia.

Based on the data shown in Fig. 8, other than the assumed growth in capacity in our adjusted capacity analysis, there will be limited increases in cell culture capacity in North America and Europe in the coming 5 years.

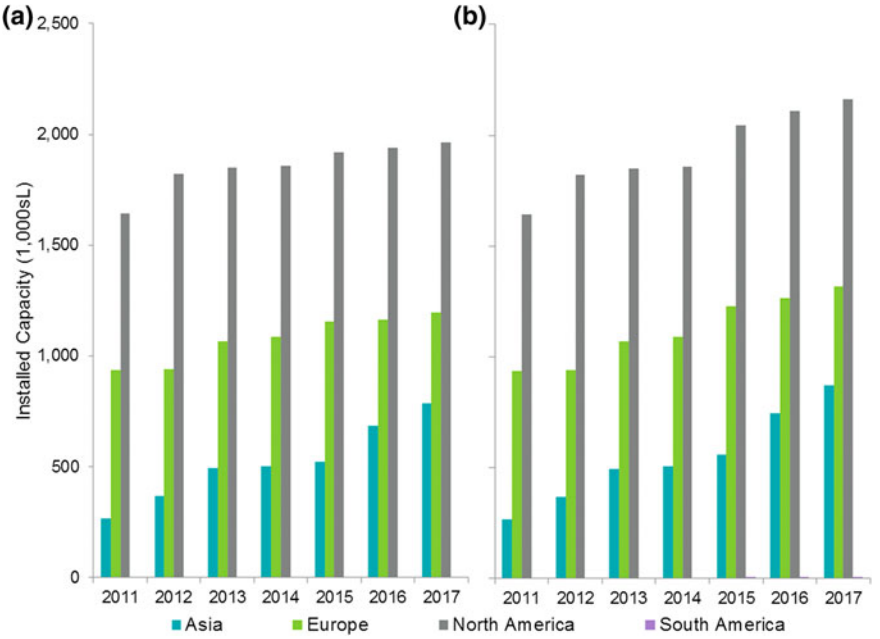


**Fig. 8** Geographic distribution of mammalian cell culture capacity. **a** Geographic distribution of mammalian cell culture capacity on an unadjusted basis. **b** Geographic distribution of mammalian cell culture capacity on an adjusted basis. Capacity shown is for the production of products for global markets; capacity for use only in ROW/developing markets is not included

**Table 9** Closed or mothballed mammalian cell culture facilities

| Company[a] | Capacity type | Location | Total capacity (L) | Year of closure |
|---|---|---|---|---|
| Amgen | Product | West Greenwich, RI | 64,000 | 2008 |
| Bayer | Product | Richmond, CA | 2,000 | 2007 |
| *BioUetikon* | *CMO* | *Dublin, Ireland* | *2,000* | *2012* |
| *Fujirebio* | *CMO* | *Gent, Belgium* | *500* | *2008*[b] |
| GenMab | Product | Brooklyn Park, MN | 22,000 | 2010 |
| GenMab | Product | Plymouth, MN | 750 | 2010 |
| Inotech Bio | CMO | Basel, Switzerland | 2,100 | 2010 |
| Lilly | Product | Branchburg, NJ | 30,000 | 2010 |
| Lonza | CMO | Baltimore, MD | 500 | 2007[b] |
| Merck KGaA | Product | Ness Ziona, Israel | 500 | 2004 |
| Merck KGaA | Product | Billerica, MA | 350 | 2009[b] |
| Progenics | Product | Tarrytown, NY | 150 | 2011 |
| QSV Biologics | CMO | Edmonton, Canada | 1,300 | 2009 |
| Roche | Product | Penzberg, Germany | 75,000 | 2010 |
| Roche | Product | San Diego, CA | 6,000 | 2007 |
| Roche | Product | Vacaville, CA | 200,000 | 2010 |
| Roche | Product | Nutley, NJ | 12,000 | 2008 |

(continued)

**Table 9** (continued)

| Company[a] | Capacity type | Location | Total capacity (L) | Year of closure |
|---|---|---|---|---|
| Roche | Product | Houston, TX | 1,500 | 2007 |
| Vaxgen | Product | S. San Francisco, CA | 1,000 | 2008 |
| WuXi Apptec | CMO | Philadelphia, PA | 3,500 | 2009[b] |

[a] Companies in italics had perfusion culture capabilities. For these companies, some or all of their total capacity has been multiplied by a perfusion factor of five as described previously
[b] Facility decommissioned and repurposed

**Table 10** Divested mammalian cell culture facilities

| Divesting company[a] | Capacity type | Location | Total capacity (L) | Year of divestment | Acquiring company | Capacity type |
|---|---|---|---|---|---|---|
| Amgen | Product | Freemont, CA | 32,000 | 2011 | Boehringer Ingelheim | Excess |
| AstraZeneca | Product | Montreal, Canada | 3,500 | 2007 | PnuVax[b] | Vaccines |
| AstraZeneca | Product | Sodertalje, Sweden | 250 | 2008 | Cobra Biologics | CMO |
| Cobra Biologics | CMO | Oxford, UK | 130 | 2011 | Oxford Biomedica[b] | Gene Therapy |
| Dow Contract Pharma[c] | Product | Smithfield, RI | 20,000 | 2006 | Alexion | Product |
| *Lilly* | *Product* | *Bothell, WA* | *10,500* | *2007* | *CMC Biologics* | *CMO* |
| Merck & Co. | Excess[d] | Research Triangle Park, NC | 2,870 | 2010 | FujiFilm[e] | CMO |
| *J&J* | *Product* | *St. Louis, MO* | *11,500* | *2010* | *Gallus Biopharmaceuticals* | *CMO* |
| Pfizer | Product | Ringaskiddy, Ireland | 5,500 | 2011 | BioMarin Pharmaceuticals | Product |
| Roche | Product | Oceanside, CA | 4,500 | 2010 | Gilead Pharmaceuticals | Product |
| Roche[f] | Product | Porriño, Spain | 40,000 | 2006 | Lonza | CMO |
| Stryker | Product | West Lebanon, NH | 5,725 | 2010 | Olympus Biotech | Excess |
| Xoma | Excess | Berkeley, CA | 8,250 | 2012 | CMC Biologics | CMO |

[a] Companies in italics have perfusion culture capabilities. For these companies, some or all of their total capacity has been multiplied by a perfusion factor of five as previously described
[b] Facility divested to company not manufacturing recombinant therapeutic biologic products
[c] At the time of its sale, facility had only microbial fermentation capacity. Cell culture capacity added by Alexion
[d] Merck is a product company; divested facility was an excess facility acquired through the acquisition of Schering Plough
[e] Facility acquired by Fujifilm to establish Fujifilm Diosynth Biotechnologies, a CMO
[f] Facility owned by Genentech, independent of Roche, when divested

In fact, over the past few years several companies have closed or "mothballed" manufacturing facilities in these geographies. These facilities are listed in Table 9 and include large commercial manufacturing facilities such as the Roche Penzberg, Germany and Vacaville, CA facilities and the Amgen West Greenwich, RI facility, as well as smaller pilot manufacturing facilities such as the Lonza Baltimore, MD facility and the Progenics Tarrytown, NY facility. In total, nearly 450,000 L of capacity at 20 facilities have been removed from manufacturing within the past 8 years.

In addition to the closed facilities listed in Table 9 there have also been several facilities that have changed hands in the past few years. A representative list of these facilities and the acquirers are listed in Table 10. Interestingly, most of these facilities involved a CMO as buyer or seller. Of these, Dow sold its facility to Alexion and exited from the contract manufacturing marketplace. Alexion converted the facility to mammalian cell culture and now uses it for the commercial manufacture of Soliris. Merck sold its Diosynth facility to FujiFilm as part of a strategic investment by FujiFilm to enter the contract manufacturing business. This facility was part of the Merck Manufacturing Network comprised of Diosynth Biotechnology and Avecia Biologics, two CMOs acquired by Merck in the past couple of years. The acquisition of the Diosynth facility by FujiFilm gave the new CMO 2,870 L of cell culture capacity as well as significant microbial fermentation capacity. Lilly also sold the former Icos facility, which had operated as a CMO, to CMC Biologics as part of CMC's expansion business in the United States which was then followed by CMC's acquisition of Xoma's 8,250 L in Berkeley, CA early in 2012. J&J sold the former Centocor manufacturing facility in St. Louis to Gallus Biopharmaceuticals, a new CMO who bought the facility to enable their entry into this market. In 2006, Roche (then Genentech) sold its Porriño, Spain facility to Lonza as part of a deal in which Genentech received rights to purchase a Lonza facility in Singapore then under construction. Roche exercised this option in 2009, incorporating the facility into its Singapore manufacturing operations. Boehringer Ingelheim purchased the Amgen Fremont CA site as part of an expansion of its CMO capacity within the United States. In addition to acquiring the manufacturing site, Boehringer Ingelheim also took over production of Amgen products currently manufactured at the facility. Stryker divested its West Lebanon, NH facility and recombinant bone growth products in 2010 to the Olympus Corporation to form Olympus Biotech, who recently began offering CMO services.

The majority of facilities with large-scale mammalian cell culture capacity will remain in the United States and Europe for the coming years, however, we forecast a higher growth rate for cell culture capacity in Asia during this same time period. This growth is the result of several facility construction projects recently completed or scheduled for completion in the next few years. These 14 new and expanded facilities will add a total of over 421,000 L of capacity to the existing capacity. Nearly 90 % of this new capacity will be added by four companies: Novartis, Celltrion, Samsung Biologics, and Innovent. Novartis has recently reinstated plans for a new facility in Singapore with 80,000 L of capacity. In 2007 Novartis had announced plans to invest in a large-scale cell culture facility in Singapore, but this

project was put on hold in 2008. Celltrion is expanding its Incheon, Korea facility by adding 90,000 L and Samsung Biologics will expand its new 30,000 L facility in Seoul, Korea with an additional 90,000 L of capacity. Innovent Biologics is planning to build nearly 75,000 L of capacity in Shanghai, China. The new manufacturing facilities under construction by Samsung Biologics, Celltrion, and Innovent reflect the growing interest in biosimilars and in biomanufacturing in general in areas outside the United States and Europe. Additionally, Novartis' expansion represents the growing interest by US and European companies in shifting manufacturing to potentially lower cost or tax-advantaged regions, following Roche's decision to close their Vacaville, CA facility and buy the Lonza facility in Singapore for which they had purchase rights [16].

Aside from the closing of facilities in North America and Europe, the future slowdown in the growth of mammalian cell culture capacity worldwide is also the result of steady increases in productivity and over-building by industry during the previous decade, generally through initiation of projects to ensure ability to meet market demands before productivity gains were assured.

## 7 Projections for Clinical Versus Commercial Manufacturing Capacity and Bioreactor Scale

The current distribution of clinical versus commercial capacity as well as the unadjusted and adjusted forecast increases in this capacity are shown in Fig. 9. As seen in Fig. 9, the total volume of cell culture capacity with commercial manufacturing capability far exceeds that dedicated to clinical production, and this is expected to continue through 2017. Furthermore, consistent with the increasing number and sales of marketed biopharmaceutical products, the volume of capacity devoted to commercial production is forecast to grow at a faster rate than that used for production of clinical trial materials.

A further breakdown of mammalian cell culture capacity dedicated to production of clinical trial material by capacity type is shown in Fig. 10. In 2011, just over 40 % of the total installed capacity used for production of clinical supplies was utilized by product companies, with excess capacity facilities and CMOs facilities controlling 27 and 32 % of this capacity, respectively. By 2017, however, the total capacity controlled by CMOs and product capacity companies on an unadjusted basis will have grown significantly so that CMOs will have approximately 154,000 L of capacity and product companies will have nearly 165,000 L of manufacturing capacity devoted to the production of clinical trial material. With excess capacity companies possessing nearly 105,000 L, a total of nearly 423,000 L of capacity will be devoted to production of clinical trial material in 2017.

In a similar manner, the distribution of commercial manufacturing capacity across company types is shown in Fig. 11. In 2011, nearly 80 % of the commercial manufacturing capacity worldwide was controlled by product companies, 14 % by

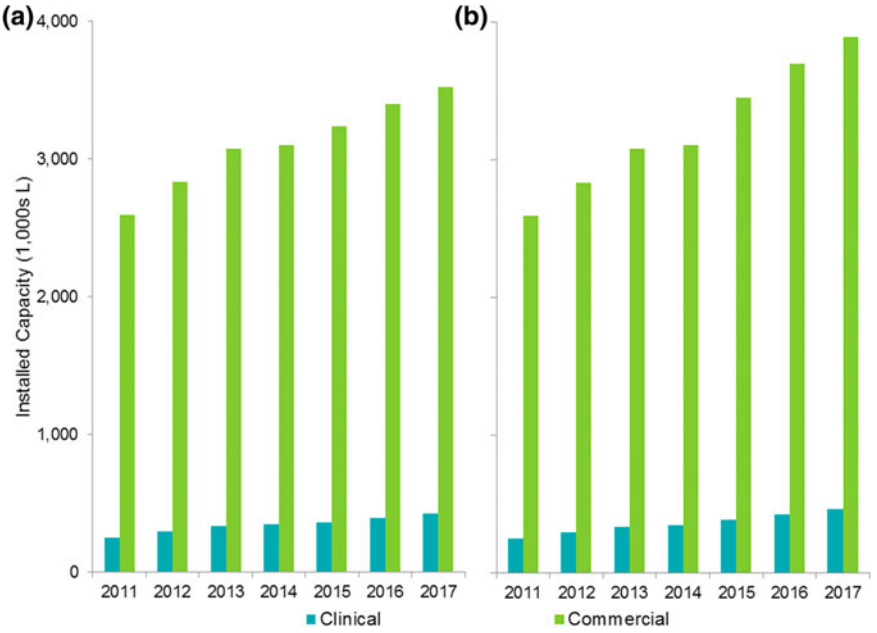


**Fig. 9** Clinical versus commercial mammalian cell culture capacity. **a** Unadjusted distribution of clinical and commercial mammalian cell culture capacity. **b** Adjusted distribution of clinical and commercial mammalian cell culture capacity

excess capacity companies, and the remainder by CMOs. By 2017 this distribution is forecast to remain about the same, with product companies controlling nearly 75 % of the commercial capacity, excess capacity companies controlling approximately 15 %, and CMOs still controlling 12 %. This distribution of clinical and commercial manufacturing capacity reflects an industrywide preference to outsource the production of early-stage clinical trial material and bringing manufacturing in-house for commercial products.

The numbers of companies with greater than 100,000 L, 10,000–100,000 L, and 1,000–10,000 L of total installed capacity are all forecast to increase by 30–45 % in the coming 5 years (see Fig. 12). Much of this growth is anticipated to come from product company facilities and from CMOs as products manufactured by these companies move to later stages of development and require greater volumes of capacity to meet the anticipated demand. Currently, there is one CMO (Lonza), one excess capacity company (Boehringer Ingelheim), and seven product companies with installed capacity greater than 100,000 L. By 2017, this category will expand to include an additional CMO (Samsung Biologics) and three additional product companies (Bristol-Myers Squibb, Celltrion, and Novartis). During the same period, the number of companies with total installed capacity in the range of 10,000–100,000 L is expected to grow by two each for the CMO and excess capacity companies and by three for the product companies.


**Fig. 10** Distribution of clinical supply capacity by facility type. The distribution of mammalian cell culture capacity used for production of clinical trial supplies among CMOs, product companies, and excess capacity facilities is shown on an unadjusted basis

As also shown in Fig. 12, those product companies who have chosen to invest in mammalian cell culture capacity have established significant capabilities in this area, with the majority having large-scale, commercial-capable facilities with capacities in excess of 10,000 L. By contrast, the majority of CMOs have much less installed capacity per company on average, as many are focused on production of clinical trial material requiring significantly smaller volumes than commercial manufacturing. This is consistent with the recent trend towards outsourcing, particularly by smaller companies, for production of early-stage clinical trial supplies. Although we also see a trend of smaller companies establishing manufacturing capabilities through increased use of single-use technologies, we believe that most small- and medium-sized biopharmaceutical companies will continue to rely heavily on outsourcing for production of clinical trial material in the coming years.

## 8 Single-Use Technologies in Biopharmaceutical Manufacturing

Over the past decade, the incorporation of single-use or disposable process technologies in the manufacture of biopharmaceutical products has increased


**Fig. 11** Distribution of commercial supply capacity by facility type. The distribution of mammalian cell culture capacity used for production of commercial trial supplies among CMOs, product companies, and excess capacity companies is shown on an unadjusted basis

significantly with applications ranging from Lonza's use of Wave Bioreactors in the inoculum train for their large-scale production bioreactors in their Portsmouth, NH facility [17] to Shire's reliance on single-use production bioreactors and other disposable technologies in the upstream portion of their new ATLAS commercial manufacturing facility [18, 19]. Increasingly, companies are considering manufacturing facility designs incorporating single-use and disposable technologies at all stages of manufacturing [20, 21].

The many driving forces for the adoption of single-use technologies in biopharmaceutical manufacturing, particularly in multiproduct facilities, include increased flexibility, reduced requirements for expensive critical utilities (e.g., WFI and clean steam), decreased requirements for equipment cleaning and cleaning validation, lower capital investments, and shorter facility construction times [22, 23]. Of these, the lower capital investment required to construct manufacturing facilities and the shorter timelines for facility construction, start-up, and validation are the most important factors driving rapid adoption of disposable technologies [24, 25]. These advantages have been demonstrated in several recently completed facility construction projects in which the construction and validation timelines for facilities incorporating single-use technologies were significantly shorter than the timelines for building conventional facilities using

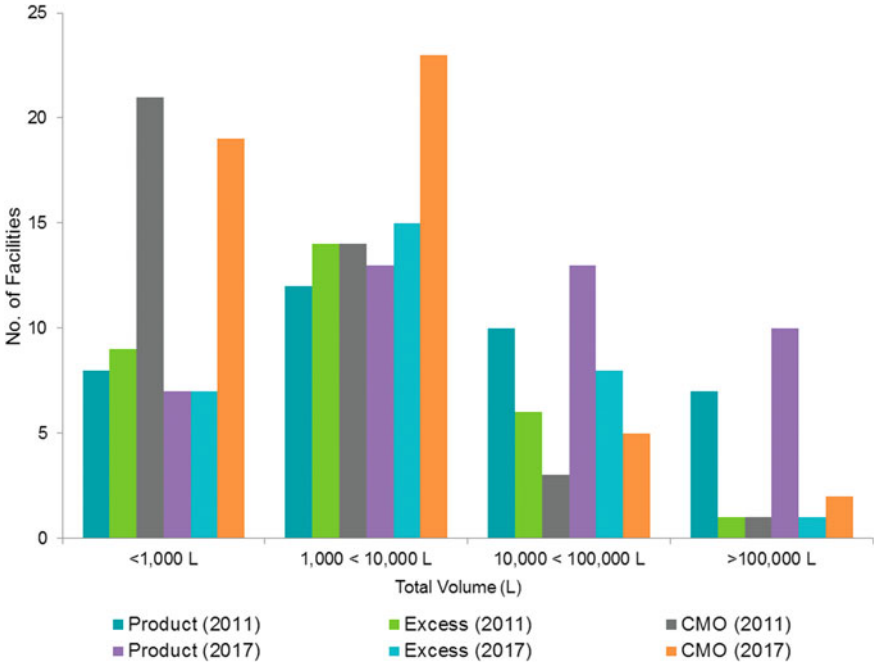


**Fig. 12** Installed mammalian cell culture capacity by bioreactor scale. The number of companies with installed bioreactors of the indicated scale are shown for CMOs, product companies, and excess capacity companies for 2011 and 2017

reusable stainless steel equipment [18, 26, 27]. Such conventional facilities typically require 3–4 years or more from the initial design and planning stages to final commissioning and qualification whereas construction timelines for similar-sized facilities incorporating single-use technologies are approximately 18–24 months. The shorter construction timelines for facilities using single-use technologies result largely from the reduced critical utilities requirements and the separation of facility construction from procurement and installation of process equipment such as production bioreactors. Single-use bioreactors and the other single-use unit operations are generally pre-fabricated by the suppliers of such equipment and do not require extensive hard piping as part of their installation. For example, single-use bioreactors up to 2,000 L can be purchased from the vendor with all instrumentation and control elements fully assembled on a portable skid. Because the utility requirements for such single-use bioreactors are significantly reduced or nonexistent, the need to install significant lengths of high purity stainless steel piping is eliminated or reduced, saving both time and money in the overall construction costs. Capital cost reductions on the order of 40–50 % have been reported for single-use–based facilities [18, 26].

The driving forces for implementation of single-use and disposable technologies in biopharmaceutical manufacturing are compelling, however, some

**Table 11** Companies with existing or planned single-use bioreactor capacity where single-use bioreactors represent 50 % or greater of total capacity

| Company[a] | Capacity type | Region[b] |
|---|---|---|
| 3P Biopharmaceuticals | CMO | Europe |
| Acceleron Pharma | Product | North America |
| Angel Biotechnology | CMO | Europe |
| BioInvent International | Product | Europe |
| *Biovian* | *CMO* | *Europe* |
| Catalent Pharma Solutions | CMO | North America |
| Celonic | Excess | Europe |
| City of Hope Center for Biomedicine and Genetics | CMO | North America |
| Cobra Biologics | CMO | Europe |
| EuBioPharm Co. Ltd | CMO | Asia |
| Florida Biologics | CMO | North America |
| *Gallus Biopharmaceuticals* | *CMO* | *North America* |
| Genhelix | CMO | Europe |
| Kalon Biotherapeutics | CMO | North America |
| *Korea Biotechnology Commercialization Center* | *CMO* | *Asia* |
| LFB Group | Excess | Europe |
| Macrogenics | Product | North America |
| Meridian Life Sciences | CMO | North America |
| Minapharm Pharmaceuticals | Excess | Europe |
| NCI Biopharmaceutical Development Program | Excess | North America |
| *Novasep* | *CMO* | *Europe* |
| Oncobiologics | Product | North America |
| PacificGMP | CMO | North America |
| Paragon Bioservices | CMO | North America |
| ScinoPharm Taiwan | Excess | North America |
| *Shire* | *Product* | *North America* |
| SynCo Bio Partners | CMO | Europe |
| Therapure Biopharma | CMO | North America |
| Vivalis | Excess | Europe |
| WuXi Apptec | CMO | Asia |
| Xbiotech | Product | North America |

[a] Companies in italics have perfusion culture capabilities
[b] Region in which single-use bioreactors are installed; company may have additional capacity in other regions

challenges and concerns related to the use of these new technologies remain, including the increase in ongoing operating costs associated with the purchase and disposal of single-use equipment, the potential for leachable and extractable substances from the product-contact surfaces to contaminate products, sourcing limitations for the purchase of single-use equipment, lack of consistent and uniform standards for the numerous connectors required to install and run single-use equipment, and the lack of regulatory experience with many of these technologies. Nevertheless, biopharmaceutical manufacturers ranging from small start-up companies to large product companies and CMOs are increasingly installing

single-use and disposable technologies, including single-use bioreactors, buffer and media storage bags, and downstream processing equipment, for the manufacture of biopharmaceutical products for both clinical trials and commercial sale.

Table 11 lists those companies whose disposable capacity represents 50 % or more of their total production capacity based on already installed single-use bioreactors and/or announced plans to bring disposable bioreactor capacity online in the next few years. Although over 80 % of the companies comprising Table 11 are CMOs or excess capacity companies, we believe product companies are also rapidly adopting these technologies and have already installed or will install single-use bioreactors in the coming years. Inasmuch as the addition of such capacity is relatively fast and requires significantly less capital investment than the installation of conventional stainless steel bioreactors, product companies, especially larger companies with existing manufacturing capabilities, may not publicly announce the installation of such capacity. Our database includes only publicly announced capacity and planned expansions, therefore this capacity may not be fully captured in our analysis. Based on the publicly available information, we estimate that single-use bioreactor capacity for mammalian cell culture amounts to approximately 4 % of the total industrywide installed capacity. Even accounting for the fact that some companies may have installed single-use capacity without publicly announcing such installations, this capacity is still a very small portion of the currently installed global capacity for mammalian cell culture. However, we believe that the adoption of single-use bioreactors for pilot and commercial manufacturing will continue to increase and that the portion of total capacity represented by single-use bioreactors will continue to grow rapidly.

Because single-use bioreactors are more flexible and portable than conventional stainless steel bioreactors, tracking installations with single-use bioreactor capacity is more challenging than tracking manufacturing capacity based on conventional stainless steel bioreactors. Nevertheless, single-use bioreactors are now beginning to affect the overall balance of supply and demand for mammalian cell culture manufacturing capacity. As the trend towards single-use bioreactors evolves, we will continue to include such capacity in our databases to enable us to track further penetration of these bioreactors in future clinical and commercial biomanufacturing facilities.

## *8.1 Single-Use Bioreactors in Biopharmaceutical Manufacturing*

Single-use bioreactors are currently available in volumes up to 2,000 L, and have been used in production of clinical trial material at scales ranging from 20 to 2,000 L. The earliest single-use bioreactor widely available for use in bioprocess applications was the Wave Bioreactor (now owned by GE HealthCare Life Sciences, Uppsala, Sweden), a self-contained disposable bag with agitation/mixing provided by an external platform rocker [28]. The platform rocker-type production vessel contains ports for the input of culture components such as the feed media

for a fed-batch process, chemicals to control pH level, and antifoam. Sufficient aeration is generated by the rocking motion and mammalian cells can grow to densities up to $10^{10}$ cells/L. When they were first introduced in the mid-1990s these bioreactors were available with up to 20 L of capacity, making them unsuitable for production. In recent years larger-scale platform bioreactors up to 500 L have been introduced and used by some companies for production of clinical trial supplies, primarily for early-stage clinical trials. A more common application of the Wave Bioreactors, however, is their use at small scales (typically <100 L) as part of the seed train prior to inoculation of large-scale stainless steel or single-use production bioreactors [29].

Due to their limited scalability and potential quality differences between product produced in a Wave Bioreactor compared to a conventional stirred-tank bioreactor, several companies have developed single-use bioreactors that resemble the standard, cylindrical stirred-tank reactor design of traditional large-scale stainless steel bioreactors used in the biopharmaceutical industry today. Xcellerex (GE HealthCare Life Sciences), Thermo-Fisher Hyclone, and Sartorius are the current market leaders in supplying single-use bioreactors with a stirred-tank geometry. For these single-use bioreactors, a support structure for the bioreactor along with all the associated addition pumps and process control computers are installed on a single skid that is installed in the production facility and connected to the necessary utilities. The disposable component of the bioreactor is provided by the supplier as a pre-sterilized bag containing all the necessary addition and sampling ports, agitator, and harvest port that can be inserted into the fixed support structure [30]. Agitation of the cell culture is provided by a pre-installed impeller that attaches to a motor in the support structure. Monitoring of pH, dissolved oxygen, temperature, and the like within the bioreactor is accomplished through various probes that are installed in dedicated ports in the bioreactor bag. Several studies have shown that cell culture processes and the resulting critical quality attributes of the product produced by these processes are comparable whether produced in single-use or stainless steel bioreactors of the same size [31]. In a recent study, high-intensity cell culture processes at up to 1,000 L using either CHO or NS0 cells resulted in comparable product regardless of whether the processes were run in single-use or stainless steel bioreactors [32]. In this study, the seed train, harvest, and all downstream processing steps were identical so the only variable was the type of bioreactor used for production.

In addition to the single-use bioreactors with conventional stirred-tank geometry, several companies have recently developed alternative single-use bioreactor systems that employ different geometries and/or mechanisms for agitation, mixing, and control of nutrients and pH. Bioreactors with cubic designs as well as other shapes are currently on the market and all have been shown to support the growth of mammalian cells with good productivity [33]. To our knowledge, these newer geometries are not widely used for production of biopharmaceuticals that are currently in the clinic, but companies are capable of evaluating the options and selecting the one or more bioreactor types that provide the best ability to support their mammalian cell culture processes.

To fully realize the potential of single-use bioreactors in the manufacture of biopharmaceutical products, a number of areas and opportunities for improvement have been identified by both users and suppliers of these systems. These include the development of better disposable sensor elements, with particular emphasis on sensors that can maintain calibration over the extended runtimes of today's high-performance cell culture processes [34], the introduction of larger transfer ports to facilitate more rapid harvesting of large single-use bioreactors and other large volume transfers, and improved construction of the disposable bags used in single-use bioreactors to reduce leakage failure rates. This last issue is of great concern to companies implementing single-use bioreactors inasmuch as bag leakage can result in costly losses during production runs. To help address this issue, ATMI recently introduced a new test system as part of their release criteria for single-use bioreactor bags to confirm the bag integrity prior to shipment to a customer [35]. The test method involves flooding a fully assembled single-use bioreactor with helium and noting any escape of the gas with specialized sensors mounted on the exterior of the bioreactor bag. ATMI claims the method can detect holes as small as 10 $\mu$, making the method much more sensitive than other bag leakage tests. These and many other innovations in development are certain to continue to improve single-use products and technologies in the future and facilitate their implementation.

## *8.2 Single-Use Technology for Downstream Processing*

The development of single-use downstream processing products capable of effective and economical operation at scales required for clinical or commercial manufacturing has lagged the development of disposable bioreactors but significant efforts by both established and new suppliers of bioprocess equipment and separations products in the last few years have introduced a wide range of scalable, single-use products for downstream processing in the manufacture of biopharmaceutical products [36]. These products have enabled the development of purification processes incorporating some, if not all, single-use components. We therefore anticipate that many sound and feasible options for employing single-use and disposable technologies in both the cell culture and the recovery and purification of biopharmaceutical products will soon be introduced in the pilot and commercial scale manufacture of these products. Eventually, we anticipate the use of disposable and single-use technologies for the entire upstream and downstream processing of biopharmaceutical products, including use in continuous process operations [37].

## 8.3 The Impact of Single-Use Technologies on Manufacturing Capacity

As outlined above, approximately 4 % of currently installed global mammalian cell culture manufacturing capacity is based on single-use bioreactors. We expect the use of disposable bioreactors will continue to increase well into the coming decade (with an estimate of 6 % or higher of the total global capacity in 2017) and that they will become routine for production of pipeline products for clinical trials. In addition, we expect that single-use bioreactors will also be increasingly used in commercial manufacturing facilities. In a recent analysis of the economic aspects of single-use systems for biopharmaceutical and vaccine manufacturing, Lee concluded that although single-use systems are more economical for manufacture at small scale, at production scales above approximately 8,000 L, conventional stainless steel bioreactor systems become more economically attractive [38]. In agreement with this, we anticipate that commercial production in single-use bioreactors will be limited in the near term to those products where commercial production can be accomplished with multiple 2,000-L single-use bioreactors [20]. For other products requiring larger production volumes, commercial manufacturing will remain primarily in stainless steel bioreactors due to the scale of manufacturing required, the large installed base of stainless steel bioreactor capacity, and process considerations.

Finally, companies in many developing markets, particularly in Asia (including the Middle East) and Latin America, have entered or are planning to enter the biopharmaceutical market to develop either biosimilar products or innovative biopharmaceuticals. A clear majority of these firms are planning to construct their initial facilities based solely or largely on single-use and disposable equipment because the timeline and capital savings are particularly compelling in this market segment. Because Asia is forecast to be the fastest growing segment for bioreactor capacity in the coming years [16], the overall market penetration of single-use bioreactors will be driven in part by this trend.

It is interesting that in September of 2012, GE HealthCare Life Sciences announced the launch of KUBio facilities [39], an "off-the-shelf" range of prefabricated modular biomanufacturing facilities designed for the manufacture of monoclonal antibodies ready for manufacture in less than 2 years.

Based on all of the potential applications for single-use and disposable technologies and on the compelling capital and time savings that can be realized when implementing these technologies, we expect the percentage of biopharmaceutical manufacturing facilities incorporating single-use and disposable technologies will increase. It is difficult to forecast longer term market penetration rates in this area, partly because facilities can be built so quickly, but we expect the number of facilities using single-use bioreactors for mammalian cell culture to increase over 50 % from the current estimated level of approximately 4 % to well over 6 % in the next 5–7 years.

## 8.4 Supply Chain Challenges and Opportunities for Single-Use Technologies

For companies using single-use or disposable technologies and products in their biopharmaceutical manufacturing facilities, control of the supply chain for the disposable components is of significant importance [27]. Reliance on others for the production and supply of single-use components, pre-manufactured solutions (such as bagged buffer and media), and other nontraditional material places a greater burden on many aspects of the supply chain, especially for facilities located in areas that are remote from supporting vendors. Even in the United States and Europe, many facilities are located a great distance from suppliers of pre-manufactured buffer or media solutions, increasing the shipping times and costs for these materials. To a much greater degree than with conventional facilities, supply chain logistics should be considered in the siting of manufacturing facilities incorporating single-use and disposable technologies.

Despite the fact that several studies have shown that facilities based on disposable technologies may actually have a lower environmental impact than conventional facilities [40, 41], there is a growing recognition of the need to develop effective and environmentally sustainable approaches to managing solid waste streams from these facilities. Many companies are increasing their focus on sustainable strategies to recycle materials from single-use process equipment [42], and guidelines for disposal of single-use systems have been developed by the BioProcess Systems Alliance (BPSA) [43].

Another important requirement in the supply of single-use components and products is the standardization of connection fittings, materials, and other design factors. Standardization among companies and products is an important need for any industry to deploy new technologies efficiently. In conventional biopharmaceutical facilities, the use of standardized sanitary-style fittings and of materials meeting a specified standard (e.g., 316 L stainless steel) helped enable efficient deployment of equipment from different suppliers and using different technologies. In addition to standardization, the development of new supporting technologies, such as improved disposable sensors is needed for industry to utilize single-use process equipment more fully. As with many important trends, implementation of one technology drives development of new supporting technologies, and this process is certainly underway with single-use technologies. The result should be more effective solutions to a broader range of applications that will continue to drive market penetration and increased market share.

Finally, given the importance of the supply chain to successful operation of single-use facilities, the need for supply chain risk mitigation strategies for these operations is high. Such strategies may include qualification of secondary suppliers, development of optimized inventory management strategies, implementation of disaster recovery plans, and other tools. A critical component of the management of the supply chain for all single-use and disposable equipment used in biopharmaceutical manufacturing, as it is with many other raw materials and

components used in the manufacture of biopharmaceutical products, is qualifying and establishing quality agreements with key vendors and suppliers [44–46]. A quality agreement with suppliers of critical raw materials and components reinforces the importance of their product in a manufacturing process and establishes the need for these vendors and suppliers to maintain suitable and auditable quality systems. These quality agreements help companies meet the regulatory requirements and expectations for proper control of raw materials and components in biopharmaceutical manufacturing as well as provide important controls and delineation of responsibilities between a company and its vendors and suppliers. To assist companies implementing single-use and disposable technologies, the BPSA has recently formed a task force to develop a quality agreements template that covers such key issues as identification of critical changes, change control and notification, subcomponent supplier qualification, component origin information, customer involvement in changes to standard products, levels of disclosure, and custom-product quality specifications [47].

Despite the fact that the implementation of single-use and disposable technologies in biopharmaceutical manufacturing is still relatively new and that several significant challenges and hurdles must still be overcome to enable a fully disposable manufacturing process, these technologies are already having an impact on the manufacture of biopharmaceutical products and on the mammalian cell culture capacity landscape. We forecast the continued increase in the adoption of single-use products and technologies in biopharmaceutical manufacturing in the coming decade, particularly as higher titer processes are developed and implemented, driving down the scale of operations necessary to meet commercial demand for future biopharmaceutical products produced in mammalian cell culture. As viable single-use solutions for all of the unit operations commonly involved in biopharmaceutical manufacturing are developed and improved standardization of fittings and connectors required to implement these technologies in a simple, cost-effective manner become commonplace, we expect to see fully disposable manufacturing processes in the not too distant future.

## 9 Capacity Utilization Today and in the Future

Although a detailed explanation and analysis is beyond the scope of this chapter, we regularly compare our current estimates and future forecasts for the supply of mammalian cell culture capacity with the current and forecast demand for this capacity. This enables an industrywide assessment of the current balance of the supply and demand for mammalian cell culture capacity along with a projection of the potential increase or decrease in utilization rates in future years. This analysis also provides a better understanding of trends and future directions in this highly dynamic field. Given the high degree of uncertainty in predicting the success or failure of biopharmaceutical products, forecasting future demand for mammalian cell culture capacity is challenging. However, by carefully analyzing trends in

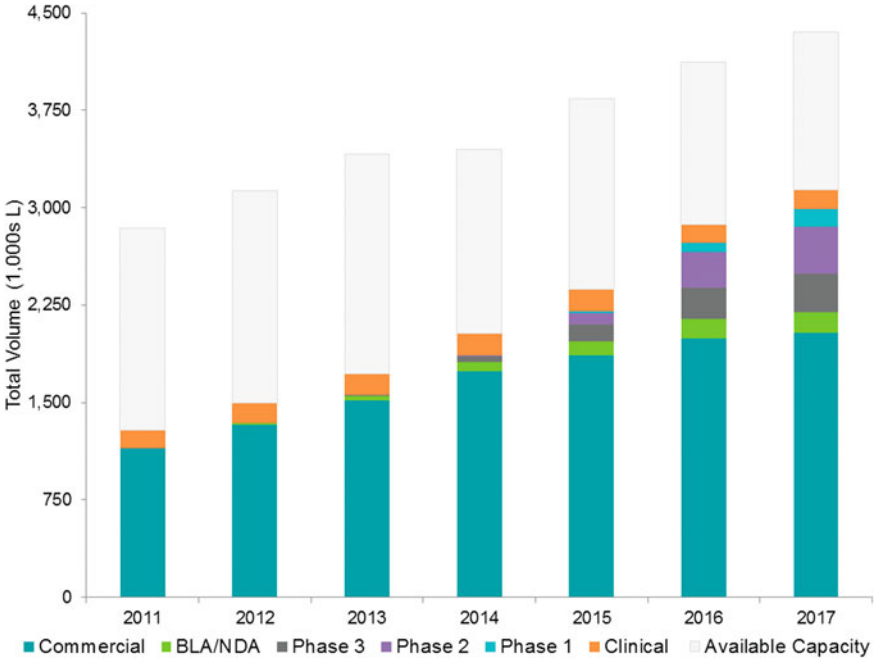


**Fig. 13** Baseline supply and demand forecast for mammalian cell culture manufacturing capacity. Demand for mammalian cell culture capacity is shown overlaid with total available adjusted capacity. This analysis assumes a maximal capacity utilization of 20 batches/bioreactor/year

process yields, the probability of success of large-volume driver products in late-stage development, and the impact of biosimilars, we can estimate future capacity utilization and assess the potential need for additional mammalian cell culture capacity in the future.

Our projected balance of future supply and demand for mammalian cell culture manufacturing capacity is shown in detail in Fig. 13. The full bar for each year represents the total installed volumetric cell culture capacity for the indicated year. Overlaid upon this graph is the demand for cell culture capacity from existing commercial products, new products that are expected to be approved from the existing clinical pipeline, and for supplying products in clinical trials. In Fig. 13, those bars indicated as BLA, Phase III, Phase II, and Phase I represent the mammalian cell culture capacity demand for products currently in these stages of development after they reach approval and commercialization. These estimates take into account the overall probability of success of products currently in clinical trial as they move through the development pipeline. The bars labeled Clinical represent the estimated demand for cell culture manufacturing capacity for the production of clinical trial supplies for each year. For each year, the remaining unused available capacity supply is shown in gray at the top of each column.

**Table 12** Forecast utilization rates of mammalian cell culture capacity based on baseline supply–demand analysis

| Years | 2011 | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 |
|---|---|---|---|---|---|---|---|
| Utilization (unadjusted) (%) | 45 | 48 | 50 | 59 | 66 | 76 | 79 |
| Utilization (adjusted) (%) | 45 | 48 | 50 | 59 | 62 | 70 | 72 |

In Fig. 13, the demand for manufacturing capacity has been adjusted forward 1 year to account for the fact that bulk product is typically made well ahead of actual sales (on which demand calculations are based). Hence, for the majority of product sold in 2011, for example, the bulk drug substance was actually manufactured in 2010 or earlier.

As shown in Fig. 13, there is currently sufficient mammalian cell culture capacity worldwide to meet the total industry demand for this capacity. According to our baseline analysis, in 2011, only 45 % of industrywide mammalian cell culture manufacturing capacity was utilized, resulting in 55 % unused capacity. If this capacity were evenly distributed among companies who needed capacity for the manufacture of all biopharmaceutical products on the market, this excess would be significant and likely to result in further facility closings or divestitures. However, as discussed in Sect. 4, the uneven distribution of manufacturing capacity implies that some companies may actually experience shortages of capacity or difficulties in accessing capacity despite the apparent large surplus of capacity available on an industrywide basis.

Our baseline analysis of capacity utilization also indicates that although manufacturing capacity in general is projected to grow in the coming years, the demand for this capacity will grow at a greater rate so that by 2017 industrywide capacity utilization will grow to 72 % on an adjusted basis or 79 % on an unadjusted basis (see Table 12). We estimate that individual companies will begin experiencing capacity shortages at an industrywide utilization rate of 75–80 %, so this forecast suggests that the industry will likely need additional capacity by 2017. As a result, we expect to see additional announcements of companies adding new or expanded capacity to come online post-2015.

The overall utilization of mammalian cell culture capacity is forecast to increase in the coming years, however, the current capacity utilization is significantly lower than it was in late 1990s and early 2000s for a number of reasons. These include the significant amount of new or expanded manufacturing capacity that has come online during the past 5 years as a result of plant construction decisions that were made early in the previous decade, dramatic increases in expression levels and overall process yields that have significantly improved productivity per unit of capacity, and a slowdown in the rate of new product approvals for "volume driver" (i.e., ton-scale) products. The increase in capacity utilization in the coming years will be driven by the continued growth in sales of existing products and approval of new volume-driver products, as well as a slowdown in construction of additional capacity and the mothballing of some existing capacity.

## 10 Outlook

Absent significant continued improvements in productivity or addition of more than modest levels of capacity in the coming 5 years, we forecast an overall increase in the level of utilization of mammalian cell culture capacity by the biopharmaceutical industry. If the currently installed mammalian cell culture capacity were evenly distributed among all companies needing such capacity, the modest tightening of capacity would likely have minimal impact on the overall capacity utilization and not cause companies to build significantly more additional capacity. However, the uneven distribution of capacity may make it more likely that companies without existing capacity will be unable to find suitable capacity for production of products currently in development. This may result in more facility construction than if mammalian cell culture capacity were a more liquid commodity. Furthermore, although the majority of mammalian cell culture capacity currently exists in the United States and Europe, we forecast a higher growth rate for new capacity in Asia. This is a trend that we believe will continue for some time, resulting in an increasing percentage of worldwide capacity in Asia later in the decade.

In addition, the availability and acceptability of single-use bioreactors for mammalian cell culture at scales suitable for clinical and, in some cases, initial commercial production will both enable smaller companies with limited financial resources to build pilot plants for early-stage production and allow all companies to establish more flexible manufacturing capacity. In fact, this trend has already begun with companies such as Acceleron Pharma building pilot facilities based on single-use bioreactors for the production of clinical trial material [48] and Shire building commercial manufacturing facilities employing single-use bioreactors [19]. As a result, we predict that facility construction will continue to trend towards less capital-intensive approaches, such as single-use technologies, for the foreseeable future.

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Adv Biochem Eng Biotechnol (2014) 139: 227–243
DOI: 10.1007/10_2013_239

Published Online: 15 August 2013

# Transcriptomics as a Tool for Assessing the Scalability of Mammalian Cell Perfusion Systems

**Karthik P. Jayapal and Chetan T. Goudar**

**Abstract** DNA microarray-based transcriptomics have been used to determine the time course of laboratory and manufacturing-scale perfusion bioreactors in an attempt to characterize cell physiological state at these two bioreactor scales. Given the limited availability of genomic data for baby hamster kidney (BHK) cells, a Chinese hamster ovary (CHO)-based microarray was used following a feasibility assessment of cross-species hybridization. A heat shock experiment was performed using both BHK and CHO cells and resulting DNA microarray data were analyzed using a filtering criteria of perfect match (PM)/single base mismatch (MM) $> 1.5$ and PM–MM $> 50$ to exclude probes with low specificity or sensitivity for cross-species hybridizations. For BHK cells, 8910 probe sets (39 %) passed the cutoff criteria, whereas 12,961 probe sets (56 %) passed the cutoff criteria for CHO cells. Yet, the data from BHK cells allowed distinct clustering of heat shock and control samples as well as identification of biologically relevant genes as being differentially expressed, indicating the utility of cross-species hybridization. Subsequently, DNA microarray analysis was performed on time course samples from laboratory- and manufacturing-scale perfusion bioreactors that were operated under the same conditions. A majority of the variability (37 %) was associated with the first principal component (PC-1). Although PC-1 changed monotonically with culture duration, the trends were very similar in both the laboratory and manufacturing-scale bioreactors. Therefore, despite time-related changes to the cell physiological state, transcriptomic fingerprints were similar across the two bioreactor scales at any given instance in culture. Multiple genes were identified with time-course expression profiles that were very highly correlated ($> 0.9$) with bioprocess variables of interest. Although the current

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incomplete annotation limits the biological interpretation of these observations, their full potential may be realized in due course when richer genomic data become available. By taking a pragmatic approach of transcriptome fingerprinting, we have demonstrated the utility of systems biology to support the comparability of laboratory and manufacturing-scale perfusion systems. Scale-down model qualification is the first step in process characterization and hence is an integral component of robust regulatory filings. Augmenting the current paradigm, which relies primarily on cell culture and product quality information, with gene expression data can help make a substantially stronger case for similarity. With continued advances in systems biology approaches, we expect them to be seamlessly integrated into bioprocess development, which can translate into more robust and high yielding processes that can ultimately reduce cost of care for patients.

**Keywords** BHK · Cell culture · CHO · Perfusion · Scale-down · Transcriptomics

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## 1 Introduction

Systems biology tools, such as DNA microarrays, have emerged as powerful techniques for probing gene expression on a global scale. Previous basic research in academic and clinical settings has driven the evolution of these tools, which are now maturing to a stage where they can be applied for biopharmaceutical development. In the biopharmaceutical industry, mammalian cell lines continue to be the primary workhorse for manufacturing complex therapeutic proteins [1]. Yet, there are multiple industrially relevant mammalian cell lines for which genomics resources are lacking.

The baby hamster kidney (BHK) is one such cell line. Despite being both a prominent basic research model and a commercially relevant recombinant host, BHK has very limited genomics resources. BHK cells were first isolated from Syrian hamsters, *Mesocricetus auratus*, in 1961 for Polyoma virus research [2]. Since then, they have been widely used as laboratory standards for growth and characterization of numerous viruses [3] and as hosts for production of recombinant proteins, such as blood clotting factors VIIa [4], VIII [5], and IX [6]; antibody/interleukin fusions [7]; human lactoferrin [8]; human antithrombin [9]; and CD4/immunoglobulin G fusion against human immunodeficiency virus [10]. Although shotgun sequencing of the Syrian hamster genome has been recently completed by the Broad Institute (http://www.ncbi.nlm.nih.gov/nuccore/APMT00000000), gene prediction and annotation remain largely incomplete.

For transcriptome analysis when species specific microarrays are not available, heterologous cross-species hybridizations have been proposed as a viable alternative [11–14]. However, imperfect homology between sequences in the sample and the probe in cross-species hybridizations bring a host of challenges, including potential for reduced signal intensity and increased cross-hybridizations [11]. Indeed, reduced signal intensity has been suggested as a possible reason for identification of fewer statistically significant genes in previous experiments [14, 15]. Cross-reactivity by probes has been attributed as a potential cause for discrepancy between results obtained from different microarray platforms [16, 17]. It is therefore imperative that cross-species hybridization experiments be carefully designed. In addition, robust data analysis techniques incorporating steps to filter out spurious data must be used.

To ensure the highest chance of success in cross-species hybridization, the species being probed must be genetically as close as possible to the species for which the array is designed. Among species or cell lines related to *M. auratus*, the mouse, *Mus musculus*[18]; the rat, *Rattus norvegicus* [19]; and, to a lesser extent, the Chinese hamster, *Cricetulus griseus* [20] and CHO-K1 [21] genomes are well characterized. Although the mouse and rat genomic data are substantially more advanced, the CHO genome is preferable to support BHK studies because it is more likely to be closer to the BHK genome. In fact, previous studies have suggested that *M. auratus* and *C. griseus* share a common ancestor for up to 10 chromosome fission/fusion events after it had diverged from the *M. musculus* lineage [22].

In this study, we test the feasibility of using Chinese hamster Affymetrix GeneChip arrays to profile the BHK transcriptome. The array was initially developed as part of an industry–academia collaboration (Consortium for CHO Genomics) to encourage the use of genomics tools in industrial CHO-based processes [23]. We then employ this approach to characterize laboratory- and manufacturing-scale BHK cell perfusion processes. Although numerous previous studies have attempted to use DNA microarrays as a tool to gain molecular insights into the cellular machinery, we demonstrate a simpler but highly relevant industrial application of such tools for evaluating system scalability.

## 2 Materials and Methods

### 2.1 Cell Line and Medium

Recombinant protein producing BHK and CHO cell lines were used in this study. The BHK cells were cultivated in a proprietary protein-free and chemically defined cell culture medium developed by Bayer HealthCare. For experiments with CHO cells, a commercially available chemically defined medium was used (CD-CHO, Gibco, Carlsbad, CA).

### 2.2 Heat Shock Experiments

Cultures for heat shock experiments were maintained in 250-mL shake flasks with 50-mL working volume. Flasks were shaken at 125 rpm on an orbital shaker placed inside a temperature-controlled incubator maintained at 5–7 % $CO_2$ and 37 °C, except during heat shock treatment described herein. Cells were subcultured once every 2–3 days, each time seeding at $0.4 \times 10^6$ cells/mL (for 2-day subculture) or $0.3 \times 10^6$ cells/mL (for 3-day subculture). For heat shock treatment, cells in mid-exponential culture were subjected to an elevated temperature of 42 °C for 2 h and then returned to 37 °C for 30 min before samples were taken. In each case, a parallel culture without the heat shock treatment was maintained to generate the control samples. All samples were taken in duplicates to assess reproducibility of microarray data.

### 2.3 Cell Expansion for Bioreactor Inoculation

A cryopreserved vial containing BHK cells was thawed and transferred into a proprietary animal component-free and chemically defined medium in a 125-mL flask at an initial seeding density of $1 \times 10^6$ cells/mL. With subsequent cell growth, cells were expanded into multiple and progressively larger shake flasks with target seeding densities of $0.3 \times 10^6$ and $0.4 \times 10^6$ cells/mL for 3- and 2-day passages, respectively. After 1–2 weeks of cell expansion in shake flasks, adequate cells to inoculate a laboratory-scale (15 L) perfusion bioreactor were obtained.

### 2.4 Perfusion Bioreactor Experiments

Cells expanded in shake flasks were used to inoculate a 15-L perfusion bioreactor, which was operated at a working volume of 12 L. The inoculation density was

**Table 1** List of samples taken for the bioreactor scale comparison study

| Time point | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Laboratory scale | X | X | X | X | X | X | X | | | |
| Manufacturing scale | | X | X | X | X | X | X | X | X | X |

$1 \times 10^6$ cells/mL. After an initial cell accumulation phase, the target steady-state cell density was maintained by automatic cell discard based on online cell density measurements using an online optical density probe that was calibrated with off-line cell density measurements using a Cedex system (Roche Diagnostics Corporation, Indianapolis, IN). Bioreactor temperature was controlled at 35.5 °C and the dissolved oxygen (DO) at 50 % by aeration using an oxygen–nitrogen mixture. A $NaHCO_3$-free medium and buffering system were used to maintain bioreactor $pCO_2$ close to physiological levels [24] and bioreactor pH was controlled at 6.8 by automatic addition of 6 % $Na_2CO_3$. The 15-L laboratory-scale bioreactor was used to inoculate the manufacturing-scale bioreactor, and operating conditions were the same in both bioreactors.

## 2.5 Analytical Methods

Both laboratory and manufacturing-scale bioreactors were sampled daily and bioreactor pH, $pCO_2$, and $pO_2$ were externally verified using a Rapidlab 248 blood gas analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY). Cell density, viability, and cell size were determined in a Cedex analyzer (Roche Diagnostics Corporation, Indianapolis, IN), whereas glucose and lactate concentrations were analyzed using a YSI 2700 analyzer (YSI Life Sciences, Yellow Springs, OH).

## 2.6 RNA Extraction and Microarray Analysis

Cell pellets were collected from both the laboratory-scale and manufacturing-scale bioreactors at multiple points over the course of the cultivation (Table 1) for mRNA extraction and subsequent DNA microarray analysis. Cell pellets were stored at -70 °C until further processing. For mRNA extraction, cells were lysed using QIAshredder reagents and mRNA extraction was performed using an RNeasy mini kit (Qiagen, Valencia, CA) per the manufacturer's recommendations. Microarray experiments were conducted using version 2.0 CHO microarrays based on the Affymetrix GeneChip platform developed by the Consortium for CHO Genomics [23]. Microarray analysis including RNA labeling, hybridizations, image processing, and preliminary data analysis including quantile normalizations were performed by AltheaDX (San Diego, CA).

## 3 Results

### 3.1 Feasibility Assessment of Cross-species Hybridization

The initial goal of this study was to test the feasibility and reliability of cross-species hybridization of BHK samples on a CHO Affymetrix microarray. The Affymetrix array contained probe sets corresponding to 23,020 CHO transcripts. Each probe set was typically composed of 11 short probe pairs (23-bp long), each containing a perfect match (PM) sequence to the CHO transcript and a single base mismatch (MM) sequence. In cross-species hybridizations, only a subset of all PM sequences will generate substantially higher signal intensity than their corresponding MM sequence. Previous cross-species hybridization studies [25, 26] have successfully employed a simple filtering criterion of PM/MM $> 1.5$ and PM–MM $> 50$ to exclude probes with low specificity or sensitivity for cross-species hybridizations (probe masking). We used the same filtering criteria for our analyses, and only probe pairs passing this criterion were used in the MAS5 condensation algorithm to estimate overall intensity and gene expression. In cases where data from multiple hybridizations in one study were analyzed together, averaged PM and MM values from all relevant hybridizations were used for the above filtering to ensure that a single common set of passing probe sets could be used for further analysis rather than slightly differing sets arising from each hybridization.

To test the feasibility of BHK-CHO cross-species hybridizations, we performed the same heat shock experiment in BHK and CHO cells as described in the Materials and Methods and Fig. 1. The resulting BHK and CHO samples were both hybridized onto the CHO microarray to compare cross-species with same-species hybridization. For comparability, both the BHK and CHO datasets were subjected to the same data processing algorithm, including probe masking. Only those data from probe sets with at least four probe pairs passing the above-mentioned probe masking criteria were used for further analysis. For the BHK cross-species hybridization dataset, 8,910 probe sets (39 %) passed this cutoff. For the CHO same-species hybridization dataset, 12,961 probe sets (56 %) passed the same cutoff. This clearly demonstrates that cross-species hybridization results in a smaller, albeit useful, dataset for further biological interpretation.

The principal component analysis of this dataset revealed that 91 % of the variability in the data was captured by the first component. The loadings plot (Fig. 2) shows that the first component (PC-1) separates BHK from CHO samples. The fact that PC-1 accounts for over 90 % of the variability in the data is unusual but not surprising; gene expression in two different cell types under different media conditions is expected to be substantially different than any other underlying variability. Although PC-2 accounts for only 7 % of the data variability, it is clear that this separates heat shock–treated samples from untreated ones. Notably, the distance between heat shock–treated and untreated samples was greater for CHO samples than for BHK, reiterating the effect of loss of specificity due to cross-species hybridizations.

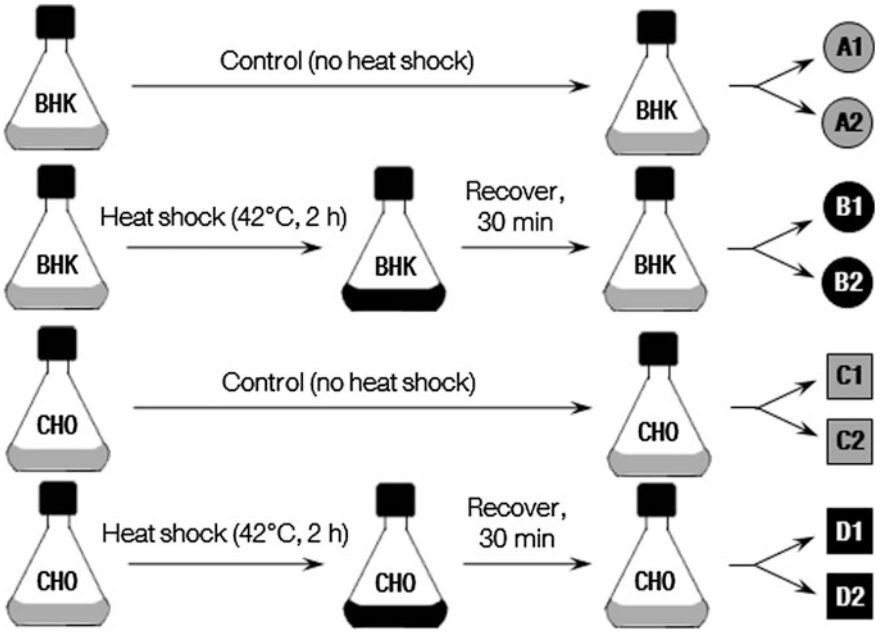


**Fig. 1** A schematic of the heat shock experiment conducted in BHK and CHO cells to assess the feasibility of BHK-CHO cross-species hybridization

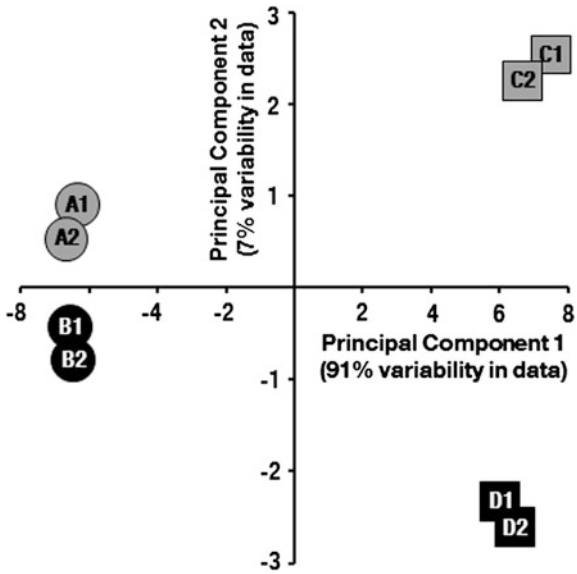


**Fig. 2** First principal component (PC-1) versus the second principal component (PC-2) from principal component analysis (on samples) of the heat shock dataset. The plot shows that data are reproducible and that PC-2 clearly separates heat shock treated and untreated samples for both BHK and CHO cells

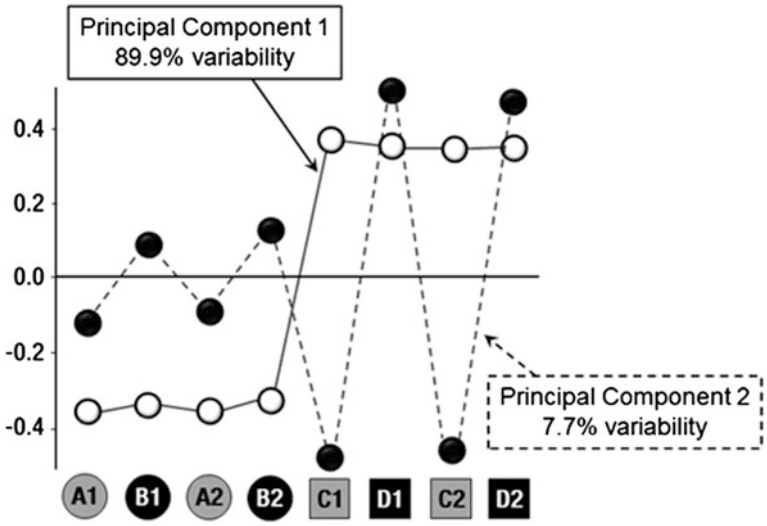


**Fig. 3** Loadings plot from principal component analysis (on genes) of the heat shock dataset. The plot shows that the first principal component (PC-1) is the cell type (BHK vs. CHO) differentiator, whereas the second principal component (PC-2) is the treatment type (heat shock vs. control) differentiator

A complimentary principal component analysis on the transposed dataset (Fig. 3) led to similar findings, with PC-1 contributing to ~ 90 % of data variability. The corresponding loadings plot showed that although a large number of genes (PC-1) have dramatically different gene expression levels between BHK and CHO cells, the differences in gene expression between heat shock–treated and untreated samples are also quite visible (PC-2). Based on the data shown in Fig. 3, those genes that have a high magnitude positive value along PC-2 axis will be upregulated due to heat shock. Fig. 4 shows a PC-1 versus PC-2 plot with this dataset. The annotations marked in the plot show that many of the genes with high PC-2 values do indeed have a heat shock protein (HSP) or an HSP-like annotation. Based on these analyses, we can conclude that cross-species hybridizations of BHK samples on CHO arrays indeed provide substantial biologically meaningful information.

## *3.2 Gene Expression Analysis in Perfusion Bioreactors*

Once it was established that BHK-CHO cross-species hybridizations can yield meaningful results, we employed this approach to characterize and compare a manufacturing-scale BHK cell-based perfusion process with a laboratory-scale system. Time-course samples were taken from both the laboratory and manufacturing-scale bioreactors (Table 1) and analyzed using the same Affymetrix DNA

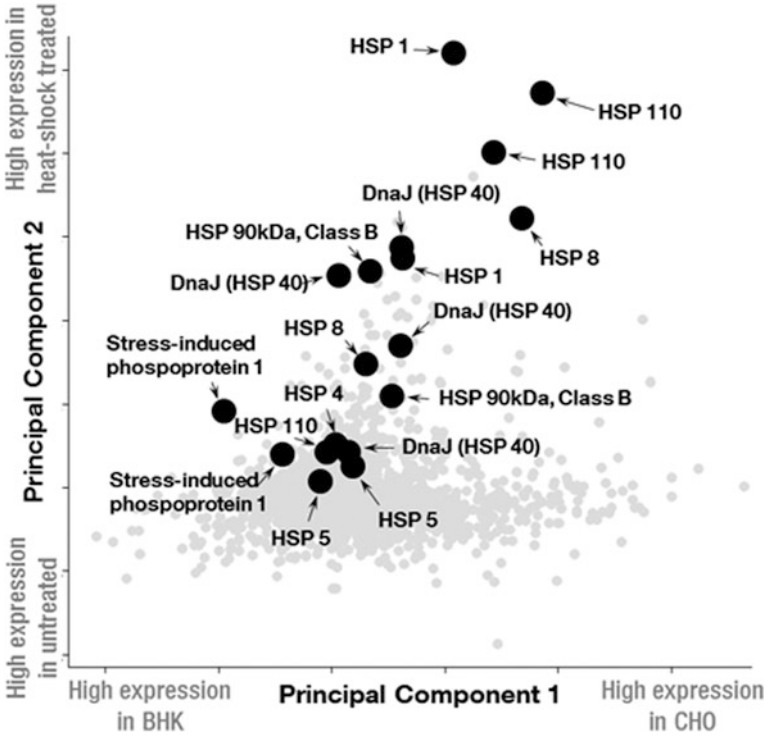


**Fig. 4** The first principal component (PC-1) versus the second principal component (PC-2) from principal component analysis (on genes) of the heat shock dataset. The plot shows that several heat shock relevant genes are identified as upregulated in both cross-species and same-species hybridizations

microarray platform as for the heat shock experiments. For data analysis, a similar approach as indicated above was used, except that a stricter filtering criterion of using data from only those probe sets providing a minimum of six probe pairs passing the probe mask cutoff (using averaged PM and MM values) was used. The stricter criterion was used because, unlike the heat shock study described earlier that contained a mix of cross-species and same-species data, this dataset was composed of only cross-species data.

A total of 7047 probe sets passed the above-mentioned criterion and were considered for further analysis. Principal component analysis on the samples (Fig. 5a) shows that PC-1 contributes to 37 % of the variability in the data. A plot of PC-1 values against cell age (Fig. 5b) shows that trends observed in the manufacturing-scale and laboratory-scale bioreactor were very similar. Therefore, from a macroscopic gene expression perspective, the scale-down model reasonably mimics cell behavior in the manufacturing scale bioreactor. We note here that higher order principal component values (PC-2 and higher) did not correlate as well. It is quite possible that higher order principal components reflect noise in the

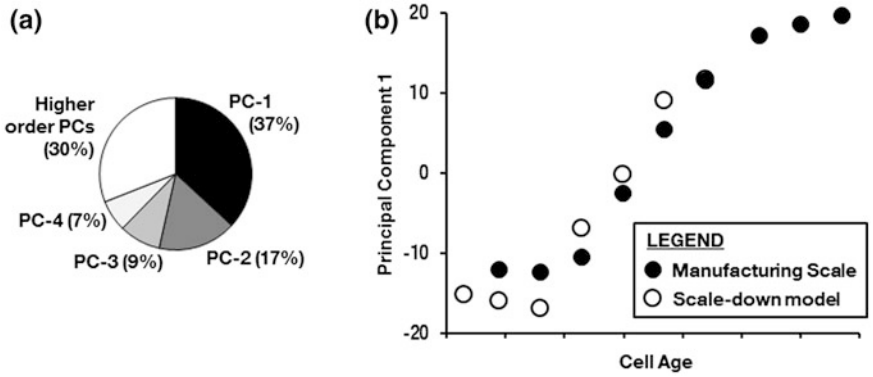


**Fig. 5** **a** Distribution of process dynamics explained by each principal component in the bioreactor dataset obtained by principal component analysis on samples **b** Loadings plot of the first principal component (PC-1) showing that PC-1 values in different bioreactor scales are comparable but cell age-dependent

data set (if the PC-1 trend is the only prominent pattern in bioreactor operation) and hence are not conserved across bioreactor scales. Another important observation from Fig. 5b is that the gene expression profile (as defined by PC-1 values) in the early stages of culture is substantially different from later stages. In fact, there is a monotonic change in PC-1 values with cell age. This implies that the overall physiological state of the cells based on gene expression gradually changes over time and that this change is conserved across bioreactor scales.

We then compared PC-1 values to all routinely measured cell culture process variables such as cell density, growth rates, nutrient consumption, metabolite production, and protein productivity. One particular process attribute—henceforth referred to as the key process attribute X (KPA-X)—was highly correlated with PC-1 values. The correlation coefficients of KPA-X versus PC-1 were 0.913 and 0.995 for the laboratory-scale and manufacturing-scale reactors, respectively. Based on these observations, we can conclude that the laboratory-scale model provides a representative reflection of the manufacturing scale bioreactor, especially to study age-dependent behavior changes and to analyze the temporal variation of KPA-X. Although Fig. 5b implies that this could be a cell age-dependent trend, it is plausible that the effect is, in fact, bioreactor culture age-dependent. The relative shift in plots of Fig. 5b when the *x*-axis is changed to bioreactor culture age is not substantial considering the magnitude of the *x*-axis scale. To decouple bioreactor culture age from cell age, future experiments will need to assess gene expression from cultures inoculated into bioreactor after a relatively long time after vial thaw.

Next, we sought to identify individual genes whose expression profiles correlated with age and KPA-X. For this, principal component analysis was performed (separately for the laboratory-scale and manufacturing-scale bioreactor datasets) on the transposed matrix where columns represent genes and rows represent

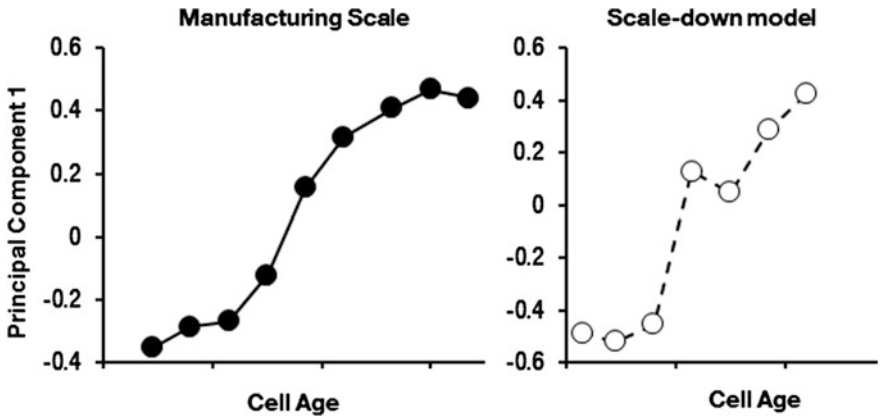


**Fig. 6** Loadings plot of the first principal component (PC-1) resulting from separate principal component analysis on genes for each of the laboratory-scale and manufacturing-scale bioreactor data

samples. This analysis is similar to that performed on the heat-shock dataset to generate Fig. 3. The loadings plot from this analysis is shown in Fig. 6. As before, genes with high magnitude positive value will have an expression profile strongly positively correlated with the trend shown in Fig. 6. Similarly, genes with high magnitude negative value will show an expression profile negatively correlated to the trend shown in Fig. 6. Some example genes with such high correlations are shown in Fig. 7. Although the biological functions of these genes are as yet unclear, especially in the context of bioprocess operation, we expect to revisit this data in the future when additional annotation information becomes available.

## 4 Discussion

The concept that the process defines the product forms the basis for approaches such as quality by design in bioprocess development. Clearly, mechanistic understanding of cell culture processes is important, but it is also quite challenging. Until recently, the tools available for interrogation of these processes were rudimentary and analysis was typically limited to measurement of attributes related to cell growth, metabolism, and product productivity along with product characterization. Systems biology tools that have already radically altered primary research and clinical study landscapes hold enormous potential for biopharmaceutical process development. However, compared to tools available for human and mouse models for clinical/research use, those available for industrially relevant mammalian cell lines are still in their infancy and publication of the CHO genome [21] is a major step in filling this knowledge gap. Yet, even with past shortcomings, researchers have employed systems biotechnology tools to study

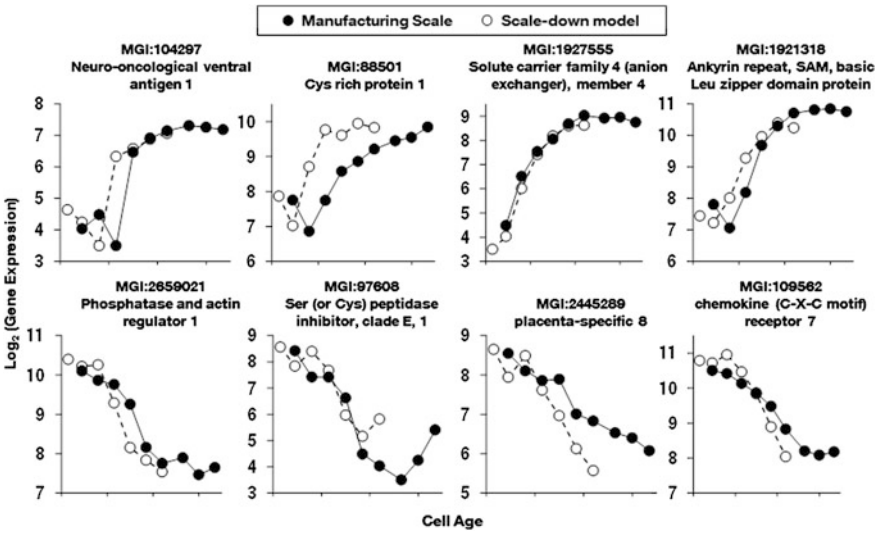


**Fig. 7** Gene expression profiles of the top four genes with the highest and lowest first principal component (PC-1) values in both laboratory- and manufacturing-scale bioreactors

apoptosis, cell growth, metabolic shifts, and productivity-altering strategies in industrially relevant mammalian cells (reviewed in [27, 28]). Although most of these studies were focused on identifying genes or gene sets that could potentially improve growth characteristics, product titers, and/or quality, few were conducted in the experimental settings typically encountered in commercial biomanufacturing. Herein, we described the application of transcriptomics to characterize an industrially relevant perfusion bioreactor process. Unlike many previous reports, we focus on bioprocess fingerprinting as a simple yet pragmatic application of systems biology where DNA microarrays are employed for process comparisons from a molecular perspective.

The BHK cell line used in this study was reasonably well characterized from a cell physiology perspective but lacked extensive genomic resources. In such situations, cross-species DNA microarray hybridizations have been used as an imperfect but adequate solution [11, 26, 29]. We have shown that despite sequence homology concerns, BHK samples on CHO microarrays can yield a substantial amount of reproducible biologically relevant information. A common concern with use of cross-species hybridizations is the possibility of low signal intensity and/or cross-reactivity arising from lack of probe specificity. Although various data filtering techniques have been described in the literature [11], balancing filtering stringency with the amount of meaningful information that can be obtained is critical. We minimized these concerns in our experiments because no single gene or small sets of genes were used to make major conclusions. Rather, the dynamics of the overall macrolevel gene expression profile was tracked across long-term perfusion culture of BHK cells to assess similarities/differences in different bioreactor scales.

Scale-down model validation is a highly relevant and important aspect of bioprocess development. Often, numerous experiments—all of which cannot be performed at manufacturing scales—are simulated in scaled-down systems. However, it is imperative that such scale-down models adequately mimic the manufacturing process as well as any underlying problems associated with it. Traditional approaches for scale-down model qualification rely on matching certain mechanical and fluid dynamics aspects of the systems [30–32] as well as comparing a limited set of routinely measured process variables (e.g. cell density, viability, product, metabolite rates) across different scales. Here we show that systems biology tools can greatly expand that repertoire of process variables, from a handful of measured growth and metabolism parameters to expression levels of thousands of genes. A simple approach would be to assess a baseline gene expression variability associated with day-to-day operations and then evaluate a new condition (e.g., a scaled-down process) to assess if its gene expression profile (using some multivariate distance metric) is substantially different from the original condition. This approach can be used to discover outliers in simple steady-state experiments or instances where the same or similar profile is expected for each sample. However, practical situations, including many perfusion cultures, may not operate as true steady-state systems over long time periods. In our example, each time-course sample turned out to be slightly different from its neighboring sample. In such cases, multivariate analysis tools, such as principal component analysis, can be employed to resolve time-dependent components for further comparisons. Using transcriptomics data, we showed that our scaled-down system was representative of the larger bioreactor based on PC-1 values, particularly in the context of studying temporal changes encountered in our process. While we have demonstrated this approach for perfusion cultures, it is also applicable to fed-batch systems, which are inherently transient and consequently associated with more dynamics in gene expression that can be compared under differing operating scales and conditions.

## 5 Conclusions

Systems biology tools are increasingly finding their way into bioprocess development, and the recent publication of the CHO genome will accelerate applications in this area. Among the multiple systems biology tools, transcriptomics is one of the more mature approaches, allowing routine estimation of thousands of intracellular variables without substantial effort. Although DNA microarrays were used in this study and issues related to cross-species hybridization had to be addressed, newer techniques such as RNASeq are gaining popularity and have considerable advantages over DNA microarrays.

Conceptually, systems biology tools can be broadly applied in two areas for bioprocess development: (1) for the mechanistic understanding of a desirable phenotype or a first-principle-based understanding of cellular physiology, which

can lead to bioprocess and/or genetic engineering modifications to achieve the desired outcome, or (2) using omics tools as a fingerprinting technique to assess similarity or differences (even in the absence of adequate biological information, such as gene annotation). Although the first area of focus offers the most promise, it is clearly more challenging and requires a substantial investment of time and resources to complete the cycle from hypothesis generation to proof of concept and eventually to application. The latter fingerprinting approach is more pragmatic and arguably has a lower barrier for success, which leads us to believe that it can provide the initial rationale for widespread adaptation of systems biology approaches in the industry. Such a collective effort, coupled with parallel advancements in sequencing, assembly, and annotation, can help realize the full potential of systems biology for the development of next-generation biopharmaceuticals. We believe we are at a juncture where we can realistically apply a subset of available systems biology tools, just as any other routine biochemical or molecular assay, to probe process development experiments and to monitor and diagnose commercial manufacturing processes.

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Adv Biochem Eng Biotechnol (2014) 139: 245–256
DOI: 10.1007/10_2013_258

Published Online: 7 November 2013

# Lifecycle Management for Recombinant Protein Production Using Mammalian Cell Culture Technology

**Enda Moran and Patrick Gammell**

**Abstract** Product lifecycle management refers to the oversight process and activities carried out to fully realize the commercial potential and value of a product in the marketplace. It is typical for many changes to be introduced to the production processes and testing methods for biopharmaceutical drugs over their lifetime in the commercial marketplace. Technology lifecycle management, as discussed here, refers to the management of the different phases or generations of processes and methods used to make and test the active biopharmaceutical ingredient or drug product, and the adoption of different devices used to present the drug product to patients. The factors to consider when making changes to a commercial biopharmaceutical manufacturing process as part of a technology lifecycle management program are discussed. A case study outlines one approach taken in bringing forward a major process change to a cell culture process for the production of a therapeutic recombinant protein.

**Keywords** Cell culture technology · Post-approval changes · Technology lifecycle management

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## 1 Introduction

Product lifecycle management generally refers to the process of maximizing and prolonging a product's or brand's value in the marketplace. Within the pharmaceutical industry, a drug product lifecycle management strategy may involve many facets such as the introduction of new indications, new delivery devices, marketing and publicity campaigns, expansion to new markets, and so on. The typical pharmaceutical drug lifecycle, commencing at the early research phase and progressing through regulatory approval to market introduction is shown in Fig. 1. The market growth and market maturity phases for a biotechnology-derived drug are relatively long compared to a traditional pharmaceutical exposed to generic competition, often extending to 15 years or longer. There are a number of reasons for this including the high cost of entry for competitors, a period of patent exclusivity for the drug innovator, patient loyalty factors, and the complex and still-maturing regulatory environment governing the introduction of biosimilar drugs. It is typical for many changes to be introduced to the production and testing processes for biopharmaceutical drugs owing to their extended period of time on the market. These changes may be motivated by many factors including the need to improve process consistency and robustness, reduce costs of manufacture, move manufacture to an alternate location, introduce a new drug product delivery device, or improve process yields to supply growing markets. Major process changes are managed in a rigorous and systematic manner to assure there is no impact on the safety and efficacy of the drug, and can take many years to implement and receive regulatory approval in the different regions around the globe. We use the term technology lifecycle management to refer to the process by which process changes are proposed, triaged, developed, and implemented into the licensed biopharmaceutical drug production process. This chapter highlights the factors to consider when making changes to a commercial biopharmaceutical manufacturing process as part of a technology lifecycle management program. The case study describes one approach taken in bringing forward a major process change to a cell culture process for the production of a therapeutic recombinant protein.

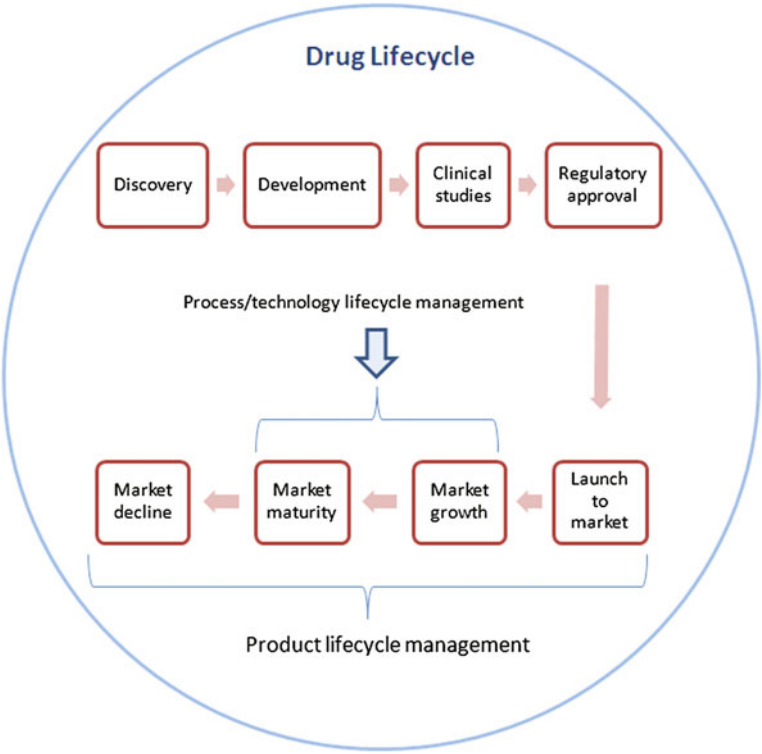


**Fig. 1** The biopharmaceutical drug development and commercialization lifecycle. Product lifecycle management focuses on maximizing and extending the value of the drug in the marketplace through multiple different strategies. Certain product lifecycle management strategies may even be started before the drug is approved and launched. Process or technology lifecycle management is used here to refer to the oversight of the different phases or generations of processes and methods used to make and test the active biopharmaceutical ingredient or the drug product for the market

## 2 Factors Driving Technology Changes During the Product Lifecycle

Multiple factors within the current pharmaceutical business environment may drive technology changes for the manufacture and testing of biopharmaceutical products.

### *2.1 Technology Advances*

Scientific and technology advances may drive changes throughout the product lifecycle. A classic example of technology driving change would be the development and subsequent widespread availability of chemically defined nutrient

media formulations that can support cell growth and productivity without the requirement for additional, animal-derived raw materials (ADRMs) such as bovine serum. Many companies have changed cell-culture–based manufacturing processes to remove ADRMs in response to health authority pressure to improve process safety, to reduce process variability, to reduce the risk of introduction of adventitious viruses to manufacturing facilities, and to reduce the cost and sourcing problems often associated with ADRMs. The equipment technology by which biopharmaceuticals are produced is also changing. For example, in the recent past the biotech industry has been increasingly focused on the use of disposables technologies for bioprocessing. These technologies have a number of advantages over stainless steel process equipment and systems due to their ease of set-up and use, reduced utilities requirements for cleaning and sterilization, and rapid turnaround times in the manufacturing operations environment. Other advancements that have prompted technology lifecycle changes for certain biotech products include the introduction of microcarriers for anchorage-dependent cell cultivation enabling the move away from roller bottle production systems, and new media/suspension cell combinations to improve upon traditional egg-based production systems for viral vaccine manufacture.

## *2.2 Decreased R&D Productivity*

Despite increasing investment into R&D within the biopharmaceutical industry, output as measured by the number of product approvals relative to industry investment has diminished significantly since the 1980s. An outcome of this decrease in output from R&D is that companies are increasingly dependent on a decreasing number of blockbuster products (defined as typically earning revenues greater than $1 BN/annum) that are coming to the end of their patent exclusivity periods. Without new product approvals to replace these blockbusters, it is a strategic play of many biopharma companies to maintain market share for existing products and to manage costs as these drugs come off patent. This situation has had a significant impact on lifecycle management within the industry as companies develop lean manufacturing systems, replace expensive raw materials, and develop more productive and more cost-efficient ways to manufacture these drugs.

## *2.3 Price Pressure from Payers*

The situation whereby the pharmaceutical industry has had meaningful control over pricing for drugs is coming under increasing pressure as payers have to deal with escalating costs due to aging populations, the growth of chronic illnesses, greater patient influence, and changing government policies. This increasing

pressure has led to novel pricing structures that can even link product price to patient outcomes indicating a major shift in the balance of bargaining power from the pharma industry to payers. The global financial crisis has also resulted in an impact on the ability of governments, who remain the biggest payers, to cover the costs of expensive biopharmaceuticals as they cut costs elsewhere within nationalized healthcare systems. These price pressures in the market inevitably filter back to the manufacturer who must look for new or modified ways to make the same product cheaper and faster.

### *2.4 Multiproduct Manufacture and Market Supply*

As the biotech industry developed in the 1980s and 1990s the companies with the first breakthrough products built dedicated facilities for their manufacture. As the industry was just developing, there was little focus on aspects of sustained, robust "manufacturability" of the product, and no great pressure to consider manufacturing needs for a broad portfolio of products. Today, there is driving pressure for companies to modernize these production processes largely for productivity enhancement reasons to liberate production capacity to manufacture multiple products in the same facility. Protein products produced in cell culture systems in the 1990s were produced in low concentrations (approximately 100–300 mg/L). For certain successful products in the market demanding 500–600 kg/annum or more, this level of productivity would be insufficient to economically supply the marketplace. Market needs therefore have driven technology advancements in production processes: in the case of cell culture technology, advances in media and cell line development have been significant enablers in this regard.

## 3 Key Considerations When Making Technology Changes During the Product Lifecycle

As described above, change is sometimes driven within the biopharmaceutical industry to take advantage of evolving process and analytical technologies. These changes allow companies to safeguard the supply of important medicines to patients and can help protect market share and competitiveness as products approach the end of patent exclusivity. On occasion, changes may not be discretionary and are forced upon companies, for example, when a particular raw material is discontinued or no longer fit for purpose. Despite the many benefits associated with change, however, it can be disruptive, expensive, and may add technical and regulatory risk. This section reviews some of the factors that need to be considered before engaging in technology change during a product's lifecycle.

## 3.1 Clinical Considerations

Manufacturers of biopharmaceuticals typically make post-approval manufacturing changes to marketed products based upon a demonstration of comparability between the product prior to the change, and the product after the change. For well-characterized biotechnology products, this comparability will consist of the existing release tests in addition to a battery of detailed characterization and in vitro functional tests to demonstrate that there has been no adverse impact on the quality, safety, or efficacy of the product following the change. The expectations for demonstration of comparability are well described in FDA, EMA, and ICH guidance documents [1–4]. This comparability approach is justified as appropriate because manufacturers of existing products have extensive knowledge about the process used to manufacture the product and the relationship between the process and the product along with supporting clinical experience. The totality of this knowledge and experience can be combined with an appropriate development package in support of the proposed changes to the manufacturing process.

There may be occasions, however, whereby a manufacturing process change will need additional in vivo studies using appropriate animal models or a human patient clinical trial. The decision to execute human clinical trials has a significant impact on project timelines, cost, and human resources required. Therefore, this is typically only pursued if the implementation of a process change results in changes to product critical quality attributes (CQAs) that are outside existing clinical experience and/or if there are no relevant or sufficiently sensitive in vitro assays to assess the impact of the variation of the product quality attribute.

Sometimes the requirement for additional human clinical trials can be negated if the biochemical comparability data package is satisfactory and the company makes a commitment to execute a post-authorization safety study (PASS) or a pharmacovigilance surveillance program designed to examine trends in the incidence of adverse events (AEs) following the introduction of the product post-change. This type of surveillance would be supplemented by the routine pharmacovigilance activities designed to look for evidence of autoimmunity, allergic reactions, and lack of efficacy.

## 3.2 Regulatory Considerations

Implementation of process changes during the lifecycle of a product will invariably involve interaction with regulatory agencies, and depending on the markets in which the product is sold, this can range from as little as one to more than eighty independent boards of health! In situations where there are a large number of jurisdictions to deal with, the assessment of whether a process change will be implemented will partly depend on the "regulatory" impact on the company and the product franchise. For example, a high-impact process change to double

process yield at half the original costs of manufacture may well be worth the time, cost, and effort of a company's global regulatory organization to pursue filings and approvals around multiple regulatory regions. However, smaller changes that cause similar disruption may not be worth the effort unless packaged or coupled with other changes that increase the payback relative to the effort, cost, and time to implement. In addition to this, regulatory submissions for a single product often must be staged and strategically planned such that at any one time, a company is not "locked out" of particular key jurisdictions that restrict the number of simultaneous submissions that they will examine for a particular product. For projects that are prioritized for important strategic reasons linked to the product lifecycle such as localization to a tax-advantaged site of manufacture or technology improvements to align with internal platform standards, and so on, it is often prudent to leverage opportunities to seek regulatory agency scientific advice prior to submission or completion of the development activities to support the change to the dossier. In the European Union and the United States, there are formal mechanisms to obtain this advice prior to submission.

Another aspect to consider from a regulatory perspective following a process change is the requirement for agency interaction after submission: these interactions can include provision of responses to follow up questions from the agency prior to approval of the change, post-approval commitments to revise specifications from a statistically significant number of commercial manufacturing batches, and so on. These types of regulatory interactions and commitments can add delay and uncertainty to approval and implementation timelines which can have a negative impact on the business case for the project and in the case of postapproval commitments, these can require additional budget (resource, capital, etc.) that was not previously considered. These regulatory risks are important to assess prior to initiation of lifecycle process change management.

## *3.3 Risk Factors*

When considering implementation of a process change as part of the lifecycle management of a biotech production process, the level of risk the company is prepared to carry should be evaluated. These risks include product supply disruption that may be incurred due to facility modifications and the use of manufacturing facility capacity and time to support demonstration/validation batches. This risk can be generally managed through strategic use of held inventory. There is also a risk that despite a significant investment of time and resource to develop and characterize a new process, the change may be held up by regulatory delays in review and approval: some of this risk can be mitigated through early interaction with regulatory agencies (Sect. 3.2) but may not be completely removed. Quantification of this risk through a success probability score can be complex and highly subjective due to the number of unknowns that factor into the calculation. Similarly, costing these risks can be equally complex as the costs can include:

- Opportunity cost lost to other programs that are delayed or deferred
- Impact on business case and net present value (NPV) for the project can have a particular effect as products approach loss of exclusivity and product sales volumes decrease, therefore increasing the time required to pay back the cost of the process change
- Additional unplanned development and/or clinical costs.

Successful technology lifecycle management will be dependent on a company's ability to capture all of the risks associated with implementation of a change and to combine them to score the overall risk in terms of a probability of technical and regulatory success. This ability to score programs in a quantitative fashion is key to deciding on which technology lifecycle programs should be prioritized based primarily on a risk/reward criterion.

## *3.4 Costs and Resources*

The use of platform technologies emerged in biopharmaceutical product development programs during the 1990s and primarily focused on monoclonal antibody production processes. There are multiple benefits of using platform approaches for development and manufacturing. Standardization of approaches and tools across multiple products leads to improved quality and consistency, substantial cost savings primarily as a result of more efficient resource utilization (equipment/people), and faster process and product development.

The resources required to support major process changes such as alignment with platform manufacturing processes or to support technology transfer to a new location are a significant determining factor for the implementation of such changes. As an example, the process development resources required for a significant process change are usually heavily engaged with ongoing process support for marketed products, troubleshooting, and developing processes for new products. The deployment of resources to support a manufacturing process change should ideally follow a prioritization process that considers multiple facets such as the potential for manufacturing-based cost savings, quality improvements, and so on. The relative impact of these different drivers should be considered: for example, those changes that are requested by regulatory agencies to improve the safety profile of a product, such as through removal of an animal-derived raw material from the process, would likely be a higher priority than changes to deliver small savings in the costs of manufacture. Similarly the magnitude of the change is an important determinant because a major process change that involves significant redevelopment, process characterization, pilot-scale runs, and significant changes to the dossier would consume much time and resources from multiple functions such as process development, engineering, validation, regulatory, and quality. This potential impact on resources should be assessed as an opportunity cost with respect to other ongoing and pipeline projects.

## 4 A Major Cell Culture Process Change for the Production of a Marketed Therapeutic Recombinant Protein

The case study that follows describes a major process change executed in the lifecycle of a marketed biotech product. A significant improvement to the manufacturing process for the production of a commercially distributed recombinant Fc-fusion protein produced in Chinese hamster ovary (CHO) cell culture was carried out. The primary motivation for this change was the need to remove animal-derived material from the cell culture medium used in the upstream process to reduce cost, eliminate raw material supply problems, and improve the risk profile with respect to risk of ingress of adventitious agents to the manufacturing process. The process improvement was under development for approximately four years and comprised primarily of upstream process changes including the creation of a new cell bank, modified early scale-up steps using different media and disposables technology, and significant changes to the shape and operation of the fed-batch production culture. The steps taken in the overall program are illustrated in Fig. 2. A significant post-approval technology change such as this is a major undertaking for any biopharmaceutical enterprise. In this case, it demanded resources from multiple functions (process development, manufacturing operations, regulatory affairs, engineering, supply chain, etc.) over many years, and required capital investment in the manufacturing facility to enable a "fit" of the new process. The early planning phase, therefore, aimed to inform the organization up front of all the activities required to bring this change through to regulatory approval, thus ensuring "all-in" commitment before taking on the project.

The early process development work assessed the feasibility of removing the animal-derived raw materials from the cell culture media used in the upstream process. The CHO cell line was fastidiously dependent on these materials as nutritional sources. The cell line was adapted to a proprietary protein-free medium formulation using an in-house adaptation protocol. The cell line exhibited an improved growth rate in the protein-free medium at the end of the adaptation; see Fig. 3.

The protein biopharmaceutical product, which has been marketed for many years, is defined by a well-established product profile and a series of specifications describing its critical characteristics or attributes. Therefore, the process improvement was developed to deliver a product comparable to the currently marketed therapeutic protein. The development was executed in bench-scale (5 L), and pilot-scale systems, ranging from 150 to 1600-L scale: these pilot systems were shown to be representative of manufacturing-scale operations (12500 L) for the previous version of the manufacturing process for this protein. Quality by design (QbD) principles, tools, and approaches were applied in this technology development program [5]. The outcome at the end of process development and process validation in the commercial-scale production plant was a more robustly operating upstream process, absent of ADRMs, and a mean increase (60–70 %) in batch productivity; see Fig. 4.

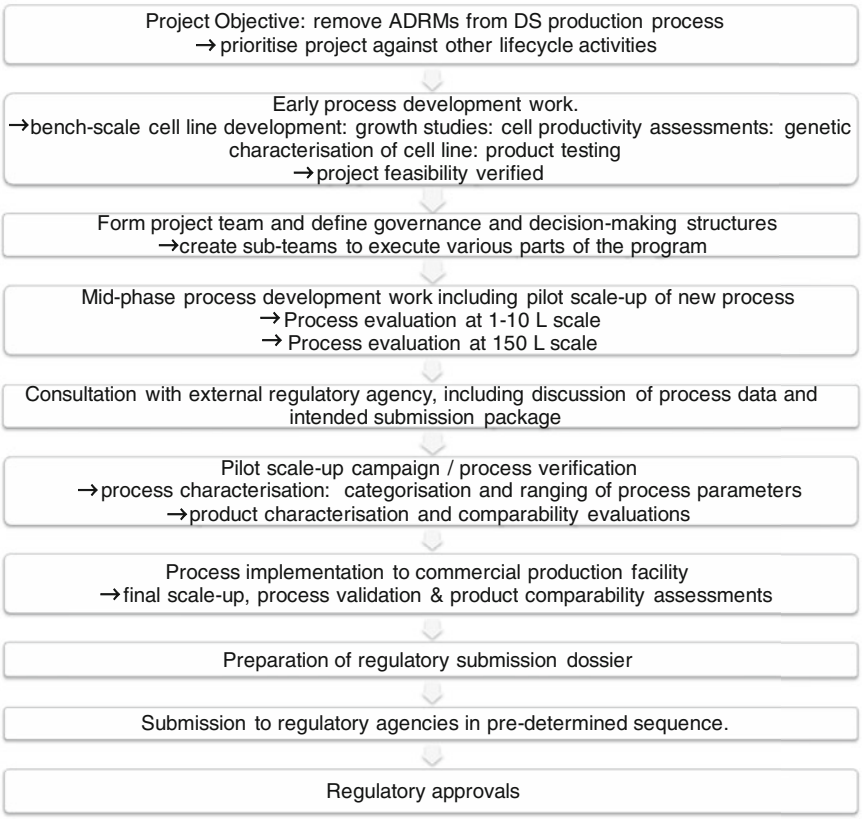


**Fig. 2** The sequence of events adopted towards project execution and regulatory approval of a major postapproval process change for production of a biopharmaceutical product

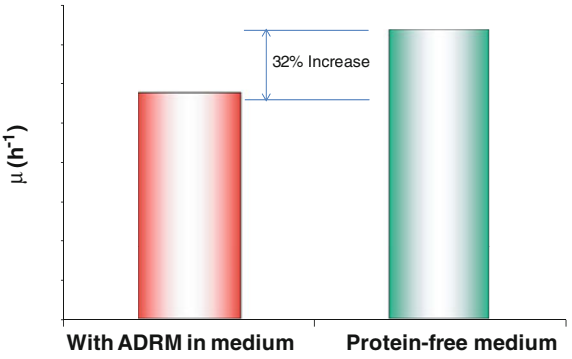


**Fig. 3** Growth rate of the CHO cell line in flask culture after a lengthy and successful adaptation to protein-free conditions.
ADRM = animal-derived raw materials

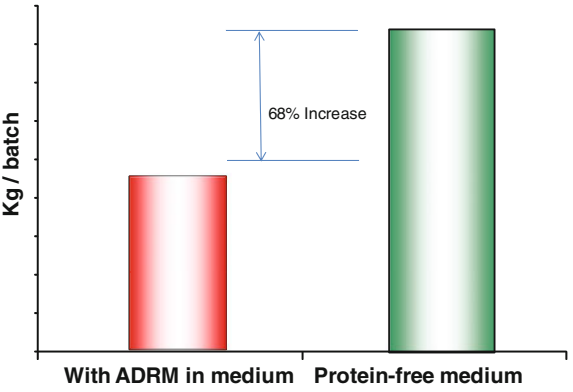


**Fig. 4** Overall process (batch) productivity improvement after process development across bench-scale (5 L), and pilot-scale systems ranging from 150 to 1,600 L. The data shown compare performance of the process pre- and postchange at the eventual manufacturing scale of 12,500 L. ADRM = animal-derived raw materials

The process of gaining regulatory approvals for this technology change in multiple regions around the globe extended for many more years. In general, each agency or board of health has the same foundational expectations around product comparability, process validation, and the like, informed by the ICH and other guidances. However, certain country-specific nuances and expectations are not uncommon. Although all questions and requests for information could be dealt with by the organization, this lengthy regulatory process demanded continued input from various organization functions and disciplines long after the large-scale process validation was completed in the commercial production plant.

## 5 Summary and Conclusions

Technology changes during the lifecycle of a biopharmaceutical product may be motivated by multiple factors such as the need to modernize technology in the production process, achieve improved levels of robustness or consistency, drive down costs of manufacture, and so on. Post-approval changes to biopharmaceutical manufacturing processes are major projects for the sponsoring company to execute in terms of time, money, and resources. Complex changes may also carry a risk of unexpected events or issues arising, either associated with manufacture of the product, or with the use of the product in the patient population. As such, technology change during the lifecycle of a commercial product should be managed in a structured and formal manner to evaluate the potential benefit of the change versus the costs and risks associated with its implementation.

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Adv Biochem Eng Biotechnol (2014) 139: 257–259
DOI: 10.1007/978-3-642-54050-9


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