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Preface

Since the introduction of recombinant human growth hormone and insulin a quarter century ago, protein therapeutics has greatly broadened the horizon of health care. Many patients suffering with life-threatening diseases or chronic dysfunctions, which were medically untreatable not long ago, can attest to the wonder these drugs have achieved. Although the first generation of protein therapeutics was produced in recombinant *Escherichia coli*, most recent products use mammalian cells as production hosts. Not long after the first production of recombinant proteins in *E. coli*, it was realized that the complex tasks of most post-translational modifications on proteins could only be efficiently carried out in mammalian cells. In the 1990s, we witnessed a rapid expansion of mammalian-cell-derived protein therapeutics, chiefly antibodies. In fact, it has been nearly a decade since the market value of mammalian-cell-derived protein therapeutics surpassed that of those produced from *E. coli*. A common characteristic of recent antibody products is the relatively large dose required for effective therapy, demanding larger quantities for the treatment of a given disease. This, coupled with the broadening repertoire of protein drugs, has rapidly expanded the quantity needed for clinical applications. The increasing demand for protein therapeutics has not been met exclusively by construction of new manufacturing plants and increasing total volume capacity. More importantly the productivity of cell culture processes has been driven upward by an order of magnitude in the past decade. For the biochemical engineering community, especially the researchers and bioprocess professionals engaged in cell culture engineering, this technological advancement is a cause for celebration.

Although the emergence of cell culture engineering is relatively recent, the demand for a robust manufacturing platform has propelled tremendous advances in a short decade. In the course of transforming from an exploratory technology to a robust instrument generating life-saving medicines, cell culture processes have evolved to almost uniformly employed stirred tank bioreactors for large-scale operations. Batch and simple continuous cultures are seldom employed, except on research scales. Fed-batch cultures and continuous processes with cell retention, also commonly referred to as perfusion culture, have become the norm. To capture the essence of those advances and to highlight products that have been the driving force for the expansion of cell culture

processes, three chapters in this volume are devoted to those topics: antibody products, fed-batch cultures and perfusion processes.

In the past decade, we have seen the specific productivity of recombinant antibodies by high-producing cells approach that of natural antibody secreting cells in vivo. This has been largely accomplished through random screening of clones with higher productivities. As we strive to generate hyper-producing cell lines that exceed the production capabilities of native antibody secretors in our bodies, screening processes will likely incorporate more rational approaches. Use of targeted markers, known to confer hyper-productivity traits will likely prevail. The complex trait of hyper-productivity is most probably a composite of many physiological characteristics, including robust growth, efficient energy generation, reduced susceptibility to reactive oxygen species, and enhanced protein processing and secretion machinery. Identifying the potential target genes for desired traits is a key to cell engineering. This issue also includes a chapter that gives a comprehensive review of the current state and future prospect of metabolic engineering of mammalian cells.

Looking to the future of cell culture engineering, we anticipate the productivity to continue to swing upward at a pace greatly exceeding the increasing demand to reduce the cost of goods. We are likely to see the next phase of process enhancement integrate renovations in process engineering with innovations in cellular engineering. Process renovations will continue to draw out the full capacity of fed-batch and perfusion processes. With imagination, one may see cell engineering efforts induce revolutionizing changes in cell culture processes. As genomic and proteomic tools become more readily available for cell culture engineering research and our understanding of the physiological basis of hyper-productivity further advances, such imagination will become closer to reality.

Minneapolis, July 2006 Wei-Shou Hu

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Established Bioprocesses for Producing Antibodies as a Basis for Future Planning

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Abstract In the early years of monoclonal antibody production for human therapy and diagnosis the methods used were arrived at by individual organisations. However, there is now an accumulating body of information on antibodies and fragments that have been produced by processes approved for human use. This information is becoming available at a time when the number of potential antibody-based medicines is growing sharply.

The review addresses the reported production routes, their scale and the titres achieved. It identifies the performances of fed-batch and perfusion culture versus batch

culture, and compares processes for the production of antibodies for diagnosis and for antibody fragments.

The analysis defines the likely routes of future production in a sector where demanding regulations constrain new technology. It also indicates what levels of performance new approaches will need to meet to be competitive.

Keywords Antibody production/manufacture · Antibody fragments · Mammalian cell culture · Therapeutic and diagnostic antibodies · Transgenic

1 Introduction

Following the important development of hybridoma technology for producing monoclonal antibodies by Kohler and Milstein in 1975, immediate breakthroughs in the treatment of human diseases were expected. The early monoclonal antibodies, originally labelled as "magic bullets", faced clinical disappointments owing to their murine origin which induced the human anti-mouse antibody (HAMA) immune response. However, monoclonal antibodies currently represent the second largest single category of biopharmaceutical substances under investigation as therapeutic drugs [1, 2]. This can be attributed to the recent genetic engineering methods for constructing chimeric, humanised and human monoclonal antibodies that circumvent the HAMA response. Consequently, the full potential of monoclonal antibodies, with their unique binding specificity and potential to be produced in unlimited quantities, is rapidly becoming recognised in biomedical research, diagnosis and therapy.

Over the past decade, monoclonal antibodies have been responsible for several of the important advances in pharmacotherapy; agents such as Synagis™, Herceptin™ and Remicade™ have transformed the treatment of infectious diseases, cancer and autoimmune diseases, respectively [3]. However, biopharmaceuticals are amongst the most expensive of all drugs. For example, the annual cost per patient for antibodies like Rituxan™ and Enbrel™ is $10 000–$15 000 [4, 5]. Given the time, cost and risk associated with biopharmaceutical drug development, the ultimate success of new antibody candidates will partly depend on pharmacoeconomic issues. Increasing pressure from healthcare providers, as well as the much disputed anticipated capacity shortage [6], has triggered a drive to reduce manufacturing costs at the commercial scale by an order of magnitude from $1000s per gram to $100s per gram [7]. These pressures have encouraged the search for alternative production technologies, such as the use of transgenics, as well as the use of detailed molecular engineering to alter the effectiveness of the antibody. For antibodies to reach their full commercial and medical potential, all improvement efforts need to demonstrate that they can bring down the cost of antibodies.

This paper analyses the manufacturing processes used for marketed antibodies for therapeutic and diagnostic uses and assesses likely routes for future antibody production.

Antibody engineering efforts are initially summarised in Sect. 2. In Sect. 3, the investigation of commercial production methods for monoclonal antibodies is presented, and generic processes are derived in Sect. 4. Future processing routes that may be used commercially are discussed in Sect. 5 based on antibodies in development. Finally, a summary of the findings is provided.

2 Antibody Engineering Efforts

Antibodies are populations of protein molecules, known as immunoglobulins, synthesised by an animal in response to a foreign macromolecule, an antigen. The development of hybridoma technology in mice by Kohler and Milstein [8] allowed, for the first time, the production of monoclonal antibodies recognising specific antigens of choice and led to their widespread application in research and development [9]. However, the use of such murine antibodies as human therapeutics has been limited by their immunogenicity in humans (e.g. [10]). Genetic manipulation of murine monoclonal antibodies began in the 1980s to reduce their immunogenicity. These techniques were used to generate mouse–human chimeric antibodies [11] and then humanised antibodies with 90–95% human content [12]. They have been shown to be significantly less immunogenic than murine antibodies, and with a longer half-life in the body. More recently, transgenic mice have been genetically engineered to generate fully human antibodies [10, 13]. A number of fully human antibodies are now in clinical development with the aim of eliminating the problems with immunogenicity.

Various antibody fragments can be derived that are proving to be of practical use in therapy and diagnosis. Some of these antibody fragments were originally derived from whole antibodies by enzyme proteolysis. However, more recent developments in recombinant DNA technology mean that these fragments can also now be produced using expression hosts such as *Escherichia coli* [14]. All of the small fragments (Fv, scFv, Fab, $F(ab')_2$) are useful because they are still capable of binding to antigens and in some therapies this is sufficient. Their smaller size can, in certain situations, improve their diffusion or penetration properties [14, 15].

3
Investigating Marketed Monoclonal Antibodies

3.1
Method of Investigation

This investigation focused on reviewing products approved between 1980 and 1999. By 1999, the FDA (Food and Drug Administration) or the European Commission (on the advice of the European Medicines Evaluation Agency, EMEA) had together approved eight therapeutic and ten diagnostic monoclonal antibodies. Attempts were made to elucidate the manufacturing processes for these marketed monoclonal antibodies and, wherever possible, to follow the changes made to the process during manufacture for the clinical trial phases. Manufacturing details were found for all eight of the approved therapeutic monoclonal antibodies (OKT3™, ReoPro™, Rituxan™/MabThera™, Zenapax™, Simulect™, Synagis™, Remicade™, Herceptin™) and six of the diagnostic ones (CEA-Scan™, Tecnemab-K-1™, Myoscint™, ProstaScint™, LeukoScan™, Humaspect™).

The remainder of this section focuses on the manufacturing processes employed by commercial manufacturers of monoclonal antibodies and highlights the main differences discovered. The data were mainly derived by reviewing product approval information by the FDA and EMEA unless otherwise indicated. The key reports used were FDA "Summary Basis for Approval" (SBA) reports, FDA "Product" reviews, FDA "Chemistry, Manufacturing and Controls" reviews or EMEA "European Public Assessment Reports" (EPAR) for each product. Most of these sources were found on the FDA and EMEA websites (www.fda.gov/cber/products.htm and www.emea.eu.int/htms/human/epar/epar.htm (formerly www.eudra.org)).

3.2
Results of Investigation

This section provides a summary of the use, nature and manufacture of the approved antibodies investigated. The companies, indications and approval dates associated with the antibodies investigated are listed in Tables 1 and 2. This information highlighted the fact that antibodies have been approved for the following therapeutic indications: transplant rejection (three); cancer (two); cardiovascular disease (one); respiratory disease (one); and autoimmune disease (one). This indicates that antibodies have become successful in clinical trials for a range of indications. Currently, approximately 200 antibodies are in clinical trials [2]. Examining the antibodies in development (e.g. PhRMA database in [1]) suggests that in future the major therapeutic applications of antibodies will be for cancer and autoimmune diseases, such as arthritis. Most of the diagnostic antibodies approved were for imaging of can-

Table 1 Therapeutic monoclonal antibodies and fragments approved by 1999 and investigated in this study[a]

Product name	Manufacturer	Marketer	Indication and approval date
			Transplant rejection
Orthoclone OKT3 *Muromonab-CD3*	Ortho Biotech	Ortho Biotech (subsidiary of Johnson & Johnson)	Treatment of acute kidney transplant rejection (1986) Treatment of acute heart and liver transplant rejection (1993)
Zenapax *Daclizumab*	Hoffmann-La Roche	Hoffmann-La Roche	Prevention of acute kidney transplant rejection (1997)
Simulect *Basiliximab*	Novartis Pharmaceuticals AG	Novartis Pharmaceuticals Corp.	Prevention of acute kidney transplant rejection (1998)
			Cancer
Rituxan/MabThera *Rituximab recombinant*	IDEC Pharmaceuticals; Genentech	Genentech (USA); Zenyaku Kogyo Co. (Japan); Hoffmann-La Roche	Treatment of non-Hodgkin's lymphoma (1997, USA; 1998, EU)
Herceptin *Trastuzumab*	Genentech	Genentech (USA); Roche (outside USA)	Treatment of metastatic breast cancer (1998, USA; 2000, EU)
			Cardiovascular disease
ReoPro *Abciximab*	Centocor	Centocor (USA); Eli Lilly (outside USA); Fujisawa Pharmaceutical Co., Ltd. (Japan)	Prevention of blood clots with high risk coronary angioplasty (1994) Prevention of blood clots in refractory unstable angina when coronary intervention is planned (1997)

Table 1 (continued)

Product name	Manufacturer	Marketer	Indication and approval date
			Respiratory disease
Synagis *Palivizumab*	MedImmune, Inc. (USA); Boehringer Ingelheim Pharma KG (EU)	MedImmune, Inc. & Abbott Laboratories (USA); Abbott Laboratories Ltd. (outside USA)	Prevention of respiratory syncytial virus (RSV) infection in paediatrics (1998, USA; 1999, EU)
			Autoimmune disease
Remicade *Infliximab*	Centocor	Centocor (USA); Schering Plough (outside USA); Tanabe Seiyaku, Ltd. (Far East)	Treatment of Crohn's disease (1998, USA; 1999, EU) Treatment of rheumatoid arthritis (1999, USA; 2000, EU)

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm
NB: Centocor is a wholly owned subsidiary of Johnson & Johnson

Table 2 Diagnostic monoclonal antibodies and fragments approved by 1999 and investigated in this study[a]

Product name	Manufacturer	Marketer	Indication and approval date
			Imaging of tumours
CEA-Scan *Arcitumomab*	Immunomedics; Pharmacia Inc.	Immunomedics	Detection of recurrent and metastatic colorectal cancer (1996)
Tecnemab-K-1 *Anti-melanoma MAb fragments*	Sorin Biomedica Diagnostics SpA	Sorin Biomedica Diagnostics SpA	Staging and follow-up of metastases from confirmed cases of melanoma (1996)
ProstaScint *Capromab pentetate*	Cytogen	Cytogen	Detection, staging and follow-up of prostate cancer (1996)
Humaspect *Votumumab*	Intracel Corp.	Organon Teknika	Detection of recurrent and/or metastatic carcinoma of the colon or rectum (1998)
			Imaging of cardiovascular diseases
Myoscint *Imciromab pentetate*	Centocor	Centocor (USA); Mallinckrodt (outside USA)	Detection of myocardial infarction (1996)
			Imaging of infectious/inflamed sites
LeukoScan *Sulesomab*	Charles River Laboratories; Immunomedics Inc.; Pharmacia Inc.	Immunomedics	Detection of bone infections (1997)

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm

cerous tumours (four); additional uses included imaging of cardiovascular disease (one) and infections (one).

3.2.1 Product Nature and Cell Types

A summary of the nature of the marketed monoclonal antibodies investigated, as well as characteristics of the culture systems employed, is given in Tables 3 and 4. These are discussed further below. Examination of these tables indicates that to date most therapeutic monoclonal antibodies have been whole and intact (seven out of eight). However, for diagnostic applications the use of antibody fragments conjugated to radioisotopes tends to be more common than the use of whole antibodies (four out of six).

At present all marketed monoclonal antibodies are expressed in mammalian cells. This can be attributed to the fact that other cells, such as *E. coli*, lack the cellular machinery required to secrete antibodies and accomplish necessary post-translational modifications, such as glycosylation. These mod-

Table 3 Antibody nature, cell type and culture system of the therapeutic antibodies investigated in this study[a]

Product name	Product nature		Cell type	Culture system	
	Whole Mab [b] vs fragment	Murine vs human		in vivo	in vitro
Orthoclone OKT3	Whole Mab	Murine	Hybridoma	Yes	–
ReoPro	Fab fragment	Chimeric	Mouse myeloma (SP2/0)	–	Yes
Rituxan/ MabThera	Whole Mab	Chimeric	Chinese hamster ovary (CHO)	–	Yes
Zenapax	Whole Mab	Humanised	Mouse myeloma (GS NS0)	–	Yes
Simulect	Whole Mab	Chimeric	Mouse myeloma	–	Yes
Synagis	Whole Mab	Humanised	Mouse myeloma (NS0)	–	Yes
Remicade	Whole Mab	Chimeric	Mouse myeloma (SP2/0)	–	Yes
Herceptin	Whole Mab	Humanised	Chinese hamster ovary (CHO)	–	Yes

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm
[b] Mab = monoclonal antibody

Table 4 Antibody nature, cell type and culture system of the diagnostic imaging antibodies investigated in this study[a]

Product name	Product nature Whole Mab [b] vs fragment	Murine vs human	Cell type	Culture system in vivo	in vitro
CEA-Scan	Fab fragment conjugated to ^{99m}technetium	Murine	Hybridoma	Yes	–
Tecnemab-K-1	Fab and F(ab)$_2$ fragments conjugated to ^{99m}technetium	Murine	Hybridoma	Yes	–
Myoscint	Fab fragment conjugated to ^{111m}indium	Murine	Hybridoma	–	Yes
ProstaScint	Whole Mab conjugated to ^{111m}indium	Murine	Hybridoma	–	Yes
LeukoScan	Fab fragment conjugated to ^{99m}technetium	Murine	Hybridoma	Yes	–
Humaspect	Whole Mab conjugated to ^{99m}technetium	Human	Human cell line immortalised by Epstein–Barr virus (EBV)	–	Yes

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm
[b] Source: [29]

ifications are believed to be necessary for various antibody effector functions and may also influence antibody half-life in the body; therefore, the regulatory agencies are concerned about a reproducible glycosylation pattern (e.g. [16]). Analysis of the marketed antibodies revealed that three cell types have been adopted (Tables 3 and 4). Murine monoclonal antibodies were produced in hybridoma cells (derived from mouse myeloma cells), whereas genetically engineered antibodies (chimeric or humanised) were produced in either Chinese hamster ovary (CHO) cells or mouse myeloma cells (NS0 or SP2/0 myeloma cell line).

Orthoclone OKT3 (Ortho Biotech 1986) was the first monoclonal antibody approved as a biopharmaceutical (for treatment of organ transplant rejection). It is the only therapeutic antibody produced in hybridoma cells (Table 3). In contrast, most of the diagnostic antibodies are produced in hybridoma cells (five out of six) and are hence murine in nature (Table 4). This

suggests perhaps that the HAMA response from the use of murine antibodies has been more critical in therapeutic antibodies with repeated dosing than diagnostic ones. However, Scheinberg and Chapman [17] pointed out that immunogenicity of murine antibodies could be a significant problem if recurrent diagnostic imaging tests were used.

Following OKT3, subsequent therapeutic antibodies were all genetically engineered and cultured mostly in murine myeloma cells. ReoPro (Centocor 1994) was the first chimeric therapeutic antibody and half of the therapeutic antibodies approved by 1998 were chimeric, produced primarily in mouse myeloma cells rather than CHO cells (three out of four). Rituxan/MabThera (IDEC/Genentech 1997) was the only chimeric antibody expressed in CHO cells. However, Birch et al. [18] indicated that both cell types have been widely studied in industry and lend themselves to large-scale processes since they grow in suspension culture. There is extensive experience in the use of CHO cells for the production of other recombinant proteins for therapeutic use [19]. Although the specific titres attained for each of the approved products were not disclosed, reports of investigations in industry (Lonza Biologics, formerly Celltech and Merck) provide an indication of titres achieved commercially [20–22] during 1985–1995. These are summarised in Table 5. This indicates that higher titres have been achieved in myeloma cells than CHO cells. However, commercial production of recombinant antibodies from CHO cells may suggest that it is now possible to attain higher biomass levels in fermentations, comparable to those obtained with myelomas. A 2002 survey of biomanufacturing directors at biopharmaceutical companies indicated that the median level of expression obtained for monoclonal antibody products in all phases is 600 mg/L [23]. Almost all participants in the survey expected expression levels to increase; more recently titres of 5 g/L have been reported in the literature.

Three of the eight therapeutic antibodies were humanised, two of which were expressed in NS0 myeloma cell lines and one in CHO cells. Zenapax (Hoffmann-La Roche 1997) was the first humanised antibody approved for

Table 5 Typical antibody titres for each cell type during 1985–1995

Cell type	Titre
Hybridoma	150–500 mg/L [a]
Myeloma	500 mg/L, 1–2 g/L [a,b]
CHO	90–550 mg/L [a]
EBV-transformed human	90 mg/L [c]

[a] Source: [20]
[b] Source: [21]
[c] Source: [22]

therapeutic use. It is a second-generation molecule for a similar indication to OKT3, but it offers the advantage of repeated dosing with less immunogenicity. The EMEA report for Zenapax indicated that early clinical trial material was prepared from the mouse myeloma cell line SP2/0. Due to limited production yields, the antibody was re-expressed in the mouse GS-NS0 myeloma cell line and bioequivalence was established. Herceptin (Genentech 1998) was the latest humanised antibody approved of those reviewed here (for breast cancer). In addition to differences in titre, the choice of cell line is also probably influenced by the experience of the staff within a company. For example, an examination of all the Biologic License Applications (BLAs) based on mammalian cell culture submitted by Genentech revealed that they all used CHO cells.

Examination of the diagnostic imaging antibodies in Table 4 revealed that all but one of the six investigated were murine; Humaspect (Intracel Corp./Organon Teknika 1998) was the only human antibody approved for use by 1998. None of the diagnostic antibodies was humanised.

3.2.2
In Vivo Versus In Vitro Techniques

Early monoclonal antibodies were produced by the classical method in vivo where mammalian cells (hybridomas) were injected into mice or rats and the ascites, containing the secreted monoclonal antibodies, collected. The first approved therapeutic monoclonal antibody (OKT3 1986) and three of the approved diagnostic monoclonal antibodies (CEA-Scan 1996; Tecnemab-K-1 1996; LeukoScan 1997) were produced in mouse ascites fluid.

The ascites technique is adequate for small quantities of antibodies but becomes less appropriate with an increase in scale. Although the high concentration of monoclonal antibodies in ascites fluid is high at 10–15 g/L [18, 24], the volume is very small so that the actual yield from a mouse is small (50–75 mg). Hence, scale-up requires enormous numbers of animals and is costly. A further drawback is that the product is contaminated with significant levels of various mouse proteins, thus complicating the downstream processing [24].

The alternative to ascites is cell culture in vitro. In vitro cell culture reduces the chance of introducing adventitious agents and irrelevant antibodies from the animal host. For therapeutic antibodies, in particular, most manufacturers have now adopted this technique (Table 3).

3.2.3
Comparison of Commercial Antibody Production from Ascites

A comparison of the manufacturing processes adopted to recover and purify the antibody from the ascites is illustrated in Table 6. Examining the clarifi-

Table 6 Comparison of commercial production of monoclonal antibodies from ascites[a]

OKT3 (1986) Ortho Biotech	CEA-Scan (1996), LeukoScan (1997), Tecnemab-K-1 (1996) Immunomedics & Sorin Biomedica SpA
Ascites production in mice	Ascites production in mice
Harvesting ascitic fluid	Harvesting ascitic fluid
Centrifugation	Centrifugation
Stored frozen	Stored frozen
NH_4SO_4 fractionation	Ion-exchange chromatography[b]
Anion-exchange chromatography	Ion-exchange chromatography[b]
	Affinity chromatography[b]
	Pepsin digestion
	Two chromatography steps
	Reduction of $F(ab)_2$
Formulation	Formulation
Sterile filtration (0.2 μM)	Sterile filtration (0.2 μM)

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm
[b] Order of these three chromatography steps not disclosed

cation stages indicates that centrifugation rather than filtration was used in all cases. This can be attributed to the fact that ascites fluid has a high content of solids and tends to block membrane filters [18]. Examination of these processes revealed that the main differences lie in the purification methods employed. OKT3 was approved 10 years before the other three diagnostic antibodies and is manufactured using the traditional method of antibody purification, where chromatography was typically used after an initial ammonium sulphate fractionation step. However, performing fractionation on a large scale in a contained and sanitary manner is difficult [18]. The diagnostic antibodies derived from ascites relied solely on chromatography for purification, suggesting that the fractionation step is no longer considered necessary as an intermediate purification step and can be eliminated. The EPAR reports (EMEA 1996, 1997) indicate that a combination of two ion-exchange steps and one affinity chromatography step is used. At first glance the manufacturing process for the diagnostic drugs also appears to have more manufacturing stages. However, this is only due to the additional requirement to digest the antibodies to form the desired fragments.

Although drugs such as OKT3 have been on the market for over 10 years, and despite the regulatory agencies' preferences for in vitro cell culture, these marketed antibodies continue to be manufactured using ascites technology. Castillo [25] commented on this apparent conservatism in the biopharmaceutical industry against introducing process changes so as to save time and avoid the need for extensive and costly re-testing in toxicology and efficacy.

In the next two sections, details of the in vitro cell culture production processes for the therapeutic and diagnostic antibodies are presented and discussed.

3.2.4 Comparison of In Vitro Cell Culture Methods

Regarding the marketed antibodies produced by in vitro cell culture methods, the analysis illustrated that different companies have opted for various suspension culture systems. These are summarised in Table 7. Suspension culture can refer to either batch/fed-batch processes or continuous perfusion systems [26]. Therapeutic antibodies use both methods. However, manufacturers of diagnostic antibodies evidently prefer perfusion systems.

Traditionally perfusion systems, such as hollow-fibre culture, have been used for small-scale applications and batch/fed-batch culture for larger-scale applications [18]; this suggests that in some cases the choice of methods has depended on the scale required. Diagnostics have tended to be required in smaller quantities, thus supporting this. However, the therapeutic drugs, ReoPro (cardiovascular disease) and Remicade (autoimmune diseases), must be produced in tens to potentially hundreds of kilograms a year (shown later in Table 13); they are produced using continuous perfusion. Given this, it appears that certain perfusion systems can be considered as a viable option for large-scale applications. These include the 500-L spin-filter culture utilised by Centocor for its marketed antibody products; the design and operation of a 500-L spin-filter reactor are described by Deo et al. [27]. Bibila and Robinson [21] indicated that fed-batch cultures were often simpler to operate and offered higher titres. On the other hand, perfusion systems have higher productivities and continually expose the cells to fresh nutrients while removing growth-inhibitory by-products. However, they suffer from higher contamination risks due to the longer cycle times. The choice between batch and continuous methods may also be dictated by the ability of the cell line to grow under either of these cultures, as well as the product quality achieved.

Further comparisons of cell culture modes revealed that a variety of reactors were used for the commercial production of monoclonal antibodies. For the batch/fed-batch culture systems, stirred tank reactors were the most common. However, examining the history of particular drugs revealed that certain antibodies were cultured in different reactors during clinical trials than at product launch. For example, during drug development, Rituxan/MabThera was produced at different facilities with slight modifications of the manufacturing process. According to the EPAR report for MabThera (EMEA 1998), Rituxan/MabThera is commercially produced by “suspension culture” but clinical trials assessed “suspension culture produced antibody, hollow fibre

produced antibody and stirred tank produced antibody". Since suspension culture can refer to either batch or continuous culture in any reactor, it is not clear which type of bioreactor system is referred to as suspension culture in this case. Amongst the four drugs produced using batch/fed-batch culture, only one stated explicitly that fed-batch fermentations were employed. Fed-batch mode is typically used so as to attain higher cell densities. For example, Bibila and Robinson [21] reported on feeding strategies that achieved up to a 12-fold increase in titre over a batch process resulting in a titre of 1.8 g/L.

The bioreactor sizes are also indicated in Table 7. This reveals that production scales of 400 L up to 12 000 L are being employed. This reinforces the fact that some antibodies are now required in larger quantities. That also became evident when examining cumulative doses per patient and the potential num-

Table 7 Comparison of in vitro suspension culture systems[a]

Product	Suspension culture system	Bioreactor train scale[d]
Therapeutic		
ReoPro	Continuous perfusion—spin-filter[b]	10–500 L[b]
Rituxan or MabThera	Batch/fed-batch	Not disclosed
Zenapax	Batch/fed-batch—stirred tank	30, 80, 400, 2000 L
Simulect	Continuous perfusion—membrane	Not disclosed
Synagis	Fed-batch—stirred tank	400–10 000 L
Remicade	Continuous perfusion—spin-filter	10–500 L[b]
Herceptin	Batch/fed-batch—stirred tank	80–12 000 L
Diagnostic		
Myoscint	Continuous perfusion (assumed)[c]—spin-filter	10–500 L[b]
ProstaScint	Continuous perfusion—hollow-fibre	AcuSyst-XCell, six hollow-fibre cartridges
Humaspect	Continuous perfusion—hollow-fibre	Not disclosed

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm
[b] Source: [27]
[c] Assumption: Centocor uses the same cell culture technology for all its antibodies
[d] It is possible that multiple fermenters at the given scale may be employed during manufacturing

ber of patients to be treated (shown later in Table 13). Schenerman et al. [28] provided an indication of the scale-up and facility changes possible during drug development. During the course of clinical manufacture of Synagis, data from lots produced in 20-, 45-, 100-, 200- and 500-L bioreactors were used for product registration; Synagis was then commercially manufactured at a second facility using 400-, 2000- and 10 000-L bioreactors.

Concerning the perfusion systems used for commercial production of monoclonal antibodies, the drugs that utilised hollow-fibre perfusion systems (ProstaScint, Cytogen; Humaspect, Intracel Corp.) were both diagnostics. Cytogen utilised the AcuSyst-XCell hollow-fibre perfusion cell culturing systems [29]. The bioreactor was filled with six hollow-fibre cartridges. However, this mode of cell culture was not employed in earlier clinical trials since the SBA report (FDA 1996) indicated that Cytogen had three processes during drug development. Although details of the first two processes were not disclosed, differences in the production medium and bioreactor technology were indicated and equivalence tests performed. The remaining drugs produced by perfusion include Centocor's three drugs (ReoPro, Remicade, Myoscint) and Simulect (Novartis). For two of the Centocor drugs (ReoPro, Remicade) continuous perfusion was specified; however, for Myoscint the mode of cell culture was not indicated. It was therefore assumed that the manufacturers used the same technology for Myoscint. Erdmann [30] reported that Centocor had developed a generic process for the manufacture of its antibodies using a continuous perfusion system. However, it is not clear if this was for future candidates or was established earlier. The cell culture train includes 10- and 500-L spin-filter perfusion reactors [27].

3.2.5
Comparison of Downstream Processing Sequences

In contrast to the variation in bioreactor technology employed by these different antibody manufacturers, the range of downstream process sequences was more restricted. Details on the manufacturing processes employed by commercial producers of monoclonal antibodies are shown in Tables 8, 9, 11 and 12.

3.2.5.1
Downstream Processing of Therapeutic Whole Antibodies

Tables 8 and 9 show the information collated for the bulk manufacture of therapeutic *whole* antibodies using batch/fed-batch culture (four) and perfusion culture (two). The initial inoculum grow-up typically occurred in spinner flasks before passing onto a train of seed and production fermenters. Roller bottles were also mentioned once.

Table 8 Comparison of in vitro commercial bulk manufacture of therapeutic *whole* monoclonal antibodies using batch/fed-batch culture [a]

Product Manufacturer	Rituxan/MabThera IDEC	Zenapax Hoffmann-La Roche	Synagis MedImmune, Inc.; BI	Herceptin Genentech
Inoculum grow-up	• Spinner flasks	• Spinner flasks	• T-flask and spinner flasks	
Cell culture [b]	• Batch/fed-batch culture	• Batch/fed-batch stirred tank culture	• Fed-batch stirred tank culture *18–22 days*	• Batch/fed-batch stirred tank culture
Recovery	• Depth filtration	• Concentration	• Microfiltration *Millipore plate and frame system, tangential flow*	• Harvesting *Filtration (assumed)*
	• Hold (at 2–8 °C if > 24 hours)		• pH and conductivity adjustment	
Purification (ROUND 1)	• Affinity chromatography *Protein A*	• Anion-exchange chromatography *Q-Sepharose* *Flow-through collected*	• Cation-exchange chromatography *Antibody binds* *Reduces volume, BSA, DNA*	• Affinity chromatography *Protein A* *Removes unwanted protein, potential endotoxin*
			• Enzymatic treatment *Benzonase to remove DNA*	• Virus inactivation *Incubation at low pH (< 3.7)*
Purification (ROUND 2)	• Anion-exchange chromatography *Removes impurities, e.g. Protein A, host cell DNA*	• Cation-exchange chromatography *S-Sepharose*	• Affinity chromatography *Removes benzonase, cell-derived contaminants*	• Cation-exchange chromatography *Removes aggregates, fragments, CHO impurities*
		• Virus inactivation *pH 3.6–3.8, 30–35 min*	• Virus removal filtration *Nanofiltration*	
		• Concentration/diafiltration	• Virus inactivation, pH incubation	
		• Virus removal filtration *Nanofiltration, Pall DV50*		

Table 8 (continued)

Product Manufacturer	Rituxan/MabThera IDEC	Zenapax Hoffmann-La Roche	Synagis MedImmune, Inc.; BI	Herceptin Genentech
Purification (ROUND 3)	• Anion-exchange chromatography *Removes host cell impurity proteins, endogenous & putative adventitious viruses*	• Anion-exchange chromatography *Q-Sepharose-II* *Flow-through collected*	• Anion-exchange chromatography *Flow-through collected* *Reduces BSA, transferrin, endotoxin*	• Anion-exchange chromatography *Removes DNA, endotoxin and retrovirus*
	• Virus inactivation *(order not disclosed)*	• Virus removal filtration *Millipore Viresolve*	• Diafiltration *Buffer exchange* *(order not disclosed)*	
	• Virus removal *(order not disclosed)* Dilution & filtration	• Ultrafiltration		
Purification (ROUND 4)		• Gel filtration chromatography *S-300*	• Hydrophobic interaction chromatography *Phenyl* • Diafiltration/concentration	• Hydrophobic interaction chromatography *Removes aggregates, fragments, CHO proteins*
		• Ultrafiltration *Concentration*		

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm

[b] The type of bioreactor for Zenapax and Herceptin was not specified in the regulatory reviews but the use of stirred tank reactors was confirmed by industrialists

Table 9 Comparison of in vitro commercial bulk manufacture of therapeutic *whole* monoclonal antibodies using perfusion culture[a]

Product Manufacturer	Simulect Novartis	Remicade Centocor
Inoculum grow-up	• Roller bottles	
Cell culture	• Continuous membrane perfusion culture *First harvest discarded*	• Continuous spin-filter perfusion culture *35–60 days*
Recovery		• Filtration *Clarification, dead-end filtration*
Purification (ROUND 1)	• Dilution	• Affinity chromatography *Direct product capture on Protein A, bovine IgG removal*
	• Ion-exchange chromatography *Antibody binds to column*	• Freeze, thaw, pool
	• Virus inactivation *pH treatment followed by pH adjustment*	• Virus inactivation *Solvent/detergent*
	• Filtration	
	• Pooling of purified harvest lots	
Purification (ROUND 2)	• Chromatography *Protein A (assumed as problem of leaching mentioned)*	• Cation-exchange chromatography
	• pH Adjustment	• Virus removal *Millipore Viresolve system*
Purification (ROUND 3)	• Ion-exchange chromatography *Flow-through collected, DNA removal*	• Anion-exchange chromatography *Flow-through collected*
	• Dilution & in-process filtration & storage	
Purification (ROUND 4)	• Ion-exchange chromatography *DNA removal, antibody binds*	• Anion-exchange chromatography *In-line dilution, bovine IgG removal*
	• Concentration/diafiltration & storage	• Ultrafiltration *Final 0.2 μm filtration*

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm

The culture broth is first subjected to primary recovery operations. These serve to remove particulate matter, such as cells, that would otherwise foul the subsequent purification stages that rely on column chromatography, and to remove water so as to allow sensible volumes to be handled by chromatographic steps.

Turning to commercial methods employed to clarify batch/fed-batch cultures (Table 8), the FDA and EMEA reports only mentioned a filtration stage explicitly. However, clarification is often accomplished using centrifugation, and concentration can also be achieved using techniques such as precipitation and aqueous two-phase partitioning [31]. It is not clear whether filtration was preferred over centrifugation for clarification for scientific reasons, or whether it was based on the fact that staff designing the processes were more familiar with filtration. As mentioned earlier, centrifugation was the clarification method of choice for crude antibody preparations from ascites owing to its high solids content. In contrast, crude antibody preparations derived from mammalian cell culture are generally easier to clarify owing to a much lower cell density and lower solid, lipid, DNA content [18]. Hence clarification can be achieved using either centrifugation or filtration [18]. There have been safety concerns in the past with centrifugation of large volumes because of aerosol contamination risks, although these are diminishing with new designs. In addition, Sofer and Hagel [31] indicated that complete cell removal is not always achieved with centrifugation, and thus a subsequent filtration step is required prior to chromatography to avoid clogging of columns. Regarding concentration by precipitation, the disadvantages of this technique on a large scale have been alluded to earlier. Microfiltration and ultrafiltration are well-proven techniques for concentration but their performance can be limited by concentration polarisation and fouling. In two cases the filtration systems were specified, indicating that either depth filtration or tangential flow microfiltration were suitable to achieve primary recovery of the product from culture broths.

Regarding primary recovery operations for perfusion cultures, one (Remicade) of the two cases in Table 9 indicated that dead-end filtration was used for clarification. For the other case (Simulect), it is not clear whether the cell retention in membrane-perfusion cultures obviated the need for a further filtration step or whether the step was just not mentioned in the FDA and EMEA approval reviews.

The antibody in clarified fermentation broths is only a minor component of a complex mixture of molecules that need to be removed, such as lipids, DNA, proteases, pyrogens, viruses and modified forms of the desired antibody [18]. The purification procedures for antibodies derived from batch/fed-batch or perfusion cultures were all similar. Purification was achieved using a series of chromatographic separations, viral inactivation and removal steps, as well as intermediate ultrafiltration steps.

Birch et al. [18] highlighted the fact that purification strategies usually aim to achieve major purification in the first chromatography step, so that later steps can be optimised for the removal of small quantities of a few specific impurities. For the therapeutic whole antibodies reported here, four chromatography steps were typically used. Half of the manufacturers of these antibodies utilised affinity chromatography as the first chromatographic step, while the other half employed ion-exchange chromatography. The use of affinity chromatography as a first step can be attributed to its very high purity levels, often greater than 95% [18]; however, it suffers from expensive matrices and ligand leakage. In addition, Ultee and Rea [32] pointed out that another problem was that many antibodies required a fairly low pH to elute from affinity matrices. This problem renders this technique unsuitable in cases where antibodies cannot withstand exposure to these conditions. Ion-exchange chromatography was also popular as a first purification step. Possible reasons for this are that the matrices are typically an order of magnitude cheaper than affinity matrices, have a high capacity and can be selected to bind either the antibody or impurities. However, lower purity levels may be achieved and conditioning steps using pH adjustment/dilution/buffer exchange were often required prior to ion-exchange separations (Tables 8 and 9). For ion-exchange steps, success depends on whether there is a sufficient difference in isoelectric points between the target and the impurities [18, 32].

Further examination of Tables 8 and 9 revealed that the three therapeutic purification processes that began with affinity chromatography were followed by two or three ion-exchange steps. For the ion-exchange-based processes, one was followed by two more ion-exchange steps and the other two were followed by an affinity step and one or two ion-exchange steps. Indications of the matrices employed were sometimes disclosed: for affinity separations Protein A was commonly used, and for ion-exchange steps S-Sepharose was mentioned once as the cation-exchange matrix and Q-Sepharose as the anion-exchange matrix. Cation-exchange steps were typically used to bind the antibody to the column, whereas anion exchange was often used to bind impurities as the flow-through was collected. In some cases the functions of these steps were also mentioned. These included the use of cation-exchange steps for removal of antibody aggregates, fragments and host cell proteins, and anion-exchange steps for removal of DNA, endotoxin, retrovirus and leached Protein A where applicable. In addition, Centocor is reported to use 1-m-wide chromatography columns and high-capacity ion-exchange matrices that increased product loading from 20 to 100 g/L [30, 33]. This resulted in a significant increase in purification capacity to 8–16 kg and enabled each perfusion culture to be purified in one lot.

In three cases shown in Table 8 (Zenapax, Synagis and Herceptin) the fourth chromatographic step was either gel filtration chromatography or hydrophobic interaction chromatography. Hanna et al. [34] and Birch et al. [18]

commented that gel filtration chromatography (or size exclusion chromatography) was suitable as a final step to remove antibody aggregates and act as a buffer-exchange step into the formulation buffer. One of the EMEA reports indicated that the final hydrophobic interaction chromatography step served to remove antibody aggregates, fragments and host cell proteins. Ultee and Rea [32] commented that hydrophobic interaction chromatography could be an ideal complement to ion-exchange chromatography and perhaps deserved more attention in industry. The remaining processes in Tables 8 and 9 used ion-exchange chromatography as their final chromatographic step followed by a diafiltration/concentration step. The lack of gel filtration chromatography as a final step in all but one case reflects a trend in the biopharmaceutical industry to replace the final polishing chromatography step with an ultrafiltration step that concentrates and diafilters the batch. Centocor [33] are reported to have modified an existing process that included gel filtration followed by diafiltration with a single ultrafiltration step. This resembles the process for their product Remicade that was deduced (Table 9) from the product approval information. Kurnik et al. [35] compared the use of size exclusion chromatography and tangential flow filtration (TFF) for buffer exchange. They concluded that TFF offered a greater range of buffer exchange and improved the process economics; however, they commented that size exclusion chromatography would be favoured in situations where protein denaturation occurs in TFF.

Regarding steps between each chromatographic separation, some of the licence approval information supplied by the regulatory agencies disclosed when intermediate operations were required for pH adjustment, buffer exchange or to reduce the volume loaded onto a column. However, the method to achieve these functions was not always indicated. In some cases ultrafiltration and diafiltration were specified (e.g. Zenapax, Synagis in Table 8; Simulect in Table 9). The process for Remicade (Table 9) did not require such intermediate filtration steps. An article based on material from the manufacturers of Remicade (Centocor) [33] reported that they introduced new technologies into an existing process that increased yield by 25% and decreased process lead times by 50%. One of the actions that facilitated this was the elimination of diafiltration steps prior to ion-exchange steps and their replacement with in-line dilution using ion-exchange chromatography. Ultee and Rea [32] also indicated that it was possible to develop conditions to allow the product stream from an ion-exchange column to flow directly onto a hydrophobic interaction column without intermediate handling; ion-exchange chromatography uses a low-ionic-strength load and a high-ionic-strength elution and hydrophobic interaction uses the reverse.

Turning to virus clearance, all the processes for the therapeutic antibodies in Tables 8 and 9 had at least one specific step dedicated to this. Half had two specific viral clearance steps: virus inactivation and removal. Techniques used for virus inactivation included treatment with either pH or solvent/detergent.

Table 10 Examples of purification steps used for viral safety[a]

Zenapax Hoffmann-La Roche	Remicade Centocor	Herceptin Genentech
Cation-exchange chromatography	Affinity chromatography	Affinity chromatography
Virus inactivation	Virus inactivation	Virus inactivation
Virus removal filtration	Virus removal filtration	Cation-exchange chromatography
Anion-exchange chromatography	Anion-exchange chromatography	Anion-exchange chromatography
Virus removal filtration		Hydrophobic interaction chromatography

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm

Nanofiltration systems were typically used for virus removal. The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production [36]. The ICH[1] guidelines on viral safety have been adopted by regulatory agencies such as the FDA, and suggest the use of several different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance. A drug product is generally considered safe when there is less than one potential contamination in every million doses [36]. Total process clearance is determined by adding up the log reduction factors in virus titres of the individual steps identified to clear viruses. The licence approval documents for the antibodies investigated indicated other steps in the purification process that served to ensure viral safety. These are summarised in Table 10. This highlighted that typically four or five steps contributed to viral clearance, where existing chromatographic steps in the purification process were used, in addition to specific viral clearance steps, to demonstrate a reduction in the virus particle titre.

In addition to the above steps found in most of the therapeutic processes examined, one company also used enzymatic treatment to remove nucleic acids using Benzonase. Treatment with such enzymes typically reduces the high viscosity caused by nucleic acids such as DNA, but adds an extra con-

[1] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

taminant to be removed by subsequent steps. In all other cases, DNA removal was achieved primarily by anion-exchange chromatography.

3.2.5.2 Downstream Processing of Diagnostic Imaging Whole Antibodies

Table 11 summarises the process details found for the bulk manufacture of two diagnostic whole antibodies. The primary recovery operations were very similar to those described above for therapeutic antibodies. Turning to the

Table 11 Comparison of in vitro commercial bulk manufacture of diagnostic *whole* monoclonal antibodies[a]

Product Manufacturer	ProstaScint Cytogen	Humaspect Intracel Corp.
Cell culture	• Perfusion hollow-fibre culture *60 days*	• Perfusion hollow-fibre culture *10 weeks*
Recovery	• Filtration *Clarification*	
Hold/pool	• Storage *at 2–8 °C*	• Pooling and freeze at – 70 °C *Each batch eight harvests* *(weeks 1 & 2 discarded)*
	• Pooling of harvests	
Purification (ROUND 1)	• Filtration *Clarification and concentration of pooled harvests* • Gel filtration chromatography *Sephadex G-25* *(assumed for buffer exchange)* • Affinity chromatography *Protein A*	• Virus inactivation 1% *Triton-X-100, 1 h,* *ambient temperature* • Affinity chromatography *Protein G, more than one cycle*
Purification (ROUND 2)	• Anion-exchange chromatography *DEAE-Sepharose*	• Anion-exchange chromatography *Q-Sepharose*
Purification (ROUND 3)	• Cation-exchange chromatography *S-Sepharose*	• Anion-exchange chromatography *Q-Sepharose* *Residual DNA binds to column*
	• Dead-end filtration 0.22-μm *filter*	• Virus removal filtration *(order not disclosed)* *Viresolve 180,* *remove HSV-1 and poliovirus*
		• pH and conductivity adjustment

[a] Sources: FDA approval information on http://www.fda.gov/cber/products.htm, EMEA reports (EPAR) on http://www.emea.eu.int/htms/human/epar/epar.htm

purification steps, it can be seen that only three chromatographic separations were employed for purification, whereas four such steps were typically used for therapeutic applications. This suggests perhaps that diagnostics have less stringent purity requirements. For example, Sofer and Hagel [31] suggested that for a diagnostic it could be acceptable to have a purity level of 95%, whereas for therapeutics purity levels generally exceed 99%. A slightly unusual step is the use of gel filtration as the first step in purification at Cytogen for ProstaScint when it is usually recommended as a final polishing step. It was assumed that the main function of this gel filtration chromatography was not for purification but for buffer exchange. The matrix employed (Sephadex G-25) supported this assumption. Regarding the matrices employed for diagnostic antibodies, the use of Protein G for affinity separations and DEAE-Sepharose for anion-exchange chromatography was also mentioned in addition to those disclosed for therapeutics.

The regulatory reports for these diagnostics (ProstaScint and Humaspect) did not disclose information on whether intermediate ultrafiltration steps were required between the chromatography steps for buffer exchange and/or concentration. It can therefore either be assumed that the chromatographic steps were carefully designed so that the antibody was washed or eluted in a buffer suitable for the next chromatographic step or that the conditioning steps were used.

With regard to viral clearance, one of the two diagnostics in Table 11 mentioned specific viral clearance steps of a similar nature to those employed for therapeutic antibodies. However, none was mentioned for ProstaScint and it is possible that existing purification steps were also used to ensure viral reduction.

3.2.5.3 Downstream Processing of Antibody Fragments

Although the use of *E. coli* fermentation was mentioned earlier as a suitable method for the production of antibody fragments, the two marketed fragments were both made by mammalian cell culture at Centocor. As indicated in Table 12, the processes are very similar to those for whole antibodies but include papain digestion steps to create the antibody fragments. Once again it can be seen that the process for the diagnostic antibody (Myoscint) is shorter than that for the therapeutic antibody (ReoPro).

4 Generic Antibody Production Processes

From the analysis of the operations commonly used in monoclonal antibody manufacture, potential generic processes can be constructed using either an

Table 12 Comparison of in vitro commercial bulk manufacture of monoclonal antibody *fragments*

Product Manufacturer	ReoPro [a] Centocor	Myoscint [b] Centocor
Inoculum grow-up		
Cell culture	• Continuous spin-filter Perfusion culture	• Fermentation *At least five fermenters; assume continuous as same manufacturer as ReoPro*
Recovery	• Filtration *Dead-end*	• Filtration *Dead-end (assumed like ReoPro)*
Purification (ROUND 1)	• Affinity chromatography *Protein A*	• Affinity chromatography *Protein A*
	• Papain digestion	• Papain digestion
	• Affinity chromatography *Protein A*	• Affinity chromatography *Protein A*
	• Virus inactivation *Solvent/detergent*	
Purification (ROUND 2)	• Ion-exchange chromatography	• Anion-exchange chromatography • DTPA-coupling reactions
Purification (ROUND 3)	• Ion-exchange chromatography	• Gel filtration chromatography
Purification (ROUND 4)	• Ion-exchange chromatography	
Purification (ROUND 5)	• Gel filtration chromatography • Virus removal filtration	

[a] Assumptions: Few details were provided in FDA reviews for ReoPro; the process details were deduced assuming parts to be similar to Myoscint and Remicade as they are all produced by the same manufacturer (Centocor)
[b] Source: FDA approval information on http://www.fda.gov/cber/products.htm

affinity-based process or an ion-exchange-based process with specific viral clearance intermediate filtration steps. These are indicated in Fig. 1.

Generic processes have the advantage that they greatly reduce the development time and streamline regulatory aspects of processing [37]. Several companies claim to be adopting a generic purification strategy such as Amgen (Thousand Oaks, CA) and Cambridge Antibody Technology (CAT; Cambridge, UK) [37]. Amgen highlights the need to keep robustness in mind when designing a generic purification process. Robustness studies are key to demonstrating that the process performs adequately within its control limits and consistently provides material of defined purity, quality and yield.

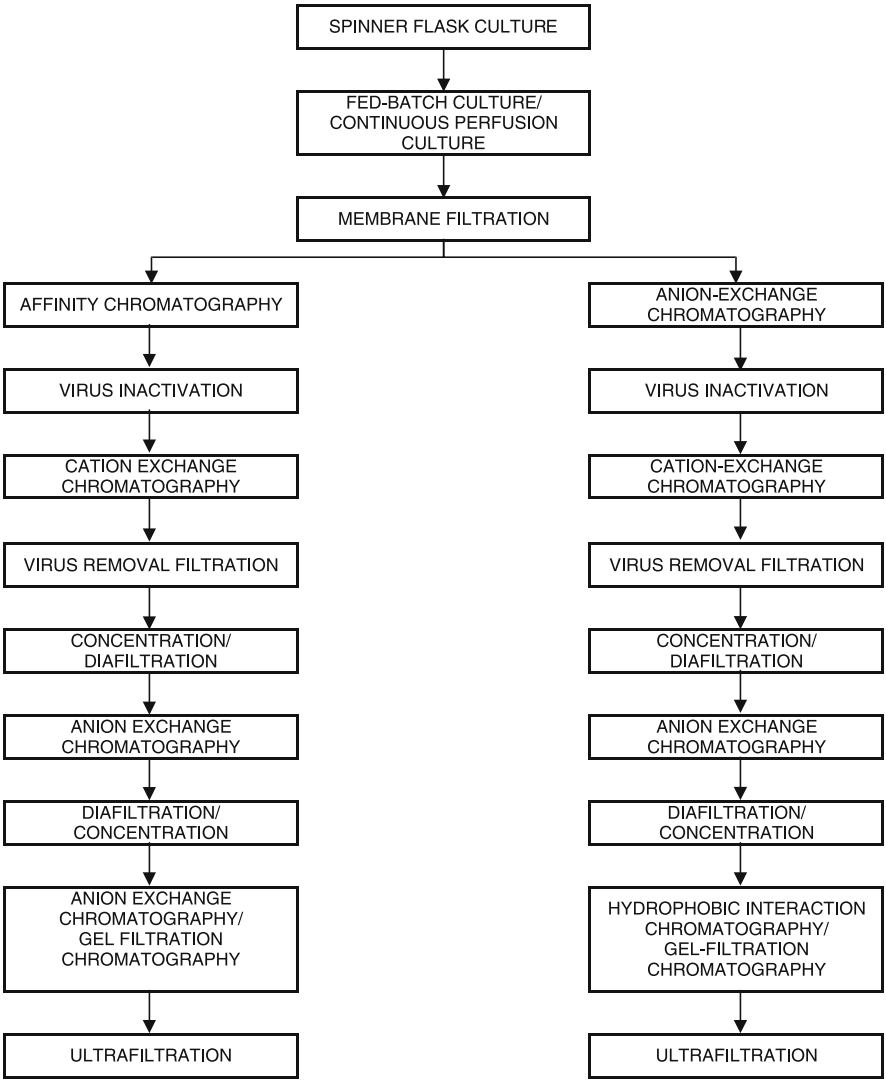

Fig. 1 Generic antibody production processes based on mammalian cell culture. An affinity-based purification and an ion-exchange-based process are defined

However, CAT cautioned that since all antibodies are slightly different the generic processes just represent a starting point that requires modification for effective scale-up [37]. Industrial-scale antibody production strategies must balance the needs of robustness, purity, yield and economics.

Table 13 Typical cumulative dose, price and market potential of therapeutic antibodies investigated in this study

Product name	Typical cumulative dose/patient (mg) [a]	Typical price/patient/ treatment ($) [b]	Typical unit selling price ($/g)	Potential annual US market (# patients) [d]	Potential annual US demand (kg)
Orthoclone OKT3	50–70	3000–4200 [c]	60 000	7300 [e]	0.3–0.5
ReoPro	30	1690	56 300	309 600 [f]	9
Rituxan/ MabThera	2800–3000	14 000–15 000	5200	68 600 [g]	192–205
Zenapax	350	6120	17 500	11 800 [h]	4
Simulect	40	3160	79 000	15 400 [e]	1
Synagis	600–900	7150–8580	10 500	53 300 [i]	32–48
Remicade	350–1050	2530–7590	7200	54 000 [j]	20–57
Herceptin	5740	33 350	5800	12 800 [g]	73

Sources:
[a] Doses from product labels available on www.fda.gov and www.emea.eu.int
[b] Prices for the drugs (except Orthoclone) were obtained from the International Pharmaceutical Services website (www.internationalpharmacy.com)
[c] [83]
[d] Market size derived from annual/quarterly sales reported by the marketing company, where available, or other sources in 2000/2001 (except Zenapax—1998)
[e] Organ Transplant Association website (http://organtx.org/imm/sim.htm)
[f] Eli Lilly 2000 Annual Report (www.lilly.com/about/investor/00report/english/letter.html)
[g] Genentech 2001 Second Quarter Report (http://www.gene.com/gene/investor_relations/financial_info/sec_forms/10q200106.pdf)
[h] [29]
[i] [45]
[j] Johnson & Johnson quarterly sales reported in 2001 (http://media.corporate-ir.net/media_files/nys/jnj/reports/websales.pdf)

5 Dose and Market Potential as Drivers of Quantities Needed

Reviewing the trends in doses and market potential of therapeutic antibodies is key to assessing what levels of performance new approaches will need to meet to be competitive.

For the therapeutic antibodies, the cumulative dose per patient has been derived in this review from clinical studies and product labels. The cumulative price per patient per course of treatment was also found and these data are summarised in Table 13. This indicated that antibody doses are increasing to gram quantities rather than milligrams, with a concomitant increase

in the price of the treatment. All but one of the antibodies require repeated dosing. The quantity needed is also a reflection of the indications that antibodies are capable of treating; these now include chronic conditions (e.g. Herceptin for breast cancer) as well as indications which potentially affect tens to hundreds of thousands of patients (e.g. Remicade for rheumatoid arthritis). Table 13 also contains estimated demands of each drug based on the number of patients to be treated. Attempts were made to derive figures that reflected the potential market share captured by each drug. This was achieved by determining the number of patients based on sales reported for each drug. The average annual demand for marketed antibodies was estimated to be 46 kg and is expected to increase as each drug manages to penetrate its market further. It has been claimed that the demand for increasingly large quantities of antibodies is beginning to outstrip supply of cGMP production capacity, which could result in antibodies in the development pipeline not reaching the market. However, at present there is considerable controversy regarding the existence of a shortfall in worldwide capacity [37]. The estimates largely depend on the average titre assumed for the currently available cell culture facilities. The disputed capacity crunch, along with increasing pressures to drive down the cost of antibody therapy, has led to several developments to address these issues. These are discussed in the next section.

6
Future Trends in Antibody Manufacture

Having analysed processes for approved antibodies, attention is now turned to some of the technologies being used for antibodies in development in order to assess future trends in antibody manufacture.

6.1
Bioreactor Choice

Regarding cell culture bioreactors, the Wave Bioreactor™, introduced by Wave Biotech in 1998, offers an alternative disposable culture system up to 500 L. Singh [38] indicated that batches ranging from 100 mL to 580 L have been run for monoclonal antibodies with productivity and maximal cell densities comparable to those of stirred tank bioreactors. This reactor offers an alternative to the more expensive stainless-steel bioreactors, with claims of being an order of magnitude cheaper [38], and has the advantages that come with the use of disposable components, such as not requiring cleaning and sterilisation. However, the limitations of its scale may pose problems during late phase clinical trials and launch if scale-up results in a switch in bioreactor technology, as well as the accompanying bioequivalence studies.

For large-scale reactors ($\geq$ 1000 L), an alternative could be to run several bag reactors in parallel; however, scaling out rather than scaling up will also require further tests to determine the effects of pooling fermentation broths. Many firms now use disposable bags for inoculum preparation [39]. The Wave Bioreactor has also been developed to operate in the perfusion mode [40].

6.2 Transgenic Organisms

Although mammalian cell culture has emerged as the chosen method of production for marketed antibodies, alternative systems are being developed for antibodies with projected annual marketed demands of several hundreds of kilograms, if not tons. In particular, the use of transgenic plants and animals as culture systems is attracting attention in process development circles, for applications requiring very large amounts. Candidates for the source of antibodies include the milk of transgenic mammals, the eggs of transgenic chicken, the seeds/leaves/tubers of transgenic plants and the cocoons of silkworms. Increasing interest in these transgenic organisms can be attributed to claims of a competitive cost of goods, a lower capital investment, the flexibility to modulate capacity and the ability to assemble more complex antibodies, when compared to mammalian cell culture.

6.2.1 Transgenic Mammals

The key companies that specialise in transgenic mammals employ transgenic mice, rabbits, goats, sheep and cattle. GTC Biotherapeutics (Framingham, MA; formerly Genzyme Transgenics) is the main firm actively engaged in antibody production in transgenic milk. It has developed transgenic goats to provide large-scale production at a potentially lower cost than mammalian cell culture [41]. In order to mitigate risk, biopharmaceutical companies making antibodies are partnering with transgenic companies to evaluate the potential of transgenics in parallel with clinical trials of their cell-culture-derived products. Their strategy is to make the transition from mammalian cell culture to transgenically derived product at late stage clinical trials or post-approval so as to meet market demand [42, 43].

At present no antibody products have been licensed using transgenics, but GTC Biotherapeutics has worked on developing transgenic versions of antibodies for companies such as Abbott Laboratories (Abbott Park, IL), Bristol-Myers Squibb (New York, NY), Centocor and Elan Pharmaceuticals (Dublin, Ireland). Most of these transgenic antibodies are in pre-clinical trials whilst their cell culture versions are either in Phase II clinical trials or are marketed [42]. For example, Centocor collaborated with GTC Biotherapeu-

tics to jointly explore the production of Remicade for rheumatoid arthritis in transgenic goats [44]; Centocor also evaluated the potential of transgenics for other monoclonal antibodies in its portfolio as a long-term strategy for large volume products [44, 45]. Lonza Biologics, the second largest contract manufacturer of cell culture biologics, also worked with GTC to develop methods for purifying transgenically produced monoclonal antibodies and proteins [45].

Young et al. [41] and Pollock et al. [46] provide a generic process for the production of biopharmaceutical proteins, such as monoclonal antibodies, from the milk of transgenic dairy animals (Fig. 2), drawing on their experience at GTC Biotherapeutics. Immunoglobulin purification from milk does not appear to pose particular problems [47]. The purpose of the initial separation steps is to achieve a fat-free, caesin-free and lactose-free protein concentrate [46]. Although standard dairy procedures of centrifugation and precipitation followed by ultrafiltration can be used to achieve this, the method of choice is to remove these components in a single or a tandem microfiltration and ultrafiltration step [41, 48]. The purification steps involve the

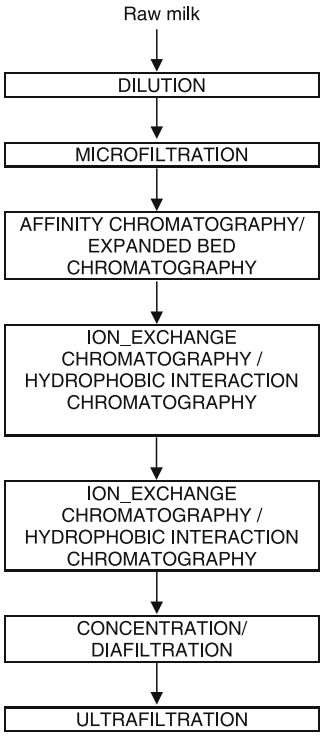

Fig. 2 Generic process for the production of biopharmaceutical proteins, such as antibodies, from transgenic milk (adapted from [41, 46])

separation of endogenous whey proteins from the target antibody and uses a combination of affinity (or pseudo-affinity), ion-exchange, and hydrophobic interaction chromatography [45]. Young et al. [41] and Pollock et al. [46] indicate that it is possible to attain yields of 65% and a final product purity of 99.99%.

Although significant progress has been reported with transgenic dairy animals, a number of hurdles remain to be overcome. Concerns regarding the use of transgenic animals include the lead time for building up herds; goat and sheep breeding has a turnaround of approximately 18 months [46], while for cattle it is 3 years. The risk of product contamination with prions and viruses is another concern that requires careful hygiene standards [44]. Ethical problems associated with gene manipulation in animals and the use of animal materials also exist [49]. Some human therapeutic proteins could be detrimental to animals' health when expressed in their mammary glands [50, 51].

The optimism regarding the use of transgenic technology to drive down the costs of monoclonal antibodies has to be tempered by the fact that a substantial portion of the cost of monoclonal antibodies is associated with downstream processing and fixed facility costs [4]. Experts from biopharmaceutical companies have found the costs of transgenic-derived products and cell-culture-derived products comparable; the savings are only moderate as the culture stage only affects 20–30% of the capital expenditure and 20–30% of the cost of goods at production levels of 50–300 kg/year [52].

6.2.2 Transgenic Chickens

Companies are also developing methods for creating transgenic chickens. For example, TranXenoGen (Shrewsbury, MA) launched agreements to produce monoclonal antibodies in egg whites for three partners in 2001 (KS Biomedix (London, UK), Abbott Bioresearch Center (Abbott Park, IL), Amgen (Thousand Oaks, CA)). Potential advantages of this technique compared to dairy animals include the following: faster and less expensive breeding cycles (21 days to hatch and 5–6 months to mature); a contained and sterile environment for production within eggshells; an established manufacturing infrastructure including clean facilities, pathogen-free birds and fully automated equipment; and experience with regulatory authorities in examining egg-derived products (e.g. flu vaccine) and the production facilities [53]. However, the production of transgenic chicken is still several years behind the transgenic mammalian technology [50]. Furthermore, clarification of the egg white still poses challenges; a number of procedures are being tried including precipitation or shearing to thin the egg white (personal communication with TranXenoGen). Once this has been done, purification protocols similar to those used for cell-culture-derived products can be used.

6.2.3 Transgenic Silkworms

The generation of transgenic silkworms that spin human recombinant proteins as a component of the cocoon has been reported recently [54]. The protein production capacity of silkworms can compete with that of most industrial systems in use; each silkworm can produce up to 300 mg of protein per gland in 4 days. In addition, the glands produce an almost pure product and Wurm [55] indicates that purification should be a rather simple process. Concerns arising from potential contamination by adventitious agents are substantially less. Wurm [55] states that such silkworm systems could become a major technology for the production of high-value proteins. However, product quality may be an issue related to the limited glycosylation performed by insects.

6.2.4 Transgenic Plants

Producing antibodies in transgenic plants may represent a cheaper alternative than transgenic animals [44] that poses fewer health risks from pathogen contamination and is easier to scale up to produce tons rather than kilograms of antibodies per year [56]. Further advantages of transgenic plants include: the capacity to efficiently fold and assemble complex secretory antibodies (sIgA) unlike mammalian cell culture [57], and the ability to target antibodies to seeds or tubers that are stable [58]. The FDA is expecting an increasing proportion of product submissions to be based on transgenic plant expression systems [59].

Worldwide there are more than 20 companies that have developed various plant expression systems. Currently most of the antibodies and antibody fragments expressed in plants, termed "plantibodies", have been in tobacco or corn [59]. The most advanced product is CaroRX™ produced in tobacco by Planet Biotech (MountainView, CA); its drug is a chimeric secretory antibody that has been shown to reduce tooth decay in animal models and pilot phase II clinical trials [57]. Epicyte Pharmaceutical (San Diego, CA) is developing a pipeline of plantibodies to treat inflammatory and infectious diseases through passive immunotherapy applied topically. Its antibody against herpes simplex virus was initially produced in soya bean for pre-clinical trials, but rice expression systems are being used for clinical trials [60]. The remaining antibodies in Epicyte's pipeline are being produced in corn [60] through collaborations with Dow and Dow AgroSciences (Indianapolis, IN) [61]. Meristem Therapeutics (Clermont-Ferrand, France) has used tobacco and corn systems to produce antibodies and has signed an agreement with Goodwin Biotech (Plantation, FL) to act as a contract manufacturer of monoclonal antibodies [62]. To mitigate risks, it will offer to test the feasibility of transgenic

plants for production simultaneously with Phase I/II clinical trials using cell-culture technologies. Monsanto Protein Technologies (St Louis, MO) has also transgenically modified corn to produce monoclonal antibodies for clients.

Although transgenic tobacco has been widely employed for research, full-scale commercial production will probably involve grain crops, e.g. corn, rice and wheat, as well as oilseed crops, e.g. soya bean and oilseed rape [56, 58]. They have a lower content of toxic compounds and there is an existing infrastructure for crop cultivation, harvesting, distribution and processing [56, 58]. The storage properties of seeds provide added flexibility in processing management and batch production. Many companies developing transgenic plant expression systems have chosen corn after surveying various crops for potential recovery and the economics of production [58]. When extraction and purification are necessary, the current procedures of crushing and milling may be adapted for the extraction of recombinant products [63]. The final steps will usually consist of standard chromatography procedures [60]. A generic process flow sheet for Meristem's production of recombinant products from transgenic plants [62, 64] is illustrated in Fig. 3.

In addition to the downstream processing, several issues remain to be resolved: environment control, disposal of biomass with residual antibody, post-translational modifications of protein products and the time to establish stocks. However, many of these are being addressed. For example, strategies

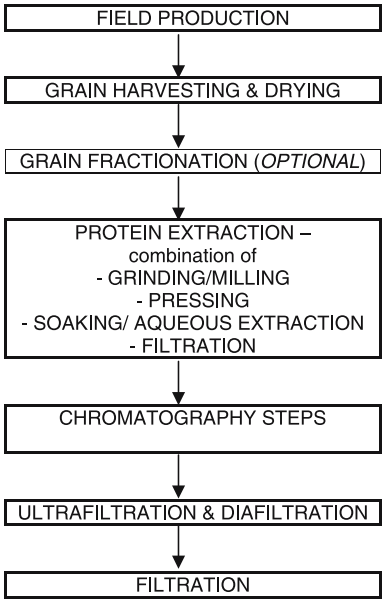

Fig. 3 Generic process flow sheet for Meristem's production of recombinant products from transgenic plants (adapted from [62, 64])

for containment include self-pollinating crops and male sterility (no transgenic pollen) [60]. Where glycosylation is considered critical, it may be possible to develop more suitable transgenic plants from mutants lacking some of the enzymes in the glycosylation pathway [58]. Alternatively, drugs can be chemically modified after isolation. Neose (Horsham, PA) is developing systems that can be used to glycosylate proteins derived from any biomanufacturing process [50]. With regard to timelines, plant production companies are working with pharmaceutical companies to make these mutually compatible [65]. Plants are comparable with animal systems; it takes approximately 20 months from transfection to GMP-quality clinical supply production [14].

6.3 Alternative Expression Systems for Antibody Fragments

Antibody fragments have been of limited use for many therapeutic applications because of their short in vivo half-lives. To enhance in vivo delivery and pharmacokinetics, fragments have been fused to lipids and polyethylene glycol (PEG) [66]. Further methods to enhance their functional affinity include engineering Fab and sFv into conjugates that are dimeric (diabodies), trimeric (triabodies) and tetrameric (tetrabodies) through the use of chemical or genetic cross-links. In addition, bispecific antibodies that contain two different binding specificities fused together have shown promise in early stage clinical trials for cancer [66].

Although licensed antibody fragments have been produced using mammalian cell culture to produce whole antibodies, with proteolysis steps to derive the Fab fragments, other systems are being developed for fragments in development. For example, companies are employing microbial expression systems, such as *E. coli*, and yeast expression systems, such as *Pichia pastoris*.

Bacteria tend to be favoured for expression of small, non-glycosylated Fab and scFv fragments. *E. coli* systems offer the advantages of producing high levels of product at a fast rate, cheap and simple media, and the capability of generating multi-ton yields of product annually. Antibody fragments can be expressed by *E. coli* as soluble proteins found either intracellularly in the cytoplasm or secreted into the periplasm if preceded by a signal sequence. The latter offers benefits over the accumulation of insoluble inclusion bodies in the cytoplasm, which require efficient re-folding and renaturation processes [14]; such steps often lead to higher downstream costs than mammalian systems. Erdmann [30] reported that Genentech preferred using *E. coli* to generate antibody fragments. A schematic of the process details provided by Erdmann [30] on Genentech's approach is given in Fig. 4.

E. coli has also been used to generate antibody fragments that can be "PEGylated" to enhance in vivo half-lives. Details of the process used for the production of PEGylated Fab fragments for research purposes by Celltech are given in the literature [67, 68]. Celltech (Slough, UK) has developed

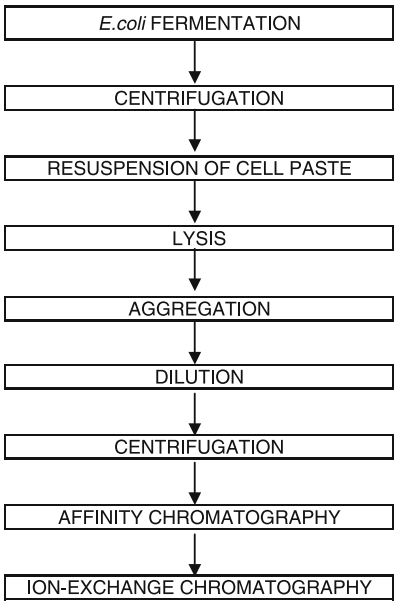

Fig. 4 Production of antibody fragments using an *E. coli* based process (Source: [30])

a large-scale process to produce such fragments under GMP conditions for clinical trials [68]; Celltech has products using this technology in pre-clinical and clinical trials for cancer, rheumatoid arthritis and Crohn's disease. The GMP process employed by Celltech at the 1000–10 000-L scale differs slightly from the process used to produce research reagents; the main difference is that ion-exchange chromatography is favoured over protein G affinity chromatography at the large scale (A. Chapman, Celltech, Slough, UK, personal communication). The GMP process to generate PEGylated antibody fragments is illustrated in Fig. 5. Comparing the process flow sheets in Figs. 4 and 5, it can be seen that periplasmic extraction is preferred over mechanical cell disruption, such as homogenisation, which can be time-consuming and result in the loss of heat-labile proteins. In addition, periplasmic extraction leaves the protoplast intact whereas homogenisation ruptures the whole cell. It is interesting to note that the number of processing steps to produce PEGylated antibodies is similar to that typically used to recover antibodies from mammalian cell culture.

Efforts are also being focused on eukaryotic cell cultures, such as the yeast *P. pastoris*, to allow efficient production of fully processed scFv fragments and Fab fragments [66]. Examples of protocols for *Pichia* production processes exist for 1-L fermentations where the antibody fragments are secreted into the culture medium. Recovery and purification typically involve a combination of centrifugation, ammonium sulphate precipitation, and affinity, ion-exchange and gel filtration chromatography [69, 70]. However, Marty et al. [71] found

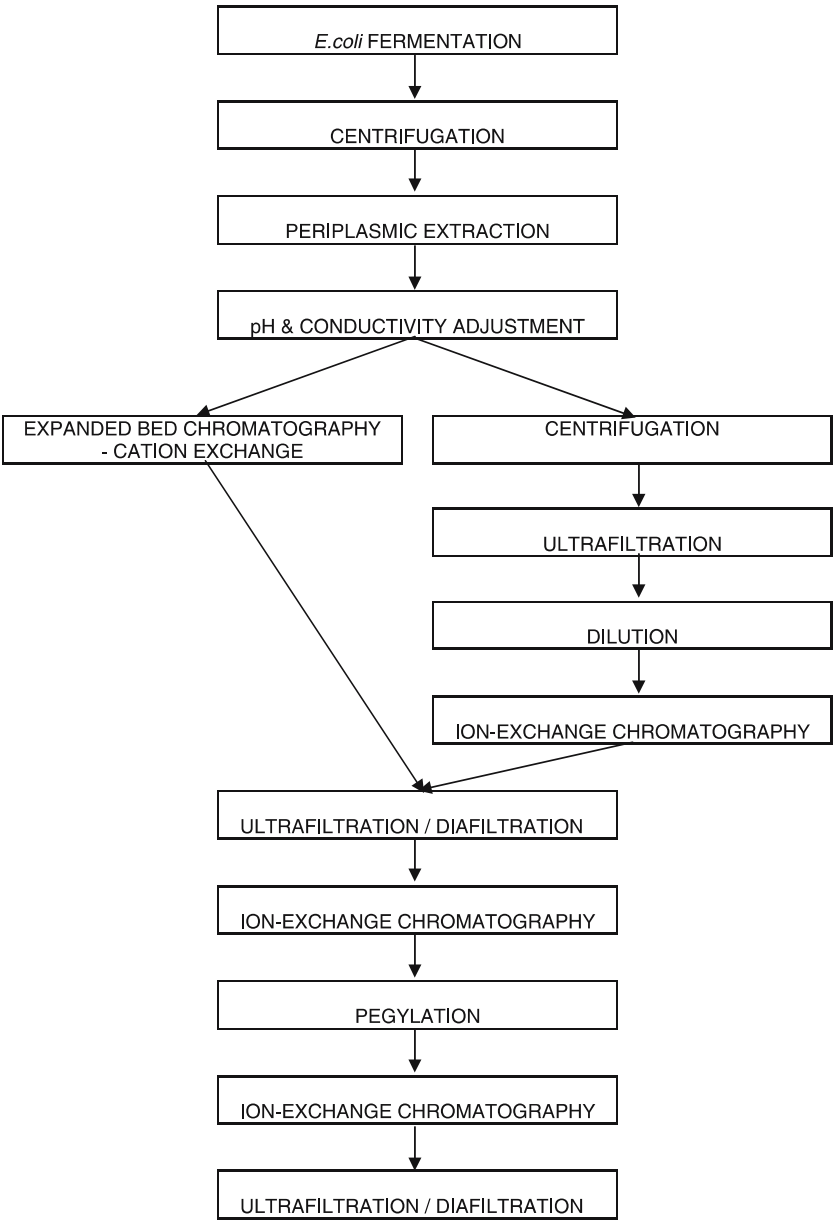

Fig. 5 Production of PEG-conjugated antibody fragments based on Celltech's *E. coli* process (Source: [67, 68]; A. Chapman, Celltech, Slough, UK, personal communication)

ammonium sulphate precipitation detrimental to their antibody fragments and favoured purification based on chromatography alone. Hence it is predicted that product recovery and purification protocols for *Pichia*-derived

antibody fragments will be similar to the generic processes for mammalian cell-culture- derived antibodies indicated in Fig. 1.

6.4 Newer Downstream Processing Approaches

Regarding novel approaches in downstream processing, expanded bed chromatography has been gaining interest as it can combine clarification, concentration and initial purification in one step. Such processes allow culture broths to be applied directly onto the column without clogging it. Industrial, as well as academic, evaluation of expanded bed chromatography has been executed. For example, Celltech uses expanded bed chromatography to replace a centrifugation, filtration and chromatography step, as can be seen in Fig. 5. Celltech has found that expanded bed chromatography gives better overall recoveries than the conventional product recovery route (A. Chapman, Celltech, Slough, UK, personal communication). Centocor [33] and Genentech [72] are also reported to be investigating the use of expanded bed processes for monoclonal antibody production in an attempt to reduce manufacturing costs. GTC Biotherapeutics also indicates that it is possible to subject the whole milk from goats or the whey concentrate to direct capture by expanded bed chromatography [46]. Fahrner et al. [73] at Genentech showed that expanded bed Protein A affinity chromatography was an efficient method for purifying a recombinant humanised monoclonal antibody from unclarified CHO cell culture, and that it provided purification performance comparable to that using a packed bed column. However, they indicate that several questions still remain, such as the process economics and the trade-off between higher overall yields and lower cycle limits.

Affinity chromatography is often the first chromatography step because of its high resolution. However, natural affinity ligands have several drawbacks, which include their high cost, their biological origin, ligand leakage and poor stability to sanitising agents (e.g. [74]). These problems, combined with the fact that fairly large volumes of harvest fluid are loaded onto this column step, have resulted in the emergence of competing alternatives. Recognising the benefits of Protein A purification of antibodies, synthetic low molecular weight compounds have been developed as Protein A mimics using chemical combinatorial libraries and molecular modelling [74, 75]. Research efforts to design and test Protein A mimetics to purify antibodies from serum, ascites fluid, human plasma, egg yolk and cell culture supernatants are reported in the literature (e.g. [76–80]). These studies illustrate that the Protein A mimetics can achieve comparable purities to those of Protein A (90–95%) and yields in the range of 60–95%. Further improvements are anticipated in binding capacity [74], yield and purity [79]. Mimetics have also been shown to be robust and resistant to harsh sanitising agents [76–80]; hence they are

expected to have longer lifetimes than Protein A ligands. In addition, a comparison of synthetic and biological Protein A ligands indicated that synthetic ligands eliminate biological contamination from protein ligands and thus facilitate validation [81]. One of the commercial suppliers of Protein A mimics (ProMetic BioSciences Ltd, Cambridge, UK) claims that its mimetic ligands are also inexpensive (about one-third the cost of Protein A). Increasing pressures to reduce production costs may result in several companies switching to synthetic affinity ligands. ProMetic claims that several companies are already using their mimetic ligands for purification of antibodies at different stages of clinical development [82].

7 Conclusions

The unrivalled specificity of monoclonal antibodies, combined with considerable genetic engineering efforts to make them less immunogenic, has renewed confidence in antibodies as a promising class of therapeutics. This paper has highlighted the main differences and similarities found in the commercial bulk production of monoclonal antibodies and reviewed future trends in antibody manufacture. Examination of the nature of the marketed monoclonal antibodies indicates that to date most therapeutic antibodies are whole, whereas for diagnostic applications antibody fragments tend to be more common. The use of in vivo animal versus in vitro cell culture techniques was also examined. Early monoclonal antibodies were produced in mice or rats. However, for later antibodies, therapeutics in particular, most manufacturers have adopted cell culture for more effective scale-up and simpler downstream processing. Comparing the manufacturing processes based on cell culture revealed that the main differences were in the cell type (hybridoma, myeloma or CHO cells) and the cell culture method (batch or perfusion) selected. However, the sequence of downstream operations was found to be very similar, relying primarily on filtration and chromatography operations. The processes for diagnostic antibodies were found to be shorter and marketed antibody fragments required extra steps for antibody digestion. Generic processes can be envisaged and used as a starting point for purification of each drug to speed up development times.

A review of the trends in doses and the market potential of therapeutic antibodies indicates that new approaches may be needed to be capable of producing the tens to hundreds of kilograms a year of antibodies at lower costs. Assessment of future trends in antibody manufacture has highlighted some of the significant challenges that lie ahead. Stirred tank bioreactors may be replaced by the Wave Bioreactor for modest needs with the advantage of disposable bags. However, the limitations in scale suggest that it will only be used for clinical trial material preparation, inoculum preparation and

commercial production for fermentation up to the 1000-L scale for drugs targeting a small market and with low doses. The use of transgenic mammals, chicken and plants as culture systems is actively being pursued for antibodies with projected annual marketed demands of several hundreds of kilograms, if not tons. Despite higher productivities, competitive timelines and lower costs, significant regulatory hurdles still need to be resolved. Antibody fragments are being engineered to enhance in vivo half-lives and have been produced successfully in *E. coli* and *P. pastoris* for candidates in preclinical and clinical trials. The processes used are similar to those used for mammalian cell-culture-derived products. In *E. coli* additional steps are required for periplasmic extraction or cell lysis. In both cases centrifugation is used for product recovery rather than microfiltration, and purification is primarily achieved using chromatography steps, although precipitation is still sometimes used as well. Regarding novel approaches in downstream processing, expanded bed chromatography has been gaining interest as it can combine clarification, concentration and initial purification in one step. However, its use may depend largely on process economics. A further development in purification has been the emergence of synthetic ligands to compete with Protein A. Increasing pressures to reduce production costs may result in several companies switching to these cheaper synthetic affinity ligands. In addition, eliminating Protein A also removes the problem of Protein A leaching, which should also reduce the validation burden on companies. Industrial antibody production processes and all the advances being made must demonstrate that they meet the demands for antibody products and improve the overall process economics so that the drugs are not so costly as to preclude wide use.

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Fedbatch Culture and Dynamic Nutrient Feeding

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Abstract In the past decade, we have seen a rapid expansion in mammalian cell based therapeutic proteins reaching clinical applications. This increased demand has been met with much increased productivity through intensive process development. During this time, fedbatch culture processes have emerged as the predominant mode for producing recombinant proteins. In this review, we discuss the fundamentals of fedbatch culture process design, focusing on the use of stoichiometric nutrient requirements for feed medium formulation, and articulating the need and potential means for devising rational dynamic feeding schemes. Incorporation of on-line nutrient measurement will play a key role in further refinement of process control for the development of a much sought after generic feeding strategy that can respond to the changing demands of different cell lines in a fluctuating culture environment. The future of process engineering will likely require a combination of current process engineering strategies along with a better understanding and control over cell physiology. Process development will likely to entail not

only optimizing traditional engineering parameters but also engineering cell lines with desired characteristics. The integration of cell engineering and process intensification will likely provide the stimuli that propel the limits of growth and productivity to the next high level.

Keywords Cell culture · Process optimization · Process control · Metabolism · Cell engineering

Abbreviations

B	base concentration
C	liquid phase oxygen concentration
C^*	liquid phase oxygen concentration in equilibrium with the aeration gas
F	volumetric nutrient feed rate
F_B	volumetric base feed rate
G	glucose concentration
G_F	glucose concentration in feed
H^+	proton concentration
k	proportionality constant relating turbidity to cell concentration
K_La	liquid phase mass transfer coefficient for oxygen in the bioreactor
q	specific nutrient consumption rate
q_i^{est}	estimated specific nutrient consumption rate
q_G	specific glucose consumption rate
q_L	specific lactate consumption rate
q_O	specific oxygen consumption rate
$S_{i,t}$	concentration of nutrient i at time t in the bioreactor
$\widetilde{S}_{i,t}$	cumulative amount of nutrient i produced or consumed at time t
S_{Fi}	concentration of nutrient i in the feed medium
S_i^{min}	minimum required concentration of nutrient i
S_i^{max}	maximum required concentration of nutrient i
t	time
$\overline{t}$	representative culture time $(1/2(t_1 + t_2))$
Tb	turbidity
V	culture volume
ΔV_k	volume of feed added in the k^{th} feed addition
x	cell concentration
$\alpha_{i,\overline{t}}$	stoichiometric ratio of nutrient i to the chosen reference nutrient at time $\overline{t}$
α_{LO}	stoichiometric ratio of lactate consumption to oxygen production
γ	correction factor for non-linearity
μ	specific growth rate

1 Introduction

The production of recombinant proteins for human therapeutic agents in mammalian cells brought about a major resurgence of bioprocess engineering in the last decade of the twentieth century. Not since the initial expansion

of antibiotic production capacity have process engineers played such a key role in bringing a large array of products to therapeutic use in such a short time. The increased output required to meet the expanding market was not accomplished by merely increasing the total culture volume. A large part was achieved through improving yields by process renovation, as opposed to process innovation. Only a decade ago, an antibody titer in the hundreds of milligrams per liter was the norm. Now, concentrations of a few grams per liter are common. With the increasing development of new products and the growing need for large quantities of each new therapeutic, it is prudent to reassess the technological advances made in the past decade and to pursue innovative ideas that will ease the task of meeting future demands.

The final product concentration is primarily affected by the specific productivity of cells, the maximum cell concentration, and the duration that high viability can be sustained. For batch processes, the low level of nutrients that can be tolerated by cells limits the final cell and product concentration. Cells are simply unable to attain and sustain high cell concentrations with the resources available in a typical growth medium. To overcome nutrient limitation, fedbatch processes have been widely practiced and are currently the norm for most cell culture processes. In fedbatch cultures, concentrated medium is added during cultivation to prevent nutrient depletion, prolonging the growth phase and increasing cell and product concentrations. Continued addition of medium past the peak of cell concentration also increases the final titer significantly by allowing cells to be kept viable at high concentrations and continue to produce product for a longer time.

Efforts to enhance the performance of fedbatch culture have traditionally focused on medium development, process control, and manipulation of cell metabolism by control of the culture environment. With recent advances in genomic research tools and a more global understanding of cell physiology, metabolic engineering may emerge as a more prominent strategy to increase productivity. Even with the promise of creating superior host cells through cell engineering, pushing the limits of productivity will always require an intensive process engineering effort to accommodate the increased demands of higher cell and product concentrations. This review will summarize current practices and articulate the developmental needs of fedbatch culture to meet these future challenges.

2
Different Forms of Fedbatch Culture

Fedbatch processes are widely used in multi-purpose, multi-product facilities because of their simplicity, scalability, and flexibility. A variety of fedbatch operations, ranging from very simple to highly complex and automated, are seen in current production facilities. To illustrate the basic operation principles

of fedbatch cultures as compared to a typical batch operation, time profiles of cell, nutrient, and product concentrations for batch (Fig. 1a), intermittent-harvest fedbatch (Fig. 1b), and traditional fedbatch cultures (Fig. 1c) are shown.

In general, fedbatch processes do not deviate significantly from batch cultures. For both intermittent-harvest and traditional fedbatch cultures, cells are inoculated at a lower viable cell density in a medium that is usually very similar in composition to a typical batch medium. Cells are allowed to grow exponentially with essentially no external manipulation until nutrients are somewhat depleted and cells are approaching the stationary growth phase. At this point, for an intermittent-harvest fedbatch process (Fig. 1b), a portion of the cells and product are harvested, and the removed culture fluid is replenished with fresh medium. This process is repeated several times. This simple strategy is commonplace for the production of viral vaccines produced by persistent infection, as it allows for an extended production period. It is also used in roller bottle processes with adherent cells.

For production of recombinant proteins and antibodies, a more traditional fedbatch process (shown in Fig. 1c) is typically used. While cells are still growing exponentially, but nutrients are becoming depleted, concentrated feed medium (usually a 10–15 times concentrated basal medium) is added either continuously (as shown) or intermittently to supply additional nutrients, allowing for a further increase in cell concentration and in the length of the production phase. In contrast to an intermittent-harvest strategy, fresh medium is added proportionally to cell concentration without any removal of culture broth. To accommodate the addition of medium, a fedbatch culture is started in a volume much lower than the full capacity of the bioreactor (approximately 40% to 50% of the maximum volume). The initial volume should be large enough for the impeller to be submerged, but is kept as low as possible to allow for a maximum extension of the cultivation period.

In batch cultures and most fedbatch processes, lactate, ammonium, and other metabolites eventually accumulate in the culture broth over time, inhibiting cell growth. Other factors, such as high osmolarity and accumulation of reactive oxygen species, are also likely to be growth inhibitory, and certainly contribute to the eventual loss of viability and productivity. The effects of lactate and ammonia on cultured cells are complex. Detectable changes in growth, productivity, and metabolism have all been documented [1]. Additionally, metabolite accumulation has been found to affect product quality. In recombinant erythropoietin producing CHO cells, high ammonia concentration has been reported to affect the glycoform of the product [2].

By minimizing metabolite accumulation, the duration of a fedbatch culture can be even further extended and higher cell and product concentrations can be achieved. Reduced metabolite accumulation in fedbatch culture is traditionally accomplished by limiting the availability of glucose and glutamine using controlled feeding strategies that maintain glucose at very low levels.

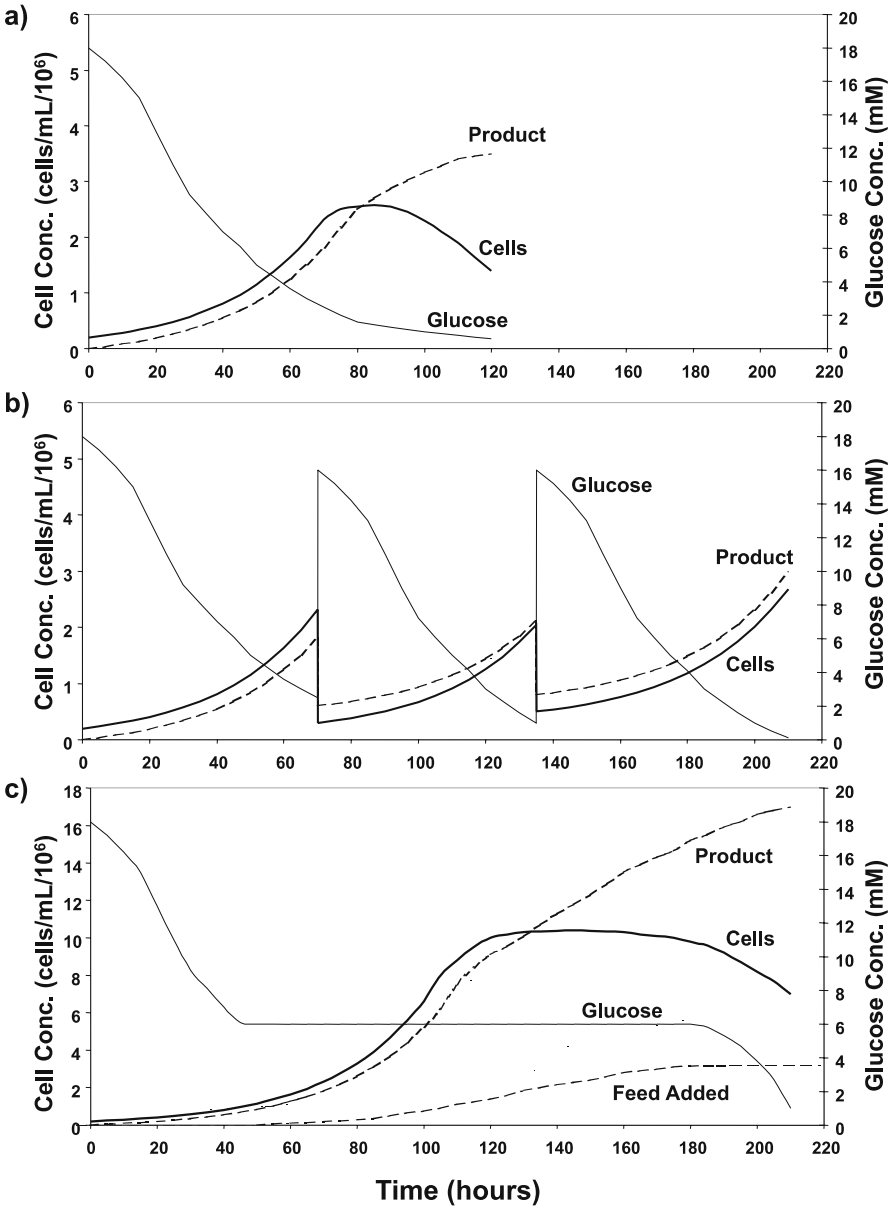

Fig. 1 Representative cell, nutrient, and product concentrations for a typical **a** batch culture, **b** intermittent-harvest fedbatch culture, and **c** fedbatch culture with dynamic feeding. As compared to a batch culture, the strategies shown in Figs. **b** and **c** extend the duration and productivity of a culture run by re-supplying depleted nutrients. In fedbatch culture (**c**), feed is added continuously to sustain nutrient levels. Much higher cell and product concentrations are achieved

After extended exposure to low glucose concentration, cell metabolism is directed to a more efficient state, characterized by a dramatic reduction in the amount of lactate produced. Such a change in cell metabolism from the normally observed high lactate producing state to a much reduced lactate production state is often referred to as metabolic shift. The observation of such changes in metabolism was made more than two decades ago [3–7], yet its application in fedbatch culture was not realized until much later [8]. Extending the methodology to controlling both glucose and glutamine at low levels, both lactate and ammonium accumulation can be reduced [7, 9–11]. By applying such a control scheme in fedbatch culture, lactate concentration was reduced by more than three fold, and very high cell concentrations and product titers were achieved in hybridoma cells [8].

Figure 2 compares the time profile of cell growth, glucose concentration and lactate concentration for two hybridoma fedbatch cultures growing under different metabolic states. Shown in Fig. 2a is a culture in which the glucose level was controlled in the range of 1.0–4.0 mM, a relatively low concentration. In many cultures, glucose concentration is controlled at even higher levels, in the range of 10 mM. In these ranges of glucose concentration, cells behave very similarly, having a high lactate production rate. As a result, the level of lactate accumulated eventually requires the addition of base to maintain pH. To supply nutrients to the culture, feed medium was added approximately proportionally with the base addition rate, since lactate production is indicative of the metabolic demands of the culture. This feeding strategy will be discussed in more detail in Sect. 4.2.2. A final cell concentration of 7.5×10^6 cells mL^{-1} was obtained with lactate accumulating to nearly 70 mM in the final culture volume. In the culture shown in Fig. 2b, the set point of glucose concentration was at 0.03 mM. Feed medium was added based on the oxygen uptake rate (OUR), which is estimated on-line. This strategy will also be discussed further in a later section (4.2.4). The continuous exposure to very low glucose concentrations allowed cells to shift their metabolism to a state where little lactate was produced. The final lactate concentration only accumulated to 40 mM. With the control of glucose concentration at low levels, the reduced lactate concentration, and the elimination of base addition, a final viable cell concentration of more than 11.5×10^6 cells mL^{-1} was achieved.

Historical data from several batch and fedbatch hybridoma cultures, including those shown in Fig. 2, were analyzed to generate the values in Table 1. Direct comparison of the values between cells in different metabolic states illustrates that the stoichiometric nutrient consumption and metabolite production for cells is notably changed in different metabolic states. Under typical culture conditions, where nutrients are supplied in excess, more than half of the carbon in glucose and at least one fourth of the nitrogen in glutamine consumed is excreted as lactate and ammonium [5, 12]. For hybridoma cells in a high lactate producing state, this observed stoichiometric ratio is be-

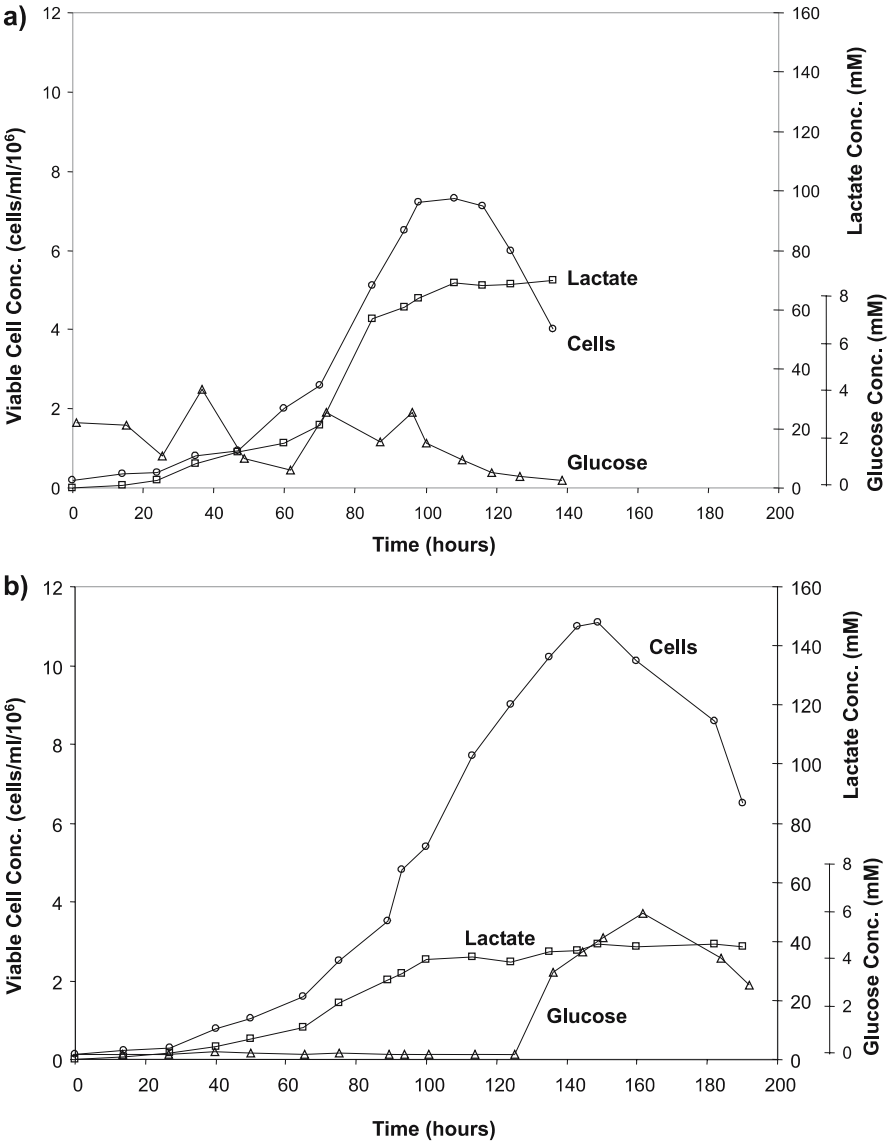

Fig. 2 Time profiles of cell, lactate, and glucose concentration for a hybridoma fedbatch culture with cells growing with **a** high-lactate producing metabolism, and **b** metabolic shift. Metabolic shift was achieved by control of glucose concentrations at 0.03 mM

tween 1.4–2.2 moles lactate produced per mole glucose consumed. For the same cells cultured in a metabolically shifted state, a very low ratio of less than 0.5 moles of lactate produced per mole of glucose consumed is observed. The ratio of ammonia produced per glutamine consumed is also compared

Table 1 Characteristic Stoichiometric Ratios of Key Nutrients for Cells Growing in Different Metabolic States

Stoichiometric ratio (mmole/mmole)	Without metabolic shift	Metabolic shift	Lactate consuming cells
lactate/glucose	1.4 – 2.2	0.05 – 0.5	0.4 – 1.0
ammonia/glutamine	0.5 – 1.3	0.1 – 0.3	–
alanine/glutamine	0.2 – 1.3	0.01 – 1.3	–
oxygen/glucose	1.0	1.0 – 2.0	–

in Table 1, showing a dramatic reduction from 0.5–1.3 moles ammonium per mole of glutamine to 0.1–0.3 mole per mole under metabolically shifted conditions. In later stages of fedbatch cultures, lactate consumption, as opposed to production, is occasionally observed, although this phenomenon is not well documented in published literature. In such cases, an approximate ratio of lactate to glucose consumption is between $\sim$ 0.4–1.0 moles of lactate consumed per mole of glucose consumed. While this observation seemingly contradicts the role of lactate as an inhibitory molecule, it illustrates the flexibility of mammalian cells to adapt their behavior for survival under a wide range of conditions. With this repertoire of available cell behavior, fedbatch culture strategies that provide conditions that reduce metabolite accumulation is a field of fedbatch culture technology still warranting further development.

3 Designing Feed Medium for Fedbatch Cultures

The design of feed medium is critical for the implementation of a successful fedbatch process. A well-designed feed medium should ensure cell growth and product formation are not limited by depletion of any medium component or inhibited by excessive nutrient concentration or metabolite accumulation. To achieve this, a good estimate of the rates of consumption of medium components is required. For most processes, a feed medium that is 10 to 15 times the nutrient concentration of basal medium is used. With this simple design, the consumed nutrients are replenished, and the growth and production phases are prolonged; however, many components will likely be supplied in excess, while others will be in limited supply [13].

The nutritional requirements for mammalian cells are very complex. Most media contains glucose, vitamins, and virtually all amino acids. Among the amino acids included, 13 are deemed “essential” for cultured cells, as most cell lines cease to grow in their absence [14, 15]. This requirement for cultured cells is higher than the 11–12 essential amino acids required for survival

of mammals. The other (non-essential) amino acids can be synthesized or inter-converted from essential amino acids by cells. Even though they are not essential, they are often included in culture medium, and have been shown to improve growth and product formation. When supplied in excess, amino acids can contribute significantly to energy metabolism, especially glutamine, which, in some cell lines, has been shown to supply more than half of the energy derived from the TCA cycle [5].

Because many amino acids are inter-convertible, their consumption rates (especially for non-essential amino acids) can be highly variable, even among similar cultures of the same cell line. For the purpose of feed medium design, representative, working values for consumption rates, as opposed to precise quantities, should be estimated and used to prepare an appropriate feed medium. In designing feed medium, both absolute concentration and the consumption rates of nutrients must be taken into consideration. A nutrient may be present at a high concentration for a different reason than it is consumed quickly. Sometimes high concentrations are needed to provide a chemically balanced environment, as is the case with potassium, sodium and phosphate. These components are primarily supplied as inorganic salts, and their concentrations greatly exceed the levels of the more rapidly consumed organic nutrients. At a cell concentration of 5×10^6 cells mL^{-1}, the consumption of most salts is hardly measurable. Essentially all nutrients are taken up by cells through transporters present in the cell membrane, for which the transport rate is affected by the concentration of the nutrient in the medium. Some slowly consumed nutrients may need to be sustained at high concentrations to facilitate sufficient transport across the cell membrane. This may be particularly relevant in amino acid utilization, for which transporters are often shared by a group of amino acids with similar properties. For example, leucine and phenylalanine use the same amino acid transporter (transporter L) as glutamine. Since glutamine is often present in such high concentrations, low concentrations of those other amino acids may affect growth and production, as they must compete with glutamine for a sufficiently high transport rate.

The absolute concentration of nutrients maintained in a fedbatch culture can be a key factor in the overall performance of a fedbatch process. In batch cultures, nutrients are present at higher concentrations and are not significantly depleted until cells reach their peak concentration. In contrast, for fedbatch cultures some nutrients may not be fed at a sufficient rate and can reach a low concentration that is sustained over a longer period, eliciting a more apparent effect on cell growth and the final product. Product quality, specifically in terms of glycoform, has been shown to be affected in long-term fedbatch and continuous cultures, especially when lower concentrations of glucose and glutamine are maintained [16–19].

With these considerations, a logical approach for the design of feed medium is to divide the chemical species into two categories: those whose

consumption rates for growth and product formation are significant and measurable, and those whose concentrations greatly exceed the amount required for growth. Species that are appreciably consumed should be replenished by the feeding medium at same rate they are consumed to maintain their concentration in an optimal range. Conversely, nutrients that are hardly consumed should only be added to the extent that they are not diluted significantly by the volume expansion.

3.1 Feed Medium Design for Consumed Nutrients

A primary objective of nutrient feeding is to replenish nutrients that have been consumed and additionally supply what is required to sustain growth and production until the next point of medium addition. Under balanced growth conditions, the specific consumption rates of various nutrients are relatively constant, and, correspondingly, the proportionalities of consumption of nutrients or production of metabolites relative to one another are constant. These rate proportionalities are termed stoichiometric ratios. A well-formulated feed medium is designed to add nutrients at appropriate stoichiometric ratios to match their consumption rates, simultaneously keeping all nutrients within their desired concentration ranges.

Stoichiometric ratios can be calculated using historical culture data obtained from the cell line of interest, growing under relevant cultivation conditions. Typically, one medium component is chosen as a reference nutrient, and the consumption of all other nutrients are determined by ratios to that reference component. Any species consumed or produced by cells in a quantifiable amount can be chosen as the reference nutrient. Common choices for reference nutrients are glucose, glutamine, oxygen, and lactate, as they are consumed or produced in larger quantities among all nutrients and metabolites. They are also relatively easy to measure.

Using batch culture data, the stoichiometric ratio, $\alpha_{i,\overline{t}}$, for nutrient i at time $\overline{t}$ is calculated using the concentration differences at two time points, as shown in Eq. 1. In principle, the stoichiometric ratio can be calculated as the ratio of the specific rates; however, specific rates derived from culture data are often noisy, and can lead to inaccurate results.

$$\frac{S_{i,t_2} - S_{i,t_1}}{S_{r,t_2} - S_{r,t_1}} = \left(\frac{\Delta S_i}{\Delta S_r}\right)_{\overline{t}} = \frac{q_{i,\overline{t}}}{q_{r,\overline{t}}} = \alpha_{i,\overline{t}} \,. \tag{1}$$

In many cases, data are collected from fedbatch cultures (as opposed to simple batch cultures), where culture medium was added or removed during cultivation. In these cases, concentration differences cannot be used directly since the rise and fall of the measured concentration is not exclusively due to consumption or production by cells. For fedbatch culture data, stoichiometric ratios are calculated from cumulative consumption data. Cumulative

consumption of nutrient i at time t can be found using Eq. 2.

$$\widetilde{S}_{i,t} = \int_0^t q_i \cdot x \cdot V \cdot \mathrm{d}t = V_{t_0} \cdot S_{i,t_0} + \sum_{k=1}^{m} \Delta V_k \cdot S_{F_i} - V_t \cdot S_{i,t} \,. \tag{2}$$

The integral term is the cumulative consumption ($\widetilde{S}_{i,t}$). Using culture data from a culture where medium is fed intermittently, a good estimate for this integral is found using the right-most side of the equation. The first term is the total amount of nutrient i initially in the starting culture volume ($V_{t_0} \cdot S_{i,t_0}$). The second term is a summation of the amounts of nutrient i fed in each feed volume addition, where ΔV_k is the volume added in the k^{th} feed addition, and S_{f_i} is the concentration of nutrient i in the feed medium. The summation term $\left(\sum_{k=1}^{m} \Delta V_k \cdot S_{F_i}\right)$, therefore, is the total amount of nutrient i added or removed from the culture in m feed additions. The last term is the amount of nutrient in the culture at time, t. The total amount of nutrients i and r, consumed or produced are then used to calculate stoichiometric ratios, as shown in Eq. 3.

$$\frac{\widetilde{S}_{i,t_2} - \widetilde{S}_{i,t_1}}{\widetilde{S}_{r,t_2} - \widetilde{S}_{r,t_1}} = \left(\frac{\Delta\widetilde{S}_{i,\bar{t}}}{\Delta\widetilde{S}_{i,\bar{t}}}\right)_{\bar{t}} = \frac{q_{i,\bar{t}}}{q_{r,\bar{t}}} = \alpha_{i,\bar{t}} \,. \tag{3}$$

In most cases, a two-point calculations are noisy and could give inaccurate results. More accurate stoichiometric ratios can be found by plotting the cumulative consumption data for each nutrient ($\widetilde{S}_{i,t}$) versus the cumulative consumption for the reference nutrient ($\widetilde{S}_{r,t}$). Figure 3 shows representative cumulative consumption plots for several nutrients vs. glutamine (a common reference nutrient), as well as for lactate vs. glucose. The slopes of these lines are the stoichiometric ratios of the components with respect to glutamine. Since any nutrient can be chosen as the reference nutrient, plotting data against more than one reference nutrient can be used to check the accuracy of the estimated ratios. The stoichiometric ratio plots in Fig. 3, which represent actual fedbatch culture data, are not all linear, clearly illustrating that specific consumption rates can change over the course of a culture, and the magnitude of this change varies among different nutrients. The data shown for oxygen vs. glutamine and, potentially, lysine vs. glutamine, can be reasonably well fit by a single straight line. The stoichiometric ratios of alanine to glutamine and lactate to glucose change dramatically during the cultivation. If these changes are significant, excessive accumulation or depletion of a component could occur using a single feed medium throughout the culture. In these cases, it may be advantageous to change the feed medium composition for different phases of growth. For some non-essential amino acids, it is difficult to decide an appropriate stoichiometric ratio, as these nutrients can switch between being consumed to being produced in a single culture. A very rough estimate is usually sufficient for non-essential amino acids. Feed

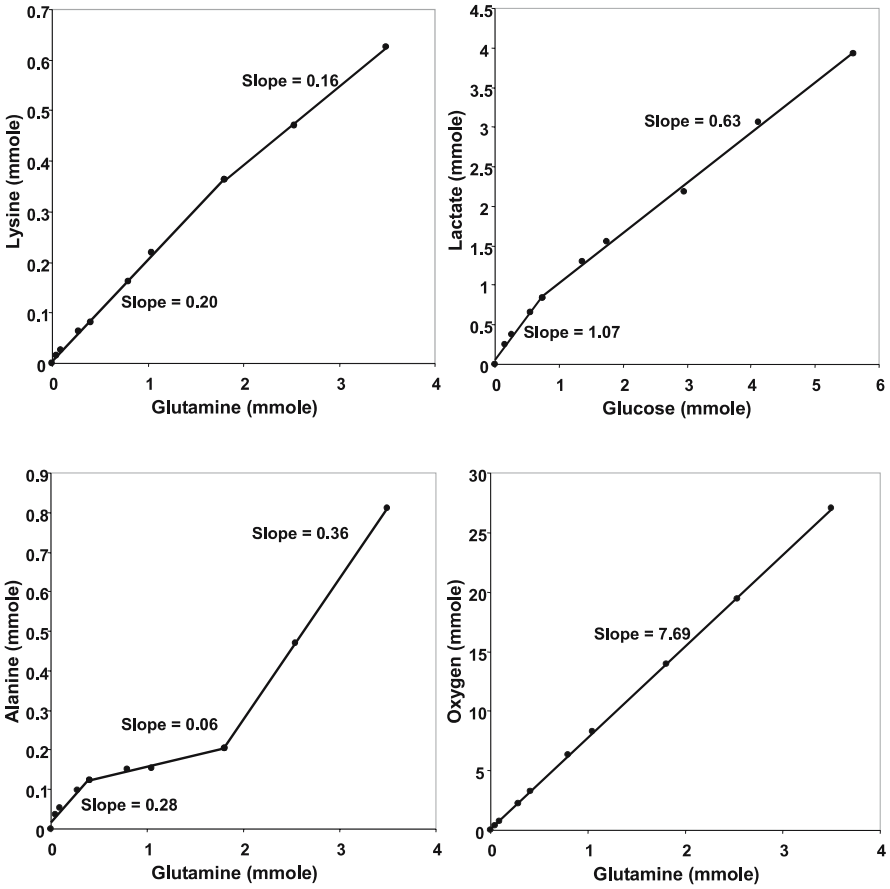

Fig. 3 Stoichiometric ratios obtained from a fedbatch culture of hybridoma cells. The slopes of fitted trend-lines represent stoichiometric molar ratios for the pair of nutrients plotted. While some ratios remain relatively constant throughout the course of a culture, others change during cultivation, warranting a change in the feed medium composition and/or feed rate

medium design using this method is best approached as an iterative process, where a design is tested, analyzed and refined until an optimal medium emerges. Using these methods, a very effective medium is typically achieved with fewer than three rounds of optimization.

3.2 Feed Medium Design for Unconsumed Components

Some medium components are consumed in such small quantities that they can virtually be considered unconsumed by cells. Many inorganic ions (i.e.

sodium, calcium, and sulfate) fall into this category. In feed medium, these components should be included at low levels (typically 1x concentration or less), with the goal of avoiding their dilution by volume expansion. For many fedbatch cultures, inorganic salts, such as NaCl, are completely eliminated from the feed medium to reduce osmolarity in culture.

The intracellular concentrations of some ions, like magnesium (as a complex with ATP), phosphate (as free phosphate, and in nucleotides), and potassium (for membrane potential), are at least ten times greater inside cells than in typical medium. At very high cell concentrations, the amount of these ions taken up by cells may cause significant depletion in the medium and it could be necessary to compensate their consumption by including them at more than 1× concentrations in the feed medium. This is also true for components like protein hydrolysates, serum, insulin, transferrin, vitamins, and lipid hydrolysates. These additives need to be replenished as they are consumed or become inactive with time.

The concentrations of these medium components are not routinely measured. Some may be taken up by cells and recycled back to the medium (e.g. transferrin), while others are taken up by cells in minute quantity and are needed only for cell mass expansion (e.g. lipid supplements). These medium components are also turned over intracellularly, and turnover rate parameters can be very difficult to quantify. A strategy for determining concentrations of such components is to aim to maintain their concentrations above a minimum required limit, but below a maximum tolerable bound. Appropriate feed medium concentrations for these components (S_{F_i}) can be determined to keep concentrations within the bounds $S_i^{\min}$ and $S_i^{\max}$, shown in Eqs. 4 and 5. Maximum and minimum values must be experimentally determined.

$$S_i^{\min} \leq \frac{S_{i,t_0} \cdot V_{t_0} + \sum_{k=0}^{m} \Delta V_k S_{F_i} + \int_0^t q_i^{\text{est}} \cdot x \cdot V \, \mathrm{d}t}{V_{t_0} + \sum_{k=0}^{m} \Delta V_k} \tag{4}$$

$$S_i^{\max} \geq \frac{S_{i,t_0} \cdot V_{t_0} + \sum_{k=0}^{m} \Delta V_k S_{F_i}}{V_{t_0} + \sum_{k=0}^{m} \Delta V_k} . \tag{5}$$

In these equations, q_i^{est} is an estimated maximum consumption rate for nutrient i, and is used to determine if the proposed concentration is sufficient to maintain concentrations above $S_i^{\min}$. The maximum concentration that can possibly accumulate in the reactor is calculated as the case where there is no consumption of the nutrient.

3.3
Idealized Fedbatch Culture Medium Design for Altered Metabolism

The methods discussed for medium design focus on the calculation of quantities required for replenishment of nutrients consumed in culture; however, replenishing nutrients is not sufficient to keep cells growing indefinitely or to physiological concentrations in a bioreactor. Inhibitory metabolites, like lactate and ammonia, often accumulate in the culture, preventing high cell and product concentrations. As shown in Fig. 2b, a fedbatch culture that controls glucose and glutamine at very low concentrations can be used to change cell metabolism to a state where the production of inhibitory metabolites are markedly reduced. Under these conditions, the changes in cell metabolism in terms of stoichiometric nutrient utilization for some commonly measured nutrients are significant. These changes among various states have been highlighted in Table 1 (Sect. 2). A more complete analysis of all consumed nutrients under metabolic shift conditions reveals that the stoichiometric utilization of many other nutrients is also altered. A comparison of ten cultures, with a wide range of lactate production rates was performed by Gambhir et al. in 2003 [11]. By calculating stoichiometric ratios and nutrient consumption rates for cultures having similar growth rates, it was found that the specific consumption rates of most nutrients are reduced in lower lactate producing cultures. Additionally, other stoichiometric ratios, in addition to those in Table 1, are changed significantly. Among the compounds consumed at higher rates, the consumption of oxygen is least affected by the metabolic state. Seeing the magnitude of changes in stoichiometric ratios between cells in different metabolic states, the feed composition should be altered as cells shift between metabolic states in order to create a balanced environment. When culturing cells at low glucose and glutamine levels to achieve metabolically shifted cells, the medium composition should be gradually changed to more closely match the metabolic needs of the cells in the desired state.

While continuously changing feed medium composition would provide the most ideal environment for the cells, there are a large number of components in the medium. It is not practical to monitor and adjust the feed composition for optimized addition of each component throughout the length of a process. Additionally, the affects of frequent adjustment of medium composition on growth and kinetics of product formation are not well known. In practice, the adjustment of stoichiometric ratios in the feed is focused on changing a few important components that are consumed in large quantities (typically glucose, oxygen, and glutamine). As the metabolic state changes from a high to low lactate producing state, a strategy involving the combined adjustment of feed rates and medium composition for those few components can be used to sufficiently avoid over-feeding and provide the long-term, stable environment required to elicit metabolic shift. The appropriate adjustments are made by

monitoring the $\Delta L/\Delta G$ ratio throughout the culture and using historical data to estimate the requirements of cells in their current metabolic state.

A challenge of achieving a metabolically shifted fedbatch culture is designing a feed medium that can be used to direct cells to the desired state. For a first estimate, one can assume that the two main energy sources for cells are glucose and glutamine. Under the most ideal metabolic state (with no lactate formation), it can be assumed that all of the glucose and glutamine consumed that is not incorporated into biomass and product is completely converted to CO_2, H_2O, and NH_3 through the TCA cycle. All of the other nutrients consumed are incorporated directly into biomass and product, which can be estimated using cellular composition data [11, 20, 21] and the amino acid composition for an IgG molecule [11, 22]. This ideal state is represented at the left-most side of the graph in Fig. 4, which illustrates the relationship between nutrient consumption rates and the observed $\Delta L/\Delta G$ ratio. The amount of glucose and glutamine utilized for each unit of biomass formed is relatively constant in all metabolic states. In contrast, the excess amount

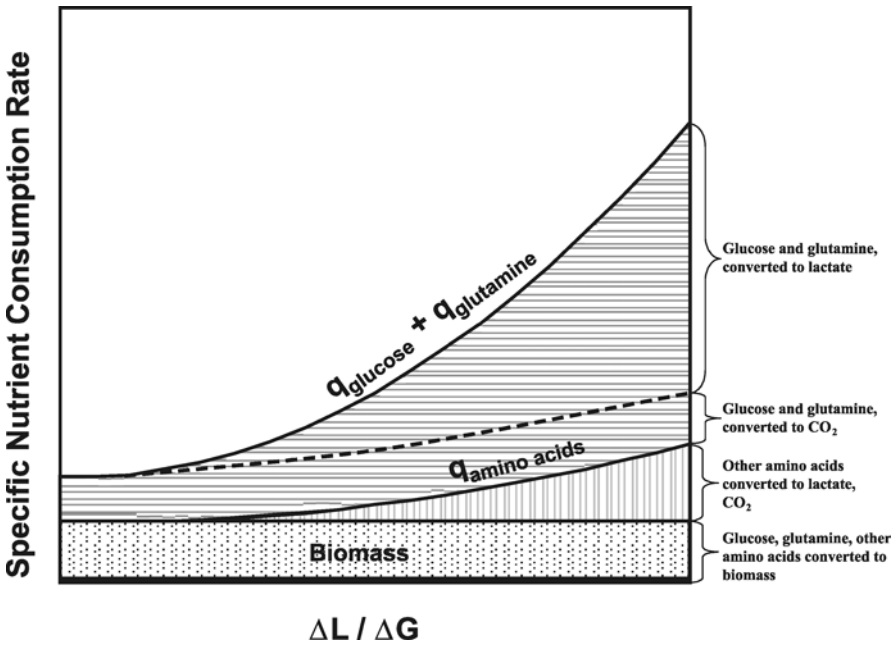

Fig. 4 The fate of carbon obtained from consumption of glucose, glutamine and other amino acids at different metabolic states, as characterized by the molar ratio of lactate production to glucose consumption ($\Delta L/\Delta G$). The nutrient consumption rate changes with the observed metabolic state. The relative fractions of nutrients contributing to biomass and product formation, energy generation, and metabolite production can be inferred from this diagram

of nutrients consumed by cells increases as the metabolic state shifts toward higher $\Delta L/\Delta G$ ratios. Experimental observation has shown that this shift toward high glucose/glutamine consumption and lactate/NH_3 production is accompanied by an increase in overall amino acid consumption. Overall, the portion of glucose and glutamine converted to biomass is small for cells producing lactate at a fast rate. For cells producing less lactate, a larger fraction of the consumed nutrients contribute directly to cell and product formation.

The fraction of essential amino acids that is taken up by cells contributing directly to biomass and product formation is relatively large. In light of this, supplying enough amino acids for product synthesis should not be overlooked in medium design. For recombinant antibody production in NS0 cell lines, several groups have observed that supplementing amino acids beyond concentrations present in typical culture medium can substantially improve both cell growth and productivity [23–25]. Many current antibody production processes are said to have an antibody productivity of 40×10^{-12} g cell^{-1} day^{-1}. For cells with an average cell mass of 10^{-10} g cell^{-1} and a doubling time of one day, a high-producing cell produces 10^{-10} g of cell and 4×10^{-11} g of antibody each day. If protein comprises approximately 60% of the cell mass, each cell contains 6×10^{-11} g of protein. Essentially, cells produce antibody and cellular proteins in the same order of magnitude. Amino acids are also consumed for nucleic acid synthesis and may be accounted using cellular nucleotide composition data [11]. Phosphate is another important nutrient that must also be considered, as it is ubiquitously present in many intracellular molecules including DNA, RNA, coenzymes, and phospholipids. Supplementation of medium with phosphate, like amino acid fortification, has also been shown to increase cell concentrations and improve antibody titer in NS0 cells. The phosphate requirement can also be estimated from cellular composition data [23]. A medium based on cellular composition is the most logical starting point for designing an ideal stoichiometric fedbatch medium with the capability of achieving cultures with reduced lactate production.

4 Control Strategies for Fedbatch Cultures

In addition to feed medium design, the method of feed medium delivery is another important consideration that can greatly affect the performance of a fedbatch process. Ideally, the consumption or production rate of the reference compound can be used to establish a feedback loop that determines how much medium should be added. This can be implemented using complex online measurements paired with an automated feeding system, or using very simple off-line manual feeding and monitoring. Many processes fall some-

where between these two extremes. Combined with a good feed medium design, measurement of a single compound and addition of a single feed stream can be used to supply all essential culture nutrients in their appropriate proportions.

In the development of a feeding strategy, three elements should be considered: the control criterion (level of different nutrients to be maintained), the mode of feeding (continuous or intermittent feeding), and a control strategy for determining the timing and amount of medium to be delivered. The simplest strategy is to allow nutrient levels to vary in a wide range by adding large quantities of feed medium at widely separated time intervals (e.g. once or twice per day) based on off-line measurements or historical data. Such a periodic feeding scheme is very simple and is usually sufficient for most processes that aim to avoid depletion or overfeeding of nutrients. For more specialized cases, especially those aiming to manipulate cell metabolism, more frequent, almost continuous measurement of parameters, along with well-controlled, continuous feeding schemes, are required.

4.1 Control Criteria and Measured Variables

A common control objective for fedbatch cultures is to maintain one or more key nutrients within an acceptable range of concentration. Other components are added proportionally according to the established stoichiometric composition of the feed medium. The level and range of the controlled nutrients are usually chosen to attain desirable culture characteristics, such as high productivity, consistent glycoform, and delay of the onset of adverse growth conditions. Ideally, the controlled nutrients should be measured continuously, especially if automated feed addition is required.

Measured variables in cell culture processes typically include the concentrations of glucose, lactate, glutamine, and cells. Concentrations of other amino acids can also be measured. Even among the frequently measured variables, few are determined on-line in real time. Like microbial fermentation processes, pH, dissolved oxygen, and, less frequently, turbidity are the primary variables available on-line. Mass spectrometry based off-gas analysis is also commonly used in microbial fermentations for measuring oxygen uptake rate and CO_2 evolution rate; however, it is not commonly used in mammalian cell culture because the difference in composition between the inlet and outlet gas stream is small. This limited availability of variables measured on-line constrains the latitude of possible control objectives for mammalian cell cultures.

Presently, a sound mathematical description of the relationship between growth, productivity and measurable nutrient concentrations is not available for mammalian cells in culture. In microbial fermentations, the Monod model, although far from ideal, is often adequate for prescribing the relation-

ship between growth rate and a limiting nutrient concentration. It is common practice to control growth rates by manipulating the feed rate. For example, in the cultivation of recombinant *Escherichia coli*, the growth rate is commonly controlled below its maximum by creating a nutrient-limiting environment after cell density reaches a certain level [26]. This reduces the culture oxygen demand and decreases unwanted metabolite formation. With the complexity of mammalian cell metabolism and nutritional requirements, the dependence of growth rate on nutrient concentrations has a high dimensionality, making defining a similar control objective a complex task. Despite the decades of research and success in large-scale mammalian cell processes, there are few articulated control objectives for fedbatch cultures beyond simply maintaining nutrient concentrations at a prescribed set-point. Most fedbatch cultures for mammalian cells do not employ control algorithms explicitly, but rely heavily on heuristic explorations. Different means of implementing the most common control objectives through measurements and controlled feeding schemes are described below.

4.2 Feeding Strategies

4.2.1 Feeding by Direct Measurement of Nutrient Consumption

Direct measurement of concentrations of nutrients is the most straightforward way to determine the amount, rate, and timing of feed medium addition. Based on current concentrations of nutrients, one can determine how much medium should be added to replace nutrients and sustain cell growth until the next medium addition. The concentrations of some nutrients can be determined on-line, although off-line nutrient measurement is more widespread. Glucose and glutamine are the two nutrients most commonly measured and utilized as control objectives. The depletion of either of these nutrients in culture usually leads to a rapid and irreversible decline in viability, especially for myeloid derived cells (NS0, SP0 or hybridoma cells). Direct measurement of these compounds on-line can be implemented using an auto-sampling device in series with commercially available immobilized enzyme/membrane based measurement devices. YSI® (Yellow Springs Instruments) analyzers are among those widely used for on-line nutrient measurement and have been developed specifically for use in cell culture and fermentation processes. Such devises can measure several nutrients simultaneously including glucose, lactate, glutamate, glutamine, and ammonium. Combined lactate and glucose measurements have been used to assess culture conditions for maintenance of glucose and lactate concentrations in perfusion culture [27]. HPLC can also be implemented as an on-line approach for measurement of glucose and amino acids [28–30]. This technique requires

a series of processing steps for sample preparation before injection into the HPLC, and a significant lag time between sampling and control action is inevitable; although for mammalian cell cultures, even an hour lag time may be tolerable.

With continuous monitoring of nutrient levels, simple set point control can maintain concentrations within a desired range, provided that the control range is not very narrow and the set point is not too close to the detection limit. For example, set point control based on nutrient measurement would not be a desirable strategy to maintain glucose at a level that will elicit a shift in metabolism ($\leq 0.05\,g\,L^{-1}$). Very small, frequent adjustments in feeding rates would be required to minimize overfeeding and, more critically, avoid complete depletion of some nutrients. Long delays in the determination of nutrient concentration combined with the decreased sensitivity in discerning concentration changes at very low nutrient levels contribute to the risk associated with such a process. Additionally, at such low nutrient concentrations, stoichiometric ratios and consumption rates are very sensitive to fluctuations in nutrient concentrations, and even a short exposure to elevated glucose concentrations may cause metabolically shifted cells to revert to a less efficient state [31]. Additionally, it has been shown that a lengthy exposure to low glucose concentration is needed for metabolism to shift completely from a high lactate producing state to a very low lactate producing state. In one case using a myeloma cell line, two fedbatch cultures in series were needed to complete the shift [32]. More critically, the risk of complete nutrient depletion and a rapid decrease in cell viability makes this a risky strategy. When nutrient concentration is maintained at high levels, stoichiometric ratios are relatively constant and there is little risk of nutrient depletion. Except for cases where fedbatch culture is used to elicit metabolic shift, nutrient monitoring and a set-point control objectives provide reliable and consistent processes.

4.2.2 Proportional Feeding with Base Addition

Another widely used control strategy is to proportionally add medium according to the amount of base added to the culture. This provides the option of continuous on-line feed addition without an on-line nutrient measurement device. In most cultures, a large fraction of the glucose consumed is converted to lactic acid, which results in a decrease in culture pH and requires addition of base. In principle, to maintain culture pH, one mole of base is added to neutralize each mole of lactic acid produced. If the stoichiometric ratios between lactic acid production and the consumption of other nutrients are relatively consistent, the rate of lactate production can be used to estimate the consumption rates of other nutrients. Barring the effects of buffers such as sodium bicarbonate or HEPES, the base addition rate

is indicative of lactate production. The primary advantage of this automated method is its simplicity. Sophisticated computer control and tuning are not required.

Proportional feeding according to base addition, as with set-point control, is not well suited for processes requiring highly accurate control of feed rate, like those used to elicit metabolic changes. The method, especially when buffers are used in the medium, does not provide enough sensitivity in response to lactic acid production. For control of nutrients at low concentrations, the buffer effect introduces a pronounced risk of nutrient starvation. Reducing the buffer capacity in the culture medium can increase sensitivity enough to allow small alterations in metabolic behavior [33].

4.2.3
Proportional Feeding with Turbidity

On-line laser turbidity probes have proved an accurate way to estimate cell concentrations and implement process control strategies by using a correlation between off-line counts and optical density [34, 35]. This allows for stoichiometric ratios between nutrient consumption and the integral of viable cell concentration (IVC) to be determined and used to establish proportional feeding strategies [36]. Simple proportional feeding with turbidity works well during exponential growth phase when viability is high and specific growth rate is relatively constant; however, near the end of exponential growth phase when viability drops, an assessment of viability or metabolic activity must be used to adjust feed rates and avoid over-feeding.

4.2.4
Proportional Feeding with Oxygen Uptake Rate (OUR)

Among the on-line measurements available, OUR measurement is the most capable of delivering an accurate estimate of cellular demands. It is capable of providing a level of control sufficient to elicit metabolic changes. Oxygen consumption is a true physiological indicator and, unlike pH, is not masked by buffers or the consumption or production of other acidic culture components. Oxygen consumption is also one of the most sensitive among measured variables, and can provide an accurate indication of changes in metabolic rate. A small change (e.g. 0.02 mM) in oxygen level over a short time span can be confidently measured, providing an immediate determination of changes in metabolic rate. In a proportional OUR feeding strategy, on-line oxygen consumption data is used to calculate nutrient requirements and control continuous feeding streams. The demands are calculated using established stoichiometric ratios that are determined from accumulated historical data for a particular cell line. This relation between OUR and nutrient consumption rates determines the feeding rate.

In mammalian cell bioreactors, the oxygen concentration difference between inlet and outlet gas streams is small and usually does not provide a confident estimate of oxygen consumption. On-line oxygen uptake rate measurement (OUR) for cell culture processes is typically implemented using a liquid phase oxygen balance. The general equation for the mass balance of oxygen in culture fluid is shown in Eq. 6.

$$\frac{dC}{dt} = K_L a \cdot (C^* - C) - \text{OUR} . \tag{6}$$

For calculation of OUR using this balance, two approaches are frequently used. Both take advantage of the relatively invariant oxygen transfer coefficient ($K_L a$) in cell culture bioreactors. In the first method, periodic perturbations of oxygen concentration are introduced by changing the gas phase oxygen concentration so that it is in equilibrium with the liquid phase. A decline in the dissolved oxygen follows due to the lack of oxygen transfer and the continued consumption of oxygen by cells. Since C^* is changed to be in equilibrium with C, $(C^* - C)$ becomes zero, and Eq. 6 is reduced to:

$$\frac{dC}{dt} = - \text{OUR} . \tag{6a}$$

The change in dissolved oxygen concentration is exclusively due to uptake by cells; therefore, OUR can be estimated by determining the initial slope of the dissolved oxygen profile over time. Strictly, the driving force can only be assumed to equal zero for a short period of time. In some cases, a nonzero driving force $(C^* - C)$ must be considered, and a predetermined value for $K_L a$ must be used. In this case, the full form of Eq. 6 is integrated over time to solve for OUR.

In the second method, dissolved oxygen concentration is maintained at a set point ($dC/dt = 0$) by varying the inlet gas phase composition. This reduces the mass balance equation to the following form.

$$K_L a \cdot (C^* - C) = \text{OUR} . \tag{6b}$$

For the case where $K_L a$ is measured and is assumed to be relatively constant over time, the outlet gas phase composition (C^*) and dissolved oxygen concentration (C) allows for calculation of OUR [37]. For small reactors where the primary aeration is achieved by surface aeration and gas-permeable tubing, a proportional OUR feeding strategy works extremely well, as shown by the number of publications using OUR for controlled feeding to induce metabolically shifted cultures [8, 33, 38, 39]. In large-scale fermenters, where direct sparging is used continuously or intermittently, $K_L a$ fluctuates and must be correlated carefully with varying aeration conditions. Despite these difficulties, proportional OUR feeding in large-scale bioreactors is still useful and provides a robust fedbatch operation. The lack of reliable

$K_L a$ estimates in large-scale cultures may pose a challenge if fine control is required.

4.3 Delivery of Feed Medium

In addition to medium design and determination of the required amount to be added, the mode of medium delivery in terms of the frequency and amount fed at each interval, must be optimized. The way feed medium is delivered is constrained by both physical limitations in equipment and by feed medium composition. The feed medium usually consists of a solution of concentrated amino acids and other organic nutrients. The solubility limit for amino acids and the tendency of the concentrated solution to precipitate impose practical constraints.

The main consideration in determining the proper feeding frequency is the acceptable range of nutrient concentration (e.g. the amount of over-feeding at the time of addition and the extent of nutrient depletion immediately before the next feeding). More frequent feeding reduces the deviation from a set point. If feeding is coupled to on-line measurements such as base addition, turbidity, or OUR measurement, feeding can be continuous, and is usually implemented by computer control. When feeding is coupled to less frequent off-line measurements, medium is typically added manually, a few times daily.

Processes employing continuous feeding to control nutrient levels in a small range require substantially more effort to develop than intermittent feeding strategies. While allowing more control over environmental conditions, direct evidence of the superiority of continuous feeding in terms of extending culture lifespan and increasing productivity has not been clearly documented, except for the case where a metabolic shift has been implemented. In fact, with simple off-line monitoring, robust, intermittent feeding strategies have been shown to yield satisfactory results [40]. Most fedbatch cultures are operated in two different stages: a growth phase and a production phase. During growth phase, the primary objective is to reach maximum cell concentration in as short a time span as possible. When the cell concentration approaches its maximum, a transition to a stationary or production stage occurs, where the process objectives change from providing optimal growth conditions to providing conditions to maintaining cell viability and maximize specific productivity. During the growth stage, maintaining relatively constant, optimal growth conditions by more continuous feeding is likely beneficial. Intermittent addition of large quantities of nutrients, causing large environmental fluctuations, induces stress and may decrease the maximum growth potential. In the production phase, maintenance of steady optimal conditions may not be advantageous. In fact, it is a common strategy to enhance protein secretion by introducing sub-optimal conditions by

reduction of temperature, introduction of osmotic shock, or addition of mild cytotoxic substances like sodium butyrate [41–50]. Conceivably, intermittent addition of medium to introduce rapid changes in the culture environment may stimulate protein production. Systematic comparisons of different feeding modes will certainly illuminate the development of an optimal strategy.

5
On-line Estimation for Control of Stoichiometric Feeding

A challenging issue of controlled stoichiometric feeding is the adjustment of the feeding rate, or even feed composition in response to metabolic changes (varying stoichiometric ratios) throughout a culture [12]. This is particularly critical when fedbatch cultures are used to elicit a metabolic shift. Changes in metabolism over the course of a culture are commonly seen, as is evident by the nonlinear relationships between specific nutrient consumption rates. Many such changes bear little consequence on cell growth or productivity, but in some cases, the affects are profound. For simplicity, major changes in feed composition or feed rate are only made when profound changes in metabolism are observed. For example, in late stages of growth, some cells cease to produce lactate and consume glucose at a slower rate. In some cases, the trend of increasing lactate concentration in culture is reversed. Lactate is sometimes transported back into the cells and consumed, presumably, re-entering the energy metabolism pathways. The reversal of fluxes required for this type of metabolic change, though not well studied, warrant a change in feeding strategy.

Most major changes are reflected in the metabolic rates of glucose, lactate and glutamine, and are easily detected by tracking their stoichiometric ratios throughout the course of a culture. Detecting changes in stoichiometric ratios for these key species off-line is relatively straightforward, as many enzyme-based assays are available; however, with off-line measurements, one is limited by the sampling frequency and lag time between a measurement and detection of a true alteration in metabolism. More frequent, on-line measurement of nutrients would allow for timely detection of changes in stoichiometric ratios; however, its widespread implementation in industrial processes will require further development of reliable, automated sampling methods. Most on-line nutrient measurement methods rely on an automated sampling device that separates biomass from the medium. This can difficult to implement continuously. In the past decade, advances in continuous cell removal for perfusion culture has allowed for more reliable continuous production processes. Perhaps these same separation techniques can be further developed for application in continuous on-line nutrient measurement. As these technologies develop, control schemes that integrate adjustment of feed

composition and feed rate according to real-time assessments of metabolic state can be implemented.

The following example illustrates the value of such on-line measurements for specific rate estimation and adjustment of nutrient feeding in fedbatch cultures. We will consider a realistic case where the stoichiometric ratio, $\alpha_{GL} = q_G/q_L$, changes during the culture. A change in carbon energy metabolism in cultured mammalian cells is reflected in the specific rates of lactate production and oxygen uptake. With the on-line measurement of OUR, medium feed rate, base addition rate, pH and culture volume, the specific growth rate, specific lactate production rate, and specific oxygen consumption rate can be estimated and used to track changes in stoichiometric consumption of oxygen and lactate [51].

In addition to this, turbidity (or an on-line cell counting device) can be used as an on-line estimate of total cell concentration with the assumption that the measured turbidity (Tb) is proportional to cell concentration (x).

$$\frac{1}{x}\frac{\mathrm{d}x}{\mathrm{d}t} = \frac{1}{Tb}\frac{\mathrm{d}Tb}{\mathrm{d}t} = \mu \,. \tag{7}$$

At times, a non-linear proportionality between turbidity and cell concentration is observed in some concentration ranges, and a correction factor, usually an empirically fitted exponent value (γ), is used.

$$x = x_0 + k(Tb - Tb_0)^{\gamma} \,. \tag{8}$$

As metabolism changes, the specific rate of lactate production (approximated by base addition rate, and turbidity) will vary. Assuming that the protons driving pH changes are derived entirely from lactate, and that the buffering effect of bicarbonate is negligible, the change in proton concentration can be described as follows.

$$V\frac{\mathrm{d}H^+}{\mathrm{d}t} = q_L x V - F_B \cdot B \,. \tag{9}$$

As base is added to control pH, the H^+ concentration is essentially maintained at a constant concentration, and the lactate production rate (q_{LxV}) and the specific production rate (q_L) is related by the following simplification of Eq. 9.

$$q_L x V = F_B \cdot B \,. \tag{10}$$

Using the on line turbidity measurement, the following expression can be used to obtain an estimate of the specific lactate production rate.

$$q_L = \frac{F_B \cdot B}{Tb \cdot V} \,. \tag{11}$$

On-line specific oxygen consumption rate is calculated similarly using OUR and turbidity.

$$q_O = \frac{\mathrm{OUR}}{Tb} \,. \tag{12}$$

With these on-line estimations, the stoichiometric ratio of lactate and oxygen can be measured.

$$\alpha_{LO} = q_L/q_O \,. \tag{13}$$

Knowing only α_{LO} in real-time is not sufficient to obtain an accurate estimate of other important stoichiometric ratios. Consider the case that glucose feeding rate, F, is to be estimated using only on-line data. The current glucose concentration (G) and the current specific glucose consumption rate are not measured. A mass balance for glucose concentration is written as follows.

$$V\frac{dG}{dt} = FG_O - q_G x V \,. \tag{14}$$

Although q_L, q_O, G_O, x, V and the current feed rate, F, are all known, both dG/dt and the current specific glucose consumption rate, q_G, still are unknown. If the stoichiometric ratios, q_L/q_O and q_L/q_G, are constant, the following simple relationship would determine q_G.

$$\frac{q_L}{q_O} = \delta\frac{q_L}{q_G} \,. \tag{15}$$

As discussed earlier, in the case where a culture is intended to elicit a metabolic shift to a lower lactate producing state, q_G/q_O varies as cells change from fermentative type of metabolism to more oxidative metabolism, and the proportionality constant, δ, varies as the metabolism changes. Changes in specific consumption rates result in a deviation from the set-point, and dG/dt becomes nonzero. Addition of a direct on-line measurement of glucose concentration is necessary in this case for accurate feed rate estimation during periods where stoichiometric nutrient consumption ratios are changing.

In the special case that a specific cell line is cultivated under well-characterized conditions, an empirical correlation for δ can be obtained from historical data and used for such control when on-line glucose measurement is unavailable. An example of the historical data that could be used to derive such a correlation is shown in Fig. 5, where data from more than 30 hybridoma fedbatch cultures are plotted in a single chart. Such correlations are inevitably subject to a large margin of error due to differences in operation parameters. For cells in a high lactate producing state, a wide range of glucose concentrations will not significantly affect stoichiometric nutrient consumption, and the margin of error in the high q_L/q_G range is broad. The spread of data narrows significantly, as cells are cultivated under conditions with smaller $\Delta L/\Delta G$ ratios, allowing a sufficiently accurate correlation to be determined for control of glucose consumption during the transition from a high lactate producing state to a low lactate producing state.

Such archives of accumulated kinetic data for a single cell line have been used to develop a few examples of model-based, adaptive control algorithms

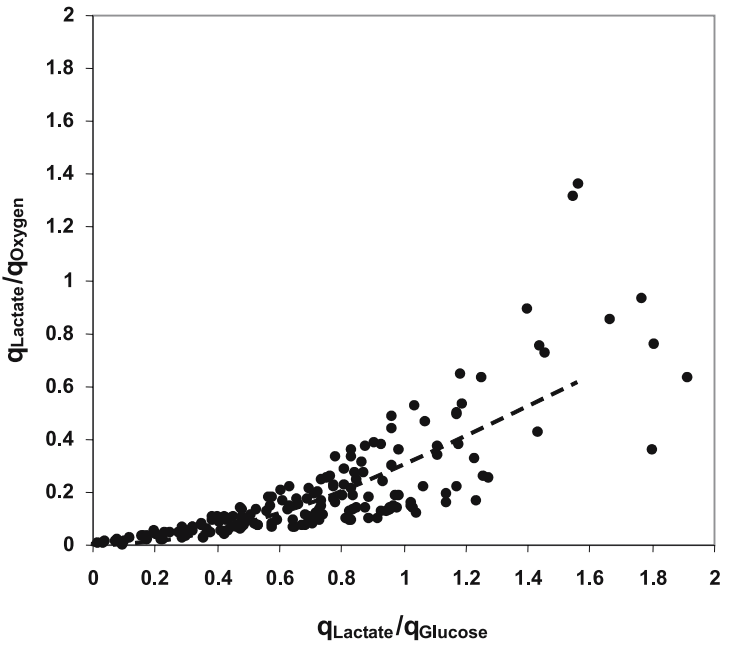

Fig. 5 Correlation of stoichiometric ratio to metabolic state ($\Delta L/\Delta G$). Data compiled from 30 fedbatch cultures illustrate that a correlation exists between the stoichiometric ratio of lactate to oxygen and the metabolic state ($\Delta L/\Delta G$)

with an ability to adjust feed rates according to detected changes in physiological state. This subject has been recently reviewed by Portner et al. in 2004 [52]. These strategies are referred to as open-loop-feedback-optimal (OLFO) strategies. Based on previous culture results, an a priori feed trajectory is determined, then adjusted during a culture according to detected changes in culture conditions (e.g. set-point deviations). In these strategies, widely spaced, intermittent off-line measurements can provide a sufficient guide for adjustment of the feed rate to match changing nutrient demands. Such strategies provide the flexibility required to for control under unpredicted conditions, but still rely heavily on accumulated data for a single cell line to be successfully implemented.

6
Factors Limiting Cell Concentration and Productivity in Fedbatch Cultures

In the past decade, intensive efforts to develop fedbatch and perfusion processes have resulted in a nearly ten-fold increase in maximum cell concentrations and an over ten-fold increase in final product titers. These substantial

improvements are largely responsible for the current success of recombinant protein production in mammalian cells. Even with these improvements, the viable cell concentration achieved still falls short of that in vivo by more than an order of magnitude. The maximum possible cell and product concentrations that can be attained through further process development remains to be seen.

With current culture technology, maximum cell concentrations are limited by environmental and biological factors that are not well understood. Excess metabolites, osmolarity, reactive oxygen species (ROS), and a wide variety of other factors can lead to cell death via apoptosis. If these biological factors were eliminated, much higher cell and product concentrations would likely result. From a practical standpoint, the maximum concentration of nutrients that can be conveniently dissolved for fedbatch medium is only about 15 times more concentrated than typical maintenance medium. If a fedbatch culture is started at 40% of its maximum volume with 1× medium, and is fed 15× medium to full volume capacity, the total nutrients provided to the culture would be 9.4× the concentration of the start up medium ($15\times0.6 + 1\times0.4$). Assuming the cell concentration attainable in 1× medium is approximately 2.5×10^6 cells mL^{-1} under typical culture conditions and further assuming that 9.4× nutrient content would support 9.4 times the cell concentration, a maximum cell concentration of 2.4×10^7 cells mL^{-1} can be expected.

This simple estimate was based on cells growing in a state characterized by a high $\Delta L/\Delta G$ ratio, where excess metabolites are produced. It is likely that an even higher concentration could be reached by operating in a more efficient metabolic state (characterized by a lower $\Delta L/\Delta G$ ratio). The average specific nutrient consumption rate for cells growing in a low $\Delta L/\Delta G$ state is approximately three fold less than that observed in cells growing with a high $\Delta L/\Delta G$ ratio. The yield of cells on 1× medium could potentially increase three-fold, allowing for a maximum cell concentration of nearly 7×10^7 cells mL^{-1}. Such an estimate of the limit of achievable maximum cell concentration is obviously subject to debate and assumption. Increasing the feed nutrient concentration, or even resorting to a feed in the form of a solid, rapidly dissolvable medium, would certainly alter this calculation.

Another obvious constraint is oxygen consumption. At an oxygen consumption rate of 4×10^{-10} mmol $cell^{-1}$ hr^{-1}, the oxygen uptake rate of a culture with a cell concentration of 7×10^7 cells mL^{-1} would be almost 30 mmol L^{-1} hr^{-1}. Although high, such a requirement is not completely out of reach. Using an aeration rate of 0.1 vvm would supply about 50 mmol L^{-1} hr^{-1} of oxygen in the gas phase. With the increased cell concentrations, and assuming that the specific antibody productivity can be maintained at high levels, such cultures could easily achieve titers on the order of 10–20 g L^{-1}. Such a concentration exceeds that typically attained from mouse ascites. Whether there will be an associated stability issue at such a high antibody

concentration remains to be seen. It is clear that the cell and product concentrations currently seen in processes can likely be raised to even higher levels by further enhancement of fedbatch processes.

To achieve the upper limits of cell and product concentrations in fedbatch cultures, a number of environmental and biological factors contributing to growth inhibition and degeneration of the culture productivity will need to be further addressed. As mentioned, the effects of excess lactate and NH_3 formation from glucose and amino acid metabolism are among the first factors to affect cell growth and metabolism. To address the potential limitations on productivity due to the cell's inherent metabolic characteristics, there have been increasing attempts of metabolic engineering to alter cells. Cell lines that grow in the absence of glutamine have been generated by introduction of the glutamine synthetase gene, which converts glutamate to glutamine. In addition to reducing ammonia accumulation by enabling cells to grow in the absence of glutamine, the glutamine synthetase gene functions as a selectable, amplifiable marker (using methionine sulfoxamine, MSX) for introduction and high level expression of recombinant antibodies or proteins in both mouse (NS0) cells [53, 54] and CHO cells [55, 56]. Its use as a selectable, amplifiable marker is referred to as the GS system. Additionally, it has been found that many cell lines can be adapted to glutamine-free medium when substituted with sufficient glutamate or other amino acids and intermediates [57, 58]. Recently, it was reported that glutamine was completely replaced by pyruvate, resulting in essential elimination of ammonia formation in attached MDCK, BHK, and CHO cell lines [59]. The use of this strategy in completely defined medium with suspension-adapted, recombinant product producing cell lines has not yet been reported.

Clearly, simultaneous reduction of both lactate and ammonia accumulation in fedbatch processes would be highly desirable. Such a reduction of both metabolites has been achieved by controlling both glucose (0.2 mM) and glutamine (0.2–0.4 mM) at very low concentrations, and was accompanied by a marked increase in cell concentration [8]. As mentioned in the preceding section, such control is difficult to implement in different cell lines without extensive effort, making its widespread use in large-scale processes difficult.

Reduction of both lactate and ammonia in large-scale bioprocesses is more likely to be achieved by advances in medium and cell line development. The use of alternative sugars (in place of glucose) is being re-visited as a practical method for reducing lactate production. Early in the development of cell culture technology, alternative sugars (i.e. galactose, fructose, sucrose, ribose, mannose) were shown to allow cell survival and growth for a wide range of cell lines [60–62]. Generally, most cells are able to survive well in medium with glucose completely replaced by other sugars; however, slightly decreased growth rates, markedly reduced lactate formation, and increased glutamine consumption and ammonia production rates were observed [3, 4, 63–67]. The

use of alternative sugars for achieving high cell and product concentrations in fedbatch culture has not been fully explored in the context of some of the more recent advances in bioprocess technology.

Recently, a fedbatch process that reduced lactate accumulation by intermittently replacing glucose with galactose, was used to increase cell concentration. A CHO cell line was first adapted to utilize glutamate in place of glutamine, resulting in reduced ammonia accumulation [68, 69]. The glutamate-adapted cells were inoculated into a fedbatch reactor in glucose-containing medium, and grown to a high density with a high rate of lactate production. When glucose was nearly depleted, a galactose containing feed medium was added. With essentially no glucose present, galactose and lactate were consumed simultaneously while cell proliferation continued [70, 71]. With the reduced metabolite accumulation, such strategies could increase the cell concentration and the duration of the production phase, leading to increased product titers.

In exploring such changes in medium design and feed strategies, one must be mindful that in the production of biologics, quality of the product is a primary concern, especially the glycoform distribution. It is known that fedbatch culture processes may produce products with glycoform different than those produced in batch culture. It is possible that with more process variables available for manipulation, products may have an even wider spread of glycan distribution over different runs. When low sugar concentrations or alternative sugars are used in a fedbatch culture, it will be prudent to further examine the effects on productivity and glycosylation. One study reports varied antibody production in hybridoma cells, when cells were cultured in fructose as opposed to in glucose [72]. Another study shows observable differences in binding kinetics of antibody produced in hybridoma cells cultured in varied concentrations of glucose, fructose, mannose, or galactose [73].

Genetic engineering of cell lines for enhanced metabolic properties is also an attractive alternative that warrants further exploration. Reduction of lactate production by disruption of lactate dehydrogenase expression [74, 75], over-expression of the cytoplasmic pyruvate carboxylase enzyme [76–78], and alteration of sugar transporters are all being explored [79, 80]. These examples, along with other work related to creating superior cell lines for recombinant protein production are reviewed in this series in detail by Seth et al., specifically regarding the development of more robust cell lines through the introduction of anti-apoptotic genes. More robust cell lines with enhanced resistance to adverse culture conditions will help in the pursuit of achieving the practical limits of cell concentration in fedbatch culture.

7 Concluding Remarks

Fedbatch culture processes have become a reliable, flexible, and scalable means for operation of most cell culture processes. Its wide adoption facilitated the surge of increased productivity of cell culture processes in the past decade, yet there is still the potential and desire for further advances in process enhancement. Better understanding of cellular physiology, improved medium design, reliable on-line instrumentation, and improved feeding rate control hold great promise in its further development. Additionally, the use of cell engineering to confer cells with many characteristics favorable for sustaining high viability and productivity, while maintaining consistent product quality is likely to contribute significantly to future process development. It is likely that a five to ten fold increase in product concentration, beyond current levels, can be accomplished.

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The "Push-to-Low" Approach for Optimization of High-Density Perfusion Cultures of Animal Cells

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Abstract High product titer is considered a strategic advantage of fed-batch over perfusion cultivation mode. The titer difference has been experimentally demonstrated and reported in the literature. However, the related theoretical aspects and strategies for optimization of perfusion processes with respect to their fed-batch counterparts have not

been thoroughly explored. The present paper introduces a unified framework for comparison of fed-batch and perfusion cultures, and proposes directions for improvement of the latter. The comparison is based on the concept of "equivalent specific perfusion rate", a variable that conveniently bridges various cultivation modes. The analysis shows that development of economically competitive perfusion processes for production of stable proteins depends on our ability to dramatically reduce the dilution rate while keeping high cell density, i.e., operating at low specific perfusion rates. Under these conditions, titer increases significantly, approaching the range of fed-batch titers. However, as dilution rate is decreased, a limit is reached below which performance declines due to poor growth and viability, specific productivity, or product instability. To overcome these limitations, a strategy referred to as "push-to-low" optimization has been developed. This approach involves an iterative stepwise decrease of the specific perfusion rate, and is most suitable for production of stable proteins where increased residence time does not compromise apparent specific productivity or product quality. The push-to-low approach was successfully applied to the production of monoclonal antibody against tumor necrosis factor (TNF). The experimental results followed closely the theoretical prediction, providing a multifold increase in titer. Despite the medium improvement, reduction of the specific growth rate along with increased apoptosis was observed at low specific perfusion rates. This phenomenon could not be explained with limitation or inhibition by the known nutrients and metabolites. Even further improvement would be possible if the cause of apoptosis were understood.

In general, a strategic target in the optimization of perfusion processes should be the decrease of the cell-specific perfusion rate to below 0.05 nL/cell/day, resulting in high, batch-like titers. The potential for high titer, combined with high volumetric productivity, stable performance over many months, and superior product/harvest quality, make perfusion processes an attractive alternative to fed-batch production, even in the case of stable proteins.

Keywords Animal cell culture · Antibody production · Media development · Perfusion process optimization

Abbreviations

CSPR	Cell specific perfusion rate (nL/cell/day)
D	Dilution rate (fermentor volumes/day)
OP	Operating point
OTR	Oxygen transfer rate (mM/L/day)
OUR	Oxygen uptake rate (mM/L/day)
QP	Specific production rate (pg/cell/day)
RT	Residence time (h)
SGR	Specific growth rate (1/day)
t	Time
V	Fermentor volume (L)
VP	Volumetric productivity (mg/L/day)
X	Cell concentration in fermentor (cells/mL)
X_H	Cell concentration in harvest (cells/mL)

1 Introduction

Over the last several years, it has become evident that the success of perfusion technology depends to a great extent on our ability to dramatically reduce the volumetric perfusion rate. Ideally, the perfusion rate would be around 1 volume/day, resulting in a high, batch-like titer and low liquid throughput. In combination with high cell densities of $20–60 \times 10^6$ cells/mL and superior product quality, this would significantly enhance the economic potential of perfusion technology.

However, the reduction of perfusion rate depends on multiple factors, including the relationship between specific productivity and specific perfusion rate, the medium formulation and cost, the half life of the product, and the dependence of product quality on fermentor residence time. As the perfusion rate is decreased, a limit is reached below which cultivation is impossible due to poor growth, decline in specific productivity, product degradation, or compromised product quality. The main directions in research to overcome these problems are: (1) development of media with enhanced "depth"; (2) systematic evaluation of the effect of ultralow perfusion rates on cell physiology and productivity; (3) protection of the product from degradation.

In the case of a stable protein, the concern about product degradation is minimal. The optimization objective is simplified to the development of a medium and a feeding strategy that enables operation at low perfusion rate while maintaining good cell growth, viability, and specific productivity. To this end, the "push-to-low" optimization technique has been developed and successfully applied. This approach involves an iterative stepwise decrease of the specific perfusion rate in highly instrumented, computer controlled fermentors. The cell density is maintained constant, at a maximum level. At each optimization step, a steady metabolic state is established, and the performance of the cell culture is evaluated. This involves monitoring of key physiological variables, including growth rate, cell death, specific production rate, as well as the concentration of selected nutrients and inhibitory metabolites. Based on this analysis, a decision on whether and how to perform another push towards lower perfusion rate is made. If necessary, the medium formulation is "in-process" modified at each step, so that medium depth progressively increases over the course of the optimization. The process continues until the lowest possible perfusion rate is reached.

The push-to-low technique was used in the optimization of a murine hybridoma perfusion process for production of antibody against TNF. Starting from standard conditions and medium, the perfusion rate was successfully decreased several fold. This resulted in a significant increase in antibody titer, while maintaining good growth and viability. A substantial improvement of the process was achieved, positively impacting the up- and downstream manufacturing steps. In general, our results suggest that for the production of

stable proteins, the operation of perfusion cultures at low feed rate is physiologically possible, economically feasible, and should be considered as a major direction for perfusion culture optimization.

2 Materials and Methods

2.1 Cell Line, Medium, and Fermentation System

Mouse-mouse hybridoma cells producing a monoclonal antibody against TNF were cultured in a proprietary medium buffered with 2.0 g/L $NaHCO_3$, and supplemented with glucose and glutamine. All experiments were conducted in 15 L fermentors equipped with external cell retention devices (Fig. 1). DO was maintained at 50% air saturation by diffusing oxygen through silicone tubing. The agitation speed was kept constant at 80 rpm and pH was controlled at 6.8 by automatic addition of 0.3 M NaOH or CO_2. The fermentors were inoculated at an initial cell density of approximately 1.0×10^6 cells/mL. Cell density was maintained at a set point of 20×10^6 cells/mL according to the control logic described below [1].

2.2 On-Line Measurements and Off-Line Analyses

DO and pH were monitored by retractable Ingold electrodes (Ingold Electrodes, MA). The accuracy of the on-line measurements of DO and pH was confirmed off-line using a NOVA blood gas analyzer (NOVA Biomedical, MA). The same instrument was used to quantify the dissolved CO_2 concentration. Cell density was monitored by a retractable optical density probe (Aquasant Messtechnik, Switzerland) calibrated to display the cell number. Calibration was checked daily and recalibration was performed when deviation from the off-line cell counts was detected. Generally, the probe performed reliably, requiring only infrequent, minor adjustments.

The fermentor and the harvest were sampled on a daily basis. The cell concentration was determined by averaging several hemacytometer counts. Cell viability was estimated via trypan blue exclusion. Cell size was determined by an electronic particle counter CASY (Scharfe Systems, Germany). The glucose and lactate concentrations were measured off-line using a YSI Model 2700 analyzer (Yellow Springs Instruments, OH). A modification of the same instrument, equipped with appropriate enzymatic membranes and software, was used for glutamine and glutamate assay. Ammonia was measured by Ektachem DT60 analyzer (Eastman Kodak, NY). Apoptosis was quantified following the standard An-

nexin V and Apo 2.7 (Clontech, CA) procedures provided by the indicator dye manufacturer.

Product concentration was determined by a nephelometric assay. To quantify and compare product quality (integrity and glycosylation) under different conditions, fermentor harvest was collected during steady state fermentation periods. Before purification, the harvest was passed through a cell separation filter, and concentrated by ultrafiltration.

2.3 Control of Cell Density

A prerequisite for the success of the perfusion culture optimization experiments is reliable long-term monitoring and control of cell concentration. Stable control cannot be achieved if the perfusion system relies on its "natural", chemostat-like equilibrium between growth and washed out cells. The drifts in the specific growth rate and in the harvest cell density often result in large fluctuations of fermentor cell density even if the perfusion rate remains unchanged. To enable robust control, an additional factor referred to as "cell discard rate", *CDR* (measured in L/day), needs to be introduced as described by the following equation:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu \cdot X - D \cdot X_{\mathrm{H}} - \frac{CDR}{V} \cdot X\,. \tag{1a}$$

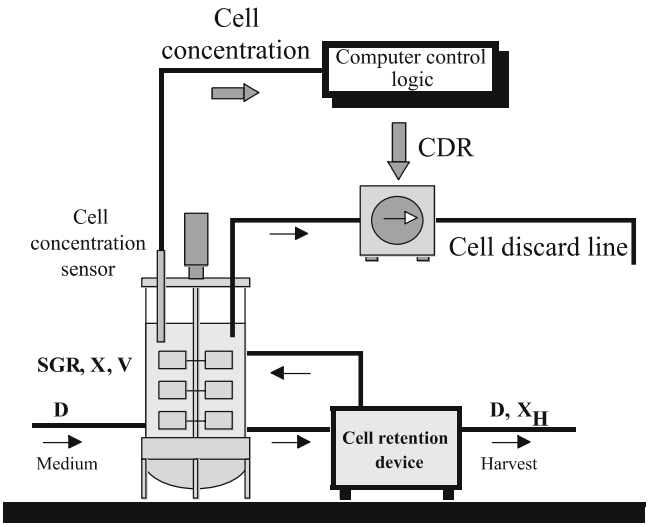

Fig. 1 Scheme of the 15 L perfusion fermentor system equipped with an external cell retention device and CDR-based cell density control

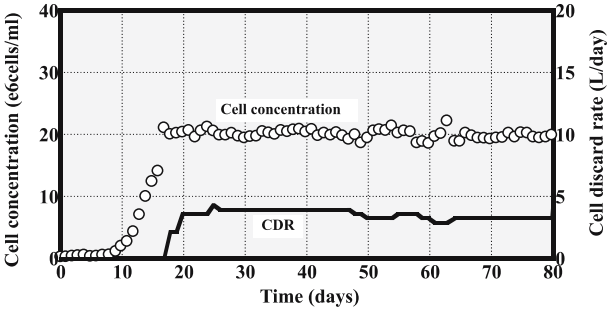

Fig. 2 Reliable CDR-based control of cell density in a perfusion animal cell culture over a period of 80 days

Assuming steady state, the expression is simplified to:

$$X = \frac{D}{\mu - \frac{CDR}{V}} X_{\mathrm{H}} \,, \tag{1b}$$

where μ is the apparent specific growth rate, X and X_{H} are the fermentor and harvest cell density, respectively, V is fermentor volume, and D is the perfusion rate (note that the term "perfusion rate" used in this paper is equivalent to "dilution rate"). The scheme of the CDR-based cell density control system is shown in Fig. 1. Cell concentration is computer controlled in a closed loop at the desired set point below the natural equilibrium by automatic removal of the extra cells from the fermentor. Excellent control can be achieved using this scheme, which guarantees long-term stable operation and high quality optimization data (Fig. 2).

2.4 Specific Perfusion Rate and Medium Depth

The cell-specific perfusion rate (*CSPR*) is a composite variable routinely used in monitoring and control of Bayer perfusion processes [2]. Its calculation is simple and requires only D and X (monitored either off-line or on-line):

$$CSPR\,(\mathrm{nL/cell/day}) = \frac{D(\mathrm{L/L} \times \mathrm{day})}{X(10^6\ \mathrm{cells/mL})} \,. \tag{2}$$

CSPR represents the volume of medium given to one cell in one day. Depending on the process, *CSPR* may vary widely, typically in the range 0.05–0.5 nL/cell/day. *CSPR* does not provide direct information about cell metabolic activity. Therefore, perfusion control based on *CSPR* remains fundamentally open loop with respect to cell physiology. The underlying assumption of the *CSPR*-based feed control is that cells are always in the same physiological state, disregarding possible metabolic changes that may occur during the process [3]. Despite its limitations, however, *CSPR* is indispensable

in quantifying and controlling perfusion cultures, conveniently "packaging" all medium components into a single entity. In comparison, other strategies, such as the glucose-based perfusion control [4], rely on a single medium component, assuming one-to-one relationship between glucose uptake and the overall cellular metabolism.

Another advantage of *CSPR* is that it links key perfusion process variables, such as titer, specific productivity (QP), cell density, and volumetric productivity (VP):

$$TITER = \frac{QP}{CSPR} \tag{3}$$

$$VP = TITER \times D = X \times QP\,. \tag{4}$$

CSPR is also closely related to the term "medium depth", which is often referred to in this paper. The medium depth is the reciprocal of the lowest possible *CSPR* ($CSPR_{\min}$):

$$MEDIUM\ DEPTH = \frac{1}{CSPR_{\min}} = \frac{X_{\max}}{D} \tag{5}$$

and represents the maximum number of cells that can be supported by 1 mL of medium in 1 day. For example, if $CSPR_{\min} = 0.1$ nL/cell/day, then medium depth is 10×10^6 cells/mL/day.

3
Conceptual Framework for Optimization of Perfusion Cultures

Before discussing the experimental data, it will be useful to outline the conceptual framework of our study. This focuses on some general aspects of fed-batch and perfusion cultivation modes. Although the issue is not new, the publications are still controversial [5, 6]. Our goal is to interpret the subject in view of some emerging trends in perfusion technology.

3.1
Application of Fed-Batch and Perfusion

Numerous publications dealing with the choice of cultivation method give the impression that one of the existing approaches – batch or perfusion – is clearly superior [7–13]. It is the authors' opinion that the question "which process is better – batch or perfusion?" is conceptually wrong, and that the right question asks when to use batch and when perfusion. At the present state of development of fermentation technology, it is unreasonable to look for a single universal answer.

There are several "easy" cases in which it is relatively straightforward to select the optimal process mode. In general, products prone to degradation require perfusion. So does a cell line that produces only in an active growth stage, the situation known as "growth-associated" production kinetics. On the other hand, a fed-batch approach may be favored in the case of high medium costs, where titer significantly affects the cost-of-goods. Fed-batch would also be the method of choice when cells secrete product in a non-proliferative state, or if the cell line is unstable, so that the production time horizon is limited. Unfortunately, many real situations fall in the gray zone between these "easy" cases, and the batch-or-perfusion decision can be difficult. The choice is often based on company tradition, existing facilities, infrastructure, and experience. Nevertheless, there is a growing interest in high-density perfusion culture, rationalized by some of the advantages of perfusion technology. These include superior product quality, steady state operation, excellent culture control, and high culture viability. Further development of perfusion technology is likely to result in more efficient processes operating at high cell densities in the range $40–80 \times 10^6$ cells/mL (providing high volumetric productivity) and ultralow specific perfusion rates below 0.05 nL/cell/day (providing batch-like titer).

3.2 Four Limiting Factors in Perfusion Culture: Determination of the Optimization Space

Perfusion culture is limited by several factors that reflect the physical characteristics of the perfusion system and the properties of the cell culture and the product. The intersection of these factors defines the process optimization space. The four most important are:

1. Maximum allowable residence time (RT_{max}) in the fermentor, defined by product stability. This corresponds to the minimum perfusion rate ($D_{min} = 1/RT_{max}$).
2. Maximum perfusion rate (D_{max}). Typically, D_{max} reflects the volumetric capacity of the cell retention device.
3. Maximum cell density (X_{max}). In most cases, X_{max} is defined by the maximum O_2 transfer rate (OTR) of the fermentor. The OTR limitation reflects the physical characteristics of the fermentor system, as well as the shear sensitivity of the cell culture.
4. Minimum cell-specific perfusion rate ($CSPR_{min}$) defined by the nutritional depth of the medium (Eq. 5).

Figure 3 illustrates the relationship between these factors. This simplified description enables one to define the zone of high D and low X (high $CSPR$, low titer, and low RT) and the zone of low D and high X (low specific perfusion rate, high titer, and high RT). These "natural" limitations are usually not

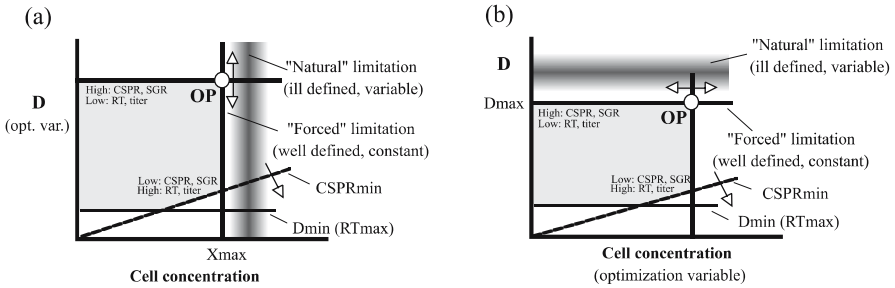

Fig. 3 Limiting factors in perfusion culture: **a** cell density limited culture, and **b** dilution rate limited culture. The optimization subspace is defined by the *gray polygon* (D_{min}, D_{max}, X_{max}, $CSPR_{min}$)

crisp. If the process is left to be controlled by them, large fluctuations would occur. For example, the volumetric capacity of the cell retention device may change over time due to various reasons, such as cell aggregation, fouling, etc. (Fig. 3b). If the perfusion rate is controlled to equilibrate the current cell retention capacity, the fermentor throughput will fluctuate. Similarly, the OTR capacity of the fermentor is likely to change over time due to antifoam addition, fouling of the silicone tubing in case of membrane oxygenation, change in the specific OUR of the cells, etc. If cell density is controlled to match the maximum OTR, then cell density will drift (Fig. 3a).

To provide stable control, the process should not be left to operate at its maximum OTR or D defined by the "natural" limiting (equilibrium) point. Instead, an artificial, "forced" limitation that will keep the process close to, but below, the natural equilibrium shell should be introduced. An example of a forced limitation is the above-described cell discard rate control (Eq. 1). In this case, the fermentation can run for many months at a stable operation point (OP). In this sense, the optimization of the perfusion process can be viewed as an upward or downward sliding of OP on the forced limitation line (Fig. 3), so that a particular optimization criteria is met. In the case of OTR limited culture, cell density will be controlled at a constant level, and *D* will be the optimization variable (OP will slide vertically). If *D* is limiting, perfusion rate will be kept constant below the natural limitation zone, and cell density will be the optimization variable (OP will slide horizontally).

3.3 Product Stability

The first critical task that has to be completed before initiating the series of optimization experiments is to determine the long-term stability of the product under real fermentor conditions. The results can force process development in one or another direction. In terms of stability, the spectra of

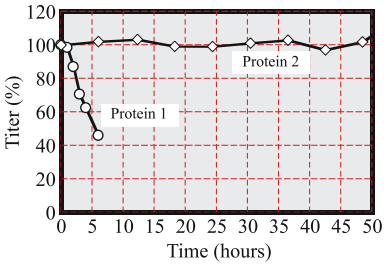

Fig. 4 Degradation of stable and unstable recombinant proteins produced in cell culture. The tests were conducted in supernatant under conditions equivalent to those in a fermentation run. *Protein 1* degrades quickly, while *Protein 2* remains stable for many days

biotechnology derived proteins is broad, ranging from stable to extremely labile molecules that degrade within hours. For example, monoclonal antibodies are usually stable, while large, heavily glycosylated molecules, such as FVIII [14] and ATIII [15], are very labile. Two examples from the authors' laboratory are shown in Fig. 4. While one of the proteins degrades quickly in a matter of hours (half life of about 5 h), the other remains stable for days. Obviously, these two molecules would require different production strategies. Often the degradation depends not only on the protein, but on the cell line itself. Degradation rates of the same protein may vary widely in different cultures [16], most likely due to proteolysis.

To quantify the degradation, a family of product concentration/quality time profiles measured in supernatants from several specific perfusion rates has to be generated. The collected data will enable the determination of the maximum allowable residence time, RT_{max}, possibly as a function of the cell-specific perfusion rate. RT_{max} defines the lowest limit of the process optimization space on the D axis (Fig. 3). In the context of perfusion technology, RT_{max} longer than 24 h defines the product as stable (RT_{max} of 1–3 weeks will be needed for batch), and opens up the bottom area in Fig. 3 for process development at low D. Then, the minimum perfusion rate from a product stability standpoint will be $D_{min} = 1/RT_{max}$.

3.4 Cell Retention

The upper limit of D is typically a result of mechanical limitations. Most often, the bottleneck is the cell retention device, which is characterized by its maximum volumetric throughput rate. In other cases, the limiting factor may be the upstream or downstream operation capacity (medium production or purification). The outcome is that D cannot increase above a certain limit D_{max}, which defines the upper end of the process optimization window for the perfusion rate (D_{min}, D_{max}).

3.5
Maximum Cell Density with Respect to O_2 Transfer Rate

The third key limitation in perfusion culture is the maximum cell density X_{max} that can be supported with respect to the O_2 transfer rate. This restriction depends on the fermentor hardware and the characteristics of the cell line (specific OUR, shear sensitivity), and graphically represents the right-side border of the optimization space (Fig. 3). Assuming growth-independent production kinetics, the volumetric productivity will be proportional to the cell density (see the antibody example below), and for optimal performance the OTR-limited bioreactor should be operated at X_{max}. Then, the key optimization parameter is the dilution rate D, which should be adjusted in the range (D_{min}, D_{max}). In general, one should try to slide the operation point OP on the X_{max} line, so that certain performance criterion is maximized. This optimization strategy, tuned up for the case of stable products, is the main focus of the present paper.

3.6
Minimum Cell-Specific Perfusion Rate (*CSPR*)

The *CSPR* (Eq. 2) cannot be reduced below a certain minimum, $CSPR_{min}$, determined by the nutritional depth of the medium (Eq. 5). Graphically, this limitation is represented by the inclined $CSPR_{min}$ line in Fig. 3. In some cases, this line may cross the $D_{min} = 1/RT_{max}$ line, and become the dominant limitation of D in the area of high cell density. Medium improvement would result in downward rotation of the $CSPR_{min}$ line, and would relax the *CSPR* limitation.

3.7
Optimization Space

The area between the D_{min}, D_{max}, X_{max}, and $CSPR_{min}$ lines defines the process optimization space (Fig. 3) for a given cell line, fermentor hardware, and medium formulation. Of practical interest is the "high X, low D" area, where perfusion culture is most productive. Therefore, in cases of stable product, the optimization will likely result in shifting OP towards the bottom right corner of the polygon.

3.8
Types of Perfusion Optimization Experiments

Table 1 outlines the four types of perfusion optimization experiments. The independent (manipulated) variables are two: the cell concentration (X) that can be easily varied using the control logic described earlier, and the per-

Table 1 Four types of perfusion optimization experiments

	Type I		Type II		Type III		Type IV	
	X	*D*	*X*	*D*	*X*	*D*	*X*	*D*
	const	const	var	const	var	var	const	var
CSPR	constant		variable		constant		variable	
RT	constant		constant		variable		variable	
Goal	Long-term stability evaluation		CSPR optimization (any product)		RT optimization (unstable product)		CSPR optimization (stable product)	

fusion rate (*D*). The dependent variables are also two: the residence time (*RT*) and the cell specific perfusion rate (*CSPR*). Clearly, these should not be perceived as output variables of the process. Instead, *RT* and *CSPR* are two factors in the beginning of the complex cause–effect cascade. Their consideration as dependent variables is practical because they represent two different aspects of the process: nutrition (*CSPR*) and degradation (*RT*). The four optimization experiments discussed below enable decoupling of the phenomena that may be taking place in the perfusion system: growth limitation due to nutrient deprivation (at low *CSPR*), and product degradation due to high exposure time to potentially proteolytic environment (at high *RT*).

Experiment Type I: This is the simplest case, when all variables are kept constant, providing a steady environment for the cells and the product. Such an experiment is most appropriate during the advanced development phase when the optimal *X* and *D* (and also *CSPR* and *RT*) have already been already determined, and the goal is to demonstrate long-term stability. The corresponding time profiles are shown in Fig. 5a.

Experiment Type II: This case is applicable to processes with stable product, not degrading up to time RT_{max}. Then, *RT* is fixed at that set point, and *CSPR* is independently optimized. The latter is varied by changing the cell concentration, as illustrated in Fig. 5b.

To achieve a reasonable variation of *CSPR*, one should target an order-of-magnitude change in cell density. The highest cell density, X_{max}, will correspond to the maximum OTR; the lowest should be in the $X_{max}/10$ range. For example, if X_{max} is 50×10^6 cells/mL, then X_{min} can be in the 5×10^6 cells/mL range. Then, if *D* is fixed at 2.5 volumes/day (*RT* = 9.6 h), *CSPR* would range from 0.05 nL/cell/day (at 50×10^6 cells/mL) to 0.5 nL/cell/day (at 5×10^6 cells/mL).

The goal of this experiment is to quantify the dependence of several metabolic rates, including specific productivity and specific growth rate on *CSPR*. This helps identify the type of production kinetics (growth-associated,

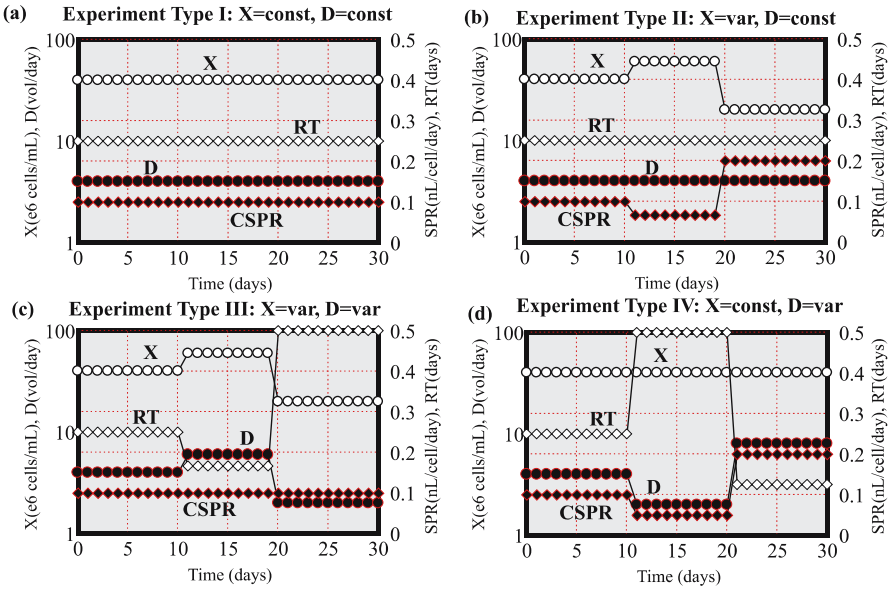

Fig. 5 Expected time profiles of *X*, *D*, *RT*, and *CSPR* in optimization experiments: **a** Type I, **b** Type II, **c** Type III, and **d** Type IV (Table 1)

non-growth-associated, inversely growth-associated). Among these, the non-growth-associated kinetics is preferred for process optimization [17].

Experiment Type III: This experiment is suitable for labile molecules, when the RT effect must be accurately determined. To decouple RT from the nutritional effect, *CSPR* is kept constant and the only variable that changes is *RT*. This is achieved by simultaneous and proportional manipulation of *X* and *D*, so that their ratio, *CSPR*, remains steady while *RT* changes as a function of *D*. It is appropriate to vary *RT* in the range 2–24 h. The left and right limits are difficult to expand. On the low end, *RT* is hard to reduce below 2 h (D_{max} = 12 volumes/day) because of the cell retention capacity (Fig. 3a). On the high side, it is usually impractical to target *RT* > 24 h (D_{min} = 1 volume/day) because the cell density must be decreased significantly to maintain realistic *CSPR*. The corresponding time profiles are shown in Fig. 5c. The key deliverable is the dependence of the apparent specific productivity and product quality on *RT*.

Experiment Type IV: This experiment can be considered when there is no concern about product degradation, so that *RT* can be disregarded as an optimization factor. *X* is maintained at the desired set point, and *D* is changed; *CSPR* and *RT* are not decoupled, and will both vary (Fig. 5d). Since cell density might be more difficult to control at various set points than *D*, the single but significant advantage of this experiment is conve-

nience. It is appropriate to keep cell density around 20×10^6 cells/mL and change *D* in the range 1–10 volumes/day, yielding *CSPR* values between 0.05 and 0.5 nL/cell/day.

3.9
Bridging of Fed-Batch and Perfusion Processes with Stable Products

Figure 6a shows the simulated time profiles of cell concentration and titer of a well developed fed-batch process. The assumptions are constant specific production rate of 20 pg/cell/day, non-growth associated production kinetics, peak cell density of 10×10^6 cells/mL, process length of 10 days, 20% increase of fermentor volume due to feeding, and stable product. Under these conditions, final titer in the fed-batch fermentor is approximately 740 mg/L.

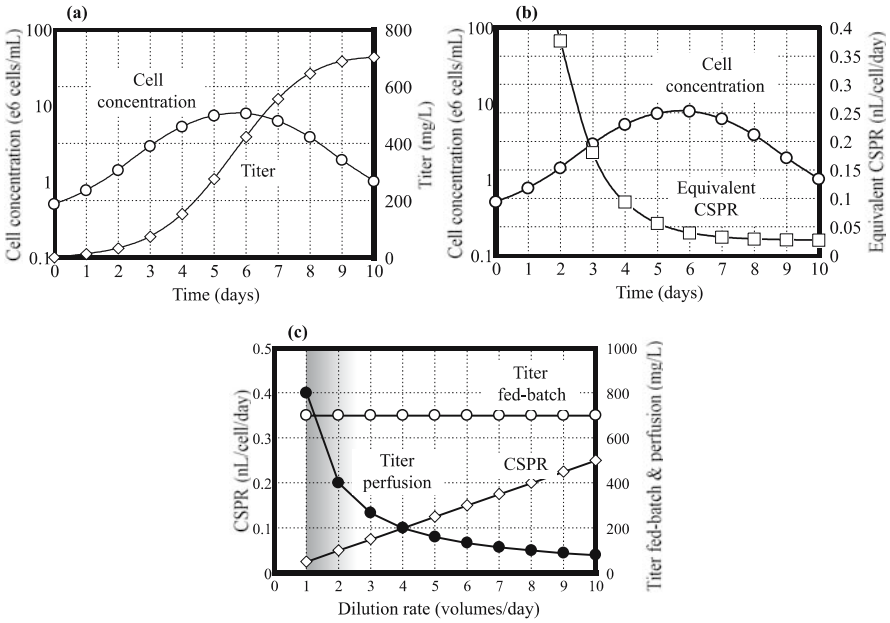

Fig. 6 Profiles of a simulated fed-batch process, and comparison with a perfusion process: **a** fed-batch cell density and titer; **b** fed-batch cell density and equivalent $CSPR_{eq}$; **c** comparison of the end-point fed-batch titer to the titer in a perfusion process run at constant cell density of 40e6 cells/mL and varying dilution rate. The *shadowed zone* indicates the area where fed-batch and perfusion titers are similar

3.9.1 The Concept of the "Equivalent Specific Perfusion Rate" in Fed-Batch Culture

While the concept of *CSPR* was originally developed for perfusion culture, it is possible to introduce a similar variable referred to as equivalent *CSPR* ($CSPR_{eq}$) for fed-batch culture:

$$CSPR_{eq}(t) = \frac{V(t)}{\int_0^t V(t) \cdot X(t)\,dt}, \tag{6}$$

where t is time and $V(t)$ is fermentor volume. This formula is more general than Eq. 2 because it accounts for the dynamic change of V and X. Equation 2 can be derived from Eq. 6 after considering $V(t)$ and $X(t)$ as constant, which is the case in perfusion culture. Equation 6 enables direct quantitative comparison of batch and perfusion processes. Note that the "titer" formula Eq. 3 is correct for both static (perfusion) and dynamic (batch) process. In the latter case, the calculation can be carried out by replacing *CSPR* with $CSPR_{eq}(t)$.

Figure 6b shows the time-profile of $CSPR_{eq}$ in the above-discussed fed-batch. $CSPR_{eq}$ gradually decreases down to an extremely low level of approximately 0.027 nL/cell/day, indicating that less and less medium remains "unconsumed" by the cells with the progress of the process. According to Eq. 3, low *CSPR* translates into low product dilution and higher titer. Considering the numerical data in Fig. 6a, the end-point titer can be calculated using Eqs. 3 and Eq. 6:

$$TITER(t_{final}) = \frac{QP}{CSPR_{eq}(t_{final})} = \frac{QP}{\frac{V(t_{final})}{\int_0^{t_{final}} V(t) \times X(t)\,dt}}$$
$$= \frac{20\ \text{pg/cell/day}}{0.027\ \text{nL/cell/day}} = 740\ \text{mg/L}\,.$$

In the context of our discussion, the reason for the high titer of fed-batch processes is the extremely low $CSPR_{eq}$ that can be achieved at the end of the run, significantly lower than the *CSPR* typically maintained in perfusion cultures.

3.9.2 Comparison of Fed-Batch and Perfusion Titers as a Function of *CSPR*

Figure 6c shows a comparison of the described fed-batch process and its perfusion counterpart. The following assumptions are made about the perfusion process: same specific productivity of 20 pg/cell/day, non-growth-associated production kinetics, constant cell density of 40e6 cells/mL. The optimization

variable is *D*, changing in the range 1–10 volumes/day. *CSPR* decreases linearly with *D* from 0.25 nL/cell/day to 0.025 nL/cell/day.

The perfusion process titer increases in a non-linear fashion with the decrease of *D*. At $D = 10$ volumes/day ($CSPR = 0.25$ nL/cell/day), titer is low (80 mg/L), but reaches 200 mg/L at $D = 4$ volumes/day ($CSPR = 0.1$ nL/cell/day). Further decrease of *D* is followed by a steep increase in titer, reaching 400 mg/L at $D = 2$ volumes/day ($CSPR = 0.05$ nL/cell/day) and 800 mg/L at $D = 1$ volume/day ($CSPR = 0.025$ nL/cell/day). At this point, the perfusion titer has surpassed the fed-batch benchmark. This is possible because the *CSPR* of the former is lower than $CSPR_{eq}$ of the latter. In general, it can be expected that if *CSPR* of the perfusion system goes below 0.05 nL/cell/day, perfusion and fed-batch process become comparable in terms of titer.

However, in reality, fed-batch would always offer the potential of lower *CSPR* and higher titer than perfusion. The reason is that in the final phase of the fed-batch process, the cell culture is sacrificed due to severe nutrient limitation. Viability decreases, often down to 0%. This is not an option in the perfusion process where maintenance of high viability is mandatory. This high viability comes at the price of higher *CSPR* and, correspondingly, lower titer. However, as long as the perfusion process is operated in the far left side of Fig. 6c, the titer difference may not be dramatic. This, in combination with the superior product/harvest quality of perfusion culture (low impurities, low residence time), makes the latter an attractive manufacturing option, even in the case of stable proteins.

3.9.3 The Push-to-Low Optimization Approach

Figure 6c shows that high titer perfusion processes can be developed by the substantial decrease of *CSPR* (low *D*, high *X*, or combination of both). A strategic way to accomplish this task is systematic medium improvement, incrementally reducing the nutrient limitation barrier.

Figure 7 illustrates graphically a concept referred to as push-to-low optimization. The name reflects the incremental in-process shifting of the cell culture towards lower *CSPR*s, typically starting at relatively high values. The process either maintains constant cell density, while *D* is incrementally decreased (Fig. 7a), or *D* is kept steady, while cell density is incrementally increased (Fig. 7c). These two options correspond to experiments Type IV and Type II, respectively (see Table 1). Good initial values of cell density and *D* are 20×10^6 cells/mL and 4 volumes/day, corresponding to a *CSPR* of 0.2 nL/cell/day.

The push-to-low optimization consists of several steps, each of which includes a stepwise decrease of *D* (or increase of *X*), establishing a new steady state, and comprehensive in-process analysis of the residual medium components and specific metabolic rates to discover possible limitations. Key

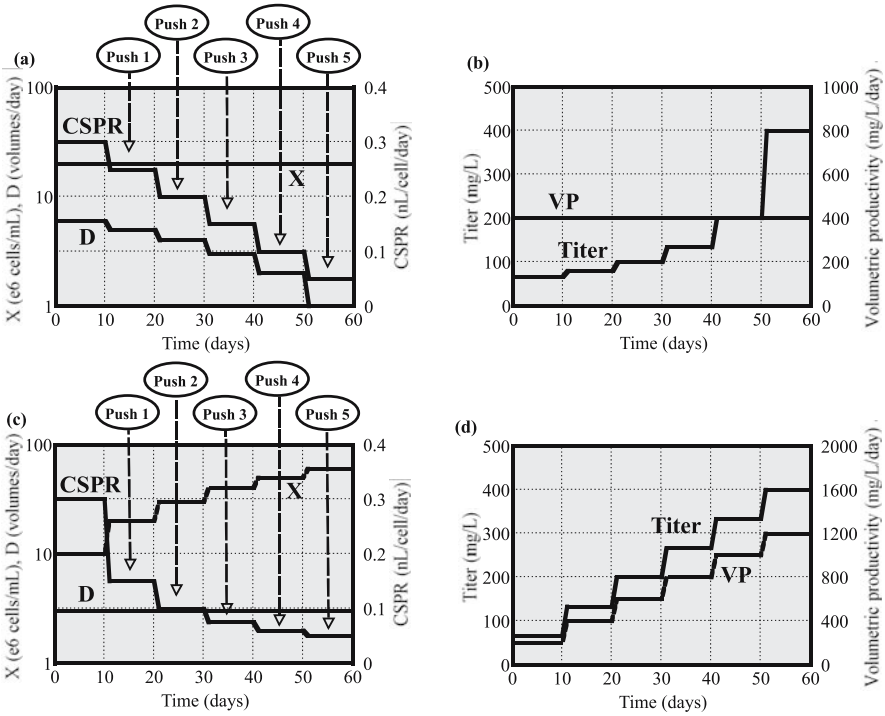

Fig. 7 The push-to-low concept: theoretical profiles of *D*, *X*, *CSPR*, titer and *VP*. **a**,**b** Case when *X* is kept constant, and *CSPR* is "pushed" downwards by decreasing *D*. **c**,**d** Case when *D* is kept constant, and *CSPR* is "pushed" downwards by increasing *X*

physiological state variables, such as specific growth rate, specific O_2 uptake rate, specific glucose uptake rate, and most importantly, the specific production rate, should be closely monitored. If limitation is identified, the medium must be improved before another downward *CSPR* "push" is made. This iterative procedure continues until no further decrease of *CSPR* is possible, and the culture inevitably crashes, even if it appears that all medium components are available. This can be caused by either metabolite inhibition or "hidden" limitation by unknown compounds. The last safe steady state before the crash defines the optimal *CSPR* at which the process should be operated.

4
Results and Discussion

4.1
Push-to-Low Optimization of Hybridoma Culture

The push-to-low optimization approach was applied to a hybridoma culture producing antibody against TNF. The cell concentration was automatically controlled at 20×10^6 cells/mL using the CDR-based control scheme shown in Fig. 1. *CSPR* of 0.3 nL/cell/day ($D = 6$ volumes/day) was considered as stan-

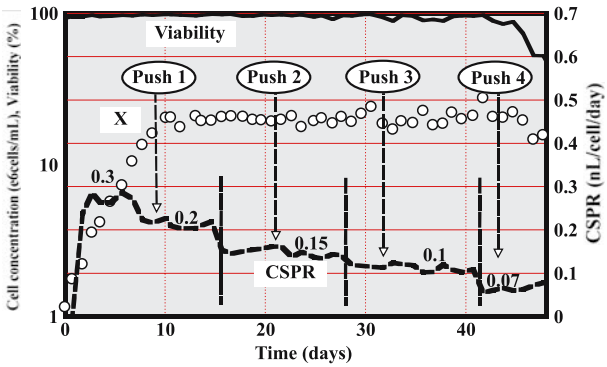

Fig. 8 Time profiles of cell concentration, viability, and *CSPR* in a push-to-low optimization run for production of monoclonal antibody against TNF. *CSPR* was reduced stepwise from 0.3 nL/cell/day down to 0.07 nL/cell/day

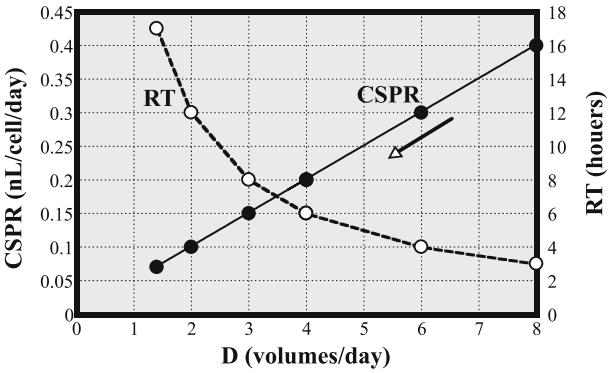

Fig. 9 Dependence of *RT* and *CSPR* on *D* in the push-to-low optimization run for production of monoclonal antibody against TNF. *CSPR* decreased linearly with *D*, which was changed from 8 volumes/day to 1.3 volumes/day. *RT* increased from 3 h to approximately 17 h

dard before the optimization began. This high *CSPR* provided long-term stable operation, but antibody titer was low, and large medium/harvest volumes needed to be stored and processed. The goal of the optimization was to increase titer by stepwise reduction of *CSPR* without compromising volumetric productivity. Figure 8 shows the time profiles of cell concentration, viability, and *CSPR* in one of our experimental runs. During this process, *CSPR* was reduced in several steps: 0.3 – 0.2 – 0.15 – 0.1 – 0.07 nL/cell/day, following experiment Type IV approach (Table 1, Fig. 7a). This was possible because the antibody was found to be stable, and *RT* was not a critical optimization factor. The major optimization variable was *CSPR*, which was manipulated by decreasing *D* from 6 to 1.3 volumes day, corresponding to an increase in *RT* from 3 to 17 h (Fig. 9).

4.2 Dependence of Key Substrates and Metabolites on *CSPR*

Figure 10 shows the dependence of glucose, lactate, glutamine, and ammonia concentration on *CSPR*. Glucose concentration decreased at lower *CSPR*, from ~ 3 g/L at 0.4 nL/cell/day to ~ 1.2 g/L at 0.07 nL/cell/day. Correspondingly, lactate concentration increased from ~ 0.5 g/L at 0.4 nL/cell/day to ~ 1.0 g/L at 0.07 nL/cell day. Neither of these values were significant in terms of substrate limitation or metabolite inhibition.

The profiles of glutamine and ammonia were similar. Ammonia increased from ~ 4 mM to ~ 6 mM with the decrease of *CSPR*, and glutamine decreased from ~ 4 mM to ~ 1 mM. These levels were not in the range where substrate limitation or metabolite inhibition is to be expected.

Steady state amino acid concentrations at *CSPR*s of 0.07, 0.10, 0.15, and 0.20 nL/cell/day are shown in Fig. 11. At each steady state, the amino acids

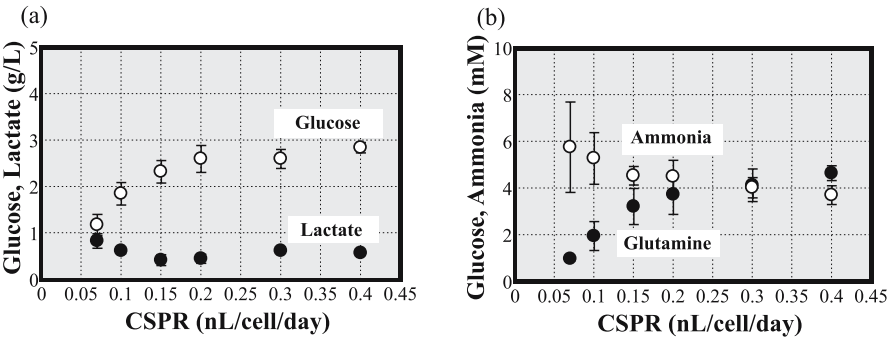

Fig. 10 Dependence of key process substrates and metabolites on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against TNF: **a** glucose and lactate, **b** glutamine and ammonia

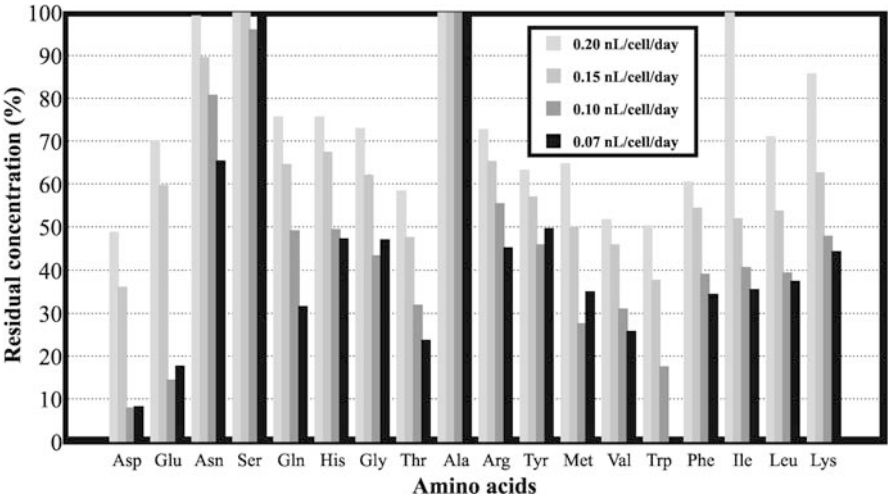

Fig. 11 Dependence of the residual concentrations of amino acids (percent of medium concentration) on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against *TNF*

were analyzed by HPLC, enabling in-process correction of the medium formulation. At all *CSPR*s amino acids were generally above 20% of initial concentration, except for asparagine and tryptophan, which were depleted after the last *CSPR* push.

4.3 Physiological Response to Low *CSPR*

The dependence of the specific growth rate (SGR) on *CSPR* is shown in Fig. 12a. Decrease in *CSPR* slows down cell growth to low levels, reaching practically zero at *CSPR* of 0.07 nL/cell/day. The simplest hypothesis – substrate limitation – was not confirmed by the glucose, glutamine, and amino acids analysis. However, the possibility of a "hidden" limitation by a non-identified medium component cannot be ruled out.

SGR inhibition caused by the accumulation of a toxic metabolite is another plausible hypothesis. Since the well-known metabolites, such as lactate, ammonia, and dissolved CO_2, did not reach toxic levels, their role in inhibition was not obvious. While the possible presence of unknown toxic compounds in the culture supernatant has attracted some attention recently [18–22] only one inhibitor (methylglyoxal) has been well characterized [20]. In any case, an understanding of the low SGR phenomenon is essential for the further development of perfusion technology. Maintenance of an active cell population with good SGR at ultralow *CSPR*s would enable substantial process improvement.

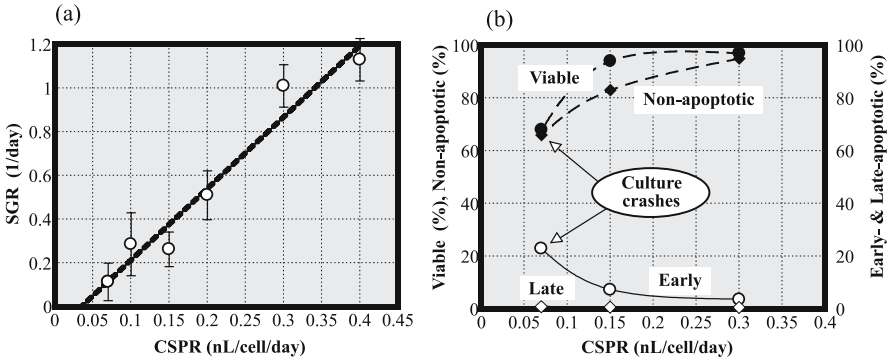

Fig. 12 Dependence of **a** SGR, and **b** concentration of apoptotic cells on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against TNF

Figure 12b shows the effect of *CSPR* on cell viability and on the portion of apoptotic cells. At low *CSPR*, the population of apoptotic cells increases. While it is unclear what the cause of this phenomenon is, the link with the reduction in SGR is obvious. Decrease in apoptosis at low *CSPR* might be achieved by identifying the underlying factor(s), use of apoptosis-resistant cell lines, or application of anti-apoptosis medium additives.

4.4 Dependence of Specific Productivity, Titer, and Volumetric Productivity on *CSPR*

The success of the push-to-low approach depends to a great extent on the type of production kinetics, which varies widely between different cell lines. For example, if specific productivity goes down with *CSPR*, then decrease of the

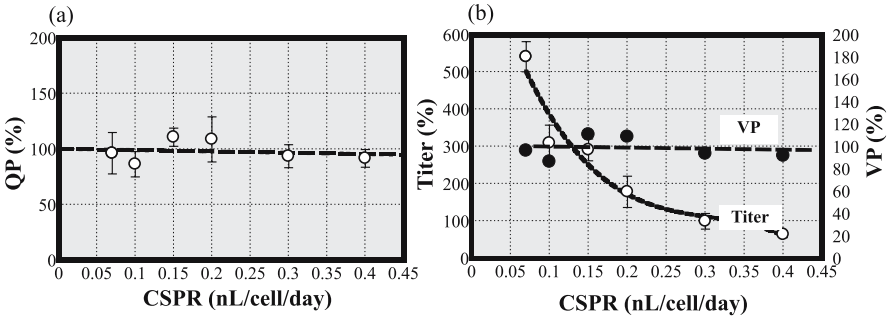

Fig. 13 Dependence of **a** *QP*, and **b** *VP* and titer on *CSPR* in the push-to-low optimization run for production of monoclonal antibody against TNF. Decrease of *CSPR* down to 0.07 nL/cell/day resulted in a ~ 500% increase in titer, while specific productivity, *QP*, and volumetric productivity, *VP*, remained unchanged

latter will likely lower the volumetric productivity. Therefore, it is essential to quantify the relationship between specific productivity and *CSPR*. Figure 13a reveals that in our case specific productivity does not depend on *CSPR*, and cells are producing at the same rate regardless of the *CSPR*-induced changes in their environment. This is the most favorable situation for the application of the push-to-low optimization approach. Since cell concentration was kept constant, decrease of *CSPR* did not change the volumetric productivity (Fig. 13b, Eqs. 3 and 4). However, improvement in titer was significant, closely following the theoretical prediction in Figs. 6c and 7b. Compared to the initial titer obtained in the non-optimized process, the final titer increased approximately 500%, resulting in a simpler process, lower liquid volume to handle, and overall reduction in cost-of-goods.

5 Conclusions

Experimental application of the push-to-low approach for optimization of anti-TNF production can be conveniently illustrated in the context of the *X*–*D* plot introduced earlier (Fig. 3). Figure 14 shows the optimization space of the 15 L development system defined by D_{max} = 10 volumes/day, X_{max} = 20×10^6 cells/mL ("forced" limitation, the "natural" limitation was far beyond 20e6 cells/mL), and initial $CSPR_{min}$ = 0.3 nL/cell/day. Due to the high product stability, there was no restriction on D_{min}. The pre-optimization

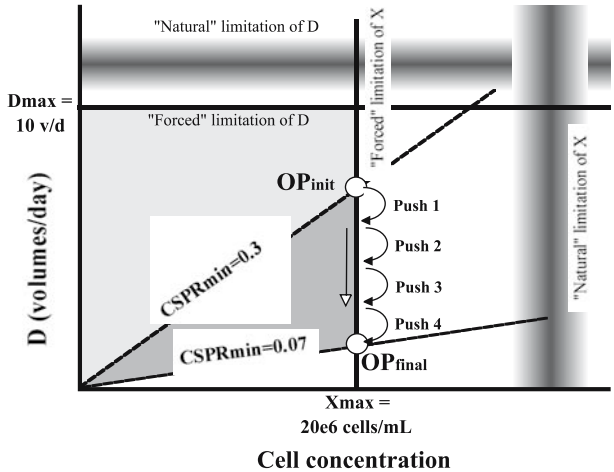

Fig. 14 Representation of the 15 L push-to-low optimization run from Fig. 8: D_{min} not restricted (stable product); D_{max}10 volumes/day ("forced" limitation); $X_{max}20 \times 10^6$ cells /day ("forced" limitation); initial $CSPR_{min}$0.3 nL/cell/day; final $CSPR_{min}$0.07 nL/cell/day

operating point, OP_{init}, was located at the intersection of X_{max} and initial $CSPR_{min}$ = 0.3 nL/cell/day. The push-to-low optimization resulted in the incremental sliding of the operating point down the X_{max} line to the lowest possible location, OP_{final}, defined by the new value of $CSPR_{min}$ = 0.07 nL/cell/day. Once the optimal operating point is determined at a small scale, scale up to production is straightforward, requiring only repositioning of the X_{max} and D_{max} lines to reflect the maximum capacity of the large scale reactors, whose optimal OP will be located at the intersection of $CSRP_{min}$ and X_{max}.

The proposed approach for optimization of perfusion cultures is most suitable to stable products for which residence time is not a critical parameter. If this is not the case, the dilution rate cannot be reduced to low levels. The push-to-low optimization procedure works with cell lines exhibiting either non-growth-associated or inversely growth-associated production kinetics. In these cases, the decrease of *CSPR* to low levels would significantly increase titer, approaching the range of fed-batch titers. This was demonstrated in a perfusion antibody fermentation, where a multifold increase in titer was achieved at constant volumetric production rate. Significant reduction of the specific growth rate accompanied with increased apoptosis at low *CSPR* was observed. This could not be explained with limitation or inhibition by the known medium components and toxic metabolites. Further improvements in titer will be possible if this phenomenon is understood and alleviated.

Successful application of the proposed optimization approach requires reliable cell concentration control, incremental in-process medium improvement, and continuous monitoring of cell physiology and product quality. The targeted *CSPR* in the case of stable product and proper production kinetics should be 0.05 nL/cell/day or lower, which will increase titer to the levels typical for fed-batch processes. The potential for high titer, combined with high volumetric productivity, stable performance over many months, and superior product/harvest quality, render perfusion processes an attractive production technology. To make high titer a standard feature of perfusion processes, further work on medium optimization, cell line improvement (apoptosis-resistant, low auto-inhibitor production), and understanding of cell physiology at ultralow *CSPR* will be necessary.

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Towards Industrial Application of Quasi Real-Time Metabolic Flux Analysis for Mammalian Cell Culture

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Abstract Cellular physiology and metabolism were monitored using a quasi real-time combination of on-line and off-line data to estimate metabolic fluxes in an established bioreaction network. The utility of this approach towards optimizing bioreactor operation was demonstrated for CHO cells cultivated in 15 L perfusion reactors at 20×10^6 cells/mL. Medium composition and dilution rates were changed to obtain several steady states with varying glucose and glutamine concentrations. When cells were restored to initial culture medium and perfusion rate conditions after being exposed to lower glucose and glutamine concentrations, the pyruvate flux into the TCA cycle was increased 30% while the pyruvate flux through lactate was decreased 30%, suggesting steady-state multiplicity. By appropriately altering cellular metabolism, perfusion bioreactors can operate at lower perfusion rates without significant accumulation of inhibitory metabolites such as lactate. Changes in glucose, lactate and glutamine uptake/production rates had significant effects on the calculation of other fluxes in the network. Sensitivity analysis of these key metabolic fluxes highlighted the need for accurate and reliable real-time sensors. Overall, rapid observation of metabolic fluxes can be a valuable tool for bioprocess development, monitoring and control. The framework presented in this study offers a convenient means for quasi real-time estimation of metabolic fluxes and represents a step towards realizing the potential of metabolic flux analysis for accelerated bioprocess optimization.

Keywords Cell culture · Metabolic flux · Perfusion · Quasi real-time analysis · Steady-state multiplicity

1 Introduction

Mammalian cells are widely used for the production of therapeutic proteins that require their ability to effectively fold and glycosylate proteins. However, productivities from typical mammalian cell processes are low and a variety of approaches have been taken to overcome this limitation. These include bioprocess engineering of perfusion culture reactors to 20×10^6 cells/mL cell densities [1, 2], and developing improved feeding strategies to optimize cellular metabolism [3, 4]. Another tool for productivity engineering is metabolic flux analysis (MFA), which determines carbon fluxes in the central carbon metabolism and related pathways [5–8]. MFA provides insights into cellular metabolism, especially under varied environmental bioreactor conditions, rapidly clarifying cellular responses to culture conditions that can influence productivities.

Most studies on MFA use either the stoichiometric approach [9, 10] or the isotope tracer approach [11] to estimate intracellular fluxes. In these studies, metabolic fluxes are computed off-line after analysis of nutrient and metabolite levels or isotopic tracer concentrations (i.e., hours or days). While this approach allows for quantification of metabolic fluxes in the chosen reaction pathway and provides information on cellular physiology and metabolism, the off-line nature limits the MFA impact and can even slow down the rate

of bioprocess diagnostics, decision-making and control. To fully realize the potential of MFA, real-time metabolic flux information should be coupled with automated process control strategies to more rapidly optimize bioreactor operation.

Early applications of on-line data for process optimization include analysis of stoichiometry [12–14], heat balances [15], and respiratory quotient measurements [16]. It is now common practice to use information on stoichiometry as well as specific uptake and production rates for on-line identification and control of bioprocesses [17]. However, this approach has not been reported using real-time information on the network of intracellular fluxes.

In the present study, we present a framework for a quasi real-time metabolic flux analysis (QRT-MFA) system and illustrate its application studying the metabolism of Chinese hamster ovary (CHO) cells in high cell density perfusion culture. This was achieved through seamless integration of LabVIEW (National Instruments, TX), the process monitoring and control environment with MATLAB (Mathworks, MA) for metabolic flux computation. Metabolic fluxes were computed for a 15 L bioreactor where glucose and glutamine concentrations were varied during the course of the experiment. Sensitivity analysis was performed on key metabolic fluxes to provide the basis for a discussion of the need for accurate and reliable on-line sensors.

2 Framework for Quasi Real-Time MFA

A schematic representation of the evolution of bioreactor monitoring and physiological state identification techniques is shown in Fig. 1. The environment in the bioreactor is characterized by several key process variables including pH, temperature, dissolved oxygen concentration and the cell specific perfusion rate (CSPR), in addition to concentrations of carbon and energy sources such as glucose and glutamine and metabolites such as lactate and ammonia. Information on cellular physiology and metabolism is obtained in the physiological state identification step which can be further subdivided into two categories, which provide extracellular and intracellular information, respectively. Extracellular information includes specific nutrient uptake and metabolite production rates, and the specific growth rate of cells. This information comprises a portion of the state vector that describes the physiology of the cell and can be used to guide bioreactor operation [18]. However, the physiological state vector defined in this fashion is limited to extracellular information. The next logical step towards obtaining more information on cellular physiology and metabolism is through the computation of intracellular fluxes. This upgrade of information content

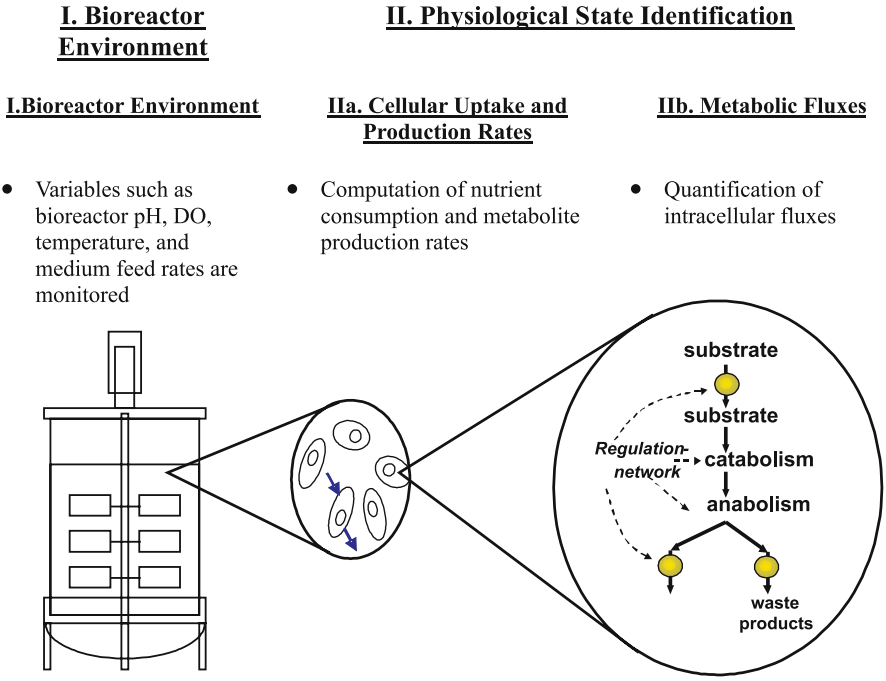

Fig. 1 Evolution of bioreactor monitoring and physiological state identification strategies from environment to intracellular fluxes

in the physiological state vector can provide for the implementation of improved bioreactor control strategies that could result in improved bioreactor performance [19].

Our motivation for developing a framework that would enable obtaining metabolic flux information in quasi real-time was to increase the quantitative information on cellular physiology and metabolism to rapidly detect and understand shifts in cellular metabolism. There is a great deal of pressure in the biotechnology industry for rapid process development and optimization. To achieve this objective, bioprocess development efforts need to be supported with rapid and high-quality information. QRT-MFA should be one such source of information that could accelerate the development of diagnostic and process control strategies.

A variety of factors were taken into consideration during the formulation of this framework. Initial work was done with simple metabolic networks that were subsequently modified to incorporate additional reactions. This resulted in the development of a metabolic network model that could be readily modified and tested. Another important feature was the provision to include both on-line and off-line experimental data as input for the computation of metabolic fluxes. While ideally QRT-MFA input data would be obtained on-

line, this is difficult to achieve in practice, especially given the extensive number of analytical tests needed. With continued development of on-line analytical techniques [20, 21], it should be possible in the future to obtain more analytical information in real-time. Given the large amount of metabolic flux data generated during long perfusion cultures (100 days or more), provisions were made to archive this information in a relational database management system. This facilitated mining for correlations between experimental variables and key metabolic fluxes as well as the comparison of results to archived cultures.

3 Materials and Methods

3.1 Cell Line, Culture Medium and Bioreactor Operation

Chinese hamster ovary cells were grown in perfusion culture using a medium with either 4.5 g/L glucose and 6 mM glutamine or with 6 g/L glucose and 8 mM glutamine (Table 1). All experiments were conducted in 15-L bioreactors (MBR Bioreactor AG, Switzerland) with a 12 L working volume and a heated water jacket. The bioreactor was maintained at 37 °C, with agitation constant at 40 rpm. Dissolved oxygen (DO) was maintained at 50% air saturation by sparging mixed oxygen and nitrogen. Bioreactor pH was maintained at 6.8 by the addition of 0.3 M NaOH. The bioreactor was inoculated at $\sim 1.0 \times 10^6$ cells/mL and cell concentrations during the experiment were maintained between 10 and 30×10^6 cells/mL by automatically bleeding cells from the bioreactor based on optical density measurements. The bioreactors were operated in perfusion mode by continuously withdrawing reactor fluid and passing it through a cell separation device. The cells were recycled back to the bioreactor while the clarified liquid was harvested for subsequent purification steps to isolate the protein of interest.

Table 1 Bioreactor conditions investigated in this study

State	Glucose in medium (g/L)	Glutamine in medium (mM)	Dilution rateflux lines (reactor volumes/day)
A	4.5	6	2.0
B	6	8	2.0
C	6	8	1.5
D	6	8	1.0
E	4.5	6	1.0
F	6	8	2.0

3.2 Analytical Methods

Samples from the bioreactor and the harvest stream were taken daily for cell density and viability analysis using a hemacytometer and the trypan blue dye-exclusion method. Cell sizes were determined using a particle counter (Casy, Schärfe Systems, Germany). These samples were then centrifuged in a Beckman CS-6 centrifuge (Beckman Coulter, CA) and the supernatant analyzed for nutrient and metabolite concentrations. Glucose, lactate, glutamine and glutamate concentrations were determined using a YSI Model 2700 analyzer (Yellow Springs Instruments, OH) while ammonia was measured using an Ektachem DT60 analyzer (Eastman Kodak, NY). DO and pH were measured on-line using retractable Ingold electrodes (Ingold Electrodes, MA). The accuracy of these measurements was verified off-line using a Stat Profile 9 blood gas analyzer (Nova Biomedical, MA). The same instrument also measured the dissolved CO_2 concentration. On-line measurements of cell density were made with a retractable optical density probe (Aquasant Messtechnik, Switzerland) calibrated with cell density estimated by the hemacytometer. Amino acids were analyzed on a HP 1090 HPLC (Hewlett Packard, CA) using the AminoQuant protocol (Series II Operator's Handbook) with pre-column derivatization by *ortho*-phthalaldehyde and 9-fluorenylmethyl chloroformate for detection of primary and secondary amino acids, respectively.

3.3 Estimation of Specific Rates

A mass balance on viable cells in the bioreactor and the cell bleed system results in

$$\mu = \frac{F_B}{V} + \frac{F_H}{V}\frac{X_H}{X_F} + \frac{1}{X_F}\frac{dX_F}{dt}, \tag{1}$$

where μ is the apparent specific growth rate (1/day), F_B the bleed rate (L/day), V the bioreactor volume (L), F_H the harvest flow rate (L/day), X_H the viable cell density in the harvest stream ($\times 10^9$ cells/L), X_F the viable cell density in the bioreactor ($\times 10^9$ cells/L), and t is time (days). Specific nutrient uptake and metabolite production rates were computed from

$$q = \frac{F_H(C_{in} - C_{out})}{VX_F}, \tag{2}$$

where q represents the specific uptake or production rates (mol/10^9 cells/day), while C_{in} and C_{out} are the bioreactor inlet and outlet concentrations (mol/L) of the nutrients or metabolites.

3.4 Estimation of Metabolic Fluxes

The bioreaction network used in the study was similar to the one proposed for CHO cells [6] with modifications to include reactions for all amino acids. It included a total of 65 reactions and 40 metabolites and the uptake/production rates of 27 of the metabolites were measured. The stoichiometric matrix was of full rank and the bioreaction network was characterized by 2 redundant measurements. A series of mass balance equations were written for each of the metabolites in the bioreaction network resulting in

$$\boldsymbol{A}\boldsymbol{x} = \boldsymbol{r}\,, \tag{3}$$

where $\boldsymbol{A}$ is the stoichiometric coefficient matrix, $\boldsymbol{x}$ the vector of unknown metabolic fluxes, and $\boldsymbol{r}$ the vector of uptake and production rates. Nutrient consumption and metabolite production rates were incorporated in $\boldsymbol{r}$ and intermediate metabolite production rates were assumed to be zero based on the pseudo-steady-state hypothesis [22]. As the stoichiometric matrix $\boldsymbol{A}$ was not square, and estimation of the metabolic flux vector $\boldsymbol{x}$ was done using the weighted least squares approach as the matrix $\boldsymbol{A}$ was of full rank

$$\boldsymbol{x} = (\boldsymbol{A}^{\mathrm{T}}\boldsymbol{\psi}^{-1}\boldsymbol{A})^{-1}\boldsymbol{A}^{\mathrm{T}}\boldsymbol{\psi}^{-1}\boldsymbol{r}\,, \tag{4}$$

where $\boldsymbol{\psi}$ is the variance-covariance matrix associated with the rate vector $\boldsymbol{r}$. Once the metabolic flux vector was estimated, the sensitivity of the metabolic fluxes to the measurements was estimated from

$$\frac{\partial \boldsymbol{x}}{\partial \boldsymbol{r}} = (\boldsymbol{A}^{\mathrm{T}}\boldsymbol{\psi}^{-1}\boldsymbol{A})^{-1}\boldsymbol{A}^{\mathrm{T}}\,. \tag{5}$$

3.5 Computer Implementation

To obtain metabolic flux estimates in a quasi real-time fashion for on-line physiological state identification, the computation of metabolic fluxes was integrated with on-line data acquisition and process monitoring. A schematic of this approach is shown in Fig. 2. Specifically, LabVIEW (National Instruments, TX) was used for on-line data acquisition and process control while all flux calculations were performed using FluxAnalyzer [23] in MATLAB (Mathworks, MA). Seamless integration between these two environments allowed transfer of specific rate data from LabVIEW to MATLAB followed by subsequent transfer of the computed metabolic fluxes in the reverse direction.

Information on cell density could be obtained in real-time from optical density measurements and this information coupled with the bleed rate helped estimate the specific growth rate of the cells. Oxygen and carbon-dioxide concentrations in the bioreactor were monitored in real-time as

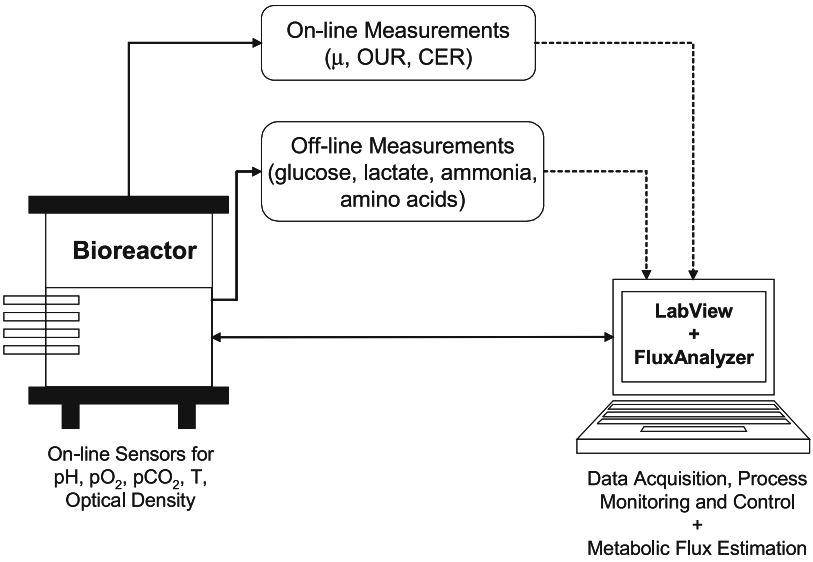

Fig. 2 Illustration of the framework for quasi real-time estimation of metabolic fluxes

well as their concentrations in the inlet and exit gas streams. This information was used in global mass balance expressions that were developed for the perfusion system to estimate the oxygen uptake rate (OUR) and carbon-dioxide evolution rate (CER). Concentrations of glucose, lactate, ammonia and amino acids were estimated off-line using the analytical techniques described earlier. Given available labor and equipment, all these measurements could be performed within one hour. This combination of on-line and off-line data was initially transferred to LabVIEW and subsequent flux estimations that were performed using FluxAnalyzer were initiated from the LabVIEW environment itself. This interface between LabVIEW and MATLAB was designed to approximate real-time MFA such that on-line information from new sensors could be seamlessly incorporated. Results of the computations included a table containing the fluxes for all the reactions in the bioreaction network along with a graphical depiction of the fluxes through the metabolic network. These metabolic fluxes were archived to observe trends of key metabolic fluxes over the course of the experiment.

4 Results

The framework presented above was applied to study the metabolism of CHO cells in perfusion culture at high cell densities. Medium composition and

perfusion rates were modified to obtain six different states designated as A through F (Table 1). The resulting metabolic responses from the cells were subsequently quantified through metabolic flux analysis. The cells were exposed to increasing amounts of glucose and glutamine when switched from state A to B. However, in C, D, and E, the cells were exposed to progressively decreasing amounts of glucose and glutamine. States F and B had identical feed medium composition and perfusion rates. This helped quantify the effect of lower nutrient concentration (in states B, C, and D) on cellular metabolism through a comparison of metabolic fluxes in states B and F.

4.1 Cell Density, Glucose, and Lactate Concentrations

Cell densities for states A through C were approximately 20×10^6 cells/mL while states E and portions of D were characterized by values in the $5–10 \times 10^6$ cells/mL range (Fig. 3). State F, which was identical to state B in terms of medium feed and dilution rate was characterized by cell densities between 10 and 30×10^6 cells/mL. These changes in cell densities were a consequence of the changes made to the cell bleed set-point. The motivation for these changes was to investigate the performance of the process in the neighborhood of 20×10^6 cells/mL, the standard operating cell density for this process.

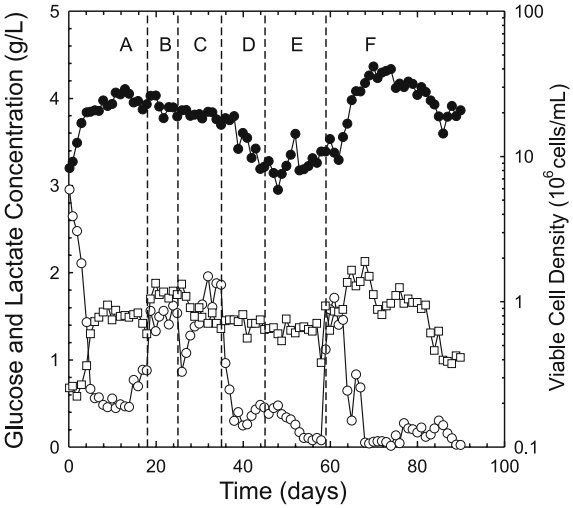

Fig. 3 Bioreactor viable cell density and glucose and lactate concentrations over the course of the experiment. Medium composition and perfusion rates of states A through F are defined in Table 1 (● bioreactor cell density; ○ glucose; □ lactate)

4.2 Metabolic Fluxes at States A through F

Recognizing the dynamic nature, especially of states D, E, and F, four to six data points towards the end of the six states A through F were used to compute extracellular rates using Eqs. 1 and 2. These calculated extracellular rates were averaged to obtain a single value for each state. This information was subsequently used to compute intracellular metabolic fluxes for states A through F from Eq. 4. A plot of the pyruvate flux into the TCA cycle versus the pyruvate flux into lactate for each of the six states is shown in Fig. 4. States A, B and C cluster in the region of high lactate and low TCA flux while states D, E and F lie in the region where the lactate flux is relatively lower and the TCA cycle flux is correspondingly higher, indicative of a more efficient metabolic state. High values of the pyruvate flux into the TCA cycle flux are desirable as this results in the production of energy. Pyruvate flux to lactate is not desirable since lactate is a waste metabolite. It appears that as the cells progressed from state B to F, there was a shift in metabolism towards a more efficient state as seen from the increase in the pyruvate flux into the TCA cycle.

Another approach to quantify the shift in metabolism due to changes in dilution rate is by examination of flux distribution around the pyruvate branch point [24]. Figure 5 shows a time course of the glycolytic flux plus the TCA cycle reflux divided by the inlet flux to the TCA cycle around the pyruvate branch point. Low values of this ratio indicate more efficient cycling of the carbon flux from pyruvate while high values correspond to increased pro-

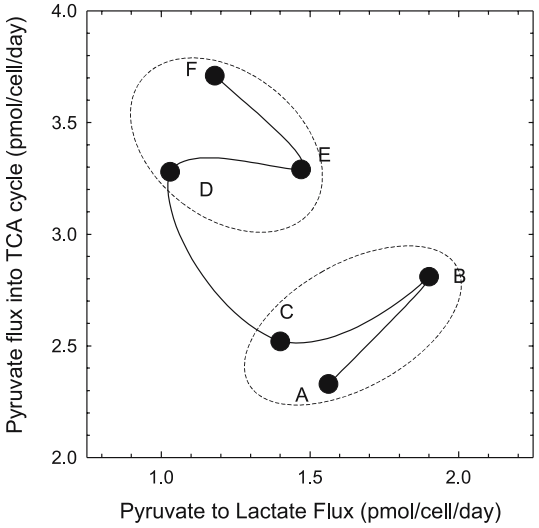

Fig. 4 Profile of the two pyruvate fluxes at states A through F

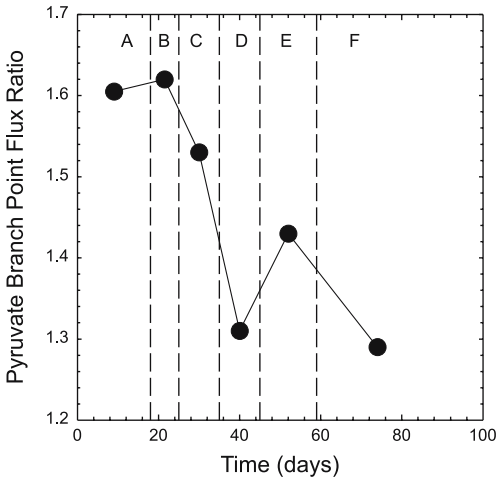

Fig. 5 Metabolic flux distribution around the pyruvate branch point during the course of the experiment. Higher values are indicative of waste metabolism while low values correspond to increased carbon flux through the TCA cycle

duction of waste metabolites such as lactate. It follows from Fig. 5 that this ratio decreases with decreasing dilution rate suggesting a shift towards more efficient metabolism at lower dilution rates.

A comparison of metabolic fluxes through the TCA cycle for states B and F that were characterized by the same medium composition and dilution rates revealed that the TCA cycle fluxes in state F were at least 30% higher than those in B. Hence, two different physiological states were observed under similar reactor operating conditions. No significant change in specific protein productivity was observed during the course of the experiment (data not shown). This has significant implications for reactor design and operation since lower production of waste metabolites such as lactate allow use of lower perfusion rates. This can translate into reduced medium costs and a more concentrated harvest stream thereby minimizing the volume of material that needs to be processed in subsequent purification steps.

4.3 Sensitivity Analysis for the Practical Realization of QRT-MFA

One approach to overcoming some limitations of metabolite balancing is through the application of sensitivity analysis where information on the effects of measured variables on key metabolic fluxes can be quantified [19]. Sensitivities of the pyruvate kinase, pyruvate dehydrogenase and citrate synthase fluxes to the various uptake and production rates are shown in Fig. 6 for states B and F. Glucose uptake rate, lactate production rate and oxygen up-

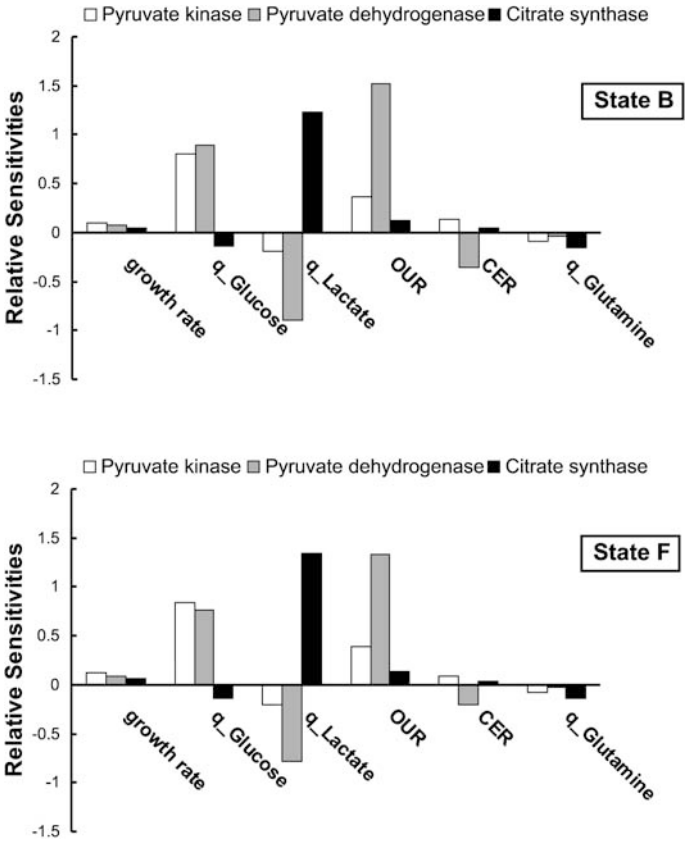

Fig. 6 Relative sensitivities of the calculated pyruvate kinase, pyruvate dehydrogenase, and citrate synthase fluxes with respect to measured specific rates. Only those specific rates with relative sensitivities greater than 0.05 are shown

take rate have the most significant impact on the above mentioned fluxes for both states B and F. For instance, a 1% change in OUR would cause a 1.5% change in the pyruvate dehydrogenase flux for state B (Fig. 6). The glucose uptake rate had a significant influence on the pyruvate kinase and pyruvate dehydrogenase fluxes but only a minor effect on the TCA cycle flux. Once important metabolic fluxes are identified, the sensitivity of these fluxes to various input data can be estimated and this information can be used to rank the input measurements. An important observation in this study was that most of the amino acids had only a minor influence on the fluxes of central metabolism. Thus if only central metabolism fluxes are of interest, off-line analysis of amino acids on a daily basis should be adequate for computing the metabolic fluxes with reasonable accuracy. This is an important simplification for the practical realization of QRT-MFA.

5 Discussion

5.1 Steady-State Multiplicity

Exposing the cells to progressively decreasing amounts of glucose and glutamine by varying either the medium composition or the perfusion rate caused a shift in metabolism towards a more efficient state as seen by increased pyruvate flux to the TCA cycle coupled with decreased lactate production. This manifested as a reduction in the pyruvate branch point flux ratio for the metabolically efficient state (F). Our observations on steady-state multiplicity are consistent with earlier chemostat studies where similar shifts in metabolism were observed at lower dilution rates [24–26].

Glucose and glutamine utilization characteristics of mammalian cells can be influenced by their respective concentrations [4, 27–29]. Generally, high concentrations of glucose result in increased glucose uptake rates with most of the glucose being converted to lactate. Even if this phenomenon is not accompanied by deceased specific protein productivities, it is undesirable because it will result in increased lactate concentrations in the bioreactor that can adversely effect cell growth [30]. Reduced lactate levels in a perfusion system can be achieved either by a decrease in cell density or an increase in perfusion rate. However, neither of these changes is desirable. As protein production is directly linked to cell concentration, reduction in cell density will decrease protein production while increased perfusion rates will result in a more dilute harvest stream. This can greatly increase the fluid volume that has to be processed in subsequent purification operations. Alternatively, if cellular metabolism can be altered to reduce lactate production when its accumulation is limiting, high cell densities can be maintained and perfusion rates can be lowered, resulting in harvest streams with higher protein concentrations.

5.2 Quasi Real-Time Metabolic Flux Analysis

We have presented results on the shifts in metabolism of CHO cells in perfusion culture as a response to changes in experimental conditions. There exist several other avenues for the application of metabolic flux analysis at the bioprocess level and these include clone selection, medium optimization and optimization of physical parameters such as pH, temperature, shear and DO among others. Moreover, metabolic flux analysis can also be used in a process evaluation and control mode where changes to set-points can be made based on the estimates of some key metabolic fluxes. However, to fully realize the potential of metabolic flux analysis for these applications, there is a need

for rapid evaluation of the fluxes, ideally, in an on-line fashion. This directly translates into a need for reliable on-line sensors for measuring concentrations of key nutrients, metabolites and amino acids.

5.3 Sensors for RT-MFA

Based on results from the sensitivity analysis described above, on-line estimation of oxygen uptake as well as glucose and lactate concentrations should be given high priority given the significant influence they exert on central metabolism fluxes. This can be followed by glutamine and ammonia concentrations that are also usually measured off-line. Given the rather complex nature of amino acid analysis, on-line analysis should only be considered under special conditions when the application demands this information.

Flow injection analysis has been used for real-time measurement of glucose and ammonia [17] while real-time measurement of glucose and lactate has been demonstrated by automatically drawing samples from the bioreactor through a circulation loop followed by sample filtration to remove biomass and subsequent analysis using standard analyzers [31]. These approaches typically require the deployment of dedicated analyzers for each bioreactor and this becomes impractical in a process development scenario where a number of bioreactors are operated simultaneously. Moreover, the presence of additional flow loops can increase the contamination risk, especially for perfusion systems given their long operation times.

There has been progress in on-line estimation of glucose, lactate, glutamine, and ammonia through the use of near-infrared (NIR) and mid-infrared (MIR) spectroscopic measurements [32, 33]. However, these measurements were characterized by high standard errors, which may limit their practical use in experiments such as those performed in this study. Specifically, lowest standard errors in the NIR measurements for glucose, lactate, glutamine and ammonia were 0.82, 0.94, 0.55, and 0.76 mM, respectively [32], while in the MIR measurements, standard errors for lactate measurements ranged from 1 to 3 mM, and those for glucose were approximately 1 mM [33]. The average concentrations of glucose, lactate, glutamine, and ammonia in this study were 4.03, 16.3, 2.74, and 3.4 mM, respectively, requiring the use of more accurate methods of analysis. Conventional off-line analytical instruments such as the YSI 7100 MBS (YSI Inc., Yellow Springs, OH) with precision on the order of 0.11, 0.22, 0.1 and 0.1 mM for glucose, lactate, glutamine, and ammonia, respectively, are better suited for analysis where the concentrations of these nutrients and metabolites are low and when increased precision is desired. Thus despite the significant progress in bioprocess monitoring over the past few decades [20], there is still a need for reliable sensors that would allow on-line estimation of the concentrations of key metabolites and amino acids.

The applicability of new on-line sensors for real-time flux analysis is currently being investigated in our laboratory.

5.4
Metabolite Balancing and Isotope Tracer Approaches as Applied to QRT-MFA

As QRT-MFA requires the rapid analysis of experimental data for estimation of metabolic fluxes, it is not practical to use isotopic tracer studies for estimating metabolic fluxes since this technique is laborious both from experimental and analytical standpoints. It is well known that metabolite balancing alone is not sufficient to estimate all the intracellular metabolic pathway fluxes because the set of linear equations defined by these mass balances is underdetermined [11, 34, 35]. Some of these limitations can be overcoming through the use of additional constraints such as including the mass balance expressions of the co-metabolites ATP or NAD(P)H, assuming the irreversibility of certain reactions, or through the use of objective functions [36]. Moreover, the metabolite balancing technique can be augmented with a few carefully planned isotope tracer studies that could validate the use of nutrient and metabolite mass balances alone for the estimation of metabolic fluxes.

5.5
Implementation of QRT-MFA in this Study

An intuitive and visual approach towards estimating metabolic fluxes was used in this study. Computation of metabolic fluxes was initiated from the process monitoring and control environment in LabVIEW through a graphical user interface. The input information consisting of rates of growth, glucose and oxygen consumption, metabolite production, carbon-dioxide evolution and uptake/production rates of amino acids was provided. While there is a provision in the software to record and use this data in real-time, a combination of on-line and off-line data was used as illustrated in Fig. 2. Subsequent metabolic flux calculations were performed using FluxAnalyzer in the MATLAB environment and the results displayed both in graphical and tabular format. The graphical display (Fig. 7) showed the metabolic flux map along with values of key metabolic fluxes. Features of the graphical display include a distinction between computed fluxes and those obtained from experimental data, and lines in the metabolic map with thickness proportional to the magnitude of the flux. The tabular depiction lists the magnitude of the fluxes through all the reactions in the metabolic pathway and has a provision for normalizing these fluxes based on any nutrient/metabolite of choice.

Seamless integration between the process monitoring and control environment (LabVIEW) and the flux computing environment (MATLAB) allowed

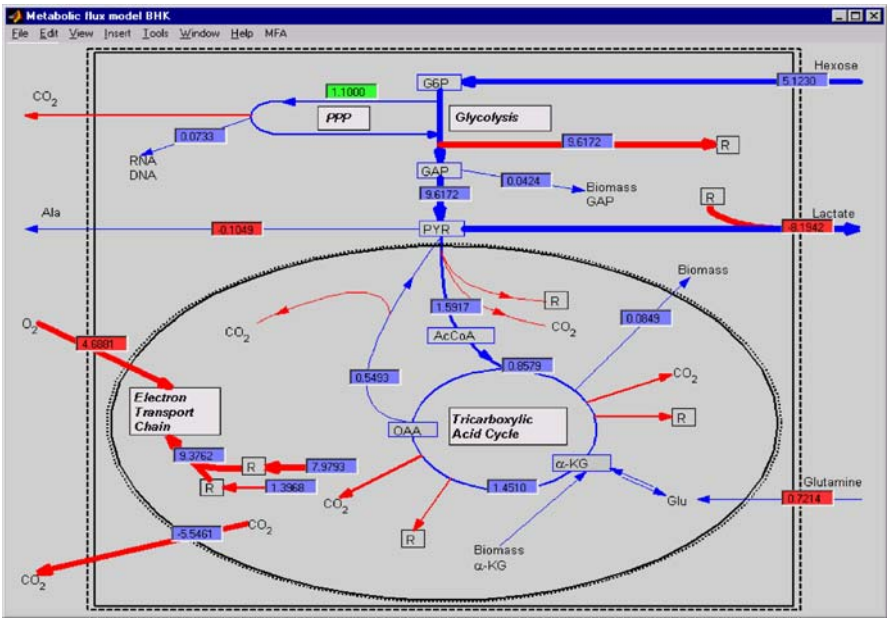

Fig. 7 Graphical representation of the results of metabolic flux analysis. Distinction is made between experimentally measured and calculated fluxes through use of *color* and the *thickness* of the flux lines correspond to the magnitude of the respective fluxes

easy access to metabolic flux information in addition to other commonly measured experimental variables such as pH, temperature and DO along with some calculated variables such as cell growth rate and specific uptake/production rates of key nutrients and metabolites. As perfusion experiments are often carried out over extended periods of time, this information can be archived allowing for trending of key metabolic fluxes. Thus changes in cellular metabolism can be readily identified and once a cause-effect relationship has been established, this information can be used to make appropriate modifications in process conditions that will result in the desired improvement in the process.

Currently, the time from sampling the bioreactor to obtaining all the necessary analytical data for computing the metabolic fluxes through a combination of on-line and off-line measurements is approximately 1 hour. Hence there is a 1 hour delay in obtaining a metabolic snapshot of the bioreactor at any given time. This delay can be reduced significantly as more data become reliably available on-line, thereby enabling the transition from quasi real-time to actual real-time estimation of metabolic fluxes.

5.6 Practical Implications of QRT-MFA

Current industrial practice for cultivating mammalian cells in perfusion culture involves bioreactor operation at desired set-points for process variables such as cell specific perfusion rate, pH, temperature, and dissolved oxygen concentration. Process characterization involves off-line estimation of primary variables such as cell density, nutrient, metabolite and product concentrations. Control schemes are typically based on values of the primary variables alone. For instance, the bioreactor cell density control loop maintains constant cell density by bleeding cells from the bioreactor. This can be readily accomplished as long there is an on-line indicator of cell density such as an optical density probe. Indirect indicators of cell density such as oxygen consumption rates could also be used for this purpose. It is important to note that current operational protocols do not rely on detailed information on cellular metabolism.

When metabolic fluxes can be estimated rapidly, a significant amount of quantitative information on cellular metabolism becomes available, which can be used in various process control loops to guide bioreactor performance in the desired direction. For instance, Europa et al. [25] have seen a decrease in the stoichiometric ratio between lactate production and glucose consumption from 1.36 mol/mol in normal culture to 0.04 mol/mol in a metabolically altered culture where the amount of glucose available to hybridoma cells in fed-batch culture was reduced. Based on their results, they suggest initiation of cultivation in fed-batch mode and exposing cells to reduced amounts of glucose so that lactate production is minimized resulting in very efficient cellular metabolism as seen by increased flux through the TCA cycle. Once this shift in metabolism has occurred, the bioreactor can be operated in a continuous mode and the advantages of efficient metabolism can be leveraged. As seen earlier, reduced lactate yields from glucose can allow for operation at lower perfusion rates resulting in a concentrated protein stream for subsequent purification processes.

For robust implementation of such a control strategy that is based largely on the metabolism of cells, it is necessary to obtain frequent snapshots of cellular metabolism that will provide valuable feedback on the efficacy of the process control strategy. In the initial fed-batch phase of the process, metabolic flux information would be necessary to follow the shift in metabolism from the high lactate producing state to one that is highly energy efficient as seen by increased flux through the TCA cycle. The feeding strategy for glucose could be based on the relative distribution of fluxes at the pyruvate branch point. During the next phase of the experiment where it is desired to maintain the efficient metabolic state of the cells, metabolic fluxes provide the information necessary to characterize the physiological state of the cells. Again glucose feeding during the perfusion phase can be

based on flux distribution at the pyruvate branch point. While it can be argued that the above control strategy can be based on glucose concentration alone, that would be representative of indirect control of cellular metabolism. With rapid estimation of metabolic fluxes, it is possible to directly control cellular metabolism by feeding glucose at a rate that ensures a fixed distribution of fluxes at the pyruvate branch point. This concept of direct estimation and control of cellular metabolism in an industrial mammalian cell bioreactor is novel and more work is necessary to demonstrate the usefulness of this approach towards accelerated process development and optimized bioreactor operation.

Other applications of QRT-MFA include medium optimization and clone selection that are usually labor-intensive and time-consuming approaches. For both these applications, initial screening is usually done in a high throughput fashion followed by evaluation of a small subset of high performers in bioreactors. Traditionally, specific protein productivity and cell growth rate and viability have been regarded as the most important indicators of cellular performance and we believe that inclusion of metabolic fluxes in this selection criteria provides an extra line of evidence for selection of a particular medium formulation or clone.

6 Conclusions

We have presented a general framework for quasi real-time estimation of metabolic fluxes in a perfusion bioreactor. The concept is general and as it relies on metabolite balancing alone, it can be readily applied to both laboratory and industrial-scale bioreactors of practically any configuration. The utility of this approach towards monitoring shifts in cellular metabolism was demonstrated using CHO cells cultivated in perfusion reactors where exposure to lower nutrient concentrations shifted cellular metabolism towards a more efficient state as seen by increased flux into the TCA cycle. This new state was characterized by lower production of waste metabolites which have significant implications for reactor design and operation. There exist several other scenarios such as clone selection, medium optimization, and bioreactor environment optimization, among others, where MFA can be applied to optimize the operation of mammalian cell bioreactors. While the framework provided in this study provides for real-time computation of metabolic fluxes, it is limited by the lack of accurate and reliable sensors for on-line estimation of key nutrient and metabolite concentrations. Once such sensors become available, they will enable the full realization of metabolic flux analysis in real-time. Implementation of MFA in this fashion will significantly increase the quality of information obtained from experiments in process development bioreactors resulting in additional insights into cellular physiology and

metabolism. This information can play a significant role in the design of operational strategies for the production bioreactor where the therapeutic protein will finally be made.

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Engineering Cells for Cell Culture Bioprocessing – Physiological Fundamentals

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Abstract In the past decade, we have witnessed a tremendous increase in the number of mammalian cell-derived therapeutic proteins with clinical applications. The success of making these life-saving biologics available to the public is partly due to engineering efforts to enhance process efficiency. To further improve productivity, much effort has been devoted to developing metabolically engineered producing cells, which possess characteristics favorable for large-scale bioprocessing. In this article we discuss the fundamental physiological basis for cell engineering. Different facets of cellular mechanisms, including

metabolism, protein processing, and the balancing pathways of cell growth and apoptosis, contribute to the complex traits of favorable growth and production characteristics. We present our assessment of the current state of the art by surveying efforts that have already been undertaken in engineering cells for a more robust process. The concept of physiological homeostasis as a key determinant and its implications on cell engineering is emphasized. Integrating the physiological perspective with cell culture engineering will facilitate attainment of dream cells with superlative characteristics.

Keywords Apoptosis · Cell cycle · Cell engineering · Cell physiology · Glycosylation · Mammalian cell culture · Metabolic engineering · Metabolism · Protein processing · Recombinant protein

1 Introduction

In the past decade, we have seen continuing growth in mammalian cell culture bioprocessing, primarily driven by the expanding antibody production industry. This expansion is not only in the number of products now available to needy patients, but also in the quantity of antibody produced. Further raising the excitement level is the growing number of prospective therapeutic products for the diseases that are likely to become treatable. Although it has been over two decades since the employment of mammalian cell culture in recombinant therapeutic production, and a decade since cell culture bioprocessing was proclaimed to be a "mature technology", the new surge in the quantities of products required and the phenomenally high investment cost for a manufacturing plant has spurred a new drive to enhance cell culture bioprocess technology. Recombinant therapeutic proteins have made a major headway in healthcare. Their future societal impacts may rival those of antibiotics, whose discovery and clinical applications have heralded the arrival of modern medicine. Like microbial fermentation technology in the 1950s allowed penicillin to become widely available, advances in cell culture processing technology have been instrumental in making these new medicines available to patients.

When assessing the biotechnological potential of process enhancement, it is instructive to look back at the history that preceded major biotechnological drugs. From its discovery by Sir Alexander Fleming and expansion into clinical applications by Edward Penley Abraham until the end of its second decade, both the product titer and the production volume of penicillin increased almost exponentially. This was followed by a steady but slower enhancement in process, in terms of the product titer, in the following half a century. The roughly three orders of magnitude increase in production volume and product concentration was the result of decades of relentless effort by process scientists and engineers through strain improvement, media development, and many other process innovations. Oxygen and heat transfer

capacity and mixing characteristics were steadily pushed upward in performance level. On-line sensors, sterility control, equipment reliability, and process control, have all been drastically enhanced over the past decades. However, the success of process technology eventually also drove down the price, as clearly seen in Fig. 1 (personal communication, Arnold D. Demain). Penicillin G is no longer produced in the United States, as the cost of production is dramatically lower in some parts of the world.

With some imagination one may see a close resemblance of cell culture products to the historical graph of penicillin shown in Fig. 1. Nearly two decades after their first introduction as therapeutic biologics, we have seen process technology make large manufacturing processes a reality, with a steady increase in titer, from tens of milligrams per liter in hybridoma cultures in the 1980s, to 4 or 5 g/L for some immunoglobulin products today. Although little published information is available, the production cost has also reduced by at least an order of magnitude since the beginning of cell culture products. On the curve shown in Fig. 1, if one were to plot such a graph for one or two decades from now, we are likely be approaching the end of the rapid exponential stage in terms of product titer and process economics. However, it is also worth noting that the graph in Fig. 1 is shown on a logarithmic scale. In terms of real quantity, tremendous process enhancement was accomplished even after the initial rapid growth phase was over. The question for bioprocess scientists and engineers to ponder is what it will take for cell culture processing to accomplish what the antibiotic industry has achieved in our society in making major medicines affordable to the world's population.

What bioprocess scientists and engineers possess today that was not available to antibiotic researchers, or even to the initial innovators in cell culture processes, is the ready availability of genomic exploration and cell engineering tools. These new genome-wide investigative tools will greatly facilitate our discovery of genes crucial for conferring cells with desired growth and production characteristics. The new methodology of targeting host genes for

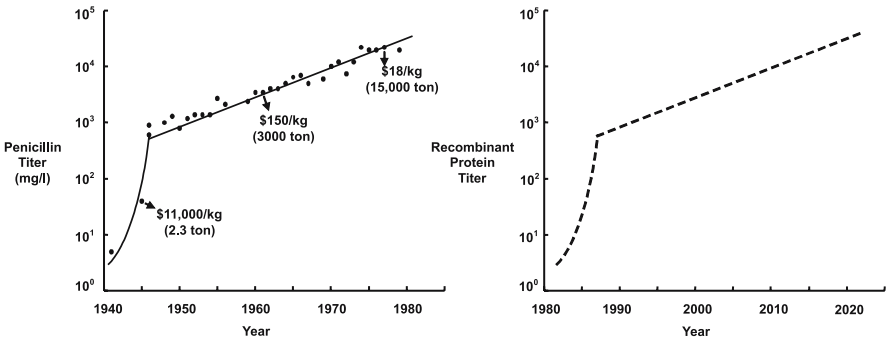

Fig. 1 Progression of pharmaceutical and biotechnological products

modulation of their expression level leading to altered cell physiology will result in greater productivity as well as increase the robustness of the process. In this article we will first discuss the wish-list of the characteristics of cells producing recombinant biologics, followed by a survey of work done on engineering cells to confer them with those characteristics.

2 Cell Line Development for Recombinant Protein Therapeutics

Cell line development typically follows a well-established scheme. Host cells are transfected with the heterologous gene of interest followed by selection of stable, high-producing clones. For recombinant protein production, selected clones are adapted to grow in suspension and in the culture environment used in production. For producing large quantities of the recombinant protein, it is preferable to grow cells in a suspension using a stirred vessel. Most of the host cells, although originating from adherent cells, have been adapted to suspension growth. The adaptation entails growing them in suspension over a period of weeks. Those originally adherent cells cease growth upon suspension in a stirred vessel. With appropriate medium, over time cells begin to grow and gradually regain normal growth rate. Adapting the host cells to suspension growth and then use for expression of heterologous gene(s) facilitates suspension growth of the selected transfectant. However, during cell transfection it is still a common practice to plate the transfected cells on a surface in the presence of serum. This not only enhances cell survival under selection pressure. It also allows surviving cells to form colonies for easier isolation of clones. Thus, after isolation of candidate producing clones, cells again undergo adaptation to suspension and serum-free medium growth as well as to other cultivation conditions.

Among the more than two dozen therapeutic cell culture products that have emerged in the past two decades, all but a few are produced by cells derived from rodents, including Chinese hamster, Syrian hamster, and mouse. Host cells used in the production of recombinant proteins fall into two categories based on their tissue origin. The first are largely hamster or human cells derived from tissues that are not specialized in secreting a large amount of proteins, such as ovary (e.g., Chinese hamster ovary [CHO] cells) and kidney (e.g., baby hamster kidney [BHK] cells or HEK 293 cells). The second are derived from professional protein secretors, mostly myeloma cells (e.g., mouse myeloma cells NS0 or Sp2/0). Although the myeloma cells used are non-immunoglobulin secretors, they retain the fully developed protein secretory machinery of the cells from which they were derived. A recombinant antibody-producing myeloma cell thus may be able to achieve a high productivity with a single copy of the recombinant immunoglobulin gene. In contrast, most non-myeloma-based host cells, when used for the production

of either antibody or other proteins, requires some degree of gene amplification to increase the transcript level and product expression rate. To achieve a high productivity, the transfected cells are typically subjected to selection pressures to induce the amplification of those product genes. A classic example is the use of methotrexate for amplifying dihydrofolate dehydrogenase genes accompanied by coamplification of the heterologous product gene.

3 Cellular Pathways Associated with Superior Growth and Production Characteristics

3.1 Energy Metabolism

Cell growth and productivity of mammalian cells has been known to be inhibited by accumulating levels of lactate and ammonia in the culture medium. The concentration of lactate and ammonia varies with the type of cell line, the mode of bioreactor operation, as well as initial glucose and glutamine concentration. Although lactate and ammonia may not be the only inhibitory substances produced in culture, they do accumulate to levels that affect growth rate. They are also the primary metabolites that need to be removed from the bioreactor in a perfusion culture. Ideally one would like to alter cell metabolism to abolish or at least minimize the accumulation of these metabolites.

Transformed cells are considered to metabolize glucose "anaerobically", meaning converting a large proportion to lactate. This wasteful type of metabolism is seen even in normal diploid cells in culture. This excessive output of metabolites is tied to excessive intake of glucose and amino acids (Fig. 2). Experimental evidence demonstrated that the excessive metabolic flux and excretion of metabolites is diminished greatly when the external concentration of glucose and amino acids are controlled at low levels, suggesting that reduced uptake through reduced external concentration leads to reduced fluxes and metabolite accumulation.

Furthermore, protein production is an extremely energy intensive process. Each peptide bond has a net energy cost of at least three molecules of ATP, or about 0.1 mol of glucose being oxidized. The productivity of a high IgG producing cell could be over 40 pg/cell-day. For an average cell of 400 pg, that is nearly the equivalent of almost 20% of cellular protein. The energetic load to cells is rather high. In studying the development of B cells to plasma cells by proteomic techniques, it was seen that energy metabolism pathways are up regulated in addition to the protein secretion machinery [1]. There has not yet been a report on comparison of energetic load of high and low producing cells.

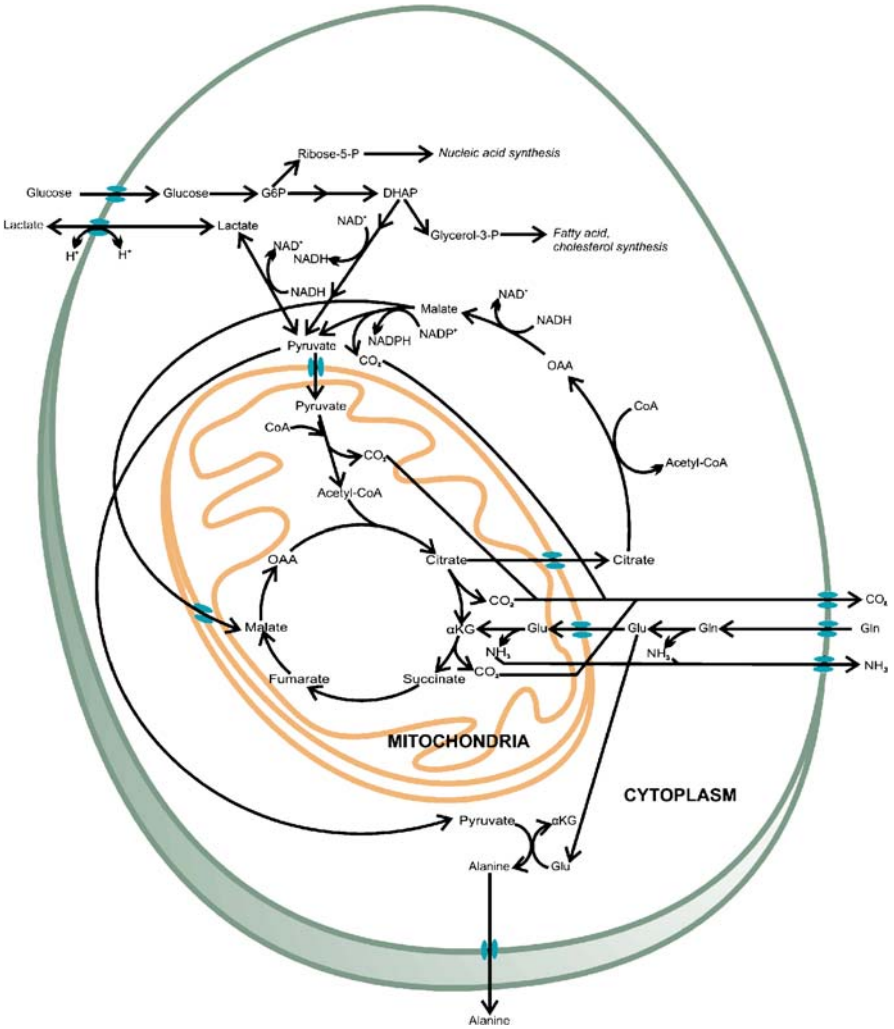

Fig. 2 Schematic representation of the pathways of glucose and amino acids. *G6P* Glucose 6-phosphate, *Ribose-5P* ribose 5-phosphate, *DHAP* dihydroxyacetone phosphate, *NAD* nicotinamide adenine dinucleotide, *OAA* oxaloacetate, *CoA* coenzyme A, CO_2 carbon dioxide, NH_3 ammonia, *Glu* glutamate, *Gln* glutamine, *αKG* alpha ketoglutarate

3.1.1 Engineering Energy Metabolism

Reduced metabolite production is certainly a desirable trait for cells in culture. Since lactate production has long been thought to be an obstacle for achieving a high cell concentration in bioreactors, there have been efforts

to reduce its production [2]. One may speculate that the accumulation of metabolites (i.e., shunting the glycolysis product to lactate instead of going into the mitochondrion for further oxidation) is the result of a constriction in the transport into the mitochondrion, or insufficient activity in the TCA cycle or oxidative phosphorylation chain. This speculation implies that if there were sufficient flux of carbons (primarily pyruvate) into mitochondrion for energy generation and sufficient efflux of chemical energy moiety (ATP) into the cytoplasm, then somehow through feedback regulation mechanisms, the excessive consumption of glucose will diminish and lactate shunt will be reduced. This is not unlike the aerobic/anaerobic metabolism switch in yeast *Saccharomyces cerevisiae*. In anaerobic growth, high glucose consumption is associated with ethanol fermentation and reduced mitochondrial activities. Upon switching to aerobic growth, glucose consumption is reduced as most pyruvate is channeled to mitochondria for energy generation.

A key control point for energy metabolism is at pyruvate. It should be noted that the pyruvate node is the juncture between glycolysis and the citric acid cycle. The pyruvate level is the balance of three fluxes: pyruvate–lactate flux, glucose–pyruvate flux and pyruvate–acetyl-CoA (or pyruvate flux into mitochondria) flux. An important target for metabolic engineering is lactate dehydrogenase (LDH), which catalyzes the production of lactic acid from pyruvate. LDH catalyses a reversible reaction that involves two reactant/product pairs, pyruvate/lactate and NADH/NAD^+. Antisense technology has been applied to knock down levels of LDH isoform A in CHO. Chimeric antisense *ldh* gene and *dhfr* were co-transfected into a *dhfr* $^-$CHO cell line. After serial selection with increasing methotrexate concentrations, a 30% decrease in LDH specific activity was observed. In another study, disruption of *ldh* gene by homologous recombination in a hybridoma cell line reduced specific lactate production and glucose consumption by half, keeping the lactate per glucose yield unchanged [4]. However, a reduction of transcript or enzyme level is no assurance of a reduction of reaction flux in the target direction. In both studies, the number of clones examined was small, but the ones reported did have the expected phenotype. A reduced LDH level resulted in a reduced flux of pyruvate–lactate. The reduced pyruvate–lactate flux also reduces the NAD^+ regeneration rate since the oxidation of NADH to NAD^+ is coupled to the pyruvate–lactate reaction. This may lead to a reduced glycolysis rate, as observed. As a result the intracellular pyruvate concentration is likely to be different and thus possibly affect other reactions. Whether the resulting phenotype also entails increased flux into citric acid cycle was not measured. Suffice to say that the transport of glucose was reduced accordingly to accommodate reduced flux. It would have been interesting to examine whether glucose transporter level was altered in the clones isolated.

Another attempt to reduce lactate production took the approach of increasing the flux of pyruvate into mitochondrion for the citric acid cycle. Yeast cytoplasmic pyruvate carboxylase gene was over-expressed in BHK-21 [5], CHO [6], and human HEK 293 [7]. Pyruvate carboxylase is the enzyme that converts pyruvate to oxaloacetate (OAA). Oxaloacetate is further converted to malate and enters the TCA cycle via the malate–aspartate shuttle. Over-expression of pyruvate carboxylase in BHK-21 cells led to a reduction in specific glucose and glutamine consumption rates by four- and twofold, respectively, in batch culture [5]. The molar stoichiometric ratio of lactate to glucose ($Y_{lac/gluc}$) was also reduced from 1.22 to 0.89 in continuous cultivation and the flux into TCA cycle was estimated to have increased by 1.4-fold. Metabolically engineered BHK-21 cells expressing pyruvate carboxylase were further transfected with a gene encoding for human erythropoietin (EPO) [8]. Under perfusion mode, cell-specific glucose consumption rate was reduced to 67% and the cell-specific lactate production rate reduced to half the amount of the control culture.

Increasing the pyruvate carboxylase activity probably decreases the pyruvate concentration, which may in turn reduce lactate formation rate. What is less clear is whether TCA cycle activities actually increased, as oxygen uptake rate was not measured to confirm the increased carbon flux into mitochondria. It is possible that OAA is transported into mitochondrion more readily. It is also possible that OAA actually remains in the cytosol in the transfectants and is converted to another compound that is not detected.

It is highly likely that the control step in nutrient flux is at the transporter level, whether it is the role of transporter proteins for uptake of glucose and amino acids into cytoplasm or the transport of pyruvate and other carbon moieties into mitochondrion. For mammalian cells in culture, an alternative route to lower lactate production is by reducing the intake of glucose, as has been demonstrated by metabolic shift [9]. Another possible means of reducing lactate production could therefore entail the reduction of glucose uptake by reducing glucose transporter. An attempt has been reported using antisense RNA expression of *glut1* [10], the gene encoding for the prevailing glucose transporter in all cells. Unfortunately the results are inconclusive as the growth rate varied profoundly with the different isolated clones. Glucose transport was significantly decreased in a gastric cancer cell line after transfection with cDNA for antisense GLUT1 [11]. Another strategy using RNA interference was employed to knock down GLUT1 expression in human skeletal myotube cultures, which led to inhibition of the serum-mediated increase in glucose uptake [12]. Although the commonly employed methods for suppressing protein expression, either via antisense or RNAi, do not allow for precise manipulation of the expression level, the concept of manipulating the transporter level for manipulating the glucose flux is still fundamentally sound.

3.2 Amino Acid Metabolism

A key issue in amino acid metabolism, like many other aspects of physiology, is homeostasis; unless all the amino acids are taken up by the cells in "right" stoichiometric ratios, interconversion among them will be necessary to meet the cellular needs. Cells can interconvert non-essential amino acids, but not the essential ones. When amino acids are taken up, they will have to be degraded by removal of an amino group to allow for catabolism of the carbon skeleton. In most cases, excess nitrogen comes from glutamine, whose carbon skeleton is catabolized through the TCA cycle. The other entry point for the amino acid carbon skeleton is through pyruvate and acetyl-CoA. Typically those amino acids are supplied in great excess. Thus excess supply of amino acids is metabolized primarily through the TCA cycle. Glutamine has also been shown to give rise to lactic acid.

If the carbon skeleton of the amino acids, especially glutamine, is catabolized primarily through oxidation in the TCA cycle to CO_2, the oxygen consumption of cells will certainly increase when the consumption of glutamine is higher. There is little evidence to support this notion. Rather, glutamine has also been reported to give rise to lactate. It is thus important to keep in mind that the drawback of excess consumption of amino acid is not only ammonium accumulation, but also potentially increased lactate production.

Although mammals have 10–11 essential amino acids, glutamine is not one of them. It is essential only for cells in culture. The expression of glutamine synthetase (GS) is also tissue-specific. It is the most abundant amino acid in blood and muscle tissue when it is synthesized. As most cultured cells are derived from other tissues, the expression level is often rather low. This low level of GS makes it essential and allowed GS to be used as a selection marker for introducing genetic elements to the cells. Glutamine is one of the more abundant amino acids in cellular proteins. It is an essential precursor in the synthesis of purine and pyrimidine. Thus, unless purine and pyrimidine are sufficiently supplied, glutamine will be required in a large quantity for nucleotide synthesis.

However, even if glutamine is required for cultured cells, the amount needed is still far below the level cells uptake from the medium. From a stoichiometric point of view, the amount taken up by cells far exceeds that needed for protein and nucleotide synthesis. A large fraction of total nitrogen intake by cells (since glutamine contributes around half the nitrogen intake, nitrogen is derived mostly from glutamine) is excreted as ammonium or non-essential amino acids, consistent with the notion that the large uptake of glutamine is not driven by synthetic cellular needs. Further support is provided by the fact that the glutamine consumed can be drastically curtailed by controlling glutamine at low levels without affecting the growth rate [13].

The question why cells consume so much glutamine thus persists. In any case, the potential detrimental effect of excess nitrogen is dampened by the interaction between nitrogen metabolism and carbon metabolism. Glutamate is used in part by aminotransferase towards alanine synthesis from glycolytic pyruvate (Fig. 2). The entry of the carbon skeleton from glutamine into carbon metabolic pool contributes to pyruvate and OAA. However, the extent of this contribution is not clear. Taken together, the experimental observations seem to indicate that the excess consumption of glutamine is neither to sustain a high intracellular concentration for nucleotide synthesis, nor is it to sustain an intracellular pool of TCA cycle intermediates or pyruvate. Rather, the excess consumption is probably due to the high uptake rate, a consequence of the abundance of amino acids transporters and high concentrations of amino acids in the medium.

3.2.1 Engineering Amino Acid Metabolism

Similar to glucose utilization, most cells in culture consume amino acids in great excess of the amount needed for synthesizing cellular and product proteins. This is evident from an analysis of published historical data [14] showing that nearly one third to a half of all nitrogen consumed by cells is excreted as ammonia and other non-essential amino acids. In another study in which metabolism of glucose was shifted from a high lactate-producing state to low or no-lactate producing state, not only the consumption rate of glucose was decreased, but also that of almost all amino acids, even while the specific growth and product formation rates were sustained. It is desirable that cells take up a sufficient amount of amino acids for growth and product formation without using them in excess and without producing excess ammonia.

A critical question is what is the "controlling" factor for reduced amino acid consumption? Intuitively one would expect the controlling reaction to be the uptake of amino acids from the feed into the reaction network. In that case the way to reduce "excess" amino acid consumption would be by restricting the uptake by either controlling the external concentration at reduced levels or by reducing the transporter concentration on the plasma membrane of those particular amino acids. Unfortunately, the relationships between the uptake of various amino acids and transporter concentrations are not simply linear. For key amino acids, the transporters involved are shared by more than one amino acid and each of the amino acids can be transported by more than one transporter. Therefore, the best way to manipulate transporters to "control" the amino acid is not so obvious.

Mammals synthesize glutamine from glutamic acid using an ATP-dependent glutamine synthetase (GS). Cultured cells have a low activity of this enzyme, making them dependent on exogenously supplied glutamine. Glu-

tamine synthetase gene has been cloned into a murine myeloma cell line, NS0, to give rise to a glutamine-independent phenotype [15]. GS is also used as a selection as well as an amplification marker for introducing heterologous genes into host cells. Since glutamine is typically supplied in culture in large excess, resulting in accumulation of ammonia, GS transfection not only eliminates the need for glutamine, but also reduces the inhibitory effect of high ammonia concentration in cell culture medium.

In addition to GS transfection, other means have also been applied to eliminate glutamine dependence. An early work on V79–56 cells demonstrated that their endogenous GS is probably silenced. Treatment with a desilencing drug, 5-azacytidine, gave rise to glutamine-independent variants [16]. In another study, mouse fibroblasts were adapted to grow in glutamine-free media supplemented with glutamate or 2-oxoglutarate [17]. Presumably, in those glutamine-independent variants GS has been activated or desilenced; however, the mechanism of the change in nutritional requirement was not studied.

Given the lack of biosynthetic pathway for amino acids of the aspartate family, mammalian cells require all four amino acids of this pathway for growth. Five reaction steps are involved to convert aspartate to threonine, with two as bifunctional enzymes. Cloning of those three enzymes from *Corynebacterium glutamicum* and *E. coli* into 3T3 cell line [18] resulted in a threonine-independent phenotype. The resulting threonine-independent cells have a high aspartate requirement, possibly due to unbalanced fluxes. This example certainly demonstrates the feasibility of over-expressing an entire pathway for synthesizing essential amino acids into mammalian cells.

3.3 Lipid Metabolism

Lipids play key roles, as lipid bilayer membranes, in many physiological functions critical to the cellular properties of particular interest to bioprocessing. In addition to affecting membrane fluidity and permeability, lipid bilayer membranes provide barriers to the environment and partition various organelles, which are critical to the synthesis of recombinant proteins, from the cytosol. Virtually all recombinant industrial proteins that are secreted get processed in the endoplasmic reticulum (ER), Golgi apparatus and are excreted via membrane vesicles. For optimal protein secretion capacity it is critical to sustain a membrane system that is capable of continual recycling. The lipid composition of ER, mitochondrial, and plasma membranes differ from each other. The plasma membrane of hepatocytes are enriched in cholesterol, however, there is less cholesterol in the rough and smooth inner ER and very little cholesterol in the inner mitochondrial membrane [19].

ER, being the primary organelle for protein secretion and intracellular distribution, occupies the largest volume among the organelles. Its prominence in volume is evident in professional secretors in the body, such as pancreatic β cells, hepatocytes, and antibody-secreting plasma cells. In hepatocytes the surface of ER constitutes about 63 000 μm^2 per cell, or about 40 times that of the plasma membrane. They synthesize 2000 molecules/s-cell of albumin (this translates to about 15 pg/cell-day) in 280–400 nm diameter vesicles delivered to the basal plasma membrane, increasing membrane surface at 0.5%/min [20]. Several lipids and membrane lipid-modifying proteins have been implicated in regulation of vesicle formation. Phosphatidic acid (PA) has been reported to be a key component defining curvature of membrane bilayer. PA was shown to be required for in-vitro formation of coated vesicles from CHO cell Golgi membranes [21].

From a bioprocessing perspective, key issues with respect to lipid metabolism are thus whether there is an optimal lipid composition or membrane structure that gives optimal growth and production characteristics; and if yes, how to manipulate the environment or engineer cells to give rise to that composition. In the following section we will discuss what cellular characteristics may be affected by lipid composition of the membrane.

3.3.1 Lipids and Cell Cultivation

Most mammalian cells in culture are provided with some fatty acids and complex lipids in their medium, although some are grown in almost lipid-free medium. Cells readily take up fatty acids, phospholipids, and cholesterol from the culture medium and incorporate them into cellular lipids. Lipids can be supplied as serum lipoproteins, as a complex with albumin, as liposomes, or otherwise solubilized, e.g., as sorbitan–fatty acid esters. Cellular uptake of fatty acids is a passive, non-energy-dependent process and intracellular conversion of free fatty acids to acyl-CoA esters is rapid, such that intracellular levels of free fatty acids remain quite low despite high extracellular concentrations [22, 23]. Cholesterol is complexed to low density lipoprotein in the body and is taken up by cells through the LDL receptor. For cells in culture, cholesterol is often supplied as a conjugate with serum albumin, or as complexes with cyclodextrin [24, 25].

Mammals cannot introduce unsaturated carbon bonds along fatty acyl chains beyond the C9 position. Thus, linoleic (18 : 2, $\Delta^{9,12}$) and linolenic acids (18 : 3, $\Delta^{9,12,15}$) are essential nutrients. The essentiality of the linoleate class of polyenic fatty acids is believed to be related to their role as substrates for prostaglandin synthesis. Prostaglandins derived from arachidonic acid act in many tissues by regulating the synthesis of the intracellular messenger molecule, cyclic AMP. Although prostaglandin is required for animals, it is not essential for cells in culture. Nevertheless, these "essential fatty acids"

(EFAs) are often regarded as the ones required for growth and proper functioning of animal cells in culture for their role in the synthesis of other polyunsaturated fatty acids. However, one may question whether they and other polyunsaturated fatty acids are absolutely essential for growth; i.e., whether or not they can be substituted by other fatty acids in the lipid bilayer membrane. Most cell types in culture do not exhibit structural or metabolic impairment when cultured in the absence of these EFAs, although some exceptions have also been reported [26, 27]. For Chinese Hamster strain CHD-3, addition of linoleic acid in place of serum albumin in media was reported to markedly improve plating efficiency [28], and linoleate was reported to be required to maintain mitochondrial function in cultured heart cells [29]. EFA requirement of various cell types in tissue culture has been reviewed elsewhere [30, 31]. In some cases, excessive unsaturates might lead to formation of lipid peroxides causing growth inhibition [32]. Overall, the extent of modification is specific to the cell line in question, the method of delivery, time of exposure to the supplemental fatty acid and its concentration, as well as to duration of the evaluation of its effect [33–35].

Cells grown under EFA-free conditions do not have measurable amounts of polyunsaturated fatty acids in membrane phospholipids; instead, they contain large quantities of two monounsaturates that can be produced biosynthetically, oleic (18 : 1) and palmitoleic (16 : 1) acids. When the same cells are grown in the presence of serum, they normally contain 20–30% polyunsaturated fatty acids [30]. In another study, cholesterol depletion in LM cells (mouse fibroblasts) was shown to be accompanied by an increase in the 18 : 1 to 16 : 0 palmitic ratio in membrane phospholipids [36]. These and other studies demonstrated that a wide range of membrane fatty acid compositions may result from altering lipid composition of the culture medium. This also indicates that cells can grow with a wide range of membrane lipid composition, even in the absence of EFAs; however, whether such changes in lipid composition of membranes has other subtle effects on growth under conditions of bioprocess interest, such as mechanical agitation [37] or direct sparging of air, is not clearly understood.

3.3.2 Physiological Effects

The lipid bilayer of biological membranes provides a matrix into which many proteins including enzymes, receptors and transporters are embedded. Lipids have no inherent catalytic activity. However, they may influence catalytic activity or biological interactions of membrane proteins. The influence of lipids on membrane protein function can either be direct, such that the binding of lipid to the protein leads to altered interaction with substrate–ligand, or indirect via modulation of the physical properties of the membrane. Depending on the type of receptor, lipid content in the membrane

can also have a specific effect on its affinity state, binding capacity, and signal transduction. Polyunsaturated fatty acid enrichment led to reduced K_m values for α-aminoisobutyrate uptake (by the Na^+-dependent high-affinity transport system) in Ehrlich ascites cells [38] and methotrexate uptake by lymphoblasts [39]. In contrast, in Y79 retinoblastoma, K_m remained unchanged for α-aminoisobutyrate uptake [40], but increased for glycine high-affinity [41]. The results indicate that all transport carriers do not respond uniformly to the same membrane lipid modifications. The bulk effects of membrane lipid organization and specific effects of individual lipids have been reviewed [42].

3.3.3 Engineering Lipid Metabolism

If one were to alter the lipid composition of the plasma membrane, the potential targets certainly include those responsible for lipid transport since transport is the first step toward modulating the composition by exogenous supplements; another group of targets are those involved in lipid biosynthesis.

Cell engineering on energy metabolism, as described above, largely entails altering biochemical reactions in the cytoplasm. In contrast, the biosynthesis machinery of lipids spans across a number of organelles; acetyl-CoA is supplied by transporting citrate from mitochondria, fatty acid synthesis occurs in cytosol and ER; cholesterol is made in peroxisome and ER, while phospholipids is synthesized on the surface of smooth ER and inner membrane of mitochondria. Overall, altering lipid biosynthesis in mammalian cells is still an underexplored territory. Much research on engineering lipid biosynthesis has focused on plants. The main goal has been to alter plant lipid composition and production of higher value seed oils. Genetic engineering in plants (reviewed in [43, 44]) has involved efforts to increase health-beneficial fatty acids found in fish oils, such as stearidonic acid (18 : 4, $\Delta^{6,9,12,15}$) and γ-linolenic acid (18 : 3, $\Delta^{6,9,12}$) [45]; increase laurate (12 : 0) content for the production of surfactants [46]; and increase production of long-chain wax esters in transgenic *Arabidopsis* [47].

Alteration of lipid metabolism genes has also been reported in other eukaryotes and animal models [48, 49]. Among the few reports of genetic alterations of lipid metabolism in cultured mammalian cells is the expression of fungal $\Delta 12$ – desaturase in cultured mouse L cells [50]. As mentioned above, unlike plants and fungi, mammals lack $\Delta 12$ – desaturase and are therefore unable to synthesize linoleic acid. With the cloning of the fungal enzyme, the authors demonstrated the capability to engineer cell lines that would no longer require an exogenous supply of EFAs. Cloning of desaturases led to increased cellular unsaturated fatty acid content, however, specific physiological changes linked to increase of a given fatty acid have not been detailed.

In recent years, studies involving cholesterol biosynthesis and its regulation have been greatly facilitated by the cloning of genes encoding enzymes that catalyze steps in the biosynthetic pathway, mostly via complementation analysis of mutations in yeast sterol auxotrophs [51]. Additionally, mutant cell lines isolated from cultured mammalian cells, Chinese hamster fibroblasts, or ovarian cells [52, 53] have played a key role in the elucidation of cholesterol homeostasis mediated by sterol regulatory element binding proteins (SREBP) [54, 55]. Recently, mouse 17β-hydroxysteroid dehydrogenase type 7 (*Hsd17b7*), encoding 3-ketoreductase of the cholesterol biosynthesis pathway was expressed in NS0 cells to relieve their cholesterol auxotrophy [56]. Interestingly, the reversal of cholesterol auxotrophy could also be accomplished by treatment with desilencing drug, 5-azacytidine to express the endogenous *Hsd17b7* [57].

Lipids play a critical role in the biogenesis of ER and other organelles central to protein secretory machinery. The transformation of B lymphocytes into Ig-secreting plasma cells is accompanied by massive expansion of ER to accommodate increased flux of secretory proteins through the organelle. XBP-1, an X-box binding protein, has been implicated in B cell differentiation and regulation of the unfolded protein response (UPR) [58, 59]. By over expressing XBP-1(S), the spliced or the active form of XBP-1 transcription factor, in 3T3 cells, a fourfold increase in phosphatidylcholine synthesis and a twofold increase in the intracellular membrane lipids phosphatidylcholine and phosphatidylethanolamine was observed [60]. The results suggest that lipids may play a role, directly or indirectly, in the development of a professional secretor. Whether such a change is also involved in the transformation of host cells to high producers after transfection and selection is not known. Nevertheless, ample evidence points to the critical role of lipids in the biogenesis of ER and other organelles central to protein secretory machinery. How one can modulate lipid composition in the medium or tinker cells with their capability to redistribute their lipid composition to favor high productivity is a question worth answering. Specific antibody production and expansion of cellular capacity to accommodate for higher production rates in mammalian cells has also been reviewed elsewhere [61].

3.4 Protein Synthesis and Secretion Machinery

3.4.1 Protein Secretory Machinery

For a high producing industrial cell line, the secreted recombinant product constitutes a very large fraction of the total protein molecules synthesized. A large portion of the protein processing capacity is devoted to the secreted protein product. It is thus instructive to review briefly the process of protein

secretion. In a professional secretor, approximately 30% of cellular proteins are destined for organelles, membrane, and secretion, and are processed through the ER. Although some proteins are translocated into the ER post-translationally, most, including typical recombinant DNA protein molecules, are translocated as nascent protein molecules. After translation initiation they are recognized by the signal recognition particle (SRP), which halts continued translation in the cytoplasm and docks the protein molecule being synthesized to the receptor on the ER membrane. It thus prevents the protein molecule from being elongated in the cytoplasm and remains unfolded without the proper environment of the ER. The nascent polypeptide is transferred to a translocon on the ER membrane. Translation elongation then resumes, allowing the polypeptide to pass through the channel of the translocon into the ER lumen. Folding of the polypeptide chain starts immediately upon translocation into the ER lumen. The signal peptide on the elongating polypeptide in the ER lumen is cleaved upon entry into the ER. The protein concentration in the ER is estimated to be about 100 mg/mL [62], a concentration at which the protein would otherwise aggregate and fall out of solution. Chaperones are major constituents of the ER. Their actions require cellular energy (ATP) and prevent protein aggregation as well as assist them in folding. In fact, an important member of the ER luminal chaperones, BiP, is also a component of the translocon complex. In addition to BiP (also known as GRP78) major ER luminal chaperones include calnexin, calreticulin, and protein disulfide isomerase.

Protein molecules that have completed the folding process are exported from the ER by inclusion in membrane vesicles. Vesicle fusion, fission and trafficking is a main form of molecular transfer from ER to different organelles (for an excellent recent review, see Lippincott–Schwartz et al. 2000 [63]). The secretory proteins in the vesicle are taken to the cis-Golgi, which along with the trans-Golgi is comprised of an array of tubules and vesicles on the opposite side of the medial-Golgi. Medial-Golgi, typically with three to seven stacks of cisternae, is the main site of glycan elongation for glycoproteins. An estimated 100 to 200 glycosyltransferases, together with various nucleotide-sugars (the precursor and substrate of the glycosyltransferases), are membrane proteins that constitute the majority of Golgi enzymes [64]. It is interesting to note that the Golgi apparatus is itself in a dynamic state, with retrograde transport to allow recycling of its own proteins and membrane. From the trans-Golgi network, secretory proteins are packaged into post-Golgi vesicles and move along a microtubule network to fuse with the plasma membrane and be secreted.

It takes a finite amount of time to process protein molecules after translation before they are excreted. With an average protein of around 350 amino acids in length, the translation takes only tens of seconds. However, the time it takes for a synthesized protein to be secreted ranges from 30 min to a couple of hours depending on the nature of the protein. α_1-protease inhibitor is

among the fastest secreted proteins with a half life of about 28 min, whereas transferrin takes a longer time of around 2 h to get secreted [65]. Furthermore, even for the same protein, the secretion time is not uniform for all molecules; rather, a distribution is seen, with some having a shorter and others a longer holding time.

3.4.2 Unfolded Protein Response and Transformation from Dormant B Lymphocyte to Plasma Cell

Cells with varying productivity may have different rate-limiting steps for achieving high productivity. Different components of the protein production machinery, from transcription to product secretion, may become a limiting factor for an increased secretion rate. It is not clear which step or which protein processing component in the machinery is limiting for product formation; nor is it understood whether the increased productivity entails increased protein secretory capacity or merely involves a more extensive use of existing capacity. This may have implication on engineering cells for better production, as enhanced production will be more profound if the "constriction" point is relaxed. Little is known about the overall transformation that host cells incur with the amplification of the heterologous gene and the overexpression of the encoded protein. However, some parallels might be drawn in the development of professional secretors in-vivo and from the cellular response incurred when cells are drawn upon to increase the load of protein secretion drastically.

ER has a finite capacity for protein processing. A sudden increase in translation rate and a surge in translocation of protein molecules into the ER may overload the protein processing capacity. This may result in accumulation of misfolded proteins in the ER and lead to the breakdown of protein synthesis machinery. Cells cope with such a surge in translation by triggering a coordinated and complex unfolded protein response (UPR); the translation rate is decreased to reduce the load of protein processing, whereas, chaperone synthesis and the protein folding capacity is increased, and the degradation of misfolded proteins (or ERAD, ER associated degradation) is increased. UPR is thus characterized by a decrease of general translation initiation rate, an increased specific expression of molecular chaperones such as GRP78 and GRP94, and an expansion in ER volume. Three proteins on ER membrane that serve as signaling molecules of ER stress are: IRE1 (inositol-requiring 1), PERK (PKR, RNA-activated protein kinase-like ER resident kinase) and ATF6 (activating transcription factor 6). All three have binding sites for BiP in the ER lumen and complex with BiP. BiP also binds to hydrophobic sites on the unfolded proteins. Under ER stress, and in the presence of excess unfolded proteins, BiP preferentially associates with the unfolded proteins, thereby releasing the sensor molecules to trigger their signaling functions [66].

The differentiation of a B cell to a professional secretor plasma cell is a case in which a non-secretor becomes one of the best protein secretors in the body. Understanding this cellular transformation and its regulation may provide clues to the mechanism of enhanced protein secretion in high producing cells. A resting B cell is low in cytoplasm and has a scarce ER. Upon antigen stimulation, the B cell is transformed into a plasma cell that secretes thousands of antibody molecules. The process of transformation is thus accompanied by greatly increased transcription and translation of an immunoglobulin gene. It also entails a series of cellular events leading to intracellular structural change in establishing a highly developed secretory machinery. An increase in cell size accompanies the cell's new secretory role. Through proteomic investigation van Anken et al. [1] followed the transition of B lymphoma cells to IgM secreting plasma cells upon activation by lipopolysaccharides (LPS). Functionally related clusters of proteins appeared in waves during the course of B cell differentiation. Mitochondrial and cytosolic chaperones were up-regulated concurrently early. Metabolic enzymes peaked later (3 days after activation). The expansion of ER appears to involve an increase in the abundance of ER proteins, including PDI which catalyses the formation of disulfide bridges, and in the level of four oxidoreductases as the B cells differentiate. Many of the redox balance enzymes in cytosol and mitochondria are also up-regulated. There is also evidence to show that Golgi increases along with ER. The first stage of ER expansion bypasses the classical UPR mechanism, apparently via signals initiated by mitogen or antigen stimulation, such as B lymphocyte-induced maturation protein 1 (Blimp-1), a key regulator of B cell differentiation [67]. Blimp-1 targets include genes involved in growth and proliferation (e.g., c-myc, cdk2, E2F-1) and genes involved in mature B cell function and activation (PAX5, MHCII, CD69). The sudden increase in synthesis of IgM subunits after 2 days and the subsequent accumulation of intracellular IgM subunits, however, does seem to trigger a classical XBP-1 ER load-dependent UPR.

One characteristic of host cell or producing cell that is most sought after is a super-high capacity of protein secretion. Protein production involves a delicate balance of many factors for its processing. From the mechanism of UPR response and B cell development to plasma cells one can certainly expect that (at least in the case of heterologous protein expression in CHO, BHK, and other non-secretory cells) the transformation from an initially transfected host cell to a hyperproducer will likely entail an expanded protein secretory machinery. In fact, in B cell differentiation the expansion of the secretory machinery arises before antibody secretion. Whether a hyperproducer of recombinant protein also develops a higher level of energy metabolism, with increased mitochondria and elevated levels of metabolic enzymes, and of lipid metabolic enzymes to supply the need of membrane lipids for organelles has not yet been examined.

3.4.3 Engineering of Protein Processing Pathways

A key role of the protein processing machinery in the ER is to facilitate protein folding or to prevent unfolded protein from aggregating. Much of the machinery is also involved in the cellular stress response. As cells are subjected to various stress conditions, such as temperature shift or exceedingly high protein synthesis rate (as discussed in the section on UPR), protein folding is also drastically affected. Cells also respond to stress conditions by expressing components of the protein processing machinery, including chaperones. Historically, many such chaperones were initially identified as stress-response proteins, including the heat shock protein (HSP) family. Many of those chaperones also play roles in both facilitating protein folding as well as in the stress response. Over-expression of HSP70 in NS0 cells under a constitutive promoter only moderately improved the viability in batch culture [68]. Surprisingly, when used as fusion partner, the frequency of hybridoma generation was significantly higher; however, the resulting murine hybridomas did not show increased productivity of a monoclonal antibody. HSP proteins constitute a large protein family, all playing roles in protein folding and stress response. Whether any other member of this family has a beneficial effect when over-expressed in a recombinant protein producer has yet to be examined.

Other components of the protein processing pathway that have been targets of metabolic engineering are GRP78/BiP, the most abundant chaperone in the ER, and protein disulfide isomerase (PDI), the ER protein which catalyzes disulfide bond formation. Expression of the stress mediator GRP78 is elevated upon glucose deprivation, unfolded protein accumulation, and inhibition of *N*-linked glycosylation. In an early study using recombinant CHO cells producing a mutant form of Factor VIII and von Willebrand factor, it was observed that the transcription of endogenous GRP78 (as distinct from the vector derived one) and another chaperone, GRP94, were actually decreased compared to wild-type CHO cells. Furthermore, over-expression of GRP78 did not lead to increased productivity of the recombinant proteins [69]. Protein disulfide isomerase (PDI) and BiP were stably expressed either alone or in combination in a recombinant CHO cell line producing a human IgG [70]. It was found that over-expression of PDI had a positive impact on specific IgG productivity, causing a moderate increase ($\sim$ 40%) in IgG secretion. However, transfection of BiP, either alone or together with PDI, resulted in a decreased productivity. In other reports, over-expression of PDI did not elicit an increase in the production of secreted protein product. Hybridoma cells stably transfected with dexamethasone-inducible PDI failed to enhance its antibody productivity [71]. Since a key role of PDI is to facilitate disulfide bond formation, another study investigated the effect of PDI over-expression on two recombinant proteins with high and low disulfide linkages, namely TNFR : Fc

and IL-15. CHO cells transfected with a dicistronic vector containing DHFR along with either a TNFR : Fc fusion protein or IL-15 were used to study the effect of PDI over-expression. Pulse chase labeling of TNFR : Fc revealed a longer retention time when PDI was over-expressed. However, the secretion was reduced. Interestingly, no evidence of increased retention was seen for IL-15 with PDI over-expression and the secretion of IL-15 was not affected [72].

These results suggest that the relationship between PDI levels and the over-expression of a secretory protein is rather complex. PDI exerts its effect by complexing with folding proteins; such complex formation is dependent on the concentrations of both PDI and all the protein molecules being processed in the ER. The same principle holds for other chaperone proteins in the ER. Over-expression of PDI or other chaperone proteins will facilitate the folding and secretion of the protein product only if the expression of the protein product takes up a large fraction of the capacity of the protein folding machinery and if the over-expressed chaperone proteins restore the appropriate stoichiometric ratio of the chaperones and the folding proteins.

In addition to protein folding and disulfide bond formation many post-translational modifications, including the initial stage of *N*-glycosylation, γ-carboxylation, phosphorylation of proteins or glycans, take place in the ER. Some of those modifications are critical to the function of some recombinant proteins. Examples include γ-carboxylation of the *N*-terminal glutamic acid residue in Factor VIII, IX, and Protein C, and phosphorylation of mannose 6-phosphate on α-L-iduronidase. Unlike IgG molecules, which are often highly expressed and produced in a number of cell types at high levels, those molecules are often expressed at a much lower level. One may be tempted to over-express proteins involved in those modifications in the hope of increasing their productivity. Vitamin K-dependent carboxylase has been stably transfected into COS-1 and CHO cells expressing Factor IX [73]. γ-Carboxylase activity was shown to have increased nearly 17- and 16-fold, in COS-1 and CHO cells, respectively.

3.5 Protein Glycosylation and Post-translational Modifications

The vast majority of recombinant proteins produced through mammalian cell culture processes are glycoproteins, with oligosaccharides attached to one or more of their asparagine (*N*-linked) or serine/threonine residues (*O*-linked). The carbohydrate content of glycoproteins can be as high as 40% of the molecular weight of the unglycosylated protein. Glycans can be comprised of a large number of different monosaccharides (Fig. 3). Protein *N*-glycosylation initiates in the ER and is further processed in the Golgi apparatus, whereas the addition of carbohydrates onto the hydroxyl groups of serine and threonine residues (*O*-linked glycosylation) has been shown to initiate in either

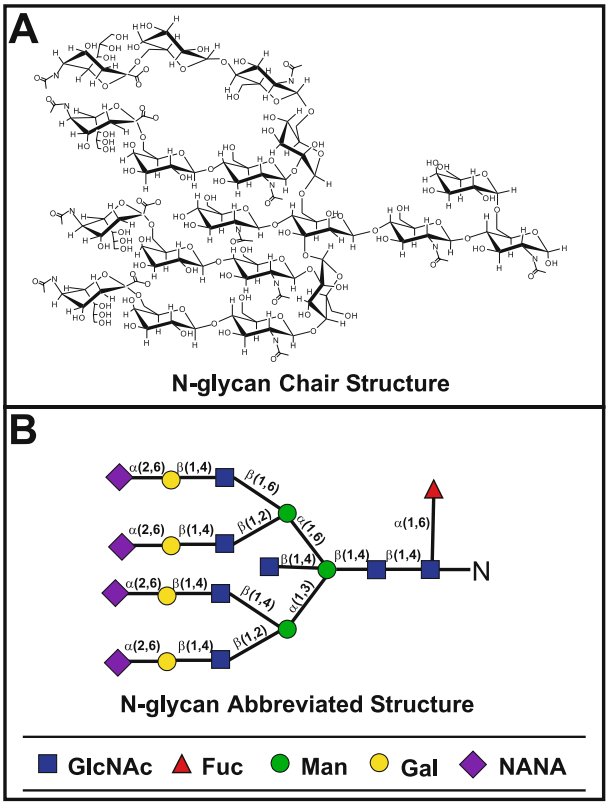

Fig. 3 Representative structure of an *N*-glycan. **a** Chair structure of an *N*-linked glycan. **b** Abbreviated structure of the corresponding *N*-glycan shown in **a**. *GlcNAc* *N*-Acetylglucosamine, *Fuc* fucose, *Man* mannose, *Gal* galactose, *NANA* sialic acid

the ER or Golgi [74]. Glycan formation requires nucleotide-sugar donors. The activated forms of these molecules arise from either salvage pathways from protein degradation in lysosomes, or direct activation through enzymatic activities. Monosaccharides including glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, mannose, fucose, and sialic acid are enzymatically linked to nucleotides, which are then available for donation onto glycoproteins. All *N*-glycans share a common core structure, whereas *O*-glycans have been documented to have up to eight core structures.

Due to inconsistencies in processing, *N*- and *O*-glycans have numerous structural heterogeneities. The differences in sugar composition of the glycan attached to the same attachment site of different glycoprotein molecules is called microheterogeneity. The difference in the presence of glycans on separate attachment sites of different glycoprotein molecules is called macroheterogeneity. Inside the Golgi, in particular, numerous factors may facil-

itate this inconsistency, including relative nucleotide-sugar concentrations, transport rate of nucleotide-sugar transporters, relative glycoconjugate enzyme activities, relative glycan concentrations, local protein structure, intra-Golgi pH, spatial glycoconjugate enzyme compartmentalization, and spatial nucleotide-sugar transporter compartmentalization [75].

Glycosylation affects protein folding while being processed in the ER. As a secreted product the presence of glycans affects the solubility, aggregation, and the stability of the glycoprotein product [76]. As therapeutic agents, the glycan structure affects the half-life during blood circulation, as well as antigenicity, immunogenicity, and other biological functions. It is well known that glycosylation affects the retention time of protein therapeutics in blood. The presence of carbohydrates delays clearance from blood, as demonstrated by a comparison of a glycosylated protein and its non-glycosylated counterpart [77]. The increased circulation half-life can be affected by both *N*- and *O*-glycosylation. Another possible mechanism of increased circulation half-life of the glycosylated therapeutics is through its increased stability, as illustrated by the decreased aggregation due to disulfide bond formation in the glycosylated product, as compared to the deglycosylated counterpart [78]. Among similarly glycosylated proteins the extent of sialylation affects the blood half-life. The effect of sialic acid content on increased circulation half-life is best documented in the case of erythropoietin (EPO). EPO molecules with higher sialic acid content were isolated by taking advantage of their being negatively charged and were shown to have higher biological activities, which were attributed to a longer circulation half-life. The decreased circulatory half-life of under-sialylated glycoproteins was thought to be caused by a higher liver uptake via the hepatic asialoglycoprotein binding protein [79]. A more recent study has pointed to the possible roles of other unidentified receptors also being responsible for the clearance of desialylated glycoproteins [76, 80]. It was also postulated that the glycosylated recombinant proteins are better trapped by the extracellular matrix, thus having a longer bioavailability in vivo than their unglycosylated variant.

Unless intended for vaccination use, immunogenicity elicited by recombinant proteins is of concern. An antibody elicited by and against the protein therapeutic can result in neutralization of the therapeutic protein and may result in reduced efficacy, and cause serious adverse clinical effects. The potential immunogenicity of recombinant therapeutics may arise from the aglycosylated protein, or from the glycan associated with them. The role of glycosylation on the immunogenicity of glycoproteins has been reviewed [81]. There are at least two mechanisms by which glycans on a protein may affect the immunogenicity of a human therapeutic: first by being a foreign glycan structure, and second by shielding a segment of protein that is otherwise antibody-inductive. Comparison of different recombinant human therapeutic proteins that are produced in different organisms and are thus differently glycosylated (as from CHO and yeast) or aglycosylated (as from *E. coli*) seems to

lead to a conclusion that the "shielding" effect of minimizing immunogenicity is affected by the nature of the protein, as well as by the source of the protein [81]. Nevertheless, evidence does indicate that "properly" glycosylated proteins (as produced in CHO) are less immunogenic. However, the variables involved in the processing of those proteins are not singularly isolated. Proteins produced in one organism may have subtle conformational difference to the others, in addition to the difference in glycan composition.

The concern about the immunogenicity of different glycoforms of the rDNA proteins produced in insect cells and in transgenic plants is one major concern about those technologies' application for rDNA therapeutic protein production. The glycosylation pathway is highly conserved in mammals. Nevertheless, divergence among different species does occur, as revealed in a glycan structural survey of IgG molecules derived from human and 12 other mammalian species and chicken [82]. A wide variation in sialylation and branch-specific galactosylation was observed. Furthermore, host cells (or host animal in the case of transgenic products) derived from other species may possess a set of glycosylation enzymes different from human. Human glycans have terminal *N*-acetylneuraminic acid (NANA), whereas other mammals have *N*-acetylglycolylneuraminic acid (NGNA). NGNA is the hydrolytic product of CMP-sialic acid hydroxylase, which is present in nearly all mammals but missing in human [83]. Thus, glycoproteins produced in CHO have some percentage of NGNA present among all sialylated glycans. Similarly, glycoproteins expressed in CHO cells have only terminal $\alpha(2,3)$-linked sialic acids, in contrast to $\alpha(2,6)$ and $\alpha(2,3)$ seen in human due to a different complement of functional sialyltransferases. Such differences in glycan composition have posed a concern; however, the antigenicity of recombinant proteins directly caused by individual glycans is still scant. Nevertheless, it has been shown that high mannose glycans may mimic fungal proteins, and can both activate and inhibit an immune response [84]. Glycans not found in humans, such as galactose-$\alpha(1,3)$-galactose linked glycans, are known to be immunogenic [85, 86], and an immune response may be mounted against recombinant glycoproteins that have this linkage.

Glycans have important cellular roles in adhesion and signaling. Glycosylation patterns have been reported to affect the efficacy of various immunoglobulin molecules. IgG molecules have a glycosylation site in their Fc region at N^{297}, which plays a key role in its effector functions. Deglycosylated IgG has been shown to completely abolish its ability to elicit antibody-dependent cellular cytotoxicity (ADCC) [87]. Lack of glycosylation at Asn-563 in IgM produced by murine hybridoma increased avidity for antigen, possibly due to modification of quaternary structure of the IgM polymer [88]. Hypogalactosylation of IgG has been shown to affect some of the effector functions of the IgG molecule including binding to complement C1q and mannose-binding protein [89]. Glycosylation sites of serum immunoglobulin A (IgA) have also been characterized [90]. Interaction of IgA with surface

receptors such as the asialoglycoprotein receptor and the Fcα receptor has been shown to be dependent on the associated sugar chain, such that oversialylated IgA bound to a lesser extent than the native or desialylated IgA [91]. The number of glycan branches has also been correlated to in vivo biological activity [92].

3.5.1 Engineering Protein Glycosylation Patterns

In the past few years, metabolic engineering has been used to modify the glycans on recombinant glycoproteins. Some have aimed to reduce or eliminate the undesired glycans that either pose potential immunogenic concern or decrease the quality of the glycoprotein product. In some cases the enzyme leading to the undesired glycan can be suppressed. Sialidase, a soluble enzyme which cleaves sialic acid from terminal glycans, has been shown to be released from CHO cells into the culture medium [93]. Its accumulation in the culture medium leads to a progressively decreasing sialic acid content, and increases the proportion of under-sialylated glycans in the product. To help prevent this, its accumulation in the extracellular media was reduced by expression of antisense RNA in CHO cells, resulting in a significant reduction of sialidase activity, and an increased sialic acid content in the glycoprotein product, DNase [94]. A glycan residue non-native to human and thus a potential target for elimination from recombinant therapeutic proteins is *N*-glycolylneuraminic acid (NGNA) (although its antigenicity in human has not yet been clearly shown). It is conceivable that a similar antisense or RNAi strategy can be applied to suppress CMP-sialic acid hydroxylase to decrease or eliminate the formation of NGNA. CHO cells were transfected with antisense CMP-sialic acid hydroxylase sequences designed from a mouse cDNA sequence [95] and shown to have a 80% reduction in hydroxylase activity. In a number of studies, over-expression of a glycosyltransferase was used to redirect the fluxes to reduce the abundance of non-human glycoforms. Glycans bearing sialic acid on humans have α(2,3)- and α(2,6)-linkages, whereas glycans produced in CHO cells have only the α(2,3)-linkage. Transfection of cDNA encoding for α(2,6) sialyltransferase enabled CHO cells to synthesize glycans with the α(2,6)-linkage [96]. This strategy was later applied to demonstrate the alteration of glycan structures of recombinant human erythropoietin produced in CHO and BHK cells [97, 98].

The examples above reflect the efforts in making recombinant proteins produced in non-human host cells resemble native glycans in human, to alleviate concerns on the potential adverse effects of the non-human glycan. In another series of efforts, the sialic acid content in glycoproteins was increased through cell engineering. Sialic acid content on therapeutic glycoproteins has been correlated to an increased circulation half-life. With the complexity of the glycan biosynthetic pathway, the rate-limiting steps in directing

glycan synthesis toward a fully processed and highly sialylated form are not clear. Nevertheless, many different approaches have been attempted. α(2,3) Sialyltransferase was amplified in CHO cells expressing a TNFR–IgG fusion protein or a modified tPA and it was shown to not only increase sialic acid content, but also to reduce microheterogeneity and enhance product consistency [99]. Others have taken a protein engineering approach to increase the number of *N*-glycosylation sites on two recombinant proteins, follicle stimulating hormone (FSH) [100] and erythropoietin (EPO) [101]. EPO (darbepoetin alfa) was engineered to increase its *N*-glycosylation sites from three to five, resulting in an increased sialic acid content compared to endogenous or recombinant human EPO [102]. The hypersialylation of the molecule was shown to have a direct correlation to increasing its half-life and in vivo biological activity, allowing for less frequent dosing in clinical applications.

However, hypersialylation or full glycosylation is not a universal optimization strategy for all products. For proteins intended for eliciting immunogenic response (as in the application of vaccines) under- or even unglycosylated glycoproteins, by their tendency to aggregate and expose otherwise shielded protein motifs, are sometimes favored. Protein engineering was applied to investigate the effect of varying occupancy of the *N*-glycosylation sites on the immunogenicity of avian influenza virus vaccine [103]. However, no significant change in immunogenicity was seen when amino acids of one or both of the two sites were altered to abolish the *N*-specific glycan linkage.

Although glycans play key roles in modulating the biological activities of glycoproteins, glycoengineering often results in changes not only in glycan structure, but also in the physiochemical properties of the glycoprotein itself. Thus, the resulting effect is not necessarily directly attributed to the glycan. A direct effect of glycan structure on the biological activities of recombinant protein has been demonstrated in the cases of *N*-acetylglucosaminyltransferase-III (GnT-III) and fucosyltransferase-VIII (FuT-VIII). With the large number of therapeutic antibodies emerging in the market, a great deal of research on recombinant proteins has focused on their glycoform. Some therapeutic antibodies confer their efficacy through blocking or neutralizing activities, while the effectiveness of others is mediated through antibody-dependent cellular cytotoxicity (ADCC). For these two types of activities, the glycan linked to Asn^{297} in the Fc region of IgG plays a key role; in particular the presence of bisecting GlcNAc has been implicated in the biological activities of therapeutic antibodies [104]. GnT-III adds a GlcNAc to the trimannosyl core of *N*-glycans of many glycoproteins through a β(1,4)-linkage. This *N*-acetylglucosamine residue bisects the *N*-glycan branches, and restricts a number of reactions in the subsequent *N*-glycan elongation. This enzyme is present in human, mouse and many other mammals, but is not detectable in CHO cells [105]. After demonstrating that the introduction of rat GnT-III into CHO cells expressing recombinant IFN-β shifted the glycoform towards more abundant bisected glycans [106],

Bailey and his coworkers successfully demonstrated that a similar strategy when applied to IgG increased its ADCC activity [107]. The same strategy was applied to engineer CHO cells producing an IgG approved for clinical use, affirming the importance of metabolic engineering of the glycoform of recombinant proteins [108].

The effector function of IgG, which is the basis of ADCC, is mediated through binding to various Fc receptors (FcγR) on leukocytes. Through a study of binding of human IgG lacking fucose on its trimannosyl core of the Asn^{297} glycan to various FcγR, it was established that unfucosylated IgG has a markedly higher ADCC activity and binding to FcγRIII [109]. It was further demonstrated that the absence of fucose is more important in increasing ADCC activity of IgG compared to the presence of a bisecting GlcNAc, which enhances ADCC only moderately, and is most effective on fucosylated IgG molecules such as those produced in CHO cells [110]. Subsequently, through sequential homologous recombination, a FuT-VIII knockout CHO host cell line ($FUT8^{-/-}$) was generated and shown to produce IgG that had significantly higher ADCC functions [111]. siRNA against the same enzyme was applied to isolate FuT-VIII knockdown variants of established antibody-producing CHO lines. Again, enhanced ADCC was demonstrated [112].

Other possible means of intervening the glycan biosynthetic pathway is by manipulating the supply of nucleotide-sugars, either through nucleotide-sugar synthesis or nucleotide-sugar transporters. Except for CMP-sialic acid, which is synthesized in the nucleus, all other nucleotide-sugars are synthesized in the cytosol. Each nucleotide-sugar is then transported into the ER and Golgi compartments by cognate transporters. Readers are referred to a recent review of nucleotide-sugar transporters [113]. CHO cells expressing interferon-γ were stably transfected with the hamster CMP-sialic acid transporter, which led to increased transporter expression levels and a moderate increase in sialylation [114]. With recent demonstration of enhanced ADCC with unfucosylated IgG molecules, it is conceivable that the GDP-fucose transporter, or enzymes contributing to the formation of GDP-fucose, will become targets of genetic engineering for recombinant IgG production.

Glycoengineering may also be applied in order to change cellular characteristics, rather than altering the properties of the secreted proteins. Overexpression of GnT-III activity led to the appearance of GlcNAc residues on the hepatocyte growth factor (HGF) receptor, c-Met, in human hepatocarcinoma cells, HepG2 [115]. Elevated levels of HGF-induced cell scattering were also observed in the GnT-III transfectants. In another study, altered cell surface glycosylation, via expression of sialyltransferase genes and *N*-acetylglucosaminyltransferase genes into human glioma cells, was shown to affect their susceptibility to different pro-apoptotic drugs [116]. Glycoengineering, when applied to host cells for recombinant protein production, has for the most part focused on changing the glycoform of the protein product, rather than altering the host cell characteristics per se. It may find more applications

in modulating the infection efficiency of host cells in virus production processes. Glycosylation may affect virus binding to cell surface receptors [117]. The host cells used in their production are usually not the native cells to be infected in vivo; they do not necessarily have the high affinity receptor(s) for efficient infection. A higher affinity of binding through protein or glycoform engineering may increase the infection efficiency and reduce the multiplicity.

Most of the reports cited above focus on redirecting glycan distributions towards the desired products. A few studies have also demonstrated improved consistency by minimizing the variation of microheterogeneity seen in rDNA glycoprotein production. However, for glycoproteins with multiple sites and high complexity, achieving consistent glycan pattern in the final product via metabolic engineering may not be an easy task. The abundance of glycotransferases has a wide variation across different cell lines derived from different tissues. How wide the distribution is amongst different host cells or producing cell lines under different culture conditions is not well known. One of these glyconjugate enzymes, GnT-I, catalyses only one reaction in the glycan pathway; while other enzymes can use many different glycans in the biosynthetic network as their substrates. Engineering of the first type of enzyme affects only a single step in the biosynthetic reaction network, the structural outcome is thus rather predictable. The effect of altering those enzymes which catalyze multiple steps in the pathway is less predictable; many steps along the reaction path will be affected. Because different glycan substrates may have different affinities for the same enzyme, and different enzymes may also compete for the same glycan substrate or nucleotide-sugar precursor, fully "designing" glycoform profiles by altering these enzymes will not be simple.

Another question frequently raised in engineering protein processing and glycosylation pathways is whether the metabolic engineering strategy will work equally well with producing cells of different levels of productivity. In other words, will the "rate-controlling step" of protein secretion or glycosylation differ depending on the protein secretion rate? We have seen a few case studies of glycoengineering being applied to cells with high productivity of antibodies [108]. In a study of BHK-21 and CHO cells producing glycoprotein product, similar glycan profiles were observed even after a 200-fold increase in productivity [118]. Obviously the effectiveness of glycoengineering on modulating the glycoform on recombinant proteins will depend on many factors. We also do not know in a high producing recombinant cell whether a given protein secretory and post-translational modification machinery is expanded to accommodate the faster secretion rate, or if a given machinery is made to work harder to produce more. Until we have a better understanding of the root of hyperproductivity some degree of empiricism will remain in metabolic engineering of the glycosylation and protein secretion pathways.

3.6
Cell Cycle Control

Cell growth is the manifestation of a delicate balance between positive and negative signals that are present both extracellularly and inside the cell. Cells receive external signals from the environment to detect the availability or absence of nutrients for DNA replication and biomass synthesis. Progression through G1, S, G2, and M phases of cell cycle is dependent on those external positive factors, including mitogenic factors such as insulin or insulin-like growth factor, fibroblast growth factor, and cytokines. Anchorage-dependent cells also receive growth stimuli through establishing contacts between surface receptors and extracellular matrices, and through the establishment of the cytoskeletal network. In addition to the mitogenic factors, there are many other factors that provide signals to cause growth arrest. An example of a diffusible factor that can signal the stoppage of cell division is the cytokine interferon, which is shown to exert an antiproliferative effect on cell lines from different origins [119]. For normal diploid cells, the cell–cell contact or contact inhibition upon reaching a confluent state has also been recognized as a signal for imposing quiescence on cells. Whether cells divide and grow, or self-destruct, is the outcome of a balancing act of the external positive and negative factors as well as that of the internal mechanisms. Loosening the controls may lead to unscheduled proliferation and transformation of cells to their malignant derivatives. Tipping the balance to favor uncontrolled growth often also results in unwarranted survival. Much understanding of the identification of targets that can be manipulated to arrest growth or improve viability of cultured cells has been gained from the study of pathways that underlie proliferation of cancer cells. The details of cell cycle control in relation to cancer progression have been thoroughly reviewed elsewhere [120]. Cancer cells typically involve mutation or alteration of genes involved in inactivation of apoptotic pathways, induction of genomic instability, self-sufficiency in growth signals, as well as insensitivity to antigrowth signals [121].

Each of the four phases of cell cycle, G1 (gap phase), S (DNA synthesis), G2 and M (mitosis), are cooperatively regulated by cyclin-dependent kinases (CDKs) whose activities are in turn constrained by CDK inhibitors. For a proper progression of mitosis, the programmed activation and inactivation of cell cycle regulators such as cyclins, protein kinases, and phosphatases is required. Each of these regulatory proteins displays characteristic periodic dynamic profiles that proceed as the cell cycle progresses (Fig. 4). Each protein's profile is the result of the interactions of the other cell cycle regulatory components – with their expression, activation, inactivation, or degradation corresponding to a specific time frame. Cell cycle events are positively regulated by the availability as well as by the post-translational modifications associated with the CDK and cyclin subunits; whereas negative control of growth is mediated by the action of CDK inhibitors (CDI), which deactivate

cyclin–CDK complexes. In order to ensure proper progression through the cell cycle, cells have developed a series of checkpoints that prevent them from entering a new phase until they have successfully completed the preceding. Figure 4 depicts this pathway and the interaction amongst the major components in the control of the cell cycle. One of the most important cell-cycle checkpoints occurs along the transition from the G1 to S phase. The pivotal player in the G1–S phase transition control is the CDK4/6–cyclin D complex. This is closest to external stimulatory or inhibitory signals. In the event that an essential mitogenic factor is absent, or an anchorage-dependent cell is kept in suspension, the cell cycle is halted at G1 phase. The activated CDK4/6–cyclin D complex can phosphorylate the regulatory protein, retinoblastoma (pRB). pRB in its unphosphorylated state binds to and inhibits the transcription factor E2F. Upon phosphorylation, pRB dissociates from E2F, leading to the activation of cyclin E transcription by E2F. E2F activation positively regulates transcription of genes involved in cell cycle progression [122, 123].

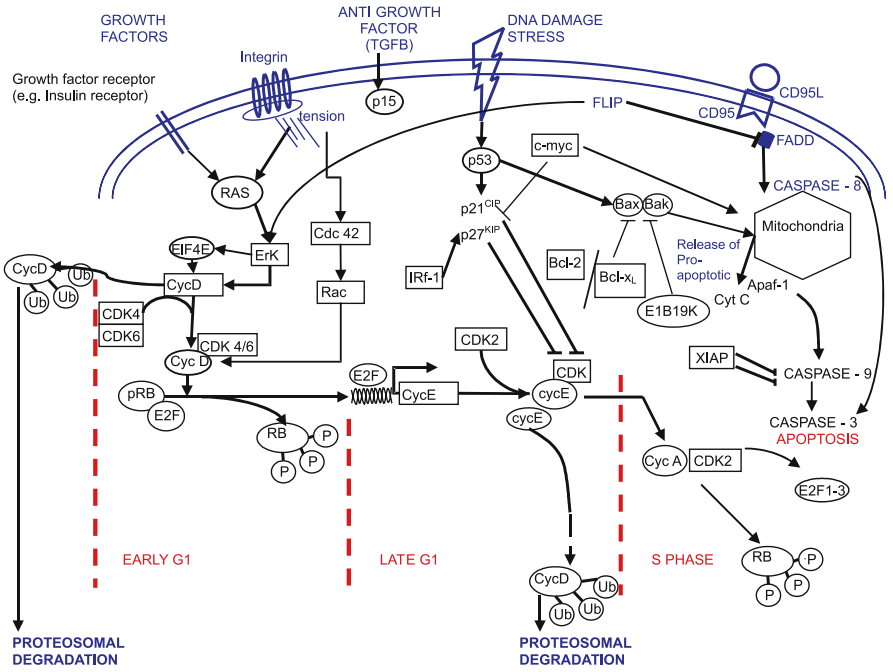

Fig. 4 Schematic representation of the interaction between cell cycle and apoptosis pathways. *CDK* Cell cycle-dependent kinase, *IRF-1* interferon-regulatory factor 1, *pRb* phosphorylated retinoblastoma, *ERK* extracellular signal regulated kinase, *FADD* Fas-associated death domain protein, *FLIP* FLICE-inhibitory protein, *Cdc42* cell division cycle 42, *EIF4E* eukaryotic translation initiation factor 4E, *Cyc* Cyclin, *XIAP* cross-linked inhibitor of apoptosis proteins, *Apaf-1* apoptotic peptidase activating factor – 1, *BAX* Bcl-2 associated X protein, *BAK* Bcl-2 homologous antagonist/killer, *Ub* ubiquitination, *cyt C* cytochrome C

Input from growth factor signaling and cell adhesion-mediated signaling are a prerequisite for driving the progression of G1 phase cell cycle. The two pathways are not independent of each other, but rather extensive cross-talk exists between the signaling pathways [124]. In normal untransformed cells, all the important growth factor signal transduction cascades are regulated by integrin-mediated cell adhesion. As a result, adherent cells rely on attachment to extracellular matrix for growth.

Cell adhesion to the extracellular matrix protein leads to downstream activation of Rho family Cdc42 and Rac, leading to cell spreading [125]. Consequently, the role of Rac signaling pathways is to activate cyclin D1 transcription during the G1 phase of the cell cycle [126].

In tumorigenesis, cells acquire activated forms of mutation in Ras signaling, which in turn leads to higher levels of cyclin D1. Cyclin D1 overexpression has also been described in pathogenesis of a wide spectrum of cancers [127] and cyclin E over-expression has been widely observed in tumors, including carcinomas, lymphomas, and leukemia. Deregulation of cyclin E expression in tumors largely involves increased E2F activity [128–130]. The most prominent members of the CDK inhibitor (CDI) family are p21 and p27. The cylin E–CDK 2 complex is targeted by p27 to prevent its activation [131], while p21 at elevated concentrations binds not only to cyclin E–CDK2, but also to other cyclin–CDK complexes to prevent the progression of the cell cycle at the G1 phase [132].

Except for vaccine productions where normal diploid human fibroblasts are employed, virtually all cells used for protein production are continuous cell lines, including CHO, BHK, HEK 293, and mouse myeloma cells such as NS0 and Sp2/0. All have lost their normal growth control, in other words, the mechanism of check point control in their cell cycle has been compromised or the regulation rendering cells to a quiescent state in the absence of mitogen has been relaxed. The mechanism of relaxation of growth control in NS0 or Sp2/0 is probably rather similar to myeloma cells (or multiple myeloma). The loss of cell cycle control in CHO and BHK cells is less studied, but is likely to fall into one of the more often seen mechanisms. While it is intriguing to alter the different components of the cell cycle in these cells, it is important to keep in mind that the cell cycle control in them is already "abnormal". A strategy for altering their cell cycle therefore, does not necessarily entail resuming their control machinery back to a normal state, but will in fact involve better understanding of the cause of their abnormality.

3.6.1 Engineering Cell Cycle Control

The temptation of grappling with the growth control from a cell's native regulation mechanism initially stemmed from the desire to bypass the mitogenic factor or serum requirements and overcome the growth arrest that occurs

naturally. As adaptation of cells to serum-free or even protein-free medium has become relatively mundane, the focus has shifted towards possibly increasing growth rate to attain a high cell concentration rapidly in the growth phase and controlling growth at a minimum rate in the production phase.

Many have examined the effect of growth rate on productivity. A rather moderate negative dependence of specific productivity to growth rate was seen for hybridoma cells [133–135]. In another study, a positive relationship has been reported between growth rate and product formation [136]. In all those cases the specific productivity was only a weak function of the growth rate; and after extrapolating the data to zero growth rate, all gave a very significant specific productivity. It thus led to the postulation that cells arrested at a non-growing state should be able to produce and secrete recombinant proteins. Ideally, cells can be engineered to allow a cytostatic element to be turned on once a high cell density has been achieved in the reactor to allow for continued production by healthy cells.

As mentioned above, the growth of mammalian cells is a response to the presence of positive signals and absence of negative signals in the environment. The mitogenic factors for cells in culture are traditionally supplied by serum or other growth factors. Furthermore, many adherent cells derive signals for cell division from cell–cell or cell–surface adhesion. Given the need to grow mammalian cells in a large-scale suspension culture in animal component-free medium, it is desirable to remove serum/growth factor and surface attachment requirements for those cells.

Since mitogenic signals relay their effects through pRB pathway in triggering entry of cells in G1 phase into S phase, members of this pathway are natural targets for cell cycle manipulation to mitigate the growth factor dependence. The possibility of alleviating the dependence on mitogenic factors through altering cell cycle control was demonstrated by microinjection of complex CDK–cyclin E complex into serum-starved quiescent fibroblast to initiate DNA synthesis [137]. Constitutive over-expression of cyclin E in fibroblasts resulted in shortened G1 phase, smaller cell size, and diminished serum requirement for the G1–S transition [138]. The realization that mitogens, such as fibroblast growth factor, affect cell cycle control through cyclins led to over-expression of recombinant human cyclin E in CHO-K1 cells [139] under a human cytomegalovirus promoter to enable them to grow in protein-free medium.

Rb complexes with E2F and acts as a negative regulator of the transcription factor activity of E2F. The controlling role of Rb is effected by its own phosphorylated inactivation and resulting dissociation from E2F, allowing E2F to turn on the transcription of cyclin E. Adherent cells kept in suspension lack Rb hyperphosphorylation [140]. E2F family proteins are thus another target that has been explored. The transcription factor E2F in mammalian cells include seven members, some act as transcription activators others as repressors. Activators promote cell cycle progression while the repressors are

thought to be required for exiting cell cycle and differentiation. E2F-1 mRNA was found to be regulated during the cell cycle, with maximal levels seen near the G1/S checkpoint [135]. Several key genes required for DNA synthesis including c-myc, dihydrofolate reductase, cdc2, c alpha, and cyclin A are under E2F regulation. Over expression of E2F-1, E2F-2, or E2F-3 in NIH 3T3 cells resulted in anchorage-independent growth on soft agar [141].

CHO-K1 cells over-expressing exogenous human E2F-1 have an extended S phase and grow in protein-free medium [142]. However, over-expression of E2F1 (a member of the E2F family) may lead to p53-dependent induction of apoptosis. The p53 protein is a transcription factor that can induce apoptosis or cell cycle arrest genes in response various stress signals, such as DNA damage, heat shock, or hypoxia. A microarray study has also shown the effect of up-regulating E2F-1 in human cell line [143]. E2F-1 regulates genes that encode components of the DNA damage checkpoint and repair pathways as well as factors involved in chromatin assembly/condensation, chromosome segregation, and mitotic spindle checkpoint. Interestingly, over-expression of cyclin E alone failed to induce growth in suspension. The over-expression of cyclin E gave rise to concurrent increase of CDK inhibitor p21 [144]. It was proposed that the sequestration of the increased p21 level by cyclin D1–CDK4/6 is still necessary for anchorage-independent growth.

There have also been attempts to engineer the cells to induce growth arrest. The premise is that at such minimum growth rate, or even growth arrest conditions, the productivity can be sustained to allow for continued product accumulation. Among the negative regulators of cell cycle control, cyclin-dependent kinase inhibitors (CDIs) are the most prominent ones (reviewed by [145]). Inducible expression of these cytostatic genes can lead to the arrest of cell proliferation. Over-expression of CDK inhibitors p21 [146] or p27 [147] in CHO cells resulted in G1-phase cell cycle arrest and a reported 10- to 15-fold [146] increase in specific productivity of the reporter secreted alkaline phosphatase (SEAP). In another study, using a Lac-switch system [148] to induce p21 protein expression in NS0 cells producing chimeric IgG4 antibody [149], a fourfold increase in antibody production was reported during G1-phase arrest.

Cell proliferation and differentiation are highly coordinated in normal cell development. In addition to playing a pivotal role in regulating proliferation, some CDKs and their regulatory cyclins and CDK inhibitors also regulate cell cycle exit and differentiation. In some systems, cell-cycle arrest is a prerequisite for differentiation initiation. Over-expression of the CDI p27 in erythroid cells inhibits CDK2, resulting in withdrawal from cell cycle and ensuing differentiation [150]. Over-expression of p21 in myelomonocytic cell line U937 causes cell cycle arrest and the appearance of monocyte-specific cell-surface markers [151]. Although growth control was achievable by over-expression of the CDK inhibitor p21 in hamster BHK-21 cells, in some cases apoptosis was inevitable under growth-arrested conditions [152].

The proto-oncogene c-myc is a central regulator of cell cycle progression and is also implicated in repressing genes that correlate with the progression of apoptosis [153]. Several target genes that are negative regulators of cell cycle progression, p27 [154] and p21 are repressed by c-myc. It is also known to accelerate apoptosis through the enhancement of cytochrome c release from mitochondria [155]. A CHO cell line transfected with c-myc shows higher apoptotic rates in perfusion culture, however, coexpression of c-myc with Bcl-2 has lead to improved viability [156].

Overall, the effort in manipulating cell growth through cell engineering has been towards arresting cell growth rather than towards shortening the cycling time to make them grow faster. Of course many cell lines employed in recombinant protein production are already growing at a fast rate, doubling every 12–15 h. The S phase for a typical mammalian cell spans over nearly 8 h. Without increasing the number of replication origins on chromosomes significantly, further increase of the growth rate will be difficult. Caution should also be taken in arresting cell growth. Apart from the fact that cell cycle growth is closely related to apoptosis, the cellular biosynthetic machinery will have to be sustained at a high fidelity and efficiency state to produce rDNA proteins. This necessitates continual renewal of the cellular machinery. The imposition of growth arrest must be accompanied by such a renewal, making cells resemble liver or pancreatic cells, quiescent and yet highly active in protein secretion over an extended period. The attempts to "control" growth rate have focused on over-expressing negative factors rather than switching cells into G0 phase. Most quiescent cells, including the professional secretors in liver and pancreas, hepatocytes and beta cells, are at a G0 stage of the cell cycle. Ideally once a cell concentration reaches a high level, cells are switched to a G0 stage; and at that "matured" and developed stage, they secrete the product while continually renewing their cellular machinery to keep themselves at a high productivity state for a period.

Cell cycle regulation is closely related to apoptosis. Its implication in cell engineering has been reviewed recently [146, 157]. c-FLIP/caspase-8-inhibitory protein together with caspase-8 are members of the death-inducing signaling complex (DISC). Both are identified as regulators of cell proliferation and cell death, primarily in hepatocytes [158] and lymphomas [159]. Primary hepatocytes undergoing the G0 to G1 transition have up-regulated levels of c-FLIP. FLIP blocks the activation of caspase-8, inactivating the clustering of death receptors and thus inhibiting death receptor-mediated apoptosis. The role of FLIP in cell cycle progression is proposed to occur through activation of NF-kappaB and Erk signaling pathways, leading to cyclin D1 activation [160].

3.7 Apoptosis

Apoptosis is a regulated cell death in response to accumulating non-lethal stresses such as nutrient depletion, growth factor deprivation, virus infection, and metabolite accumulation. It also occurs in response to developmental cues or other regulatory signals. This irreversible process is marked by cell morphology change, DNA condensation, chromatin shrinkage, and eventually membrane blebbing. The final intracellular event involves a series of cascades leading to cellular destruction. These final acts of self-destruction are similar in all apoptosis. However, the early stage of processing the "signal" differs amongst the two major intracellular apoptosis signaling pathways – namely the death receptor pathway and the mitochondrial pathway.

3.7.1 Death Receptor Pathway

In many developmental events, an individual cell's survival is often dependent on the presence of positive factors or the absence of negative effectors. In the event that such a cell survival signal is absent or the cell death signal is present, a cell would undergo self-destruction and cease to serve its function. The apoptosis of these developmentally related events (neuronal cell, developing B cells) are largely regulated by death receptors. The death receptor pathway is mediated by binding or lack of binding of ligands to death receptors (for review of the death receptor pathway see [161]) at the cell plasma membrane. One example is the role of CD95 death ligand in T cell and B cell development, which plays a role in selection of T cell repertoire and deletion of self-reactive T and B lymphocytes [162]. Immature neurons die in large numbers during early brain development. Neuronal cells require positive survival signals, lack of which leads to neurodegenerative disorders [163].

Ligand binding initiates the recruitment of adaptor molecule Fas-associated death domain (FADD) to the cytoplasmic end of the receptors. Initiator procaspases such as caspase-8 or 10, associate with this receptor, forming a death-inducing signaling complex (Fig. 4). In the receptor complex the caspase is proteolytically activated, triggering the activation of a series of downstream effector class of caspases-3, 6 and 7. The activation of these effector caspases leads to the final implementation of the cell death program.

3.7.2 Mitochondrial Pathway

In addition to its role in energy metabolism, mitochondria also sequester pro-apoptotic proteins in the intermembrane space. These pro-apoptotic fac-

tors are released in a controlled manner in stressed cells to initiate apoptosis. The Bcl-2 family that consists of over 20 pro- or anti-apoptotic proteins is a major player in the mitochondrial apoptosis pathway. The pro-apoptotic subfamily includes Bax, Bak, and Bok, which contains BH1, 2 and 3 homology domains (Fig. 4). Upon exposure to death signals, Bax undergoes conformational changes accompanied by a translocation of the protein to the mitochondria where it gets inserted to the outer mitochondrial membrane and forms channels to increase permeability of cytochrome C and other pro-apoptotic molecules [164]. Cytochrome C proceeds to form a complex with Apf-1, procaspase-9, and dATP, known as the apoptosome. In the apoptosome the inactive procaspase-9 is activated, and the active enzyme subsequently activates downstream caspases. The anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, contain four conserved domains (BH1-4) and a hydrophobic *C*-terminal domain. Bcl-2 is localized on the mitochondrial membrane and inhibits the release of pro-apoptotic molecules from the mitochondria by maintaining membrane integrity. Bcl-x_L can be localized in the cytoplasm and binds to pro-apoptosis members of the Bcl-2 family.

Activation of ER stress proteins can, in the event that UPR cannot alleviate the accumulation of misfolded proteins, lead to apoptosis through pathways that are dependent on the mitochondrial release of pro-apoptotic factors. In the mitochondrial dependent pathway, the ER senses local stresses through Ca^{2+} binding proteins, Ca^{2+} channels, and chaperones which can relay Ca^{2+} signaling to the mitochondria. The ER also contains Bcl-2 anti-apoptosis proteins, suggesting that these family members might protect cells from apoptotic activation.

In both death receptor and mitochondrial apoptosis pathways, an array of pro-apoptotic and anti-apoptotic factors are involved. The multiple factor involvement ensures tight control of the event, and also provides the amplification of the signal, as shown in the cascade of caspases, when cell destruction is indeed called for.

3.7.3
Anti-apoptosis Engineering

Since the discovery more than a decade ago that apoptosis plays a key role in regulating cell fate in *C. elegans*, there have been numerous attempts in bioprocess-related areas and in various areas of biological science to tinker with the death pathway. Specifically, the biotechnological applications aim to delay stress-induced cell death and thus potentially increase the productivity. Since the responsible molecules involve pro- and anti-apoptotic factors, cell engineering efforts also evolved around over-expression of anti-apoptotic factor and suppression of pro-apoptotic factors. In fact, in cell engineering applications for bioprocesses, manipulation of the apoptotic pathway has been one area that has incurred the most activity.

3.7.3.1 Over-expression of Apoptosis Inhibitors

Most attempts in altering apoptotic pathways have focused on over-expressing anti-apoptotic genes instead of suppressing pro-apoptotic genes. The anti-apoptotic Bcl-2 and Bcl-x_L proteins can inhibit the release of pro-apoptotic factors from mitochondria through the maintenance of mitochondrial membrane integrity [165]. Bcl-2 and Bcl-x_L have been transfected into NS0 [166], CHO [167–169], BHK [170], and hybridoma cells [171–173]. In adherent CHO-K1 and BHK cells, over-expression of Bcl-2 and Bcl-x_L prolonged their viability in glucose-free, serum-free or 50 mM ammonium-supplemented medium [170]. However, the effectiveness of those two Bcl family genes varied with cell lines as well as the stress conditions imposed [170]. The effect of over-expressing Bcl-2 and Bcl-x_L in CHO and BHK were examined upon the infection of virus harboring heterologous protein. Bcl-x_L over-expression extended the viability in both cell lines, whereas Bcl-2 was only effective in BHK [170]. In yet another study, the over-expression of Bcl-2 and Bcl-x_L was examined in CHO-DG44. The effectiveness of Bcl-2 and Bcl-x_L were seen only when the anti-apoptotic gene was amplified through methotrexate selection under *Dhfr* system in the host cell [169]. Results of the aforementioned two studies in CHO cells were seemingly inconsistent. In the latter case, amplified levels of Bcl-x_L were required to show cell death protection, whereas in the former study Bcl-x_L levels need not be amplified, but were sufficient in providing protection. This shows that there is variation in endogenous expression of Bcl-x_L or other anti-apoptotic genes in different cell lines.

Using murine fibroblasts, rat fibroblasts, and human lymphoblasts, Bcl-2 was shown to inhibit cell cycle progression by prolonging the G1 phase of the cell cycle [174]. The adverse effects on cell cycle was reverted when Bax, a negative regulator of Bcl-2 was coexpressed in one of the cell lines. Slower growth was also observed for Bcl-2 transfected hybridoma cells in a continuous culture [172]. Both findings indicated that Bcl-2 over-expression, while delaying cell death has a retarding effect on cellular proliferation.

Constitutive expression of Bcl-x_L in murine cell lines affects the fidelity of the mitotic spindle checkpoint leading to polyploidization [175]. This polyploidy effect can be minimized using an inducible expression system. Bcl-x_L was induced under a metallothionein promoter in hybridoma cell at the late culture phase, and was shown to be successful in prolonging viability while preventing adverse genetic effects associated with the constitutive expression of the gene [173].

In addition to the native form, variants of Bcl-x_L and Bcl-2 genes have also been generated by removing their non-conserved loop domain [176]. The loop region contains a regulatory domain, which after post-translational modifications, may negatively affect the biochemical function of Bcl-2 or Bcl-x_L. Also, the non-conserved loop domain contains cleavage sites for cysteine

proteases. Removal of this loop in the variant form may make it less susceptible to proteolysis compared to the native form of Bcl-2. CHO cells, constitutively expressing the Bcl-x_L truncated form, have been shown to proliferate even after being subjected to virus infection or upon serum withdrawal; whereas cells expressing wild-type Bcl-x_L stopped growing after being subjected to these insults [177]. Similar results were obtained with the truncated and wild type forms of Bcl-2 [178]. Better apoptosis protection was also reported for SP2/0-Ag14 hybridomas expressing the loop deletion variant of Bcl-x_L [179].

A novel cell death regulator, Aven binds to Bcl-x_L and also blocks the activation of Apaf-1 (apoptosis-forming complex) downstream of the apoptosis pathway [180]. Coexpression of Aven and Bcl-x_L in CHO cells, led to increased resistance to apoptosis after staurosporine treatment and serum withdrawal compared to Bcl-x_L over-expressing CHO clones [181]. However, without the Bcl-x_L over-expression background, Aven did not confer any additional resistance to apoptosis. Given that Bcl-x_L can be subjected to caspase-mediated degradation, the authors attributed the enhanced efficacy of Bcl-x_L/Aven coexpression to the increased stability of Bcl-x_L after binding to Aven.

During the infection process, viruses often subvert the apoptotic machinery of host cells, thus prolonging the lifespan of the host cells and increasing viral production. Several viral proteins from adenovirus, hepatitis B virus and Epstein Barr virus target different components of the mitochondria-mediated apoptosis pathway [182]. One of the adenoviral proteins, E1B-19K, has been reported to be equivalent to Bcl-2 as an anti-apoptosis factor. It blocks apoptosis by interacting with the death-promoting protein Bax [183] and also blocks the ability of Bak to accelerate the apoptotic process [184, 185]. NS0 cells transfected with E1B-19K performed better in glutamine-free batch culture compared to wild type NS0. In another study, under perfusion culture conditions higher antibody titer was observed with E1B-19K transfection accompanied with a higher proportion of cells in the G1 phase [186].

3.7.3.2 Down-regulating Pro-apoptotic Factors

The cellular pathway leading to apoptosis involves the activation of a family of aspartate-specific cysteine proteases – the caspases. Several members of the caspase family are involved in apoptotic cell death. Targeting these effectors of cell death provides another means of delaying death when cells are exposed to environmental stresses. One of the caspases, caspase-3 has a broad range of intracellular protein substrates and plays a key role in committment to irreversible apoptotic cell death. Caspase-3 transcript level in recombinant CHO cells was suppressed using an antisense RNA. The viability of the transfected CHO cells was extended upon exposure to sodium butyrate treatment, but the specific protein productivity did not increase [187]. In another study, ad-

herent CHO cells transfected with caspase-3 ribozyme to suppress caspase-3 reached a higher cell density compared to the parental CHO cells. The transfected cells also exhibited high viability after induced apoptosis by serum withdrawal [188].

Another way of suppressing apoptosis is by over-expressing anti-proapoptotic proteins. Inhibition of caspase activity by chemicals, including Z-VAD-fmk and Ac-DEVD [189, 190] have been shown to lessen apoptosis. Others have attempted to over-express inhibitor proteins of caspase. XIAP (cross-linked inhibitor of apoptosis) mediates the ubiquitination and degradation of downstream effector caspases, particularly caspase-3 and 7 [191, 192]. In one study, CHO cells transiently expressing two deletion constructs and the native form of XIAP were subjected to serum withdrawal and Sindbis virus infection. Expression of the wild-type XIAP and one of the deletion constructs inhibited apoptosis significantly [193].

Caspase-3 triggers cell death in some, but not all scenarios. The role of caspase-3 is dependent on cell type or death stimulus. In one study of caspase-3 knockout mice, immature T and B cells from these mice undergo apoptosis normally. In contrast, caspase-3-defective peripheral T cells are less susceptible to death receptor-induced apoptosis than wild-type mice [194]. In another study, caspase-3 knockout human MCF-7 carcinoma cell line can still be killed by various inducers of apoptosis in the absence of DNA fragmentation and chromatin condensation [195]. These studies show that caspase-3 is essential in mediating nuclear and other specific morphological changes of the chromatin. However, when other signs of cell death such as mitochondrial dysfunction and plasma membrane permeabilization are considered, caspase inhibition appears not to confer complete cytoprotection. Therefore, in these cases, expression of anti-apoptotic proteins that restores mitochondria membrane integrity is required to enhance viability.

4 Prospect of Cell Engineering for Bioprocessing

Metabolic engineering has been employed to perturb relevant pathways critical to making cells more amenable to higher productivity levels. The efforts include manipulating their growth characteristics by overriding cell cycle control, channeling their metabolic fluxes, fortifying their tolerance to stresses, and altering their internal product processing capability to enhance product quality. To a large extent the potential of metabolic engineering in enhancing cell culture technology has been demonstrated. That being said, ample opportunities for further advancements in cell culture technology still lie ahead of us.

The work to date has been limited to manipulation of a single gene or a small number of cotransfected genes in order to alter a cell's characteristics. A supe-

rior producer will have to be endowed with a host of better traits, including all the qualities discussed in this chapter. It is worthwhile to note that a producing cell would undergo a large number of population doublings before reaching production scale. Any change made to the cell, unless inducible, will be carried throughout the cell expansion process. The cell engineering efforts reported thus far have mostly focused on the performance of cells in a small number of cultivation cycles. The long term effect of "optimal" traits imposed by biotechnologists on the cells and their product is yet to be assessed.

We should also be reminded that the major workhorses for bioproduction (for example, Chinese Hamster ovary (CHO) cells and NS0 cells) are derived from different tissues. Differences in the inherent characteristics of these cells by virtue of being derived from different tissues are more significant than the species-based differences of these cell lines. Their tissue origin equips them with different protein processing and secretory capabilities. Even though as continuous cell lines these cells possess altered metabolism and growth control, their tissue origin and whether or not they are cancerous imposes differences in the apoptotic and growth control mechanisms. Cell engineering strategies will have to take such differences into consideration. Enhanced organelle biogenesis may be beneficial in enhancing productivity for one line, but not for the other; conversely, perturbation of cell cycle control may enhance longevity of one compared to the other.

Much effort has been devoted to channeling or altering a specific arm or branch of a given metabolic pathway. However, cell physiology is the manifestation of a vast, tightly regulated network of reactions. Any alteration in one branch will inevitably cause changes in others. Therefore, given that cells are in a state of homeostasis under normal physiological conditions, any artificial perturbation introduced will have to consider the more global effect across pathways in order to accomplish the goal of metabolic control. For example, channeling of glucose to minimize lactate production alters the proton and NADH fluxes as well as H^+ transport across the cell membrane, which is coupled with lactate transport. This in turn affects HCO_3^- flux across membranes as well as homeostasis of CO_2/HCO_3^- intracellularly. An overall systems analysis approach is therefore required for rational cell engineering.

Globally, intracellular environment is in a homeostatic state; locally, profound dynamism is at play. UPR, cell cycle and apoptosis are all controlled by molecular complexes whose dynamics are governed in turn by the dynamics of their components. In the past two decades we have witnessed tremendous increase in the capacity of cells to accommodate high product secretion rates and we have learned to achieve these productivity levels fairly consistently. CHO, a cell line that hardly secretes protein in its native state can now be made to produce nearly 40 pg/cell-day of IgG, a level rivaled only by professional secretors like plasma cells or hepatocytes. However, the changes in cellular machinery accompanying the transformation to high level secretors are still unclear. It is generally thought that the machinery for protein

secretion would have been expanded. The amplification process of the heterologous gene followed by cloning procedures may have led to selection for cells that can withstand the UPR response and thus have high secretory capacity. Alternatively, the amplification process may have somehow induced biogenesis of key organelles involved in protein processing – ER, Golgi, and mitochondria. Given the ambiguity of causation, attempts to enhance the secretory pathway have only met with limited success. One should be reminded that control of the protein folding process is a delicate balance among various chaperones and other protein-processing proteins. The amplification of one or a few of the key players may not reflect the "correct" stoichiometry and therefore not lead to the right balance of molecular interactions that are critical to protein processing.

To live or to die is a balancing act of many protein complexes, each of which is expressed dynamically. Amplifying or suppressing one or more gene will alter the abundance of the target protein, but may not give rise to the prescribed dynamic profiles of the protein complexes that will lead to altered growth control. Recent advances in cell engineering have accomplished much in overcoming the cellular self-destructive mechanism and have demonstrated their potential in enhancing productivity. Nevertheless, ultimate cell growth control will require a more refined strategy in controlling the dynamics of the target gene(s).

Mammalian cell-based pharmaceutical biotechnology is at a noteworthy juncture. Mammalian cell-based biologics will certainly increase in quantity, affordability, and availability to needy patients in the years to come. Much of that will be accomplished by process intensification through better cell lines and better processes. We are likely to see cell engineering play an even greater role in process enhancement. We will have to take more of a systems approach to understand the effect of genetic alteration on cell homeostasis. We will have to develop better control strategies for gene manipulation, not limited to introduction of a battery of genetic elements but able to control the temporal expression of the introduced genes to enable them to play in harmony with the entire system. Physiological insight, equipped with gene discovery through genomic and proteomic approaches and new tools in cellular engineering will lead the way to desired production cell lines.

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