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Mohamed Al-Rubeai *Editor*

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Preface

Animal cells are the preferred 'cell factories' for the production of complex molecules and antibodies for use as prophylactics, therapeutics or diagnostics. Animal cells are required for the correct post-translational processing (including glycosylation) of biopharmaceutical protein products. They are used for the production of viral vectors for gene therapy. Major targets for this therapy include cancer, HIV, arthritis, cardiovascular and CNS diseases and cystic fibrosis. Animal cells are used as in vitro substrates in pharmacological and toxicological studies. *Animal Cell Culture* is designed to serve as a comprehensive review of bioprocessing of animal cells for the production of biopharmaceuticals, covering the current status of both research and applications. For the student or R&D scientist or new researcher, the protocols are central to the performance of cell culture work, yet a broad understanding is essential for the translation of laboratory findings into the industrial production.

This book brings together the knowledge and experience of those who are using animal cells for the production of proteins. The chapters review the state of the art with in-depth assessments that emphasize the practical aspects of efficient operation of cell culture techniques. The book indicates those aspects of cell line development, optimization and large-scale production encountered in cell culture processes and shows how the recent development in cellular and molecular biology and omics technology can help the full realization of the potential of biotechnological exploitation of animal cells.

To optimize quality and quantity in the production of biopharmaceuticals from animal cells, researchers have developed many new methods. Considering that the global market is around 200 billion dollars and is growing at a compound annual rate of 13.5 % and that high clinical dose requirements over long periods of time have pushed demand, the optimization and scale-up of recombinant protein production technologies have become quite important.

Mass transfer, mixing and hydrodynamic forces in bioreactors are critical parameters that need to be controlled to provide cells with a satisfactory level of oxygen without damaging or stressing cells in culture. Two chapters are included to discuss the current knowledge with respect to these issues.

Selection of cell lines, suitable type of bioreactor system, optimal physical and chemical environment and appropriate production mode are of particular importance as an aid to achieving high and stable productivity. Many of the authors lay emphasis on such studies. The importance of DNA technologies, cell line stability and product quality, safety and efficacy are now widely recognized, and information is presented here on the methods to monitor, investigate and improve the selection, stability and productivity of cell lines. Transient gene expression is dealt with, as are hybridoma technology and baculovirus insect cell culture, which have been used to produce recombinant products. Perfusion processes and cell immobilization allow for far greater cell densities to be achieved, thus providing economic advantages as time is saved in turnaround and inoculum build-up. This book contains chapters to review the practical aspects of these processes for the production of biopharmaceuticals and vaccines.

Cell engineering is a new research approach which began in the early 1990s, coinciding with an increasing interest in apoptosis. There have been numerous innovative strategies based on cell engineering and molecular analysis, together with metabolite data aimed to optimize culture productivity. These strategies are described, including the proteomic and metabolic profiling of cell culture.

Although the adoption of process analytical technology (PAT) in the biopharmaceutical industry is in its infancy, it was felt that it is essential for a book on animal cell culture to include a discussion of PAT instruments, techniques and strategies of relevance. In the final chapter, experts provide the reader with an overview of the biopharmaceutical products that have been approved by the regulatory agencies from 1989 till the first quarter of 2014, including biosimilars.

I am grateful to the staff at Springer for their efficiency in preparing this volume and to Meran Owen and Tanja Koppejan in particular for their help and patience. I would also like to thank each contributor for their diligence and perseverance in preparing the chapters, which I am sure will be enjoyed and valued by everyone who reads this book.

Dublin, Ireland Mohamed Al-Rubeai

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Chapter 1
Cell Line Development

Hansjoerg Hauser

Abstract The majority of processes to manufacture biotherapeutics for use in humans are based on mammalian cell lines. Established cell lines are modified by recombinant DNA technologies to produce specific proteins. Such technologies together with cell culture processes have been improved over decades for today's manufacturing processes.

This chapter describes gene expression approaches to create mammalian hosts that produce the protein of choice. It focuses on methods that are currently applied or have been developed in the last years as well as technologies that are thought to be relevant for future applications. While these methods are directed towards high, stable and predictable production of the secreted protein they also form the basis for further modifications that lead to robust manufacturing processes through improvement of cellular properties.

Keywords Bacterial artificial chromosome (BAC) • Chinese hamster ovary (CHO) • Chromosomal elements • CRISPR/Cas • Double strand break (DSB) • Episomal vector • Expression cassette • Gene of interest (GOI) • Genetic modification • Homologous recombination • Integration site • Position effect • Recombinase mediated cassette exchange (RMCE) • Recombineering

1.1 Introduction

Cell line development (CLD) is positioned in the process of drug development process at the stage when protein as potential drug has been identified. CLD aims at the production of cell lines that allow the production of this protein in sufficient quantity and quality to perform preclinical tests as well as for clinical material. Since in these processes different amounts and in some cases also different qualities of the drug are needed CLD is often repeated for the same protein until the final cell line for production is created. This is why CLD should be a rapid and standardized process that can be performed on a routine basis. Current challenges concern the

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speed of construction, the yield of product, the quality of the product, the choice of the cell line, the stability of product formation, the robustness of the final cell line for fermentation and the suitability of the cell line for flexible use.

1.1.1 Product Quantity

Since production of proteins in mammalian cells requires large volumes in upstream and downstream processing and cost of goods is high the overall process is expensive. Consequently, the production should be as high as possible without affecting the quality of the biopharmaceutical. Maximum product titres are in the focus of cell line and upstream process engineering. To achieve this, the average specific productivity (Q_p) and the integral of viable cell density (IVCD) over process duration have to be regarded. These parameters define the product concentration in a bioreactor run. Cell line development can significantly contribute to both parameters by genetic manipulation of the cell line. Both IVCD and Q_p observed for a given producer clone depend not only on the upstream environmental conditions in the bioreactor but also on the intrinsic features of the particular cell clone deployed for production.

For optimizing the specific productivity the genetic manipulation of the cell lines with the gene of interest (GOI) plays the key role. The dominant parameter for specific productivity concerns the transcriptional efficiency of the GOI. If this is not optimal all manipulations downstream in the flow of protein secretion will be suboptimal. Thus, this aspect is directly treated in this chapter. However, a number of other cellular features influence productivity. These are not directly treated in this chapter since they are mostly specific for the protein and need special consideration. Table 1.1 summarizes the levels of expression control and the parameters that can be altered to achieve better expression.

One level concerns translation efficiency. It is well known that mRNA concentration does not always correlate with protein formation. Translation efficiency depends on the primary sequence and the folding of the mRNA, the availability of cellular factors, the concentration of negative and positive regulators and the existence of small non-coding RNAs. Another concerns the processing in the Endoplasmic Reticulum (ER) which is a luminal compartment of the cell involved in folding and sorting of newly synthesized secretory cargo proteins. Also, several quality control mechanisms are in place within the ER to ensure proper folding and secretion of expressed proteins. Accumulation of mis-folded protein within the ER compartment triggers the unfolded protein and ER overload responses and thereby causes an undesirable decline in cell specific productivity. The Golgi processing and glycosylation machinery is a subcellular compartment that supports proper post-translational modifications, in particular glycosylation and is a further bottleneck to optimal secretion efficiency. Other parameters concern the cell cycle control, the efficiency of energy consumption and aspects of secretion.

Table 1.1 Parameters controlling production strength and measures for improvement

Integral of viable cell density (IVCD)	
Peak viable cell density	Choice and adaption of the cell line
Cell death	Genes and compounds that block apoptosis
Nutrient limitation and depletion	(Re-) Feeding
Accumulation of toxic metabolites	Genetic control of the metabolism; continuous fermentation
pH-fluctuations; perturbations of the oxygen supply	Technical facilities and process manipulation
Average specific productivity (Qp)	
Gene copy number	Gene amplification
Transcription	Promoters, chromosomal elements
Translation	Cis-control elements; snRNA; context of start codon
ER-Processing and quality control machinery	Genes controlling ER processing and QC
Cell cycle status	Genes regulating the cell cycle
Efficiency of energy consumption	Genes encoding crucial metabolic enzymes
Golgi processing and the glycosylation machinery	Genes directing glycosylation
Secretion	Genes controlling secretion

The average specific productivity is calculated by dividing the arithmetic mean of daily product concentrations per production run by the cumulative number of viable cells per run. Q_p values are defined in pg/cell/24 h. Values up to 100 pg/cell/24 h have been reported.

Upstream product concentration per unit volume depends on viable cell density (IVCD) (Table 1.1). Cell density on the one hand site is a property of the cell line. Up to now there is no genetic approach to increase this property but adaption of cell lines to high density growth has been successful. Since cell density is a product of cell growth and death much effort has been made to increase, or regulate, cell growth and to decrease cell death. On the level of cell line engineering this concerns mainly intervention in the cell cycle and blocking apoptosis but also by altering the cellular metabolism through specific interventions to reduce the production of toxic metabolites and by-products.

Advances in mammalian cell culture technology have already increased product titres for certain therapeutic antibodies well above the 5 g/l benchmark with some companies reporting 10–13 g/l for extended culture durations. Together with the specific productivity these two parameters that determine the upstream product concentration per unit volume in a bioreactor run.

Many engineering attempts have been made to interfere with the above listed levels in order to improve specific productivity and volumetric yield for target proteins. The expression of specific proteins could improve the expression of certain GOIs. Similarly, the down-regulation of inhibitory proteins by genetic means can improve expression. To do so, antisense RNA, RNAi methods as well

as gene knock-outs are applied. The details of these manipulations are not discussed in this chapter. However, the principles for these methods – expression of genes and site-specific chromosomal alterations – are found in this chapter.

1.1.2 Product Quality

A strong focus of manufacturers of biopharmaceuticals is directed towards product quality. One of the most common of biotherapeutics produced by mammalian cell culture is product glycosylation. Each molecule of a protein drug can potentially contain *N*- and *O*-glycans that differ in complexity from those glycans attached to another protein molecule. To make things more complex, glycan sites may only partially be occupied. *N*-and *O*-linked glycans are the most frequent ones. Physicochemical micro-heterogeneity is not only a feature of naturally occurring proteins as well as those produced in recombinant cells. The terminal sugars of *N*-and *O*-glycans are important parameters that define pharmaco-kinetic and pharmacodynamic properties of biopharmaceuticals by influencing circulation half-life, clearance through receptor binding and properties like ADCC of antibodies. Since the composition and heterogeneity of glycosylation depends on the cellular metabolism, glyco-engineering methods targeting the sugar nucleotide metabolism have been developed. While it is often difficult to know were to interfere with the metabolism, the methods of engineering are the same as for production of the GOI. This is the reason why this item is not specially discussed in this chapter. The same is valid for other post-translational modifications like proteolytic processing and gamma-carboxylation.

1.2 Two Components of Cell Line Development: The Gene of Interest (GOI) and the Cell

A few host cell lines have been developed as suitable hosts for protein manufacturing. These include Chinese Hamster Ovary (CHO) cells, BHK21, Sp20, NS0 cells due to the fact that they are readily modified by genetic engineering and show robust growth in suspension in large scale bioreactors. More recently, some proteins manufactured from a human cell line called PER.C6 were introduced into clinical studies. Theoretically, many other cell lines could be used for these applications. However, the history of the last 20 years has shown that it is difficult to bring new cell lines to pharmaceutical production. This is mostly due to their inherent properties including the ability to become genetically modified, to express proteins highly, stably and authentically, and to grow in large scale fermentation reactors in protein free media. Finally, it takes considerable effort and time to render a new cell

line acceptable to industry and authorities, even if the requirements are given. The history of PER.C6 is an illustrative example.

Today, most professional cell line developers rely on CHO. The success of this cell line is not only based on the properties mentioned above. It is also based on its high flexibility, another word for instability (Lewis et al. 2013). This flexibility allows to provoke or just to wait for spontaneous phenotypic alterations. Screening for alterations of interest allows the selection of cell clones that are better suited for e.g. optimal production or growth. The most prominent example for such alterations is gene amplification. The back site of this flexibility is manifested by a certain instability of the selected cell clones (Kim et al. 2011; Chusainow et al. 2009; Dorai et al. 2012). Experience gained with the CHO cell line(s) has led to handle this property successfully.

Genetic modification of mammalian cells has originally been developed for basic research. Today, applications range from the investigation of gene function and regulation, the utilization of cells for the production of recombinant proteins, but also for designing and adapting the cells for defined purposes like drug screening and to specifically change cellular properties.

Traditional strategies to genetically modify cells rely on gene transfer without knowing what happens to the transferred DNA. In fact, DNA can remain transient in the cells, usually as episomal DNA, but gets lost upon subsequent cultivation. Transient expression has been optimized by different means and is today successfully used for a series of applications. This method will not be further discussed in this review. Interested readers can rely on comprehensive reviews (Assenberg et al. 2013; Geisse and Voedisch 2012; Baldi et al. 2007).

A specific modification of this approach makes use of genetic elements that lead to stable transmission of the episomal vectors. This method is briefly presented in paragraph 4.

Another traditional technique relies on transfer of the expression cassette together with a selection marker that identifies cells having integrated the incoming DNA and thus are able to transmit this genotype stably to their descendants. This method is based on random integration of the expression cassettes into the genome of the host cell. Due to chromosomal position effects this technology requires screening for cell clones with the expression properties of interest. The currently used applications that have been improving this method are described in the following paragraph.

Today, many researchers believe that rational modification of mammalian cells is moving towards biotechnology and biomedicine. Methods have been developed that allow to efficiently introduce genetic alterations at defined chromosomal sites with considerable accuracy. The predictable modification of cellular properties based on precise genetic engineering is possible but a proof that this concept is equal or superior to the currently used methods for the production of pharmaceuticals has not yet been shown. Since this aspect is important for the next period of biopharmaceutical research it is presented here in more detail.

While random integration combined with extensive screening for suitable cell clones combines very well with the high flexibility of the CHO cell line, the rational

approach relies on properties that are stably transmitted. It can be imagined that the rational approach is suitable to cell lines with low flexibility, or – in other words – cell lines with high stability.

1.3 The Classical Approach: Random Integration of GOI

Expression of recombinant DNA depends on regulatory elements on DNA (promoters, enhancers,. . .), as well as on RNA and the gene of interest (GOI). These elements together are called the expression cassette. Upon non-viral (physico/chemical) transfer of the plasmids into the cells, e.g. by DNA/calcium phosphate co-precipitation, electroporation, lipofection or nucleofection, DNA transfer leads to a low probability of integration into the chromosomal DNA of the host. Integration is based on cellular repair mechanisms that sense the incoming recombinant DNA. The activation of these repair mechanisms usually results in illegitimate recombination and integration of the recombinant DNA into cellular chromosomal DNA. These integration events are of interest. The resulting recombinant cells are usually isolated by co-expression of selection marker genes.

Higher efficiency of stable chromosomal integration of transgenes is achieved by retro- or lentiviral gene transfer. These viruses make use of their integrases after reverse transcription of the recombinant viral RNA that contains the genetic information of the expression cassette of choice (Blesch 2004; Delenda 2004; Matrai et al. 2010). Similarly, transposases mediate integration of expression cassettes by means of integrases (Ivics and Izsvak 2011).

Expression of the GOI is not only governed by promoters and enhancers but is strongly influenced by the chromosomal context that is the host DNA flanking the incoming expression cassettes. The nature of the integration site not only affects the level, but as well the stability of expression and eventually also its regulation. This is called the "position effect" (Batenchuk et al. 2011; Daboussi et al. 2012; Moreno et al. 2009). Integration can also cause deregulation or accidental disruption of endogenous genes. For cell line development this deregulation of endogenous genes by the integration event is not a relevant issue in most cases. Since integration of the transgenes is more or less random the context in each recombinant cell (with an integrative event) is different and so is its expression. A general tendency that is found for all gene transfer methods is that integration into euchromatin leads to much more efficient expression compared to integration into heterochromatin.

Transposase mediated gene transfer protocols show equal integrations into exons, introns and intergenic regions and less preference for promoter-near regions (Carlson et al. 2003; Yant et al. 2005; Baum et al. 2006) while retro- and lentiviral transfer leads a preference for promoter regions and coding sequences (Wu et al. 2003; Mitchell et al. 2004).

The problem of context-dependent expression of transgenes has been addressed by addition of chromosomal elements to the expression cassette. These elements are

Table 1.2 Cis-acting elements controlling transoription

Name	Properties	Mechanism of action	Position dependency
Promoter	Directing site and strength of transcription	Tethering transcription factors	+++
Enhancer	Directing strength of transcription	Tethering transcription factors; tethering enhancer to promoter	–
LCR Locus control region	Transcription enhancing by looping	DNAse 1 sensitivity	–
UCOE Ubiquitous opening elements	Transcription enhancement	Chromatin opening; 2 promoter elements flanking a CpG Island	+/–
S/MAR Scaffold/ Matrix attached regions	Transcription enhancement	Attachment to nuclear scaffold/matrix bordering transcriptional active chromatin domains	+/–

thought that modulate transgene expression upon chromosomal integration into the host. These are summarized in Table 1.2.

Overall, the application of chromosomal elements tries to construct context independent expression vectors that are able to shield the expression cassette and thereby avoid interference with the host cell's genes through cis-effects (Antoniou et al. 2013). Because of their size, that is often considerable, their handling is more difficult and often skipped in routine cell line development. Improvements are hampered by the fact that the underlying mechanisms are incompletely understood. In particular, the question how an element will work in a different genomic environment remains largely unanswered. Despite some promising reports, the predictability of all such genetic elements in routine vector application remains moderate and thus the original goal could not be reached.

An alternative approach to overcome or reduce context effects are given by bacterial artificial chromosomes (BACs) after engineering in E. coli. BAC vectors comprise large genomic regions (up to 300 kbp) and thus can encompass cis-regulatory elements. If the expression unit is located in the center of a BAC vector, random integration does not strongly affect it. Thus, its expression is only marginally governed by the features of the integration site.

BAC vectors are propagated and manipulated in E. coli. Methods to allow their modification by homologous recombination in E. coli (recombination mediated genetic engineering, so-called 'recombineering') have been developed (Thomason et al. 2007; Copeland et al. 2001; Narayanan and Chen 2011). Recombination in E. coli requires short homology regions of 50–70 bp, which are attached to the DNA piece of choice, e.g. the expression cassette, by PCR. These constructs, usually PCR fragments, are introduced into bacteria that carry the relevant BAC. Further, the E. coli clone expresses a recombinase to carry out the recombination reaction. A selection marker that is fused to the target cassette facilitates the screening for successful recombination. If this marker is flanked by recombination target sites

such as FRT or loxP the selection marker can be later excised by Flp or Cre (the site specific recombinase) leaving the recombination target site. Protocols without leaving remnants, so-called seamless manipulation were also developed (Muyrers et al. 2000; Warming et al. 2005; Zhang et al. 2003; Bird et al. 2012). BACs are quite fragile. This can result in fragmentation of the BAC DNA upon manipulation of the cells. Such BAC fragments after integration into the host genome are prone to chromosomal position effects. BAC vectors equipped with target sites for transposases (ITRs) can be transferred in the presence of the respective transposase lead to full length full length BAC vector integration (Davis and Stokoe 2010; Li et al. 2012).

The challenge for the use of BACs lies in the choice of the right BAC which successfully shields the expression units for many or all integration sites. While BAC vectors have been often used to study the gene regulation in an authentic context only few examples exist for their exploitation for biotechnological purposes. Proof of principle has been shown for the well-characterized murine Rosa26 locus (Blaas et al. 2009, 2012) and the dhfr locus (Bian and Belmont 2010).

BAC libraries from different species were generated. They accommodate 150–300 kbp of genomic DNA. BAC libraries from CHO cells are described by Omasa and colleagues (Omasa et al. 2010).

1.4 Stable Expression by Episomal Vectors

Ideally, the use of episomal vectors would overcome the problem of position effects (Argyros et al. 2011). Episomal vectors need equipment that ensures the replication and maintenance of the transgene that is synchronized with the cellular replication machinery. To avoid loss of the vector due to continuous division of cells different strategies have been developed to ensure extra-chromosomal maintenance of foreign DNA.

For the creation of artificial chromosomes natural chromosomes are chopped to manageable sizes (top-down). Alternatively, the expression cassette is complemented with components of chromosomes that are required for segregation into artificial chromosomes. Mammalian artificial chromosomes of some (6–10) megabases have been constructed. They comprise both centromere (the sites of kinetochore formation and spindle attachment) and telomeric ends. This allowed the construction of ‘synthetic’ chromosomes that are maintained in proliferating cells. So far, however, the design of such artificial chromosomes is too difficult and the modification is challenging due to their size.

Loss of episome maintenance in growing cell cultures can be overcome by adding viral sequences that act as origins of replication (Lufino et al. 2008). Some of these origins of replication require the presence of viral proteins. EBV-based episomal vectors which have been shown to provide long term gene expression in B cell lines but also in cell lines such as HEK293 and some rodent cells require the expression of EBNA-1 (Mizuguchi et al. 2000).

An example for episomal vectors that replicate in absence of viral proteins is based on non-viral elements (Hagedorn et al. 2011). S/MARs mediate the association with the nuclear matrix which is suggested to provide mitotic stability (Baiker et al. 2000). S/MAR harboring episomal vectors were shown to co-segregate with the host chromosome during mitosis (Stehle et al. 2007). S/MAR based episomal vectors provide transgene expression in various cell lines such as CHO cells (Piechaczek et al. 1999), primary cells, stem cells and transgenic mice (Papapetrou et al. 2006). However, episomal maintenance of these vectors does not rule out aberrant chromosomal integration of vector copies.

1.5 Targeted Integration and Locus Specific Gene Expression

The concept of targeted integration assumes that particular chromosomal sites exist that favorably support the expression of a recombinant cassette. Once, such a site has been identified the expression caste of choice is inserted. Ideally, such an integration site should support the expression of any cassette of interest. The repeated use of such a site will have several benefits:

1. Since screening is not required anymore, the cell line development is significantly speeded up (Schucht et al. 2011; Carrondo et al. 2011);
2. Integration of any transgene of interest can be achieved;
3. The properties of the targeted cell clone with respect to medium and cultivation requirement is preserved and further upstream development is avoided;
4. Unpredictable effects from random integration are not to be expected;
5. Molecularly defined cell clones would show less safety concerns; at least their characterization is significantly reduced.

The realization of the concept of targeted integration requires two conditions. First, such favorable integration sites with the above projected properties must exist. Second, the technologies of targeted integration must be efficient.

With regard to the first condition, the term ‘Safe harbor’ was created. Previous and ongoing discussions tackled the question of the perfect chromosomal locus to integrate a transgene. Apart from functional considerations, i.e. that the transgenes should integrate into transcriptionally active sites, safety aspects are of importance, in particular for biomedical applications (safe harbor). Theoretically, such “safe harbors” would be sites in the genome in which transgenes can be integrated without disturbing the activity of endogenous genes and thus without deleterious consequences (such as the influence on cell growth). More so, the transgenes should be expressed in such sites in a predictable manner. From these theoretical considerations, attempts have been made to define such safe harbor sites by criteria such as a minimal distance to standard genes and to tumor promoting genes (Papapetrou et al. 2011; Sadelain et al. 2012). However, such definitions do not consider the

topology of DNA, the possibility of inter-chromosomal crosstalk (Nunez et al. 2009) as well as perturbations induced by the integrated expression cassette. Thus, a prediction of safe harbors based on these simple criteria does not seem to be feasible and might even be misleading. In any case, rigorous testing is needed to validate the concept experimentally. Nevertheless, it is expected that integration sites exist that do not significantly influence the expression of incoming genes and that the integration does not affect properties needed for protein production.

1.5.1 Technologies of Targeted Integration

Homologous recombination (HR) is the key to targeted integration. HR is a natural process that occurs during meiosis or mitosis. It is possible to mimic this process by recombinant DNA technologies (Fig. 1.1).

HR was exploited for targeted integration of foreign DNA upon transfer to cells. It works rather successful in embryonic stem (ES) cells (Capecchi 2005). Consequently, HR is applied for specific modification of genes in ES cells for establishment of transgenic mice: Knock-out mice in which a specific gene is deleted and Knock-in mice in which reporter mice mimic the expression of an endogenous gene (Capecchi 2005). To target a gene of interest (GOI) into a defined genomic locus by homologous recombination the GOI needs to be flanked by DNA sequences that are homologous to the specific site. Generally, the targeting efficiency correlates with the length of homologous arms (Thomas and Capecchi 1987). Current targeting vectors usually comprise 2–6 kb of homology (Hasty et al. 1991).

The major drawback of homologous recombination for targeted genome modification is its low efficiency. HR efficiency depends on (i) the type of cells, (ii) the transfer protocol, (iii) the nature of the targeting locus, (iv) the length of homologous arms and (v) the extent of homology (i.e. isogenicity) of DNA (Cervantes

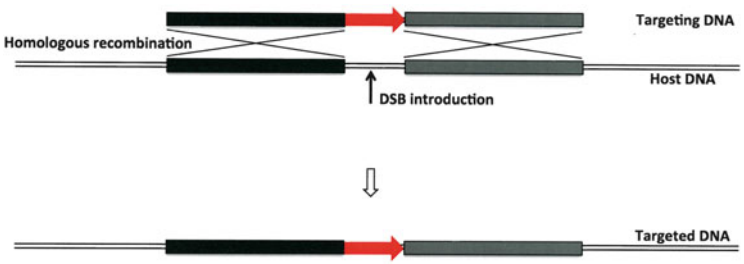

Fig. 1.1 DSB induced homologous recombination. Double reciprocal homologous recombination between the targeting DNA and the host DNA is mediated by the cellular equipment. It is only efficient if dsDNA breaks are introduced at the site of recombination (*black arrow*). This is mediated by meganucleases, ZNF, TALEN or CRISPR/Cas. *Red arrow*: GOI

et al. 2002; Hasty et al. 1991; Templeton et al. 1997; Sedivy and Sharp 1989; te Riele et al. 1992). If all parameters are optimized efficiencies as high as 10^{-1} can be achieved in mouse embryonic stem cells (Templeton et al. 1997).

In ES cells and induced pluripotent stem cells (iPS), the frequency of recombination at specific sites ranges from 10^{-3} to 10^{-6}, (Templeton et al. 1997). In somatic cells, however, the frequency of homologous recombination is significantly lower, in some cell types not detectable at all. Indeed, somatic cells preferentially undergo non-homologous recombination resulting in random integration of DNA (Tichy et al. 2010). The ratio of targeted versus untargeted (random) integration has been reported to be as low as 10^{-6} (Porteus 2007). Accordingly, routine genetic modification of somatic cells by homologous recombination is not feasible. A certain improvement of HR was achieved by the use of adeno-associated viral vectors (AAV). Up to 1 % of mammalian cells as exemplified for the HPRT locus, the collagen A1 and A2, and the Rosa26 locus were shown to be successfully targeted by the AAV vectors (Chamberlain et al. 2004, 2008; Miller et al. 2006).

Studies show that the recombination is favored in transcriptionally active sites (Thomas and Rothstein 1989).

1.5.2 Double Strand Break Induced Homologous Recombination

The rate-limiting step for HR is the presence of a double stranded break (DSB) at the intended site of recombination. HR was shown to be significantly increased by the introduction of DSB at the recombination site (Porteus and Baltimore 2003; Rouet et al. 1994; Szczepek et al. 2007; Choulika et al. 1995; Donoho et al. 1998) (Fig. 1.1). Thus, creating site specific DSB is an important tool to enhance HR and to make it suitable to somatic cell lines. Double strand break (DSB) induced homologous recombination further allows to reduce the size of homology arms which simplifies both, the construction of targeting vectors and the detection of homologous recombination events. Thus, the development of technologies to induce specific DSBs was in the center of research approaches in the last decade. The success in this field, in particular the ability to introduce DSBs at nearly any position in the genome opened a new era of genetic engineering in primary and somatic cells as well as cell lines. The currently used methods to induce double strand breaks comprise meganucleases, Zn finger nucleases, TALE nucleases and CRISPR/Cas. They are summarized in Table 1.3.

These DSB systems share the ability to introduce DSBs at sequence specific sites into the DNA. The challenge is to target the break at the site of interest. It has to be kept in mind that for mammalian species several billion potential sites per genome have to be considered and only one in haploid or two in diploid cells, should be targeted. E.g., **meganucleases** like I-SceI recognize a defined motif of 18 bp. This is not found in the genome of most mammals. Attempts to modify meganucleases to redirect them to specific endogenous DNA sequences have been made but

Table 1.3 SRe-specific DNA-break inducing systems

Name	Active component	Length of recognition sequence	Application as	Average frequency of target sites
Meganucleases	Restriction enzymes cutting dsDNA I-SceI	18 bp	DNA transfer	0
ZNF Zinc finger nucleases	Synthetic proteins composed of trinucleotide recognition modules fused to a nuclease (FokI)	2 × 9–18 bp	DNA transfer	1 in 600
TALEN Transcription activator like effector nucleases	Synthetic proteins composed of mononucleotide recognizing modules fused to a nuclease (FokI)	2 × 9–18 bp	DNA transfer	1 in 35
CRISPR/Cas	Chimeric RNA from crRNA homologous to DNA target and tracRNA + Cas9 nuclease	20 bp	RNA or DNA transfer	1 in 8

flexibility is still restricted (Seligman et al. 2002; Rosen et al. 2006), to use the meganuclease system requires that the recognition sequence has to be introduced to the site of interest before it can be targeted. This procedure has been successfully applied in various cell types (Porteus and Baltimore 2003; Rouet et al. 1994; Szczepek et al. 2007; Choulika et al. 1995; Donoho et al. 1998). In these studies, the frequency of homologous recombination could be increased significantly.

Importantly, locus-dependent effects influence homologous recombination even upon induction of DSBs (Fenina et al. 2012). This has to be kept in mind if other methods of DSB are evaluated. It is most probable that the chromatin structure influences the DSB efficiency and so the HR process.

With the advent of **Zinc finger protein linked nucleases** (ZFN) the DNA cleavage could be directed to specific endogenous sequences. These ZFNs are artificially designed proteins in which a nuclease domain is targeted to a specific DNA sequence. The DNA-binding moiety of Zn finger proteins contains modules of Zn fingers which consist of approximately 30 amino acids each. They recognize a sequence of 3–4 bp (the module) (Rebar and Pabo 1994; Choo and Klug 1994). The Zn finger domains can be designed to recognize nearly, but not all of the 64 possible nucleotide triplets. Three to six fused Zn finger modules constitute one ZFN monomer that facilitates binding to a DNA sequence of 9–18 bp. Binding of two ZFN monomers (left ZFNs arm and right ZFNs arm) thus is mediated by an 18–36 bp sequence that covers a sequence in the genome. The two ZFN monomer binding sites are not directly adjacent but are separated by a 5–6 bp spacer (Wilson et al. 2013). To induce DSBs, Zn finger domains are fused to a nuclease to form the Zn finger nuclease. For this purpose, the FokI nuclease is used. Since FokI is active as a homodimer, binding of two ZFN monomers to adjacent DNA sites is required to achieve dimerization of the FokI domains and subsequent generation of DSBs.

Another challenge in using DSBs is their degree of specificity and precision. This is defined by the frequency of cleavage at non-homologous sites ('Off target sites'). Unintended binding of the Zn finger moieties to DNA sequences that have only partial homology to the target sequence (Gabriel et al. 2011; Pattanayak et al. 2011) contribute to the lack of precision.

Not all of the possible 64 nucleotide triplets can be accommodated by a Zn finger moiety. Moreover, a significant context dependency in the binding of the Zinc finger modules was observed; i.e. a particular finger will provide affinity and specificity for a given triplet in one sequence, but not in others. Complex algorithms to predict binding have been developed (Davis and Stokoe 2010). Statistically, every 12–633 bp a binding site for Zn fingers can be found in the human genome (Segal and Meckler 2013). In vitro pre-testing is recommended to validate the ZFN pairs. Even with these pre-selections, the prediction of ZFN pairs is not satisfactory and a substantial proportion of ZFN pairs fail to act properly in vivo (Kim et al. 2012; Wang et al. 2012; Ramirez et al. 2012). To overcome the failure rate multiple sequence targets are usually generated and extensively tested in vitro.

Another factor of concern is the nuclease itself. FokI cleavage depends on dimerization. However, if two identical ZFNs bind to adjacent sites rather than to the intended heterodimers, an unspecific cleavage can occur. To overcome such problems a modified FokI domain was developed by iterative structure based design that allowed to decrease the formation of homodimers (Miller et al. 2007; Doyon et al. 2011). To increase the nuclease activity hyperactive variants of the FokI cleavage domain were employed resulting in a 15-fold increase of cleavage activity (Guo et al. 2010).

The linker domain connecting the nuclease with the DNA-binding moiety and the DNA spacer sequence between the adjacent binding sites have as well an impact on the cleavage activity of the ZFNs (Wilson et al. 2013).

In simple protocols, the ZFN encoding vectors are co-transfected together with the homology vectors (targeting plasmid). To limit the activity of the ZFN and to avoid side reactions, advanced protocols have been established for transient expression, thereby using non-integrating DNA vectors and the transfer of in vitro transcribed ZFN RNA templates (Gaj et al. 2012), the stability of ZFNs was modulated by controlled proteosomal degradation (Pruett-Miller et al. 2009).

The transcription Activator Like Effector (TALE) technology makes use of DNA-binding proteins that are secreted by the plant pathogenic bacteria genus Xanthomonas (Boch et al. 2009; Moscou and Bogdanove 2009). Recombinant TALE proteins are formed by synthetic modules. Each of these modules consists of approximately 33–35 amino acids (Deng et al. 2012) that recognize a specific nucleotide. For all nucleotides TALE modules can be constructed. Thus, the TALE DSB modules can be combined in a way that is described for the ZN modules (see above). Upon fusion of this DNA binding domain to a nuclease such as FokI the synthetic protein results in a "TALEN". With this specific DNA cleavage can be achieved.

Although theoretically TALENs can be designed for any target sequence it has been shown that custom TALENs can fail. This suggests that yet unknown rules

govern the assembly of functional repeat modules. Software programs to predict optimal TALEN binding sites were developed (Doyle et al. 2012; Cermak et al. 2011). TALENs show a largely extended flexibility in the selection of target sites if compared to the above discussed ZFNs (Cermak et al. 2011). Two studies for defined sites in human cells show that the efficiency of HR mediated by ZFN and TALEN induced DSBs is comparable (Hockemeyer et al. 2009; Mahfouz et al. 2011).

The ZFN and TALEN based strategies for inducing DSBs require the de novo design of a DNA-binding protein. However, the development of modular DNA-binding proteins is difficult, expensive and time consuming. The recently developed **CRISPR/CAS** system uses an alternative strategy that relies on an RNA guided mechanism to introduce DSBs. This strategy is based on the type II clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associate (CAS) system.

The CRISPR/Cas system was adapted to applications in mammalian cells. A short synthetic 'guide RNA' facilitates both, the binding to the DNA motif and the binding to the bacterial endonuclease Cas9. From the 100 nt spanning guide RNA 20 nt confer the binding to a complementary DNA target site. Cas9 is a DNAse that recognizes the complex of the guide RNA with the DNA target site. Cas9 induces a DSB (Jinek et al. 2012; Horvath and Barrangou 2010) into the target DNA. Upon expression of the synthetic guide RNA together with an expression of Cas9, binding of the RNA to the site of interest recruits the Cas9 endonuclease which efficiently induces cleavage (Jinek et al. 2012).

Successful cleavage by CAS9 requires that the target site contains a GNN motif located 1 bp downstream of the complementary RNA target sequence. Thus, this method allows the targeting of DNA sequences that occur every 8 bp in average, which makes it to a more powerful system compared to TALENS and ZFNs (see Table 1.3).

Customized vector systems have been introduced that facilitate co-expression of the short RNA by a polymerase III promoter (e.g. the U6 promoter) and the humanized Cas9 protein controlled by a mammalian Pol II promoter.

Although the method of CRISPR/CAS mediated genome engineering is comparably new, it was improved (Jinek et al. 2012).and can be successfully applied to various species like mouse (Wang et al. 2013) and human (Jinek et al. 2012; Cho et al. 2013; Mali et al. 2013). Of particular interest is the fact that this system has been even used to achieve simultaneous targeting of several loci: multiplexing was successful used to specific targeting of 5 loci (Wang et al. 2013).

Apart from all positive aspects the precision of the CRISPR/Cas system has been doubted. A comparably high frequency of off-target events was detected in some studies showing that even 8/20 base mismatches in the target sequence are tolerated (Fu et al. 2013; Cradick et al. 2013; Segal and Meckler 2013).

1.5.3 Random Integration Competes with HR

Two repair mechanisms of DSBs are known: (i) non-homologous end joining (NHEJ) repair that is characterized by random addition or elimination of nucleotides and (ii) homologous recombination for accurate integration of cassettes (reviewed in Urnov et al. (2010)). For all these applications the efficiency of DSB induced homologous recombination is reduced by the error prone **non-homologous end joining (NHEJ) repair** mechanism. NHEJ results in the repair of DSBs by processing of DNA ends and re-ligation, usually giving rise to mutations (insertions, deletions). Since it was found that nicked DNA does not activate the NHEJ repair mechanism but still stimulates homologous recombination nicking enzyme increasingly replace the FokI nuclease. Thus, less off-target events are observed.

1.5.4 Applications of DSB Induced Modifications

Several producer cell lines derived form CHO and HEK293 were modified by DSB induced HR. Examples concern the knock-out of the dihydrofolate reductase gene (Santiago et al. 2008) and modification of glycosylation in CHO cells (Malphettes et al. 2010; Sealover et al. 2013). Multiple gene knock-outs were achieved using a sequential ZFN-based approach in CHO and K562 cells (Liu et al. 2010; Cost et al. 2010). While much effort has been spent to improve these technologies it is to be expected that the CRISPR/Cas system will show further advantage since its handling is simpler and simultaneous targeting is possible. It thus will have rapid entry into the biotechnology field. Independent of the technology the current limitation concerns the discovery of expression-enhancing integration sites.

1.5.5 Cassette Exchange Approaches Based on Site-Specific Recombinases

The discovery of site-specific recombinases from microorganisms has opened the door towards rational modification of the genome of mammalian cells (Schnutgen 2006; Schnutgen et al. 2006). These enzymes, most of them belonging to the families of serine and tyrosine recombinases, catalyze DNA integration, excision or inversion reactions.

Cre and Flp are the most studies ones. They recognize the recombination target (RT) sites of 34 bp loxP and FRT, respectively (Buchholz et al. 1996). They catalyse recombination reactions between two identical recombination targets. Since the recombination reactions are reversible, the RTs are not altered during recombination. The position of the target sites and their orientation defines the outcome: excision, integration and inversion. (i) If a DNA element is flanked by

two directly oriented target sites it will be excised by the recombinase; (ii) if two RT sites are orientated in opposite, recombination will result in inversion of the fragment between both sites; (iii) integration is reverse to the excision reaction provided that the recombination sites are on two different DNA molecules (bimolecular reaction). ΦC31 mediates recombination between the heterotypic RT sites *attB* and *attP*. A cassette flanked by two *attB* sequences will recombine with a set of attP sites, thereby exchanging the intervening DNA sequence. As a result, hybrid *attL* and *attR* sites are generated which are not compatible for any further recombination event. Thus, the ΦC31-mediated recombination is not reversible.

Due to the intensive research in this field today these reactions are highly efficient and specific if compared to the DSB-based HR reactions.

1.5.6 Tagging and Targeting

Since the recombination target (RT) sites of the site-specific recombinases are not found in mammalian genomes modification requires the introduction of such sites into chromosomal loci. This process is called **tagging**. It can be achieved by random integration of an RT-carrying vector and screening for the relevant property of the cells or by introduction of the RT by homologous recombination. Once, a single copy RT is integrated in a cell clone, it can be targeted with a gene of interest: **targeting**. This is usually done by co-transfection of a vector harboring the recombination target site(s) and a vector encoding the recombinase. Targeting is divided in two strategies "targeted integration" and "recombinase mediated cassette exchange" (RMCE) (Wirth et al. 2007; Turan et al. 2013).

Targeted integration has been exploited for Cre an Flp (Fig. 1.2a). It requires a *single* recombinase target site (loxP, FRT) in the desired genomic location. The targeting vector contains a homologous recombination target site and the expression cassette. Upon transfer to the tagged cell line in presence of the respective

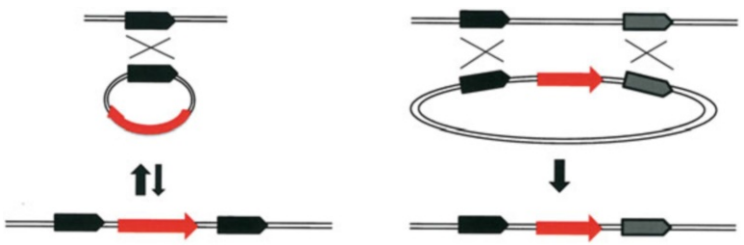

Fig. 1.2 Targeted integration and RMCE mediated by recombinases. (**a**) Targeted Integration; The integration of one RT site into the genomic DNA (*upper*) enables the recombination of a plasmid carrying the identical RT site. Note that this reaction is reversible and excision is highly favored. (**b**) RMCE: Two heterospecific (*black* and *grey*), non-interacting RT sites integrated in the genome recombine with identical RT sites on the targeting vector resulting in cassette exchange. *Red arrow*: GOI

recombinase, recombination between the two identical recombinase sites results in the integration of the whole vector into the tagged chromosomal site. To facilitate isolation of recombined cells selection markers are employed. One limitation of targeted integration is the reversibility of the integration reaction since the targeted state represents a substrate for the subsequent excision. This requires transient expression of the recombinase. Another limitation concerns the fact that not only the gene of interest is integrated but also the bacterial backbone. Since the prokaryotic sequences are frequently associated to DNA methylation, expression can be affected by silencing. The technology is frequently used (Spitzer et al. 2013; Wu et al. 2013) and commerically available.

Recombinase Mediated Cassette Exchange (RMCE) avoids the two limitations of targeted integration, reversibility and co-integration. It makes use of an exchange reaction for the DNA fragment on the targeting vector and the fragment on the chromosomal target (Fig. 1.2b). Both have to be flanked by a set of two RTs. Thereby, an exchange of the intervening sequences is achieved. A condition for the exchange reaction, namely to avoid excision, is a set of RTs that cannot recombine with each other. Such conditions are naturally provided by the ΦC31 system. For Cre and Flp mutant FRT and loxP sites were developed that show no significant cross-reactivity and thus represent ideal RTs for RMCE (Wong et al. 2005; Turan et al. 2013) Flp-based RMCE protocols turned out to be highly efficient. If combined to a stringent selection procedure 100 % targeting efficiency can be achieved (Verhoeyen et al. 2001; Schucht et al. 2006; Sandhu et al. 2011). The availability of such mutants allows to expand cassette exchange to more than one integration site in the genome, thus 'multiplexing' of targeted integration (Wirth et al. 2007; Turan et al. 2013).

1.5.7 Application of Targeted Integration and RMCE

Originally, the concept of tagging and targeting was exploited because HR methods were not available. In this approach the screening for relevant integrations sites have been essential. In contrast to the classical random integration approach it is essential to screen not only for the expression properties but also for single copy integration of the tagging construct. In many screening approaches easy detection (e.g. GFP) was used. The selected tagged cell lines serve as master cell lines for targeting cassettes of choice. Due to the fact that targeting is fast, efficient and precise, the genetic manipulation is defined and expression properties of the final producer clones are predictable. A number of studies have been performed to investigate the application of targeted integration strategies for generation of cell lines for production of recombinant proteins. They demonstrated that applying RMCE based strategy in CHO cells allows to reuse genomic sites supporting high and stable protein expression but also ensures the homogeneity in growth and productivity (Zhou et al. 2010; Kaufman et al. 2008). Examples include cell lines

producing antibodies (Wiberg et al. 2006) and G protein coupled receptors (GPCRs) (Schucht et al. 2011).

Wilke et al. used RMCE in mutant CHO Lec3.2.8.1 cells to produce glycoproteins with the well-established glycosylation pattern in a homogenous form (Wilke et al. 2011). RMCE has been successfully applied for the generation of gamma-retroviral helper cell lines. By screening and tagging chromosomal integration sites in HEK293 cells that support high level of recombinant vector production, high titer retroviral helper cell lines have been generated in which the recombinant vector can be exchanged via RMCE (Schucht et al. 2006; Coroadinha et al. 2006). By targeting different retroviral vector constructions into tagged loci, the vector design could be optimized resulting in titers of 10^7 ip/10^6 cells (Gama-Norton et al. 2010). Further, based on this principle an integrated, GMP compatible strategy for production of therapeutic retroviral vectors was established (Carrondo et al. 2011).

1.5.8 Precautions and Challenges for Targeting into Defined Chromosomal Sites

In transfection experiments it is possible that a few cells integrate the transfected plasmids randomly in addition to the recombination with the target site (Nehlsen et al. 2009). For most applications an additional integration of the vector is not a matter of concern. However, integration of the recombinase expression cassette could result in excision of the cassette in simple targeted integration but not for RMCE. Since random integration along with the appropriate targeted ones will be co-selected employment of negative selection, e.g. based on suicide genes might be of benefit (Preuss et al. 2010).

Another concern might come from the fact that the targeting strategies rely on single copy integration. With regard to high expression, single copy sites have been shown to provide high levels of recombinant protein expression. Further, single copy integrates have the advantage that they show a higher genomic stability and are less prone to rearrangements if compared to multicopy integrations. Nevertheless, the targeting concept is compatible with high copy number mediated expression. Specific sites can be exploited to integrate preformed multicopy plasmids (Kawabe et al. 2012; Crawford et al. 2013). Further, it has been shown that targeted integration can be combined with Methotrexate (MTX) based gene amplification. Upon recombinase based targeting of dhfr expressing vectors and subsequent MTX based amplification, an increase of protein expression could be achieved (Kito et al. 2002; Huang et al. 2007; Kim and Lee 2008).

Meanwhile, ample evidence is given that targeted integration of expression cassettes allows to reproducibly generate cell lines that express a gene or vector of interest. However, some studies also pointed out that defined integration sites are not totally flexible with respect to the design of the targeted cassettes. Indeed, evidence accumulates that a pre-defined site, shown to support high and efficient

transgene expression for one specific cassette, not necessarily supports other cassettes in the same way cells (Tchorz et al. 2012; Chen et al. 2011; Turan et al. 2011). Rather, the loci differ e.g. with respect to the influence of the relative orientation of targeted cassettes. Also, the composition of the targeted cassettes (e.g. the promoter element) can be unequally efficient in different loci (Gama-Norton et al. 2011; Nehlsen et al. 2009, 2011; Tchorz et al. 2012; Chen et al. 2011). Thus, for each integration site the design of the targeted cassette has to be experimentally verified. As long as methods that reliably predict the interaction of the integrated cassettes with the genome are not available, experimental validation of hot-spots or safe harbor sites is required.

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Chapter 2
Transient Recombinant Protein Expression in Mammalian Cells

Volker Jäger, Konrad Büssow, and Thomas Schirrmann

Abstract Transient gene expression has evolved into an attractive technology for the rapid production of milligram to gram amounts of recombinant proteins. This review describes the different methods for introducing foreign DNA into suitable mammalian cells with either viral or non-viral vectors. Particular emphasis is given to non-viral transient transfection which represents meanwhile the most prominent variant due to recent progress in the resulting protein productivity. Non-viral transient transfection protocols are always based on the use of specific transfection reagents or the application of an electroporation device. The corresponding methods are compared with regard to their scale-up potential, also in consideration of potential production costs. The underlying cellular pathways of plasmid DNA incorporation, cytoplasmic release and translocation into the nucleus are important details to understand the transfection principle and further improve the technology. Problems associated with the application of transient gene expression at a larger scale are also addressed. In particular, the requirement of different cell culture media conditions for plasmid DNA complex preparation (if necessary), the transfection process itself and a high titer recombinant production need to be harmonized. Strategies to improve recombinant protein productivity by increasing the cell-specific output and/or sustaining the production phase are itemized as well. This can be accomplished by enabling cells to perform episomal plasmid replication, co-transfection with other plasmids, altering the cellular metabolism, temperature reduction, supplementation of specific production enhancers or combinations thereof. A number of examples for successful applications at pilot scale are also provided.

Keywords Adenovirus • Alphavirus • BacMam • Baculovirus • Butyrate • Calcium phosphate coprecipitation • Calfection • CHO • EBNA1 • Electroporation • Flavivirus • HEK 293 • Polyethylenimine • Polyplex • Semliki Forest Virus • Sindbis

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Virus • SV40 large T antigen • Transient gene expression • Vaccinia virus • Valproate • WPRE

2.1 Introduction

Transient transfection of animal cells for both recombinant protein production and gene therapy has become more and more important with widespread applicability. Substantial progress was achieved during the 1990s which brought these technologies into the limelight. Accordingly, volume 2 of *Cell Engineering*, published in 2000, was completely dedicated to the topic of transient expression, with particular emphasis on the extremely successful baculovirus expression vector system (Al-Rubeai 2000). Meanwhile, the unsurpassed potential of mammalian cells for posttranslational modifications has further driven the development of mammalian cell-based transient gene expression systems with a remarkable increase in protein yields when compared to the low titers initially obtained. A first increase in recombinant protein productivity was achieved by introducing viral vectors. Later, non-viral systems with improved transfection efficiency and sustained productivity became available. This chapter is addressing the most recent advances in both the fundamental understanding of the transfection technology and methodological tools to further improve transfection protocols with regard to an increased recombinant protein titer and reduction of costs.

2.2 Viral Transient Transfection

Besides the baculovirus expression vector system used in combination with lepidopteran cell lines, viral transfection was initially also the method of choice for transient gene expression in mammalian cells due to the very low expression levels in non-viral transfection systems. Meanwhile, non-viral transfection strategies have been improved significantly, thus pushing aside virus-based technologies, which were always hampered by the additional requirement of virus expansion steps, as well as efforts for virus storage and particularly biosafety issues. Viruses used for this purpose are predominantly either single stranded RNA-viruses of the genus alphavirus (Semliki Forest Virus, Sindbis Virus, Venezuelan Equine Encephalitis Virus) or double stranded DNA viruses such as the Vaccinia virus, Adenovirus and Baculovirus. More recently, several flaviviruses have been used as well (Table 2.1). However, all of these expression systems have in common that the virus has to be modified to prevent uncontrolled replication as a result of an accidental release. In addition, viruses had to be either attenuated with regard to their pathogenic properties, or, as a further step, unburdened from most or all of the genes for their structural proteins to become simply replicons which require additional defective helper constructs to generate infective particles (Polo et al. 1999). Some of these

Table 2.1 Viral vectors used for transient transfection of mammalian cells

Virus vector	Virus type	Family – Genus	Vector	References
Semliki Forest Virus (SFV)	(+) ssRNA	Togaviridae – Alphavirus	Specifically modified virus	Lundstrom (2010), Fernández Núñez et al. (2013)
Sindbis Virus (SIN)	(+) ssRNA	Togaviridae – Alphavirus	Specifically modified virus	Nivitchanyong et al. (2009)
Venezuelan Equine Encephalitis Virus (VEEV)	(+) ssRNA	Togaviridae – Alphavirus	Replicon	Balasuriya et al. (2000)
Tick-borne Encephalitis Virus (TBEV)	(+) ssRNA	Flaviviridae – Flavivirus	Replicon	Gehrke et al. (2005)
Japanese Encephalitis Virus (JEV)	(+) ssRNA	Flaviviridae – Flavivirus	Full length cDNA	Yun et al. (2003)
Vaccinia Virus (VACV)	ds DNA	Poxviridae – Orthopoxvirus	Attenuated Strain	Pradeau-Aubreton et al. (2010)
ΔE1/ΔE2A Ad5 vector (Adenovirus)	ds DNA	Adenoviridae – Mastadenovirus	Specifically modified virus	Havenga et al. (2008)
Baculovirus	ds DNA	Baculoviridae – Alphabaculovirus	Specifically modified virus	Condreay et al. (1999), Ames et al. (2007)

replicons have been further modified to chimera of two related viruses. Most details of the corresponding transfection systems have already been reviewed in detail previously (e.g. Smerdou and Liljeström 2000). Because of their specific properties, replicon-based gene expression is now mostly used for experimental gene vaccine approaches rather than recombinant protein production in vitro.

Besides gene therapy applications, adenoviruses were also investigated with regard to their potential as vector for transient protein production. Due to accompanying cytopathic effects virus-susceptible cell lines such as HEK 293 are less suitable, particularly with regard to recombinant protein quality. Therefore, an adenovirus-permissive CHO cell line was generated by repeated virus infection which showed a better performance and was successfully used for production of several secreted proteins (Condon et al. 2003). Another promising approach was accomplished by deleting the viral E1 and E2A in an adenovirus vector which could be easily amplified in a PER.E2A cell line constitutively overexpressing a temperature sensitive, less cytotoxic mutant of E2A. The generated high-titer vector was subsequently used to transduce PER.C6 cells for recombinant protein production (Fig. 2.1). The efficient virus amplification process suggested a high potential for scaling-up this technology (Havenga et al. 2008).

Baculoviruses have also been studied for their ability to transfect mammalian cell lines. The inability of mammalian cells to replicate the baculovirus and to express virus-derived or recombinant DNA was originally considered as a good

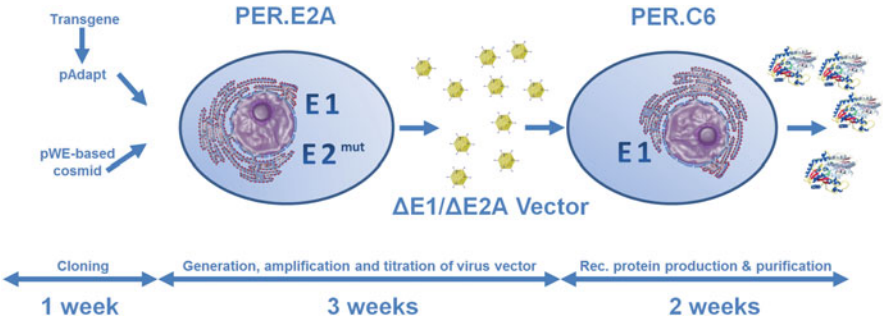

Fig. 2.1 Schematic diagram showing the use of the ΔE1/ΔE2A adenoviral vector-based transient protein production technology in PER.C6 cells including a feasible timeline (Based on Havenga et al. 2008)

sign of biosafety (Carbonell and Miller 1987). Research on protein expression in mammalian cell lines was further driven by the potential of baculoviruses as a safe gene therapy tool. Using the Rous sarcoma virus (RSV) promoter baculovirus was successfully applied to transiently transfect HepG2 cells with moderate expression levels and the requirement of a relatively high multiplicity of infection (Boyce and Bucher 1996). Since baculoviruses allow the insertion of very large genes and their in vitro propagation in lepidopteran cell lines is well-established, a continuing interest lingers on to make them also a tool for transient recombinant protein expression in mammalian cell lines. This led to the development of a substantially improved baculovirus bearing the Vesicular Stomatitis Virus glycoprotein G in its envelope, while keeping the RSV promoter (Barsoum et al. 1997). This virus was able to transfect already a larger number of mammalian cell lines. Subsequently, the technology was developing more rapidly. Another milestone was achieved using expression cassettes under control of the CMV immediate early promoter and the SV40 early promoter. This enabled the transduction of a larger number of mammalian cells including the common production cell lines HEK293, CHO, BHK-21, and HeLa (Condreay et al. 1999). Subsequently, this system was commercialized and named BacMam. An excellent in-depth overview about this expression system is provided by Ames et al. (2007).

2.3 Non-viral Transient Transfection

Despite good recombinant protein yields using virus-based transient transfection systems, they were gradually outshone by non-viral transient transfection. A large number of substantial technological improvements during the past decade meanwhile enables recombinant protein production at much higher titers and in a more sustained way. Most prominently, the use of the plasmid replication system of Epstein-Barr virus (EBV) overcame the major problem of low product titers and has

led to a more widespread application of this technology. EBV nuclear antigen 1 in combination with the viral origin of replication *oriP* allows episomal replication of plasmid DNA (Längle-Rouault et al. 1998).

The demand for applying transient transfection at a larger scale is accompanied with requirement of increasing amounts of plasmid DNA. Similar to the necessity to include an additional production step in virus-based systems in order to generate sufficient amounts of virus, substantial amounts of plasmid DNA have to be produced in suitable strains of *E. coli* bacteria and subsequently prepared at a quality compatible to mammalian cell culture applications (e.g. free of endotoxins). At small and medium scale, shaker cultures in combination with subsequent plasmid DNA purification by means of commercially available kits are the method of choice for plasmid preparation. However, with the increasing plasmid demand for transient gene expression in pilot-scale cell culture bioreactors, it became also necessary to scale-up the plasmid preparation accordingly. Thus, plasmid production in bioreactors and purification at a corresponding scale became essential prerequisites (Rozkov et al. 2008; Sun et al. 2008; Tuvesson et al. 2008; Cheng et al. 2011).

2.4 Plasmid Vector Design

Suitable plasmid vectors for transient transfections have to meet several requirements. Important criteria for plasmid vectors are:

- Yield of plasmid DNA production in *E. coli*
- Efficiency of cellular delivery upon transfection of host cells
- Strength of recombinant overexpression.

Table 2.2 provides an overview on commonly used vectors for transient transfection.

2.4.1 Plasmid Backbone and Size

Transient transcription of mammalian cells on preparative scale requires large amounts of plasmid DNA. High-copy plasmid vectors that can be prepared in high yield from *E. coli* culture are thus preferred. The pUC19 vector was derived from the pBR322 cloning vector, which was cloned from the natural ColE1-class plasmid pMB1 (Yanisch-Perron et al. 1985). Mutation of the pBR322-derived part of pUC19 (GenBank L09137.2) resulted in an ultra-high-copy number of several hundred molecules per *E. coli* cell. The pUC19 origin of replication therefore facilitates the production of large amounts of plasmid DNA in *E. coli* for transient transfection. Most vectors used for cloning and transfection have the ultra-high-copy origin of pUC19, which is characterized by the sequence ctagaagAac,

Table 2.2 Selected plasmid vectors for transient transfection

Vector	Elements	Size (bp)	Source	Reference
pTT	P(CMV), *oriP*, pUC ori, bla	5,925	NRC-BRI, Montreal, Canada	Durocher et al. (2002)
pTT5	P(CMV), *oriP*, pUC ori, bla	4,401	NRC-BRI, Montreal, Canada	Zhang et al. (2009)
pCSE2.5-hIgG1Fc-XP	P(CMV), BGH-pA, SV40ori, *oriP*, pUC ori, bla	5,038	TU Braunschweig, Germany	Jäger et al. (2013)
pCEP4	P(CMV), SV40-pA, *oriP*, EBNA1, hyg, pUC ori, bla	10,186	Life Technologies, Carlsbad, CA	Parham et al. (2001)
pcDNA3.1	P(CMV), BGH-pA, f1 ori, neo, pUC ori, bla	5,428	Life Technologies, Carlsbad, CA	
pSS185	EBNA1, *oriP*, SV40-pA, TAR, P (CMV), TK pA, hph, Tat, pUC ori, bla	11,952	Bayer Healthcare	Cho et al. (2001)
pCI-neo	P(CMV), intron, SV40-pA, neo, pUC ori, bla	5,472	Promega	GenBank U47120

(*CMV*) CMV promoter, *oriP* EBV origin of replication, *hyg* hygromycin resistance gene, *neo* neomycin phosphotransferase gene, *pA* polyadenylation signal, *bla* β lactamase gene, *EBNA1* EBV nuclear antigen 1, *PyOri* Polyoma virus origin

comprising a G > A mutation in comparison to pBR322 (Table 2.2). The pUC19 origin is often labelled 'ColE1 ori' or 'pMB1 ori' in vector maps.

Smaller plasmids are transferred to the nucleus more efficiently during transfections (Yin et al. 2005; Mairhofer and Grabherr 2008). Unnecessary parts of the vector should therefore be removed. Eukaryotic selection markers such as the neomycin phosphotransferase gene are usually not required during transient transfection. Co-expression of selection marker or reporter genes (e.g. GFP) under control of a strong promoter causes an additional metabolic burden for transfected cells, which can result in lower yield of the target protein. Antibiotic selection or cell sorting is also possible by co-transfection of separate plasmids containing these markers.

Vector stability is mainly determined by sequence and size (Oliveira et al. 2009). Larger vectors may contain a larger number of unstable regions and quality control by electrophoresis or chromatography is more difficult. Especially repetitive sequences can reduce plasmid stability, leading to mutated plasmid molecules, or may also induce plasmid degradation by nucleases in the host cell cytoplasm (Oliveira et al. 2009).

Minicircles and other minimal plasmid formats have been developed by reducing or eliminating the bacterial parts of vectors for transfections (Marie et al. 2013; Oliveira and Mairhofer 2013). The development of these systems is driven by gene therapy and DNA vaccination, which require vectors without bacterial antibiotic

resistance markers. For transient transfections, minimal plasmid systems offer improved cellular and nuclear uptake due to minimal plasmid size.

Minicircles are generated by separating the expression module from the bacterial backbone (Broll et al. 2010; Nehlsen et al. 2013). This can be achieved by site-specific recombination. Minicircles are efficiently replicated in mammalian host cells by including copies of scaffold/matrix attachment regions (S/MARs) (Nehlsen et al. 2013).

The bacterial plasmid backbone has alternatively been minimized by replacing the antibiotic selection markers. Ampicillin or Kanamycin resistance markers (830 or 890 bp) can be replaced by the smaller zeocine marker (440 bp). Plasmid propagation can be enforced without antibiotics in specially engineered bacterial strains (Oliveira and Mairhofer 2013). Plasmid propagation has been accomplished with small vector elements such as the amber suppressor tRNAs (~100 bp, pFAR system), the lacO operator (pORT system) (Williams et al. 1998) or antisense RNA (RNA-OUT system) (Luke et al. 2009). The murselect system allows propagation of completely marker-free plasmids. It relies on a bacterial strain overexpressing a regulatory RNA that recognizes the plasmid's origin of replication (Mairhofer et al. 2008).

Despite these interesting developments with focus on gene therapy, most plasmids of choice for transient transfection currently still contain bacterial antibiotic resistance markers and replication origins (Table 2.2). The design of minimal plasmids and their size reduction requires great care. Arrangement and orientation of vector elements in context to others as well as spacer sequences between these elements can dramatically effect plasmid replication and transgene expression. Particularly, DNA elements containing secondary structure information can be strongly influenced by other sequence motifs in their vicinity. Strand separation induced by negative superhelical stress (Stress-induced DNA duplex destabilization, SIDD) and B-form to Z-form (SIBZ) transition may result from a new vector design (Bi and Benham 2004; Zhabinskaya and Benham 2011). We observed that cloning of the highly repetitive EBV origin of plasmid replication (oriP) in direct neighborhood of the bacterial pUC origin of replication reduced the plasmid copy number in *E. coli*. After introduction of a 250-bp spacer between these elements, replication in *E. coli* returned to normal high levels as expected for a high copy plasmid (Jäger et al. 2013).

2.4.2 Vector Elements

Vectors for transient transfection require elements that are necessary or support recombinant protein production. Necessary elements are the promoter, the recombinant protein's coding sequence and a signal for transcription stop and polyadenylation. Supporting elements increase product yield, e.g. by stabilizing recombinant transcripts.

Antibody gene sequences were evolved for maximum expression. The coding sequence and untranslated transcript regions of other genes often benefits from optimization (Fath et al. 2011). In certain cases, codon optimization can lead to high expression of a previously undetectable gene product. Fusion partners such as antibody crystallizable fragment (Fc) or serum albumin can also strongly increase expression of difficult target proteins (Carter et al. 2010).

The human Cytomegalovirus (CMV) major immediate early promoter represents the most commonly used promoter for transient transfection. Commonly used CMV promoters comprise the region of ~610 bp upstream of the transcription start of the CMV major immediate-early gene (GenBank M60321), including the promoter's CAAT and TATA box. Mariati et al. have created vectors with additional CMV sequences upstream and downstream of the promoter, including the first exon and intron of the CMV major immediate-early gene (Mariati et al. 2010). Vectors were used for transient and stable transfections of CHO-K1 and HEK293 cells. Luciferase or EPO were produced. A beneficial effect by including the exon and the intron, but not the upstream elements, was observed.

Intron splicing enhances and stabilizes transgene expression by more efficient packaging of the transcript into ribonucleoprotein complexes and accelerates transport to the cytosol. Previous vectors used genomic immunoglobulin intron sequences. They have been replaced by plasmids containing optimized synthetic introns in the 5′ UTR (e.g. pCI-neo, Promega) or the translated secretory leader sequence (e.g. pCMV/pCMX vectors, Jäger et al. 2013).

Backliwal et al. optimized IgG production by transient transfection of HEK293 cells (Backliwal et al. 2008d). They observed a twofold increase of production when an intron was placed downstream of the human CMV promoter. The mouse CMV promoter was less efficient. A further increase was obtained by including the post-transcriptional regulatory element WPRE of woodchuck hepatitis virus. WPRE is an RNA element of ~600 bp that acts on different levels of RNA processing, transport, and translation and that can compensate for lack of an intron (Schambach et al. 2000). 5.5-fold increased IgG production was observed in HEK293E cells by including WPREs in the EBV-based episomal vector pCEP4 (Kim et al. 2009). Likewise, a threefold increase of WPRE-mediated antibody production was reported in CHO DG44 cells (Wulhfard et al. 2008).

Xia et al. expressed four genes of interest in HEK293EBNA and CHO-K1 cells using different promoters (Xia et al. 2006). The human CMV promoter with the downstream exon 1 and intron 1 of the CMV major immediate-early gene was the strongest, especially in HEK293EBNA cells, in comparison to a standard human CMV promoter or mouse or rat CMV promoters.

Promoters with synthetic enhancers represent a promising alternative to the CMV promoter. Libraries of promoters are generated by random combinations of transcription factor binding site or random sequences, followed by screening for maximal production (Ogawa et al. 2007; Schlabach et al. 2010). Synthetic promoters twice as active as CMV in transient transfections of CHO-S cells for SEAP production have been reported (Brown et al. 2014).

Transcription factor binding sites not only drive gene expression, but can also mediate the transfer of plasmid DNA from the cytoplasm to the nucleus (Gill et al. 2009). Transfection of HeLa cells using PEI lead to NF-κB-driven nuclear import of plasmids containing an NF-κB binding site (Breuzard et al. 2008). The presence of the NF-κB motif led to a sixfold increase of nuclear plasmid DNA.

2.4.3 Episomal Replication

The Epstein-Barr Virus (EBV) maintains its genomic plasmid as an extrachromosomal replicon (Lindner and Sugden 2007). The plasmid's origin of replication, *oriP*, is recognized by the viral protein Epstein-Barr Nuclear Antigen 1 (EBNA1). EBNA1 together with host cell replication factors mediate exactly one round of DNA replication per cell cycle. The viral plasmids are partitioned faithfully to each daughter cell during cell division. EBNA1 has a nuclear localization signal and contributes to nuclear retention of *oriP* containing plasmids.

oriP is located in a large region of ~2,000 bp of the viral plasmid (GenBank NC_007605, nt 7315–9312). The Family of Repeats (FR) of *oriP* is composed of 21 imperfect copies of a 30-bp EBNA1 binding site. In combination with EBNA1, FR acts as an enhancer for promoters on the same plasmid. The Dyad Symmetry (DS) element of *oriP* is the site of DNA synthesis initiation.

Transient transfection of HEK293 and CHO cells benefits from the EBV plasmid replication system (Pham et al. 2006). This requires presence of *oriP* in the plasmid vector and overexpression of EBNA1 in the host cell. It should be noted that transient transgene expression benefits from episomal replication only if production time will be longer than 2 or 3 days. Only then the effect becomes significant because non-replicating plasmids are lost during cell divisions.

HKB11 is an EBNA1-expressing human hybrid cell line derived from the fusion of HEK293S and a Burkitt's lymphoma cell line (Table 2.4). Transient transfection of HKB11 with an P(CMV) vector for overexpression of an interleukin was increased threefold by including *oriP* (Cho et al. 2001). The increase was even 18-fold when *oriP* was combined with the Tat/TAR transactivation axis from human immunodeficiency virus type 1 (HIV-1) in the vector **pSS185** (Cho et al. 2001).

Epi-CHO is a transient transfection system based on the plasmid replication system of Polyoma virus (Py). The Py origin of replication (PyOri) and the Py large T antigen (PyLT) are sufficient to launch strong episomal replication in CHO cells (Kunaparaju et al. 2005). The Py and the EBV replication systems were combined. hGH product yield was increased from 10 to 75 $mg \cdot L^{-1}$ upon transfecting PyLT-positive CHO (CHO-T) cells by including PyOri, *oriP* and EBNA1 into the vector.

The **pCEP4** vector (Life Technologies, Carlsbad, CA) contains a full-length *oriP* and the EBNA1 gene and replicates autonomously in mammalian cells (Yates et al. 1985). The *oriP*/EBNA1 combination increases protein production upon transient transfection of HEK293 cells (Parham et al. 2001). The **pTT** vector

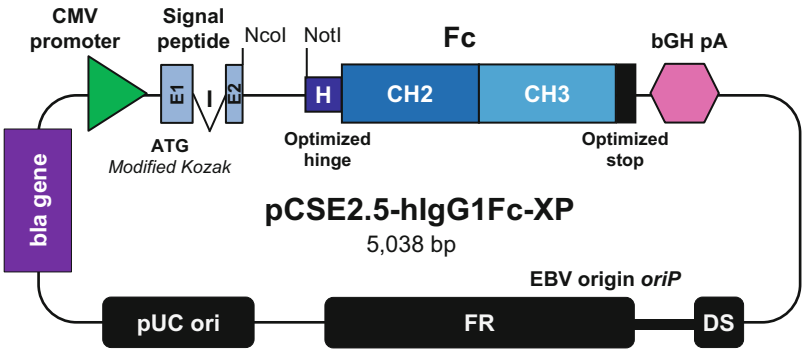

Fig. 2.2 Illustration of the optimized transient expression vector pCSE2.5-hIgG1Fc-XP (Jäger et al. 2013). The vector backbone consists of the bacterial high-copy pUC ori and the β lactamase selection marker gene. The expression cassette is under control of the immediate early CMV enhancer/promoter and the short bovine growth hormone (*bGH*) poly A (*pA*) transcription termination signal. The expression cassette is initiated by a modified untranslated 5′ region, a strong ribosomal binding site (modified Kozak sequence), and a mouse heavy chain signal peptide with two exons (E1 and 2, *light blue*) interrupted by an intron (*I*). One step cloning of single chain Fv (*scFv*) genes is possible via NcoI and NotI from HAL antibody gene libraries (Hust et al. 2011). For production of Fc fusion proteins, the vector contains a constant hinge (*H*), a CH_2 and a CH_3 immunoglobulin region derived from human IgG1. A strong stop codon with an optimal fourth nucleotide for efficient translation termination was introduced. A modified EBV oriP for episomal replication in mammalian EBNA1-positive host cells was introduced. The spacer between the highly repetitive framework region (*FR*) and the dyad symmetry (*DS*) element was reduced

contains an *oriP* but lacks EBNA1. It is used with HEK293 cell lines stably expressing EBNA1 (Durocher et al. 2002) or a truncated form (Durocher 2006). **pTT5** was derived from **pTT** and contains just the FR of *oriP* (Zhang et al. 2009).

The **pCSE** vectors were created by us for overexpression of antibody and antigen molecules as Fc fusion proteins (Fig. 2.2) (Jäger et al. 2013). HEK293-6E cells were transiently transfected with plasmids using PEI (Schirrmann and Büssow 2010). The original vector, **pCMV-hIgG1Fc-XP** was optimized in several steps to the final vector **pCSE2.5-hIg1Fc-XP**. Vector size was reduced by removal of a neomycin resistance gene. A minimized *oriP* sequence, shortened by 830 bp, was inserted. These modifications increased production 3.5-fold (Jäger et al. 2013).

2.5 Transfection Principles

2.5.1 Calcium Phosphate Coprecipitation and Calfection

Calcium phosphate coprecipitation condensing DNA into particles represented one of the first non-viral techniques for introduction of plasmid DNA into mammalian cells. Initially developed by Graham and van der Eb back in 1973 it was optimized for transient transfection of suspension cells at both spinner flask and benchtop

bioreactor scale (Jordan et al. 1998). A further scale-up to 100-L pilot scale was also successfully demonstrated using HEK 293 EBNA1 cells (Girard et al. 2002). However, it's more widespread application was always hindered by the necessity to add serum to the transfection medium, mostly to alleviate toxic side effects of the particles, and by the fact that a number of parameters have to be well adjusted and thoroughly followed to achieve good transfection and expression results. Furthermore, some of the ongoing effects were initially not completely clarified leaving plenty of optimization potential. Diminishing the amount of calcium, adjusting the amount of phosphate and using albumin as a serum replacement for protecting the transfected cells allowed also serum-free transfections (Girard et al. 2001). For CHO cells it was demonstrated that the phase of the cell cycle has a significant influence on the transfection efficiency. When arrested between G0/G1- and S-phase by mimosine and thus synchronized these CHO cells showed a better productivity of the GFP reporter gene (Grosjean et al. 2002), particularly when this was combined with a number of additional process steps such as (repetitive) glycerol shocks or other osmotic shocks momentarily increasing osmolality to approx. 2,000 m Osmol/kg (Grosjean et al. 2006). Obviously, only a proportion of cells, those which are in a specific phase of the cell cycle, are susceptible to successful transfection as shown by repeated transfections which targeted different subpopulations in the same cell culture. Despite of being quite laborious and difficult to perform at larger scale, these techniques provided principally deeper insights about intracellular plasmid DNA processing and transport following endocytosis. Further adjustments were also made with regard to a more feasible incubation period after precipitate formation as a function of temperature, pH and the calcium concentration (Chowdhury et al. 2003). The technology was further improved by omitting the extra precipitation step. This method of forming non- or micro-precipitated calcium-DNA complexes was named *calfection* (Lindell et al. 2004). Again, a successful scale-up to the 30-L scale was demonstrated, although several principal disadvantages of the technology remained. This includes the requirement of a proper adjustment of the pH to slightly alkaline conditions, a narrow range for optimum levels of calcium concentration with additional constraints due to limited reproducibility, a relatively high amount of plasmid DNA required for successful transfection (optimum expression levels at 5 mg L^{-1}) and the necessity to add serum at the time of transfection which led to additional burden during downstream processing and, together with the calcium, to an undesired increase of cell aggregation.

2.5.2 DNA Lipoplex Formation and Lipid-Based Transfection

Since the first report describing the use of lipofection with cationic lipids as a tool for DNA-transfection (Felgner et al. 1987), lipids in various forms have been

Table 2.3 Lipid-based reagents applied for transient transfection of suspension cultures

Name	Manufacturer/ distributor	Type	Specific properties, reference
Lipofectamine®2000 (LF2000)	Life Technologies	Cationic lipid	Sometimes incompatible for transfection in serum-free media
293fectin	Life Technologies	Cationic lipid	Chiou et al. (2014)
DMRIE-C	Life Technologies	Liposome formulation of 1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol	
FuGENE 6	Roche, Promega	Non-liposomal lipid-based	
Ro-1539	Roche	Modified dioleoylmelittin combined with a cationic polymer	Legendre et al. (1997), Schlaeger and Christensen (1999)
FreeStyle™ MAX	Life Technologies	Cationic lipid	
ExpiFectamine™293	Life Technologies	Cationic lipid	Chiou et al. (2014)

identified as being particularly well suited for gene delivery into mammalian cells (Table 2.3). In combination with plasmid DNA they form liposomes which are actively incorporated into cells by endocytosis (Zabner et al. 1995). This was confirmed by electron microscopy observations which also revealed significant differences in plasmid uptake between adherently growing cells and suspension cells (Labat-Moleur et al. 1996). Furthermore, the major bottleneck was identified in intracellular translocation of the plasmids into the nucleus, which resulted in the accumulation of substantial amounts of particles in the cytoplasm and was supposed to be responsible for cytotoxicity. Nonetheless, this highly efficient technology provides usually high transfection rates and is one of the methods of choice for both stable and transient transfection at small scale. This is also reflected by a wide range of commercial reagents available for this application.

Different lipid-based transfection reagents have been studied in comparative studies (also with reagents from the group of cationic polymers). The performance was shown to depend highly on the cell line used for transfection as well as other process parameters (Schlaeger et al. 2003; Rosser et al. 2005; Haldankar et al. 2006; Yamano et al. 2010). All lipid-based transfection reagents have in common that they come along with very high costs which are usually precluding applications at a larger (bioreactor-)scale as it is considered necessary for many transient recombinant protein production processes (Haldankar et al. 2006; Liu et al. 2008).

2.5.3 Cationic Polymers and Transient Transfection with DNA Polyplexes

Polyethylenimines (PEI), a family of cationic polymers were first introduced as a reagent for gene transfer by Boussif et al. (1995). Both a 50 kDa and an 800 kDa PEI not further specified (but presumably branched) were found to be suitable for transfection of embryonic chicken neurons as well as HeLa cells at an optimum N/P ratio of 9 (mole to mole ratio of the amine groups of cationic polymers to the phosphate groups of DNA). Subsequently, linear PEIs were identified to provide also good transfection results at a typically lower toxicity. However, the size of the formed PEI/DNA polyplexes was found to be highly dependent on the solution used (either 5 % glucose or 150 mM NaCl) with different optima of the polyplex size as well as the N/P ratios for either gene therapy applications in vivo or cell transfection in vitro (Goula et al. 1998a, b). Godbey et al. (1999a) investigated also smaller PEI variants down to a molecular weight of 600 but obtained best transfection results with the highest molecular weight variant tested (70 kDa). In another study a linear 22 kDa PEI provided better transfection efficiency than branched PEIs of 25 or 50 kDa (Wiseman et al. 2003). In contrast to the well-understood virus-based transfection mechanisms the principles of the PEI-based non-viral gene transfer had to be elucidated step by step (Kichler et al. 2001). A detailed study about optimum PEI / plasmid DNA ratios (based on suitable N/P ratios), cytotoxicity, stability against DNase digestion, and intracellular translocation of plasmids was performed by Oh et al. (2002). They showed that PEI polyplexes increase the cellular uptake of plasmid DNA, efficiently protect against intracellular enzymatic DNA digestion, and prolong the subcellular DNA retention, whereas a contribution of PEI alone for an improved translocation into the nucleus as suggested previously (Godbey et al. 1999b) could be excluded. Linear PEI of 25 kDa was confirmed as the most suitable variant of PEI when compared to linear PEI of both higher and lower molecular weight as well as branched PEI for either CHO DG44 cells (Derouazi et al. 2004) or HeLa and HEK293 cells (Reed et al. 2006). Moreover, linear PEI with a certain degree of polydispersity was shown to bind DNA less efficiently but provide better recombinant protein yields than more uniform fractions or fully hydrolyzed PEI (PEI "Max") (Kadlecova et al. 2012). Being currently the most preferable cationic polymer for gene transfer into cells with regard to transfection efficiency and cytotoxic side effects, PEI nevertheless may induce necrosis and apoptosis to some extent (Hunter 2006). In an approach to better understand the transfection principle, Carpentier et al. (2007) investigated the kinetics of plasmid uptake, subsequent translocation into the nucleus, transcription and protein expression. Up to 65,000 plasmid copies in both GFP-expressing and non-expressing cells were detected intracellularly 1 day post transfection, confirming previous results of Kichler et al. (2001) who found 50,000 plasmid copies per cell 7 h post transfection. However, just 1,850 copies were found in the nucleus of GFP-positive cells and 550 copies in GFP-negative cells. Variations in the total amount of plasmid DNA during transfection did not significantly change the ratio of producing and non-producing cell, thus indicating that a cellular state no further specified is of major importance for a successful transient

transfection. This is in accordance with earlier results demonstrating that recombinant protein productivity is dependent on the phase of the cell cycle during transfection (Grosjean et al. 2002).

2.5.3.1 Polyplex Uptake, Plasmid Release and Translocation into the Nucleus

The biochemical principles responsible for the successful use of polythylenimine as a transfection reagent were initially unknown and it took more than a decade to investigate and understand the most important steps of the underlying pathways. The internalization of the polyplexed plasmid DNA, its intracellular trafficking and release from the polyplex as well as its positioning into the cellular nucleus are based on a series of rather complex mechanisms. PEI:DNA polyplexes are first specifically bound to (heparin sulfate) proteoglycans, in particular syndecans, before being internalized and transported to late endosomes by two constitutive endocytic pathways. Polyplex internalization by both the clathrin- and a caveolae-dependent pathway were observed. Both the transfected cell line and the type of the polyplex appear to determine which of the pathways is used most efficiently (von Gersdorff et al. 2006). For instance, both pathways were observed in COS-7 cells but only the latter was demonstrated to result in a successful gene expression (van der Aa et al. 2007). The mechanism of a successful internalization was shown to be different to and independent of both the clathrin- and the 'standard' caveolin-mediated endocytosis by possibly bypassing early endosomes (Payne et al. 2007). Different syndecans are reported to have quite opposing properties with regard to their potential to incorporate the polyplexes. Only syndecan-1 promotes subsequent trafficking of the PEI:DNA complexes (Paris et al. 2007). Using HeLa cells, this slightly different caveolar pathway was compared to the clathrin pathway and confirmed to be the predominant uptake mechanism (Gabrielson and Pack 2009). The initial steps are based on a capture of the polyplexes by filopodia with locally clustering syndecans. By either directional surfing along these filopodia or by an actin driven filopodia retraction the polyplexes are first guided to the cell surface and then specifically internalized via caveolae. This process is far beyond a random attachment of polyplexes by simple electrostatic interactions and contributes significantly to the success of a transient transfection (ur Rehman et al. 2012).

Once in the endosome, polyplexes are proposed to be subjected to the so-called proton sponge effect with an osmotic swelling and eventual vesicle rupture as a result of the ongoing acidification of the maturing vesicle (Nel et al. 2009). Different to that, more recent findings revealed that the corresponding endosome is not disrupted and the release of the plasmid DNA from the polyplex is a more subtle process (ur Rehman et al. 2013). This is in accordance with findings that additional, free PEI molecules might play a pivotal role in the process of DNA release from endosomes (Yue et al. 2011). An early intracellular dissociation of radiolabelled PEI and DNA was also confirmed by subcellular fractionation methods (Shi et al. 2013). Upon dissociation, the relatively large plasmid DNA shows a low mobility (Lukacs et al. 2000) and, at the same time, is very susceptible to

inactivation and degradation by cytosolic nucleases resulting in an apparent half-life of just 50–90 min (Lechardeur et al. 1999). Thus, rapid translocation of naked plasmid DNA into the nucleus is paramount for a successful transient transfection process. The nuclear pore complex may play an important role in non-dividing cells targeted for gene therapy (van der Aa et al. 2006). However, nuclear transport in cultured, proliferating cells is presumably accomplished most efficiently during the temporary nuclear membrane breakdown in mitosis (Grosse et al. 2006). DNA nuclear targeting sequences of the plasmid, originally derived from SV40, might further enhance plasmid uptake into the nucleus (Dean et al. 2005), even if the cells are non-dividing.

2.5.3.2 PEI Derivatives and Other Cationic Polymers as Alternatives to PEI

Other cationic polymers such as poly(-L-)lysine (PLL), chitosan (Corsi et al. 2003) or linear β-cyclodextrin-containing polymers (Gonzalez et al. 1999) have been also used with some success and usually acceptable cytotoxic effects, but never received widespread application. Irrespective of the successful and meanwhile widespread use of PEI for transient gene expression there are continuing efforts to further improve these cationic polymers by reducing undesired cytotoxic and/or cytostatic effects and by increasing the transfection efficiency with better reproducibility. In addition patent issues regarding the use of PEI for commercial applications and a rapidly growing market for transfection reagents are further stimulating these endeavors. To name a few, this includes the use of PEI/chitosan complexes (Zhao et al. 2009), different PEI-cystamine derivatives (Wang et al. 2011), a family of biodegradable poly(amine-*co*-esters), particularly poly(*N*-methyldiethyleneamine sebacate) (PMSC) (Liu et al. 2011), functional block copolymers with PEG (Hu et al. 2013) or low molecular weight PEI600 cross-linked by β-cyclodextrin (Hu et al. 2012).

2.5.4 Transient Transfection by Electroporation

Being a well-established and reliable technology at small scale (Wong and Neumann 1982), electroporation was initially impossible to perform at larger scale as it is required for recombinant protein production by transiently transfected mammalian cells. First successful attempts on a larger scale were performed by Blasey et al. (1996) with COS cells and by Parham et al. (1998) using several suitable cell lines including HEK293 (±stable EBNA1 expression) and CHO (±stable SV40 large T antigen expression). Different to the principle of DNA internalization by means of transfection reagents, electroporation is based on the process of local membrane destabilizations resulting in an association of membrane and DNA (Phez et al. 2005). Once the plasmid DNA is internalized, the electroporation process is

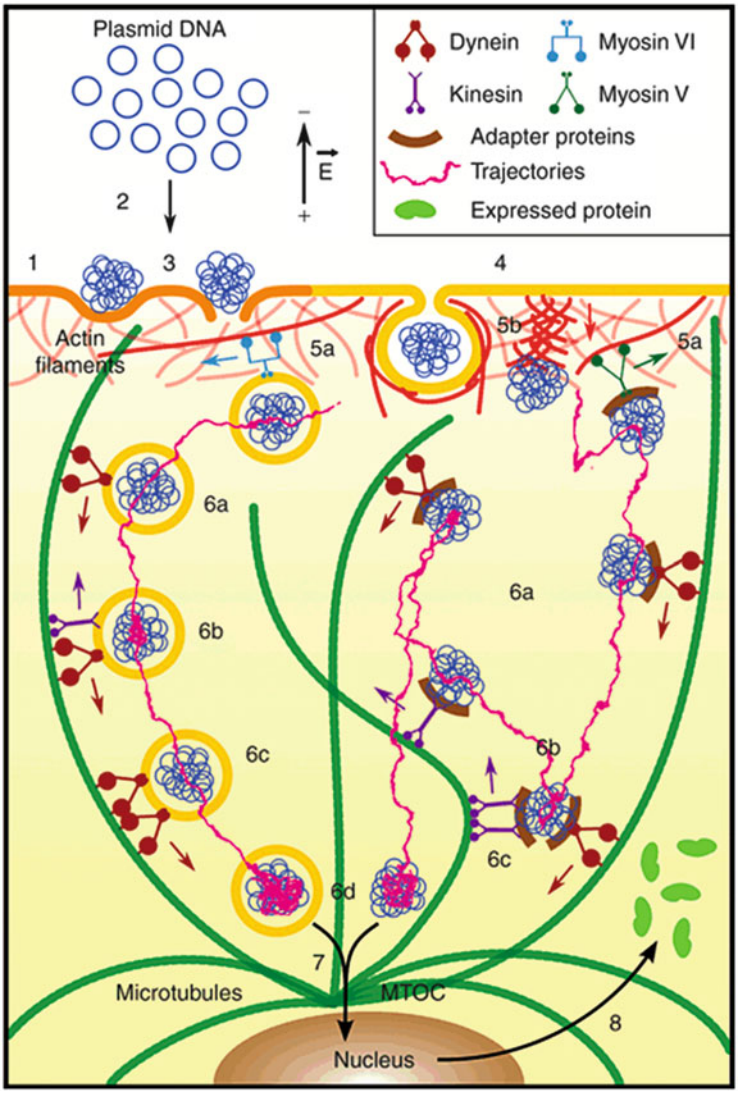

Fig. 2.3 Mechanisms of DNA electrotransfer in mammalian cells. During the application of the electric field, (*1*) the plasma membrane is permeabilized (*orange*); (*2*) the DNA is electrophoretically pushed onto the membrane side facing the cathode; therefore, (*3*) DNA/membrane interactions occur. DNA aggregates are inserted into the membrane and remain there for 10 s to minutes. After the application of the electric field and resealing of the membrane (*yellow*), (*4*) the DNA can be internalized through endocytosis (DNA in vesicles) and/or through electropores (free DNA). For gene expression to occur, (*5, 6*) DNA has to cross the cytoplasm and move toward the nucleus. Rosazza et al. found (*5*) actin-related motion that can be (*5a*) transport using myosins (in both directions) and/or (*5b*) burst of actin polymerization (actin rocketing). Further, they observed (*6*) microtubule-related motion: (*6a*) by transport through kinesin and dynein, (*6b*) by DNA interaction with oppositely directed motors, or by using (*6c*) several motors of the same type. (*6d*) Once in the perinuclear region, (*7*) DNA has to cross the nuclear envelope, after endosomal escape in case of DNA in vesicles. (*8*) Finally, DNA is expressed in proteins found in the cytoplasm. *MTOC* microtubule-organizing center (Taken from Rosazza et al. (2013), rights permitted)

additionally important for cytosolic trafficking, since it causes disassembly of the actin cytoskeleton network (Rols and Teissié 1992), which would otherwise significantly slow down the motility of molecules of the size of plasmid DNA (Lechardeur et al. 1999; Dauty and Verkman 2005). Comparable to other transfection methods, trafficking into the cellular nucleus is predominantly associated with nuclear membrane breakdown during mitosis. Correspondingly, cell synchronization in the G2/M phase was shown to increase the efficiency of transfection using cell electroporation (Golzio et al. 2002). A complete scheme describing the mechanisms of plasmid DNA electrotransfer in mammalian cells was provided by Rosazza et al. (2013) (Fig. 2.3). Meanwhile, equipment for transient transfection of up 200 billion cells has become more generally available. Suitable devices now facilitate routine electroporation with very good transfection rates and preservation of high cell viability (Johansson et al. 2013).

2.6 Cell Lines for Non-viral Transient Transfection

In viral expression systems, cells have to be susceptible to the infecting virus for subsequent gene expression. Similarly, the mammalian cell lines used in non-viral approaches have to be compatible to the plasmid DNA and the transfection technology. Initially, COS-cells (in particular COS-7) were frequently used for transient gene expression. However, their relatively low protein expression rates did always encourage the search for better alternatives such like HEK 293 cells (Berntzen et al. 2005). HEK 293 cell lines were found to be particularly well suited as they exhibit relatively low resistance against transient transfection. Nonetheless, tremendous efforts were made to use CHO cell lines as an alternative. Despite the fact that CHO cells proved to be considerably more difficult to transfect transiently, their widespread use in the production of biopharmaceuticals and the corresponding know-how in process technology as well as a posttranslational modification of recombinant proteins similar or identical to stably transfected CHO cells was deemed to justify these additional efforts. Table 2.4 summarizes some commonly used HEK293 and CHO cell lines as well as some other alternatives. Particularly CAP-T, a stably transfected human amniocyte cell line, is showing very good transfectability and high production titers. This versatile line was demonstrated to be an excellent alternative or complement to HEK293-EBNA cell lines (Fischer et al. 2012).

Unfortunately, the nomenclature for the most frequently used HEK 293 cells is considerably diverse. For instance, those cell lines which were initially established at Stanford University by stable transfection to constitutively express EBNA1 and which were subsequently both licensed to Invitrogen (originally named 293-EBNA) and deposited at ATCC as CRL-10852 (originally named 293 c18) are meanwhile described under a plethora of different names (e.g. HEK 293-EBNA, 293E, HEK293E, HEK/EBNA, HEK.EBNA, HE; Geisse 2009). It remains unclear whether these cell lines are originally derived from the same subclone or represent

Table 2.4 Mammalian cell lines commonly used for transient expression of recombinant proteins

Name	Characteristic features	Suitable plasmid vectors	Culture conditions	Culture media	References
HEK 293 cell lines					
XDC293	Clonal derivate of HEK 293 cells w. improved transfectability		Not adapted to serum-free suspension culture	DMEM w. 5 % BCS	Reed et al. (2006)
HEK 293SF-3F6	Clonal derivative of HEK 293S cells adapted to both suspension and serum-free culture	E.g. lentiviral vectors	Serum-free suspension culture adapted	Custom made low calcium SFM or HyQSFM4TransFx293	Côté et al. (1998), Swiech et al. (2011)
"HEK 293 EBNA1" (ATCC CRL-10852) or 293-EBNA from Invitrogen	Stably transfected cell line expressing EBNA1	pCEP4, pREP4, pcDNA series, pTT series	Adherent culture in serum-supplemented medium or serum-free suspension culture adapted	ExCell 293 293 SFM II or FreeStyle™ 293	Numerous applications; e.g.: Durocher et al. (2002), Geisse and Henke (2005)
HEK/EBNA-SF	EBNA1-expressing 293 c18 (ATCC CRL-10852) adapted to serum-free medium	Same as above	Serum-free adherent culture (microcarriers)	DMEM:Ham's F12-based serum-free medium or EpiSerf	Fliedl and Kaisermayer (2011)
HEK 293T/17 (ATCC CRL-11268)	Stably transfected expressing SV40 large T-antigen		Not adapted to serum-free suspension culture	DMEM w. 10 % FBS	Pear et al. (1993), Numerous applications, e.g.: Chang et al. (2007), Li et al. (2007)
FreeStyle™ 293-F	Derivative of non-transfected HEK 293 cells		Serum-free suspension culture adapted	FreeStyle™ 293 Expression Medium	Liu et al. (2008)
Expi293F™	Derivative of FreeStyle 293-F	E.g. pcDNA3.3	High density serum-free suspension culture adapted	Expi293™ Medium	Chiou et al. (2014), Vasu et al. (2013)

HEK 293SFE (HEK 293SFE41)	Stably transfected HEK 293SF-3F6 expressing EBNA1	pTT series	Serum-free suspension culture adapted	E.g. LC-SFMLB	Pham et al. (2003)
HEK 293-6E	Stably transfected expressing a truncated version of EBNA1	pTT series	Serum-free suspension culture adapted	FreeStyle™ F17	Durocher (2006), Loignon et al. (2008), Carter et al. (2010), Raymond et al. (2011)
293EBNALT 75	Stably transfected expressing murine polyoma virus large T-antigen (PyLT) + EBNA1	pQMCF plasmids (Icosagen)	Serum-free suspension culture adapted	1:1 mixture of Pro293s-CDM and 293 SFMII	Silla et al. (2011)
HEK 293S GnTI$^-$ (ATCC CRL-3022)	Lacks *N*-acetylglucosaminyltransferase I (GnTI) activity for less heterogeneous *N*-Glycosylation	Various	Roller bottle cultures w. attached cells or serum-free suspension culture	DMEM w. 1.6–10 % FBS or FreeStyle™ F17[1]	Reeves et al. (2002), Zhao et al. (2011), [1]Jäger, unpublished results
HKB-11	Somatic hybrid cell line of 293S and Burkitt's lymphoma line 2B8 expressing EBNA1	Tat/TAR-*oriP* related expression vectors	Serum-free suspension culture adapted	E.g. MICT7.0	Cho et al. (2001), (2002), (2003)
CHO cell lines					
CHO-K1-S	Non-transfected		Serum-free suspension culture adapted	CHO-S-SFM II	Rosser et al. (2005)
FreeStyle™ CHO-S		various (e.g. pCEP4, pcDNA series)	Serum-free suspension culture adapted	FreeStyle™ CHO Expression Medium	Liu et al. (2008)
CHO-T	Stably transfected expressing murine polyoma virus large T-antigen (PyLT)	Epi-CHO system, i.e. pPyEBV plasmids (Pyori, *oriP*, EBNA-1)	Serum-free suspension culture adapted	Ex-Cell 302 CHO-S-SFM II	Kunaparaju et al. (2005), Codamo et al. (2011)

(continued)

Table 2.4 (continued)

Name	Characteristic features	Suitable plasmid vectors	Culture conditions	Culture media	References
CHOEBNALT 85	Stably transfected expressing murine polyoma virus large T-antigen (PyLT) + EBNA1	pQMCF plasmids (Icosagen)	Serum-free suspension culture adapted	1:1 mixture of CD CHO and 293 SFMII	Silla et al. (2007), Silla et al. (2011)
Potelligent® CHOK1SV	Glutamine synthetase CHO K-1 cells w. Fucosyltransferase 8 knocked out		Serum-free suspension culture adapted	UltraCHO	Ye et al. (2009)
CHO-3E7	Stably transfected expressing a fusion protein composed of HSV VP16 and a truncated version of EBNA1	pTT series	Serum-free suspension culture adapted	FreeStyle™ CHO Expression Medium	Durocher and Loignon (2009), Exempl. application: Stoops et al. (2012)
Other cell lines					
CAP-T	Stably transfected expressing SV40 large T-antigen	Plasmids containing SV40ori	Serum-free suspension culture adapted	Protein Expression Medium (PEM)	Wölfel et al. (2011), Fischer et al. (2012)
VERO (ATCC CCL-81)	Non-transfected	E.g. pCEP4	Serum-free adherent culture (microcarriers)	DMEM:Ham's F12-based serum-free formulation	Fliedl and Kaisermayer (2011)

two different clones generated in parallel. In addition, long-term cultivation under various culture conditions (e.g. serum-free suspension culture vs. adherent culture in serum-supplemented medium or combinations thereof) and subsequent recloning might have resulted in substantial genetic and phenotypic diversity.

With the development of stably engineered production cell lines, recombinant protein productivity upon transient transfection was dramatically increased. This was accomplished by both better gene transcription in the nucleus and a more sustained productivity due to the episomal replication of plasmids in the proliferating cell population. However, there are also some reports recently which indicate that high level protein expression is also possible with non-engineered HEK 293 cells (e.g. Expi293F^{TM}) when thoroughly following a production protocol based on a proprietary combination of cell line, transfection reagent, culture medium and non-disclosed productivity enhancers (Chiou et al. 2014).

Besides productivity issues, specific applications of the expressed recombinant protein are also influencing the selection of the most suitable cell line. Crystallization of proteins for structural biology research for example requires very high protein homogeneity for crystal formation. This requirement is in contrast to biopharmaceutical applications where the naturally occurring microheterogeneity of glycoproteins is usually acceptable as long as e.g. immunogenicity and pharmacokinetics are not affected. Particularly, when proteins are bearing several *N*-glycosylation sites microheterogeneity might reach an extent which has to be reduced by supplementing specific inhibitors of the *N*-glycan trimming pathway such like kifunensine or swainsonine to the transiently transfected cell culture (Chang et al. 2007). A different approach is followed by cleaving the *N*-glycans from the peptide backbone with specific glycosidases (i.e. PNGaseF or Endo F series) (Lee et al. 2009). Alternatively, the selection of a cell line with altered glycosylation properties such like HEK 293S GnTI^{-} which is lacking *N*-acetylglucosaminyltransferase I and thus predominantly expresses glycoproteins with $Man_5GlcNAc_2$ *N*-glycans (Reeves et al. 2002) might overcome this problem. Meanwhile, corresponding automated production processes have been established which implement these strategies (Lee et al. 2009; Zhao et al. 2011).

To overcome limitations of CHO cells with regard to their lower recombinant protein yields, a CHO-DG44 cell line was developed stably overexpressing the anti-apoptotic protein bcl-x_L. PEI-mediated transfected of these cells resulted in a two to threefold increase of productivity and less apoptosis (Majors et al. 2008).

2.7 Cell Culture Media Conditions

The selection of suitable cell culture conditions for transient transfection processes is more challenging than for stable cell lines, because the specific requirements of the transfection step itself are also of major importance. Cell culture conditions have to be compatible to all phases of a transient transfection process:

- Complexation of plasmid DNA in a form suitable for later incorporation by target cells
- Transfection of target cells
- High titer production of recombinant proteins

Although the first step is still performed without cells, it has to be considered that the solution with the newly formed complex will be added to the cell culture afterwards and therefore, will influence also all subsequent steps. This is the reason that, instead of simple buffer solutions, cell culture media are frequently applied for this purpose since they are non-cytotoxic and contain also various nutrients and thus diminish the 'diluting' effect during transfection with plasmid DNA. As a promising alternative, transfection is also possible without a priori DNA complex formation directly in the cell suspension. This is described in more detail below.

The subsequent steps of transfection and protein production can be easily accomplished at small scale by applying first culture conditions optimized for cell transfection and separating these from a second phase optimized for recombinant protein production by inserting a simple media replacement step of either decantation (for adherently growing cells) or centrifugation (for suspension cells) (e.g. Baldi et al. 2005). However, with increasing scale a complete medium exchange is getting more and more difficult and is accompanied with an increasing expenditure of work and costs. Therefore, it is highly desirable for a reliable scaling-up of the technology that both transfection and recombinant protein production may be performed in the same culture medium thus avoiding a complete medium exchange. Serum usually does not interfere with the transfection process itself but substantially contributes to the burden of contaminating proteins in the supernatant, thus hampering protein purification and – at the same time – does not permit an animal component free process if it is required. Hence, the replacement of serum-supplemented cell culture media by suitable serum-free media is highly desirable. Both, calcium phosphate coprecipitation and calfection represent attractive methods with regard to the overall production costs and their scale-up potential was successfully demonstrated. However, for both methods serum-supplemented media (or the use of albumin as a serum replacement; Girard et al. 2001) are still an essential prerequisite. This significant drawback did finally redirect attention to other transfection technologies, as soon as inexpensive reagents such as PEI and appropriate protocols became available (Baldi et al. 2005).

Apart from serum as an undesirable cell culture supplement, there is a number of other media components which might also significantly interfere with the efficiency of several transient transfection protocols. It is obvious that ingredients such as calcium and phosphate might directly interact with the condensation and complexing of plasmid DNA. There is also evidence about other supplements which disturb transfection in a currently unknown manner. This comprises peptones which are reported to significantly increase recombinant protein productivity (Pham et al. 2003) with major peptone-specific differences in their performance (Pham et al. 2005). Unfortunately, it was also observed that they might interfere with the transfection process, thus suggesting a later supplementation to achieve

maximum transfection rates (i.e. 24–48 h.p.t.) (Pham et al. 2005). Supplementation of different peptones was also successfully applied by Kiseljak et al. (2012) with a remarkable increase in recombinant antibody production. These peptones were preferably added several days post transfection. Again, hydrolysates as well as several other common cell culture media ingredients, namely phosphate and dextran sulfate were reported to inhibited the transient transfection process (Geng et al. 2007). The negative effects on transfection efficiency were circumvented by using different, deficient media during transfection whereas detrimental effects on cell growth and viability were avoided by subsequent complementation with the complete medium.

Iron chelators also represent common ingredients of serum-free media and usually substitute transferrin (either as a natural component of serum or as a defined supplement in serum-free media) as a source of ferric ions. Iron (III) citrate, but neither ferric ions nor citrate, was identified to severely diminish transfection efficiency (Eberhardy et al. 2009). This might also occur as a result of spontaneous iron (III) citrate formation from other media components (i.e. other ferric salts and citrates).

Conditioned media in general were also shown to severely reduce transfection efficiency and recombinant protein titers when compared to transfections in fresh media (Schlaeger and Christensen 1999). This effect was also reported more recently for CHO DG44 cells (Pereira et al. 2011) and also confirmed – although to a much lesser extent – by our own unpublished results when using HEK 293-6E cells. In all these studies cationic polymers (i.e. PEI) were used as transfection reagent. By any means, the detrimental effects of these unidentified cell metabolites on transient gene expression efficiency represent a severe obstacle for the applicability of transient gene expression at a larger scale since a complete media replacement is difficult if not impossible to perform at a multi-liter scale.

On the contrary, Long R3-IGF, well known as a potent growth factor for many cell types, provides enhanced recombinant protein productivity by a factor of 2 and works synergistically with mild hypothermia for CHO cells (Galbraith et al. 2006). However, if Long R3-IGF is already a constituent of the non-disclosed basic formulation of commercially available serum-free media no further increase of productivity is expected upon supplementation.

The supplementation of compounds with no or low cytotoxicity and the potential to increase membrane permeability might also assist the incorporation of plasmid DNA. Both, lithium acetate (3 mM) and dimethyl sulfoxide (DMSO) (1.25 %) are reported to improve PEI-mediated transient transfection in Potelligent® CHOK1SV cells (Ye et al. 2009). Earlier, DMSO was already found to support transfection mediated by electroporation (Melkonyan et al. 1996).

The PEI-mediated transient gene expression in CHO-S cells was shown to be improved by supplementing prior to transfection microtubule disrupting antimitotic reagents, namely nocodazole which arrests cells in the G_2/M phase and hydroxyurea arresting cells in the G_1 phase (Tait et al. 2004). This synchronization technique increases recombinant protein productivity when compared to

unsynchronized cells but requires substantial lab work as the reagents have to be removed afterwards.

2.7.1 *Use of Productivity Enhancers*

Gene expression is based on the normal regulation of differential acetylation of nucleosomal histones resulting in either transcriptional activation (hyperacetylation) or repression (hypoacetylation). Different histone deacetylase inhibitors (HDACi) were identified to efficiently increase gene expression because of their interfering effect on this regulation (Hebbar and Archer 2003). Since its first use for enhanced protein expression (Gorman and Howard 1983) sodium butyrate is been in use for a vast number of protein production processes. Parham et al. (1998) were supplementing 2 mM of sodium butyrate to several transiently transfected cell cultures and obtained significantly increased recombinant protein yields. A significant expression-enhancing effect of sodium butyrate was also observed in BacMam transduced cells (Condreay et al. 1999). Whereas most HDACi such as trichostatin A reveal high cytotoxic secondary effects, an inexpensive alternative with a relatively low cytotoxicity was identified more recently in valproic acid or its water soluble sodium salt (Fan et al. 2005). Initially tested for viral gene transfer approaches, valproic acid was subsequently successfully tested for HEK 293 EBNA1 as well as CHO DG44 (Backliwal et al. 2008b) and has meanwhile become a standard supplement in many transient gene expression processes. Less publicized and well-known is the potential of other carboxylic acids to act as productivity enhancers. This includes pentanoic acid (Liu et al. 2001) and sodium propionate (Chun et al. 2003) which are also reported to be less cytotoxic or apoptosis-inducing.

2.8 Process Strategies for Improved Recombinant Protein Production

Numerous transfection and cultivation parameters have been varied to improve the yield of the corresponding recombinant protein production. In the following paragraphs some of the most important steps are summarized.

2.8.1 *Mild Hypothermia*

Mild hypothermic conditions represent a well known process variable to increase recombinant protein productivity of stably transfected cell lines (e.g. Weidemann

et al. 1994; Kaufmann et al. 1999). This is usually accomplished in a separate phase initiated by a temperature shift followed by decelerated cell proliferation and an increased protein production in a prolonged cultivation process. Thus, it was obvious to investigate mild hypothermia also in transient gene expression processes. Using the Semliki Forest Virus expression system, some mammalian cell lines (i.e. BHK-21 C13 and CHO/dhfr$^-$) showed much better productivity at 33 °C than at the standard temperature of 37 °C (Schlaeger and Lundstrom 1998). In the same study, HEK 293 and 293-EBNA cells were found to be less susceptible to hypothermia. These cells did not grow well at 33 °C and exhibited just slight (HEK 293) or no (293-EBNA) signs of an increased protein expression at the lowered temperature. The good response of different CHO cell lines to temperature reduction was confirmed also in a number of approaches with non-viral transient gene expression. A reduction to 32 °C increased product titers of PEI-transfected CHOK1SV cultures by a factor of 3 with an additional, synergistic effect of supplemented Long R3-IGF (Galbraith et al. 2006). Likewise, antibody expression was also increased several fold by lowering temperature to 31 °C in PEI-transfected CHO DG44 cultures (Wulhfard et al. 2008). The temperature shift was performed just 4 h post transfection, but the uptake of plasmid polyplexes was considered to be already completed. Compared to cells growing at 37 °C more cells were accumulated in the G1 phase of the cell cycle. The productivity enhancing effect was not observed when lowering the temperature 3 days post transfection or later. More recently, this process was further adjusted and optimized by performing also the PEI-mediated transfection itself at a lowered temperature of 31 °C and lessen cytotoxic effects by reducing the amount of polyplexes for transfection (Rajendra et al. 2011).

By contrast, HEK 293 cell lines are reported to be much more sensible to the stress attributed to hypothermic culture conditions (Schlaeger and Lundstrom 1998). Correspondingly, there are few reports on beneficial effects of hypothermic culture conditions using these cell lines. Productivity (and product quality) of recombinant human FVIII expressed in transiently transfected HEK293SF-3F6 cells was improved when cells were transfected repeatedly at lower DNA doses and temperature was simultaneously decreased to 34 °C (Swiech et al. 2011). Our own, unpublished results revealed that, when performing transient gene expression with HEK 293-6E cells, temperature shifts to either 32 °C or 34.5 °C did not provide higher protein expression levels but increased the cytotoxic effects of the transfection process.

2.8.2 High-Density Transfection and 'Direct' Transfection

Transfection protocols with a separate step of polyplex formation a priori are increasingly replaced by the alternative principle of generating PEI:DNA polyplexes directly in the cell culture. This strategy is providing advantages by reducing the variations associated with the polyplex formation step and simplifies

attempts to establish automated processes. This so-called *direct* transfection was accomplished with both HEK 293EBNA (Backliwal et al. 2008a) and CHO DG44 cells (Rajendra et al. 2011) and usually comes along with a transfection at high cell density. However, to achieve this high density prior to transfection these protocols stipulate a centrifugation step which complicates scaling-up. To circumvent this problem, a direct PEI-mediated transfection without centrifugation was established in combination with HEK 293-6E cells (Raymond et al. 2011). In a different approach, high cell densities of HEK293 EBNA cells were obtained using a perfusion bioreactor system (Sun et al. 2008). Several hours after transfection, which was performed using the standard method of separated a priori polyplex formation, surplus polyplexes were rapidly removed by adjusting a high perfusion rate thus lowering possible cytotoxic effects. Subsequently, cells were cultivated either in a fed-batch mode or by perfusion with an enriched medium resulting in significantly improved recombinant protein productivity when compared to previous results (Sun et al 2006).

2.8.3 Co-transfection Strategies

Co-transfection with more than one species of plasmid DNA has become a common strategy in transient gene expression approaches. First of all, this is necessary when the gene of interest is composed of more than one peptide chain and multi-cistronic vector plasmids (e.g. Li et al. 2007) are not readily available or applicable. Most commonly, suitable reporter genes for rapid and easy monitoring of the success of the transfection process such like eGFP are applied (e.g. Pick et al. 2002; Galbraith et al. 2006). Apart from these process monitoring aspects the process performance itself can be influenced by transient introduction of additional genes. The co-expression of the growth factor FGF-1 (acidic fibroblast growth factor) already increased recombinant protein titers and cell-specific productivity substantially in both HEK 293 EBNA and CHO DG44 cell cultures (Backliwal et al. 2008c). FGF-1-encoding plasmids were also used in a multi-pathway modulation in combination with additional plasmids for expression of the cell cycle regulatory proteins p18 (cyclin-dependent kinase inhibitor 2C) and p21 (cyclin-dependent kinase inhibitor 1A, WAF1), for suppression of cellular growth by arresting them in the G_1 phase. Both, the overall antibody yield as well as the cell-specific productivity of HEK 293 EBNA cells were increased several fold, reaching accumulated recombinant antibody titers in excess of 1 g L^{-1} (Backliwal et al. 2008d). Related to this strategy, Galectin-3, a lectin upregulating $p21^{WAF1}$, is prolonging the viability of cultured HEK 293 EBNA cells at a lowered biomass accumulation and an increased expression of co-transfected GOIs (Delegrange et al. 2012). Transiently transfected CHO DG44 cells behaved differently and just showed a better productivity but no extended survival.

Co-transfection with FGF-2 might also reveal expression-enhancing activity as it was shown for CHO-3E7 cells but not for HEK 293-6E (Durocher and Loignon

2009). The co-expression of an AKT mutant in combination with the supplementation of valproic acid decreases the undesired apoptosis-inducing effects of valproic acid and thus further increases recombinant protein production.

As already described, scaling-up of transient protein production requires also large-scale production and purification of plasmid DNA. If this is not possible to the desired scale, inexpensive "stuffer" or "filler" DNA might replace a certain amount of plasmid DNA without altering the transfection efficiency in PEI-mediated transfections. This allows for maintaining a minimum concentration of transfection reagent (e.g. PEI) required for successful transfection without increasing the amount of precious plasmid DNA accordingly. DNA used for this purpose is either from salmon sperm as successfully tested with HepG2, HEK 293, and A549 cells (Kichler et al. 2005) or from herring sperm as tested more recently with CHO DG44 and HEK 293 EBNA cells (Kiseljak et al. 2011; Rajendra et al. 2012). Recombinant protein production is reduced when adding this non-specific DNA but not to the same extent as the reduction of plasmid DNA would suggest. The addition of this noncoding DNA provides substantially better protein expression levels compared to a lessened amount of encoding plasmid DNA alone.

2.8.4 Large-Scale and Automated High-Throughput Applications

Scaling-up of transient transfection processes was usually started almost immediately after the corresponding cell transfection principle was developed. Initially, this was an essential part of various transient protein production projects because of the very low product titers expected. Because of the much better productivity of current transient transfection systems there was no need to further scale-up the production beyond the 100-L scale. Not surprisingly, the type of bioreactors used for transient gene expression purpose are not different to bioreactors used for other mammalian cell cultivation processes with predominantly stirred tank bioreactors operated in batch, fed-batch or perfusion mode or mixtures thereof (Table 2.5). WAVE bioreactors were also used frequently because of their ease of handling and the general tendency to implement single-use bioreactor technology, in particular when setting up new production facilities.

As mentioned earlier however, scale-up of transient transfection processes is associated with a number of individual steps which are more difficult to perform at a larger scale. Besides the preparation of bulk quantities of plasmid DNA, a media replacement prior to transfection to increase transfection efficiency and recombinant protein yield requires additional compatible equipment. Centrifugation of cells at this scale is possible but labor intensive and always associated with a certain risk of contamination. As an alternative, continuous centrifugation was introduced as an intermediate step between a 20-L preculture bioreactor and the 50-L or 100-L production bioreactors (Tuvesson et al. 2008). The use of perfusion technology

Table 2.5 Examples of large-scale transient transfections

Cell line(s)	Transient transfection system	Bioreactor system	Reference
BHK-21	Semliki Forest Virus expression system	MBR Vibromix™ bioreactor w. 11.5 L working volume	Blasey et al. (2000)
HEK 293/T17	Calcium phosphate co-precipitation	2-L stirred tank bioreactor w. 1.2 L working volume	Jordan et al. (1998)
HEK 293SF-3F6	Ad5-mediated viral transfection system	3-L (helical ribbon) stirred tank bioreactor	Côté et al. (1998)
HEK 293 HEK 293 EBNA	PEI-mediated	12- and 23-L stirred tank bioreactors	Schlaeger and Christensen (1999)
HEK 203 EBNA	Calcium phosphate co-precipitation	3-L stirred tank bioreactor w. 1 L working volume	Meissner et al. (2001)
HEK 293 EBNA SF	Calcium phosphate co-precipitation	150-L stirred tank w. 67 or 110 L working volume	Girard et al., 2002
HEK 293 EBNA	PEI-mediated	3.5-L (helical ribbon) and 14-L stirred tank bioreactors	Durocher et al. (2002)
HEK 293SFE	PEI-mediated	1-, 10- and 14-L stirred tank (pitched-blade) bioreactors	Pham et al. (2003)
CHO DG44	PEI-mediated	3- and 20-L stirred tank bioreactor w. 1.2 or 13 L working volume	Derouazi et al. (2004)
HEK 293 EBNA	PEI-mediated	50-L WAVE bioreactor w. 10 L working volume	Geisse and Henke (2005)
CHO-S	PEI-mediated	50-L WAVE bioreactor w. 10 L working volume	Haldankar et al. (2006)
CHOK1SV	PEI-mediated	20-L WAVE bioreactor w. 8 L working volume	Ye et al. (2009)
HEK 293-6E	PEI-mediated	10-L WAVE bioreactor w. 5 L working volume	Raymond et al. (2011)
HEK 293 EBNA HEK 293-T	PEI-mediated	WAVE bioreactor w. 10 L working volume or stirred tank bioreactor w. 50 or 100 L working volume	Tuvesson et al. (2008)
CHO DG44	PEI-mediated	50-L orbital shake bioreactor w. 30 L working volume	Stettler et al. (2007)
CHO DG44	PEI-mediated	50-L orbital shake bioreactor w. 30 L working volume	Wulhfard et al. (2008)
HEK 293 EBNA	PEI-mediated	2-L stirred tank bioreactor w. spin filter perfusion system	Sun et al. (2008)
HEK 293-6E	PEI-mediated	Stirred tank bioreactor w. 10 L working volume	Carter et al. (2010)
HEK/EBNA-SF	PEI-mediated	Stirred tank bioreactor w. 1.5 L working volume; Microcarrier culture	Fliedl and Kaisermayer (2011)

represents another approach to remove undesired media ingredients and establish suitable conditions for a high performance transient transfection process (Sun et al. 2008). The perfusion mode was also shown to be usable in combination with a fed-batch strategy.

The development of transient gene expression technologies with high productivity and corresponding recombinant protein yields has enabled the establishment of (semi)-automated high-throughput production facilities. With these facilities it is now possible to manufacture large numbers of smaller quantities of different proteins simultaneously for individual applications (e.g. for high-throughput screening or structural biology research). The principles of the underlying technology and the workflow of these facilities have been described (Davies et al. 2005; Lee et al. 2009; Zhao et al. 2011).

Irrespective of the targeted scale, fine tuning of transient gene expression technology with its multiple parameters affecting each other is becoming more and more important. Design of Experiments (DoE) is a common approach to address this problem and has meanwhile been successfully applied also in this context. In one study, DoE was used to optimize transient transfection of CHO-S and HEK293 EBNA cells with PEI (Bollin et al. 2011). The passage number of transfected CHO-S cells was shown to have an impact on recombinant protein production. Similar, modified PEI:DNA ratios and concentrations were influencing the maximum recombinant protein productivity. A DoE approach was also followed to further increase media performance and the corresponding cell density of HEK 293SF-3F6 resulting in a 2.4 fold increase of the protein productivity (Cervera et al. 2013).

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Chapter 3
Production of Antibodies in Hybridoma and Non-hybridoma Cell Lines

Susan McDonnell

Abstract Antibody based drugs have not only advanced the prevention and treatment of a number of life threatening diseases including cancer and arthritis, but have also provided the thrust for the continued success of the biopharmaceutical and pharmaceutical industry. Currently at least seven therapeutic antibodies are listed among the top ten revenue generating pharmaceutical products. These antibodies are generally monoclonal in origin and include, chimerised, humanised and human antibody types. Recently, new classes of antibody drugs are emerging and are expected to be among the next generation of block buster drugs within the Industry. These new classes include antibody conjugates, bispecific antibodies and antibody fragments. In this chapter, we will discuss the methods used for the production of antibodies in both hybridoma and non-hybridoma cells lines. The chapter will also focus on the successful use of these antibodies as therapeutics in cancer and rheumatoid arthritis.

Keywords Monoclonal antibodies • mAbs • Hybridoma cells • Chinese hamster ovary cells • Chimeric • Humanised and human antibodies • Cancer therapeutics • Rheumatoid arthritis therapeutics

3.1 Introduction

Biopharmaceuticals are generally referred to as biologics by the Biopharmaceutical and Pharmaceutical Industry and the worldwide growth in the biologics sector has been driven by the successful launch of therapeutic antibodies, referred to as *Monoclonal Antibodies (mAbs)* and diabetes products. The rise in total sales of biologics in the US in 2012 was 18 % which is significantly more than the growth in the pharmaceutical sector which saw only a 2.5 % increase in sales (Aggarwal 2014).

MAbs and related proteins have been shown to successfully target a wide variety of extracellular targets with an extremely high degree of specificity (El Bakri

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et al. 2010). MAbs are currently being used to treat many commonly occurring diseases, including several forms of cancer, multiple sclerosis and immunological disorders such as rheumatoid arthritis (Pillay et al. 2011; Sapra and Shor 2013). Using molecular biology methodology significant advances have been made in the design of mAbs as therapeutics that have improved their bioavailability and binding specificity and also reduced any immunological side effects (Reichert and Valge-Archer 2007). In terms of processing, production of mAbs has also seen dramatic improvements in yields, so that the average yield is now in the order of 5 g/L (Wurm 2004). Most of these improvements have resulted from process developments and monitoring as well as cell engineering (Kelley 2007; Shukla and Thommes 2010).

Currently there are at least 30 FDA approved mAbs available with the majority targeting disease like cancer and autoimmune diseases (Walsh 2010) (For more information see chapter 23). In fact, we now have a large repertoire of drugs available in our fight against cancer. If we look at the table of the top ten revenue generating biopharmaceuticals, we see that seven of these products are mAbs (Table 3.1). In addition, over 200 antibodies are currently in Phase II and III clinical trials and numerous others are in various pre-clinical and development stages. The market for mAbs currently represents approximately 20 % of all biologics and this expected to increase to around 50 % within the next 10 years (Walsh 2010). Of the 20 new biopharmaceuticals approved in the United States and/or the European Union in 2013, four were mAbs (Walsh 2013). Chapter 23 in this book includes a table with detailed information on the recently approved products currently available.

Table 3.1 lists the ten top selling biopharmaceutical for 2010–2011 with both the brand (trade) name and also the name of the active ingredient present. Information on the type of product, disease being treat, company manufacturing and also world-wide income in US$ is also presented. As we can see from this table many of the major Pharmaceutical and Biopharmaceutical companies are responsible for manufacturing and selling these products, however, they may not necessarily have developed these products. We see that many of these products are classified as block buster drugs as they generate more than 1 € billion in sales. Top of the list in terms of revenue generation is Humira® (Adalimumab) which is a human antibody used in the treatment of arthritis made by AbbVie (part of Abbott) that generated $9.5 billion in sales in 2010–2011. Not surprisingly given the world-wide increase in diabetes, Lantus® is also present in the table of top ten selling biopharmaceuticals. Interestingly, four of these top products are used for the treatment of rheumatoid arthritis and associated inflammatory disorders.

The rate of approval of new biopharmaceutical has slowed down somewhat in the past few years. In the period 2006–2010 fifty eight new biopharmaceuticals gained approval within the European Union and/or United States. Interestingly, nearly half of these new approvals were for biosimilars or reformulations of previously approved products.

Table 3.1 Ten top selling biopharmaceutical products 2010–2011

Brand name	Active ingredient	Type	Treatment	Company	World sales (USS billion)
Humira	Adalimumab	Antibody	Rheumatoid arthritis, Crohn's disease	AbbVie (Abbott)	9.5
Enbrel	Entanercept	Antibody	Rheumatoid arthritis, Crohn's disease	Amgen/Pfizer/ Takeda	8.4
Remicade	Infliximab	Antibody	Rheumatoid arthritis, Crohn's disease	Janssen Biotech/Merck	7.5
Rituxan	Rituximab	Antibody	Non-Hodgkin lymphoma	Roche/Biogen-Idec	7.1
Lantus	Insulin glargine	Protein	Diabetes	Sanofi-Aventis	6.5
Herceptin	Trastuzumab	Antibody	Breast cancer	Roche	6.3
Avastin	Bevacizumab	Antibody	Colorectal, breast, lung cancer	Roche	6.1
Neulasta	Pegfilgrastim	Protein	Neutropenia	Amgen	4.1
Lucentis	Ranibizumab	Antibody	Macular degeneration	Roche/ Norvartis	4.0
Epogen/ Procrit/ Eprex	Epoetin alpha	Protein	Renal anaemia	Amgen/ Janssen/Kirin Pharma	3.4

Source: Statista (www.statista.com)

3.2 Antibody Structure and Function

The function of the immune system is to defend the body against foreign invaders like bacteria and viruses. The immune system is composed of a network of cells and organs and the principal cells in the immune system are the lymphocytes, T and B-cells and the macrophages (Ohlin and Zouali 2003). The main organs of the immune system are the lymph nodes, spleen and bone marrow. The function of the B cells is to produce antibodies and the T-cells work with B-cells to destroy invaders (Nelson et al. 2000). The function of macrophages is to provide an immediate response and break up these foreign invaders.

An antigen is defined as anything that triggers an immune reaction and in fact the word antigen is a contraction of *anti*body-*gen*erating, which is a reference to the fact that the foreign agent provokes the immune response by inducing production of the antibody in the B-cells. Most antigens are proteins or large polysaccharides on the surface of viruses, bacteria, or foreign cells. Common examples are protein-coat molecules of viruses, parts of the capsules of bacteria and macromolecules on the surface of cells of other kinds of organisms such as protozoans and parasitic worms. Blood cells or tissue cells from other individuals (of the same or different species) also provide antigenic molecules. In fact, our immune system can recognise millions, perhaps billions of different antigens. Once an antibody binds to an antigen, it is destroyed by the immune system using the T-cells. The part of the antigen that the

antibody recognizes is called the epitope which is located on the surface of the antigen. An individual antigen can possess many different epitopes so this means that many different antibodies can be produced to the same molecule. However, each antibody is specific to a specific epitope which determines the specificity of the antigen-antigen interaction. This ability of an antibody to bind to an antigen with a high degree of affinity and specificity has led to the use of antibodies in a variety of medical applications primarily as therapeutics and diagnostic reagents.

Antibodies are glycoproteins (proteins that contain sugars) that belong to the immunoglobulin (Ig) superfamily. In mammals, there are five classes of Ig: IgA, IgD, IgE, IgE and IgM. In some mammals, IgG and IgA are further divided into subclasses, referred to as isotypes due to polymorphisms in the conserved region of the heavy chain. These different isotypes perform different roles and help direct the appropriate immune response for each different type of foreign object they encounter. Regardless of the antibody class, all immunoglobulins contain a common structural domain and are composed of two identical copies of both a heavy (~55 kDa) and light chain (~25 kDa) held together by disulphide and non-covalent bonds giving rise to the classical Y shaped molecule of ~150 kDa depicted in Fig. 3.1.

Figure 3.1 shows the basic structure of the IgG antibody and illustrates the principal parts of the basic antibody with the heavy (H) and light chain (L) containing both a constant (C) and variable region (V). The light chain consists of a variable (V_L) amino terminal portion of 110 amino acids and a constant (C_L) region of a similar length. The heavy chain is also divided into variable and constant regions; however, the heavy chain has one variable (V_H) and at least there constant regions (C_{H1}, C_{H2} and C_{H3}), each approximately 110 amino acids long (Birch and Racher 2006). The variable regions of both chains binds together to form the antigen-binding site (ABS) and within the ABS there are three hypervariable regions of between 5 and 10 amino acids in length in both the light and heavy chains referred to as the complementarity determining regions (CDRs). The CDRs

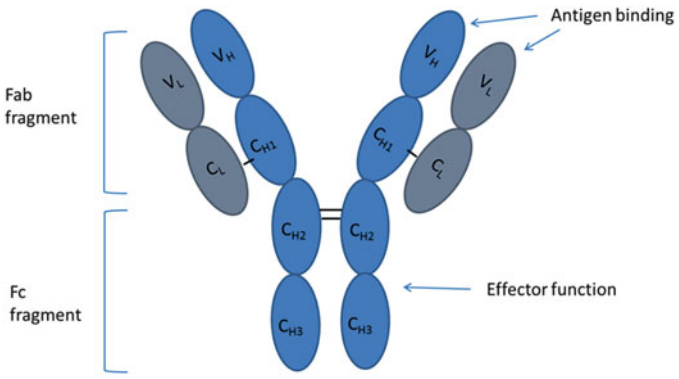

Fig. 3.1 Shows the basic structure of the IgG antibody and illustrates the principal parts of the basic antibody with the heavy (H) and light chain (L) containing both a constant (C) and variable region (V)

constitute the actual epitope binding site of the antibody and X-ray diffraction analysis has shown that each of these variable regions forms three short loops of amino acids from both the heavy and light chains to form the binding site (Conroy et al. 2009). The two arms of the antibody molecule referred to as the Fab fragment contain the antigen binding domains and the tail referred to as the Fc fragment (fragment crystallisable) controls the biological effector function such as natural killer cell activation and activation of the classical complement pathway and phagocytosis. The Fab and Fc fragments are connected by the hinge region which is rich in proline, threonine and serine and imparts rotational movement to the antigen-binding domains. Each arm or monovalent Fab fragment contains an antigen binding site thus making each antibody molecule at least bivalent.

3.2.1 Antibody Genes

At the genetic level several mechanisms are required to generate the sequence diversity needed to generate the large repertoire of potential antibody present within our body which has been estimated to be as large as 10^{12}. A diverse range of genetic steps take place including the combination of different heavy and light chains, genetic recombination within hypervariable regions, and imprecise joining during recombination (Tonegawa 1983). Following binding of an antigen to a B cell and receipt of appropriate activating signals, B cells divide and produce memory B cell as well as terminally differentiating into antibody secreting plasma cell clones. The memory B cells remain dormant until they are activated by their specific antigen. This means that when you are exposed to an antigen the second time, the antibodies are made more efficiently, thus providing the basis of vaccination.

The genes that encode immunoglobulin light chains consist of three regions: a V region that encodes the 95–96 N-terminal amino acids of the polypeptide variable region; a joining (J) region that encodes the 12–14 C-terminal amino acids of the polypeptide variable region; and a C region that encodes the polypeptide constant region (Fig. 3.2). The major class of light-chain genes in the mouse are formed from

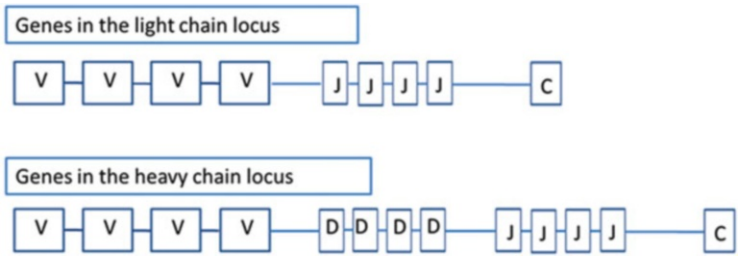

Fig. 3.2 Gene structure for the light and heavy chain locus. *V* represents variable region, *J* represents the joining region, *C* represents the constant region and *D* represents the diverse region

combinations of approximately 250 V region and four J regions with a single C region. Site-specific recombination during lymphocyte development leads to a gene rearrangement in which a single V region recombines with a single J region to generate a functional light-chain gene (Pascual and Capra 1991) Different V and J regions are rearranged in different B lymphocytes, so the possible combination of 250 V regions with 4 J regions can generate approximately 1,000 unique light chains.

The heavy chain gene locus contains the V, J, and C regions and also includes a fourth region, known as the diversity or D region which encodes amino acids lying between the V and J regions. Assembly of a functional heavy-chain gene requires two recombination events. Firstly a D region recombines with a J region, and a V region then recombines with the rearranged DJ segment. In the mouse, there are about 500 heavy-chain V regions, 12 D regions and 4 J regions, so the total number of heavy chains that can be generated by recombination event is 24,000. As this part of the gene assembles, it joins the variable coding segments with those for the constant-C segments of the heavy-chain molecule.

Combinations between the 1,000 different light chains and 24,000 different heavy chains formed by site-specific recombination can generate approximately 2×10^7 different immunoglobulin molecules. This diversity is further increased because the joining of immunoglobulin gene segments is often imprecise, with one to several nucleotides frequently being lost or gained at the sites of joining (Berek and Milstein 1987). The mutations resulting from these deletions and insertions increase the diversity of immunoglobulin variable regions approximately, corresponding to the formation of about 10^5 different light chains and 2×10^6 heavy chains which can then combine to form more than 10^{11} distinct antibodies. Still further antibody diversity is generated after the formation of rearranged immunoglobulin genes by a process known as somatic hypermutations which results in the introduction of frequent mutations into the variable regions of both the heavy-chain and light- chain genes.

3.3 Therapeutic Antibodies

The first use of the term “antibody” occurred in a text by Paul Ehrlich where the term Antikörper (the German word for antibody) appears in the conclusion of his article “Experimental Studies on Immunity”, published in October 1891, which states that, “if two substances give rise to two different antikörper, then they themselves must be different” (see review on early antibody work by Llewelyn et al. 1992). Interestingly, although the existence of antibodies was first postulated by Paul Erlich the actual structure of an antibody was not determined until the 1960s by Edelman et al. (1969).

The initial discovery of antibodies led scientists to develop a number of human and animal antisera to neutralise toxins and to treat viral and bacterial infections. Early work by Behring and Kitasato (1890) established that animals not exposed to diphtheria and tetanus could be protected by serum taken from animals treated with non -toxic doses of these bacteria. However, adverse reactions associated with the use of heterologous serum referred to as '*serum sickness*' lead to abandonment of this therapy at the beginning of the last century. The next stage in the development of antibodies as therapeutic agents was the use of immunoglobulins obtained from either normal or hyperimmune donors to be used for the prevention and treatment of autoimmune and infectious disease like measles (Stokes et al. 1944). Although these immunoglobulin infusions mimicked the normal polyclonal serum in triggering an effective immune response, there were major limitations to their use as therapeutic agents. Some of these limitations include manufacturing cost, donor availability and most importantly the potential risk of transmission of blood borne infectious agents (Llewelyn et al. 1992). Since then antibody therapy has gone through considerable and rapid developmental stages, beginning with production of murine monoclonal antibodies (mAbs), followed by recombinant antibodies, transgenic antibodies, and more recently recombinant polyclonal antibodies (Stasi 2010).

The key properties of antibodies that make them effective therapeutics is their specificity and purity. The development of antibodies to treat diseases has presented scientists and clinicians with many successes but there are still problems that remain to be overcome. In order to develop a successful therapy for a particular disease, scientists must first identify and isolate the disease target which is typically a minor component in a complex mixture of proteins and must then raise a human immune response against the disease targets. Therapeutic antibodies are thought to function through several different mechanisms including antibody-dependent cell cytoxicity (ADCC) which involves destruction of the antibody-coated cell by recruitment of effector cells (natural killer cells, macrophages) mediated by Fc part of antibody. In addition, complement-dependent cytoxicity (CDC) involves destruction through complement activation via the Fc region of the antibody.

There are currently 30 mAbs approved for clinical use (Walsh 2010) and Table 3.2 lists the properties of a selection of these mAbs. The table gives the product name (and brand name), and provides information on the antibody type, host cells, target protein, indication and year approved. Interestingly, we can see that the majority of these products are produced in CHO cells and that the products are murine, human, humanised and chimeric antibodies. The products in this table will be discussed in more detail in Sect. 3.5 of this chapter and will also be discussed in Chap. 23.

Table 3.2 Examples of approved mAbs

Product	Antibody type	Host cells	Target protein	Indication	Year approved
Orthoclone OKT3/ Muromanab-CD3	Murine	Sp2	CD3	Acute kidney transplant rejection	1986
Rituxan/ MabThera	Chimeric	CHO	CD20	Non-Hodgkin's lymphoma	1997
Remicide (Infliximab)	Chimeric	NSO	TNF-α	Rheumatoid arthritis, Crohn disease	1998
Herceptin (Trastuzumab)	Humanised	CHO	Her-2	Metastatic breast cancer	1998
Enbrel (Etanercept)	Soluble TNF-z receptor fused to IgGl Fc	CHO	TNF-α	Rheumatoid arthritis, psoriasis, ankylosing spondylitis	1998
Humira (Adalimumab)	Human	CHO	TNF-α	Rheumatoid arthritis	2002
Avastin (Bevacizumab)	Humanised	CHO	VEGF	Colorectal cancer	2004
Lucentis	Fab fragment	*E. coli*	VEGF-A	Age related macular degeneration	2006
RoActemra/ Actemra (Tocilizumab)	Humanised	CHO	IL-6	Rheumatoid arthritis	2009

3.3.1 Polyclonal Antibodies

Polyclonal antibodies are generated by repeated injection of an antigen into a suitable mammal such as rabbit or mouse. This induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen and following repeated injection the polyclonal IgG is purified from the mammal's serum. Polyclonal antibodies are heterologous and bind to a variety of antigen epitopes. This decreases their specificity but at the same time increases the possibility of binding to the antigen in a variety of different test conditions. They are most frequently used as detection reagents in enzyme-linked immunosorbant assays (ELISAs) and as diagnostic tools. Affinity purified polyclonal antibodies are frequently used in ELISAs. More recently polyclonal antibodies have been generated in camels, horse and goat.

Genetically engineered polyclonal antibodies seem to be the future of polyclonal antibodies and have been developed by a Danish company called Symphogen. These polyclonal antibodies only contain the Fab fragments and are called symphobodies. There are currently a number of these antibodies in clinical trials to prevent and treat allergic reactions, infectious diseases, and transplant rejection. Rozrolimupab® which comprises 25 genetically unique IgG1 antibodies, all of which are specific for the rhesus D (RhD) erythrocyte protein is currently undergoing Phase III clinical trials (Stasi 2010).

3.3.2 *Monoclonal Antibodies*

The landmark publication by Kohler and Milstein (1975) has been cited over 16,000 times (according to PubMed) and resulted in the award of the Nobel Prize in Physiology or Medicine in 1984. This simple and extremely elegant study gave rise to the generation of the first murine monoclonal antibodies and had a major impact on the production of antibodies as therapeutic and diagnostic reagents. Interestingly, Kohler and Milstein did not patent this technology.

The murine monoclonal antibodies were generated by the fusion of mouse B lymphocytes with mouse myeloma cells (Fig. 3.3) to produce a hybridoma. The resulting hybridoma cells are capable of producing antibody indefinitely. A key factor in the successful generation of monoclonal antibodies was the reproducibility and scalability of the selection process which led to the selection of individual cells (or clones) that express high levels of antibody productivity.

The process begins with the repeated injection of the antigen into a mouse and the subsequent removal and isolation of the spleen cells. The spleen cells which are capable of producing antibodies only have a limited lifespan in culture and the key discovery of Kohler and Milstein was to fuse these antibody producing cells with myeloma cells that did not produce antibody but were capable of growing indefinitely in culture. The cells were fused together using polyethylene glycol to generate hybridoma cells which contain a population of antibody producing cells that must be then cloned to generate individual monoclonal cells, producing a single antibody, hence the term monoclonal antibody.

The selection system utilises the presence or absence of the enzyme HGPRT (hypoxanthine-guanine-phosphoribosyl transferase) which is involved in the salvage synthesis of nucleic acids. Spleen cells are HPGRT negative whilst the myeloma cells, like the Sp2 cell line are HPRGT positive. The selection of

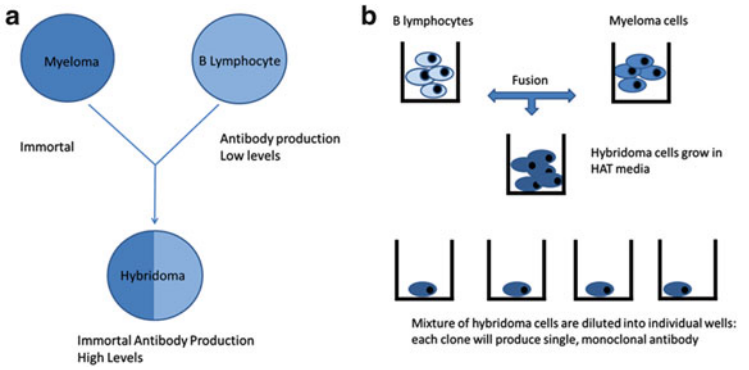

Fig. 3.3 (**a**) Generation of hybridoma cell by fused of mouse myeloma cells with B-lymphocytes isolated from the spleen. (**b**) Following selection of hybridoma cells in HAT media, individual cells are cloned by dilution and screened for antibody productivity using ELISA. Each individual selected clone produces a single monoclonal antibody

hybridoma cells was achieved by growing in media supplemented with HAT hypoxanthine, aminopterin and thymidine (HAT). The hybridoma cells are HGPRT positive and survive in the HAT media whilst the myeloma cells die as aminopterin inhibits de novo synthesis and the spleen also die as they have a limited lifespan. Following successful selection the next step is to isolate individual clones; this is usually accomplished using limited dilution in a 96-well plate where the cell suspension is diluted to 1 cell/well. Cell growth in each individual well must be monitored and after about 2–3 weeks the individual cells become small colonies containing 200–300 cells. The media from each individual clone is then removed and tested for antibody productivity using an ELISA. Clones producing high levels of antibody are then grown up and a master cell and working cell bank created and stored in liquid N_2.

Monoclonal antibodies were initially developed as a tool for studying the immune system as it allowed production of large amounts of highly specific antibodies, enabling reproducible research measurements and results. Similar to polyclonal antibodies, mouse monoclonal antibodies have been extremely successful as diagnostic reagents. Mouse monoclonal antibodies are used for identifying viruses, purifying molecules and testing for cancers and pregnancy. Orthoclone OKT3 (Muromanab-CD3®) was the first therapeutic mouse antibody approved in 1986 to treat acute kidney transplant rejection. Despite the high initial expectations of mAb therapy, OKT3 failed as a good treatment for transplantation rejection primarily because OKT3 induced severe human anti-murine antibody (HAMA) response in patients. The efficacy of murine mAbs as therapeutics has unfortunately been limited by their short circulating half-lives and difficulties inducing human immune effector response (Khazaeli et al. 1994). Currently murine mAbs represent just over 3 % of the total therapeutic antibodies currently on the market (see Fig. 23.2 in Chap. 23). More recently the main therapeutic use of murine mAbs has been to serve as targeting agents for radioisotopes that kill tumour cells. Bexxar® which is marketed by GSK for the treatment of CD-20 positive, follicular non-Hodgkin lymphoma was approved in 2003. Bexxar® contains an anti-CD020 mouse antibody (Tositumomab) linked to radioactive Iodine (I 131). Unfortunately, the sale of Bexxar® has been discontinued and marketing approval withdrawn in February 2014 due to the decline in usage (fewer than 75 patients in 2012) and the existence of other similar drugs.

A similar approach has resulted in the generation of rat, camel and many other monoclonal antibodies as well as human. The camel or camelid antibodies are particular interesting as they do not appear to contain any light chains and only contain heavy variable and light chains (Hamers-Casterman et al. 1993; Muyldermans 2001).

3.4 Recombinant Antibodies

Unfortunately, the application of murine mAbs in human medicine were limited since the mouse monoclonal antibodies were rapidly inactivated by the human immune response and prevented from providing any long-term benefits (Buelow and Van Schooten 2006). In order to eliminate the HAMA researchers were able to generate antibodies with less mouse and more human parts due to advances in molecular biology and gene cloning. These antibodies rely on the availability of the cDNA for both the heavy and light chains of the relevant antibodies. Figure 3.4 illustrates the various types of antibodies that are currently available for use as therapeutic agents and shows the percentage of either human and mouse genes. Figure 3.4 also shows the suffixes used by the Industry for the naming of these products; *omab* for mouse; *ximab* for chimeric; *zumab* for humanised and *umab* for human.

3.4.1 Chimeric Antibodies

To reduce the immunogenicity of murine antibodies in humans, chimeric antibodies with mouse variable regions and human constant regions were constructed by genetic engineering (Morrison et al. 1984). The majority of chimeric antibodies contain around 60 % human and 40 % mouse parts (Fig. 3.4). In order to generate a chimeric antibody, the heavy and light chains of the desired mouse monoclonal antibody must be isolated from the hybridoma cell line (Shin and Morrison 1989). In addition, the heavy and light chain genes must also be isolated from human B-cells. Early work by Neuberger (1983) showed that the immunoglobulin heavy chain could be successfully transfected and expressed in lymphoid cells. Morrison et al. (1984) constructed the first chimeric antibody using the procedure outline in

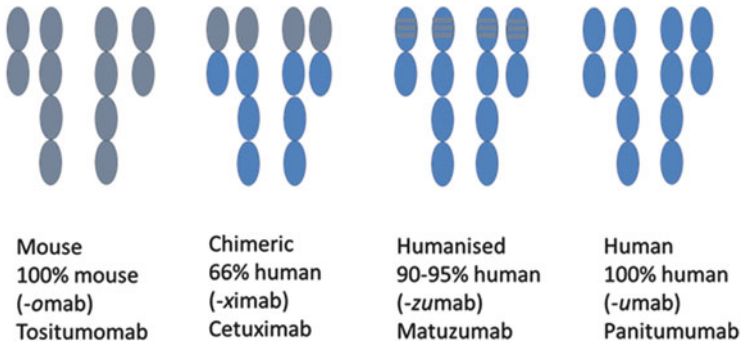

Fig. 3.4 Generation of recombinant antibodies, showing the percentage of mouse and human parts. The naming of these antibodies is also shown with examples of products from each type of mAb

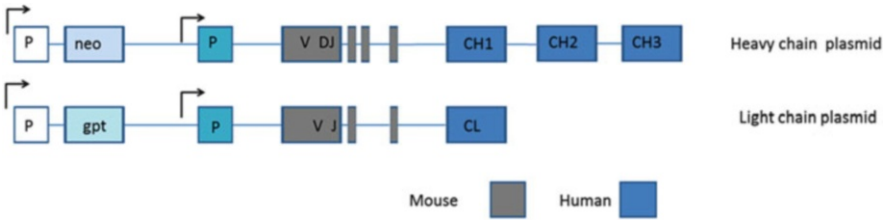

Fig. 3.5 Generation of chimeric antibodies showing separate plasmid for the heavy and light chains. Each plasmid has a promoter (P) and also selection marker, neomycin (neo) and guanosine phosphoribosyl transferase (gpt)

Fig. 3.5. The procedure involves the construction and transfection of two separate plasmids, one for the heavy chain and one for the light chain (De La Cruz et al. 2006). Each of the plasmids contains a different selection marker, e.g. neomycin (neo) and guanosine phosphoribosyl transferase (gpt). The chimeric genes are created by exchanging the variable regions (VDJ and VJ) of the human antibody heavy and light chain genes for those derived from the mouse (Boulianne et al. 1984). ReoPro® (abciximab) which targets GPIIb/IIIa was the first chimeric antibody introduced by Centocor (now Janssen) in 1994.

3.4.2 Humanised Antibodies

MAbs began to reveal their full potential in 1986 when Greg Winters in the MRC Laboratory of Molecular Biology (LMB) pioneered a technique to 'humanise' mouse monoclonal antibodies (Jones et al. 1986). Humanised antibodies are produced by grafting murine hypervariable regions on amino acid domains into human antibodies (Dall'Acqua et al. 2005). Antibody genes from murine monoclonal are isolated and sequenced. Sequences from the CDR regions are grafted onto the human gene in an appropriate expression vector (Roguska et al. 1994) and then transfected into CHO cells for production. Unfortunately, humanised antibodies bind antigens much more weakly than the parental murine monoclonal antibodies, with reported decreases in affinity of up to several hundredfold. Modification of sequences can improve their activity and made them better suitable for use as human medicines as are less likely to provoke an immune response in patients (Riechmann et al. 1988). The technique was first used in the development of Campath® (Alemtuzumab) which binds to CD52 (Riechmann et al. 1988). Campath® has been used successfully in the treatment of chronic lymphocytic leukaemia and in off-label use in multiple sclerosis. Campath® was withdrawn from the market in 2012 and despite some safety and efficacy concerns by the FDA is expected to be re-launched as Lemtrada® for the treatment of multiple sclerosis. One of the most successful humanised antibodies available is Herceptin which was developed by Genentech and approved by the FDA in 1998.

3.4.3 Human Antibodies

The first human genetically engineered antibodies were developed by Greg Winters and colleagues in 1990 using a technique called 'Phage Display' (McCafferty et al. 1990). The patented method involved initially making a library of either naive human antibodies or library to specific antigen (Gram et al. 1992). The antibody genes are then isolated and cloned into a bacteriophage (Marks et al. 1991). A crucial property of the bacteriophage is the ability to 'display' or express the proteins on the surface (Smith 1985; Barbas et al. 1991, 1992). Following several rounds of screening with the antigen, bacteriophage producing the required antibodies can be isolated, and genes cloned into CHO for production. The technique was first used in the development of Humira® (Adalimumab) by Cambridge Antibody Technology and was launched in 2003 as a treatment for rheumatoid arthritis (Table 3.2). Additional information on Humira® is provided in Sect. 3.5.2.2.

An alternative method for human antibody was also developed by Neuberger using transgenic mice with human antibody genes (Green et al. 1994). The antibody genes are knocked out in mouse and replaced with human genes to give transgenic mouse (HuMab) which is then vaccinated against the desired antigen, leading to the production of monoclonal antibodies (Bruggemann and Taussig 1997). The first product approved using this technology was panitumumab (Vectibix®), in 2006 (Jakobovits et al. 2007). Panitumumab which targets the epidermal growth factor receptor (EGFR) has been used successfully for the treatment of patients with metastatic colon cancer who express the wild-type (non-mutated) form of the KRAS gene.

3.4.4 Next Generation of Therapeutic Antibodies: Antibody-Drug Conjugates, Bi-specific Antibodies and Antibody Fragments

With the major developments in antibody engineering, the next generation of engineered antibodies will soon become available. Currently, several of the major pharmaceutical companies like Pfizer, Immunogen, Roche, Takeda, and other newer companies like Genmab and Spirogen have products currently on the market or will shortly be bringing products to the market. Principal among this new generation of therapeutic antibodies are antibody drug conjugates (ADCs), antibody fragments and bi-specific antibodies. These new antibody drug formats are expected to extend the arsenal of drugs available for treating many diseases and will have particular relevance in cancer treatment. In addition, some of these new formats will target infectious diseases.

3.4.4.1 Antibody Drug Conjugates (ADCs)

Antibody drug conjugates (ADCs) which consist of a drug attached to an antibody are proving to be new class of therapeutic which allows targeting of drug to specific cells. The fundamental design is an antibody with an inactive drug attached to it and once bound to the target cells the drug is activated. In the case of anti-tumour agents this means that healthy cells are not damaged and only tumour cells are targeted for destruction (Tian et al. 2014). ADC should significantly lower side effects. It also allows a company extend a patent on an existing compound and in some cases allows ineffective antibody to be used more effectively. Currently, there are two ADC approved for clinical use and there are at least 30 more ADC in clinical trials with about 100 in preclinical development (Flemming 2014). It has been estimated that the future market for ADCs could be as big $5 billion by 2018.

However, these drugs are quite difficult to manufacture and only small number of companies have the capability. However, more companies are developing expertise in this area and are gearing up for large-scale manufacture.

3.4.4.2 Bispecific Antibodies

Bispecific antibodies are antibodies that can bind to two different epitopes on the same or different antigens. Although the first bispecific antibodies were produced over 20 years ago, the therapeutic potential of these antibodies has only recently been achieved (Byrne et al. 2013). Bispecific antibodies can be produced through three main techniques: (i) chemical conjugation using cross-linking; (ii) fusion of two different hybridoma cell lines; or (iii) genetic manipulation of antibody genes (Parashar et al. 2011).

Removab®, the first bispecific mAb to come on the market, approved in the European Union in 2009. The antibody displays two different antigen-binding sites, a mouse-derived EpCAM binding Fab region and a rat-derived CD3-binding Fab region. EpCAM is overexpressed on the majority of epithelial tumours, and the bispecific nature of the antibody effectively brings CD3-expressing T lymphocytes into close proximity with tumour cells. Moreover, the Fc region of the antibody facilitates docking of various immune effector cells (for example, phagocytes and natural killer cells), which, in combination and in synergy with the T lymphocytes, can induce tumour cell destruction through multiple tumouricidal mechanisms. In addition, bi-specific antibodies have great potential as diagnostic reagents, as one arm can recognise the antigen and the second arm can have the detection marker (Byrne et al. 2013).

3.4.4.3 Antibody Fragments

The absolute minimum fragment of antibody that has activity is the single chain variable fragment (scFv) which contains the variable region of the heavy and light chain connected with a short linker peptide of 10–20 amino acids. The scFv has a molecular weight of 25 kDa which makes it an attractive target for drug companies from a pharmokinetic point of view. Using molecular biology researchers have created a diverse range of antibody formats and combinations, e.g. double scFv, scFv attached to Fc regions. Apparently there are up to 40 documented antibody varieties (Byrne et al. 2013).

Thus far, Fab fragment antibodies have been produced in a variety of host cells including CHO and plant cells. In addition, Fab fragments can also be produced in *E. coli* cell-based systems which makes them less expensive to produce than in mammalian cells (Holliger and Hudson 2005).

Lucentis® (Ranibizumab) was developed by Genentech and Novartis and first approved by the FDA in 2006 (Table 3.2). Genentech has the commercial rights to Lucentis® in the United States and Novartis has exclusive rights in the rest of the world. Lucentis® is a Fab antibody fragment derived from the humanised monoclonal antibody to VEGF. It has been recommended for the treatment of age related macular degeneration where the development of new blood vessels from pre-existing vasculature leads to vision impairment.

3.5 Role of Therapeutic Antibodies in Cancer and Rheumatoid Arthritis

3.5.1 Therapeutic Antibodies and Cancer

According to the World Health Organisation, cancer is currently the second biggest killer worldwide, with only heart disease killing more people each year. Cancer is a disease which can arise in any organ or tissue and results from an abnormal proliferation of any type of cell in the body (Herzig and Christofori 2002). Cancer develops in a multistep manner resulting from mutations that occur in the DNA and accumulate over time (Hanahan and Weinberg 2000, 2011). All cancer cells have a specific phenotype and show characteristic properties referred to the hallmarks of cancer which include; growing without regard to differentiation and growth control signalling, resistance to apoptosis and induction of angiogenesis.

The major treatment options available for patients diagnosed with cancer are surgery, chemotherapy and radiation. The type of treatment is largely dependent on the type and stage of cancer and generally the earlier the cancer is detected the better the prognosis. However, despite tremendous improvements in the detection and treatment of cancer, the overall survival rate is still low. The majority of traditional chemotherapeutic agents are ineffective against common solid tumours

like breast, colon and prostate. Since these agents are relatively non-specific and target both normal and tumour cells, they generally have severe side effects. In addition, many tumour cells develop resistance to standard chemotherapeutic agents (Baguley 2010).

Over the last decade mAbs have become the largest group of *new therapeutics* for cancer treatment and they have revolutionised the treatment of many common types of cancer (Weiner et al. 2010; Pillay et al. 2011). At the same time great strides have also been made in the molecular diagnosis of cancer and gene signatures have been identified for several cancer types including, leukaemias and breast cancer (Perou et al. 2000; Weigelt et al. 2005). In the following sections we shall have a brief look at some of the major success stories in cancer treatment.

3.5.1.1 Rituxan®

Rituxan® (Rituxamab) was among the first of the chimeric antibody drugs to be approved. Rituxan® is directed against the B cell-specific antigen CD20, a membrane protein that is expressed in the early pre-B cell stage and remains present in mature B cells (Pescovitz 2006). CD20 may function in normal B cell growth and activation and is expressed in more than 90 % of non-Hodgkin lymphomas and in 10 % of chronic lymphocytic leukaemia. Rituximab was initially approved by the European Medical Agency (EMEA) and the US Food and Drug Administration (FDA) in 1997 for the treatment of relapsed or refractory CD20B-cell non-Hodgkin's lymphoma (NHL). It is generally well tolerated and serious adverse events are uncommon. The effectiveness of rituximab in depleting B-cell in vivo has been shown to be by three different mechanisms: (1) promotion of B-cell apoptosis; (2) complement-dependent cytotoxity by activation of the complement cascade and (3) antibody-dependent cell mediate cytotoxicity by recruitment of natural killer cells through binding to the Fc fragment. Interestingly, rituximab may sensitize B-cells to the effect of chemotherapeutic agents. The early success of rituximab encouraged the pharmaceutical industry to look for addition anticancer drugs. More recently, the possible role of rituximab® and B-cell modulation has been investigated in patients with active rheumatoid arthritis. There have also been several studies showing off label use of rituximab® in the management of kidney transplant recipients, however, clinical studies have not vindicated this use.

3.5.1.2 Herceptin

Breast cancer is the second leading cause of cancer related deaths in women in the western world (Polyak 2001). Breast cancer mortality like many other cancer is related to metastatic spread of the primary tumour with about 50 % of all cancer patients showing evidence of metastasis at first presentation (Ahmad and Hart 1997). Human breast cancers represent a heterogeneous group of tumours that are diverse in behaviour and response to therapy (Rakha et al. 2007).

The human epidermal growth factor tyrosine kinase receptor 2 (HER-2/*neu*, c-erbB2) was initially discovered in 1985 by two independent laboratories (Coussens et al. 1985; King et al. 1985). In a seminal paper published by Slamon and colleagues in 1987 (Slamon et al. 1987) he showed that HER-2 gene was amplified in 30 % of a series of primary breast tumours. He also showed that Her-2 over expression was a significant predictor of both overall survival and time to relapse in these patients (Slamon et al. 1987). Further studies have consistently demonstrated that Her-2 gene is amplified in 20 % of breast cancers (Wolff et al. 2007). Over-expression of Her-2 has also been associated with more aggressive disease, higher mortality and resistance to treatment (Buzder et al. 2005).

Her-2 belongs to a family of cell surface tyrosine kinase receptors, the ErbB or epidermal growth factor receptor family (Hynes and Lane 2005). Her-2 activation increases cell proliferation, resistance to apoptosis, invasive growth, and metastatic behaviour through the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin and the mitogen-activated protein kinase pathways (Ignatiadis et al. 2009). Her-2 is expressed on surface of breast cancer cells and is responsible for the increased and uncontrolled proliferation of these cells.

Herceptin® was the first humanised antibody to be generated by Genentech and was approved by the FDA in 1998. Herceptin® is currently marketed by Roche under the trade name Trastuzumab and had sales of over $6 billion per year in 2010. Patients must first be tested as Her-2 positive before being prescribed the drug and the current cost of treatment is expensive and is close to ~15,000 € per month. Studies have also shown that trastuzumab is more effective when added to anthracycline- or taxane-based chemotherapy (Slamon et al. 2001). Unfortunately, there have been several reports of resistance to Herceptin® treatment.

3.5.1.3 Avastin

Malignant tumours have or acquire the ability to spread from the site of origin to distant sites in the body, a process known as metastasis (Fidler 2003). Unfortunately, the majority of patients die from metastatic spread of the disease rather than the primary tumour (Mehlen and Puisieux 2006). For many patients asymptomatic and undetectable metastases referred to as micrometastases are already present when the primary tumour is diagnosed. Death from metastases can be dependent on tumour origin, e.g. almost all lung cancer patients die from metastases while approximately one third of breast cancer patients die in the same way (Van't Veer and Weigelt 2003).

The mechanism of metastasis formation has been well documented and begins when the tumour cells move from the site of origin and degrade through the extracellular matrix into the circulation. Upon reaching its new site, the cells begin to grow and once the tumour reaches a certain size it recruits angiogenic factors to stimulate new blood vessels ensuring its survival (Geho et al. 2005). One of the most well documented angiogenic factors is vascular endothelial growth factor (VEGF).

Avastin® (Bevacizumab) is a humanised antibody directed against VEGF that was originally approved by the FDA in 2004. Avastin® is manufactured by Roche in CHO cells and generated over $6 billion in sales in 2010. Avastin® was initially approved for use in colorectal cancer which refers to cancer of the colon or large intestine and rectum. Colorectal cancer is the second most common type of cancer occurring in men and women. Risk factors for colorectal cancer include age, family history, diet, smoking and condition such as Crohn's disease and ulcerative colitis. Colorectal cancer generally occurs in individual over the age of 50 with 64 being the mean age of presentation.

Avastin® was subsequently approved for use in non-small cell lung and renal cell cancers. Recent clinical trials found that Avastin® was not effective in treating newly diagnosed glioblastomas. However, there has been some controversy as although the trials found that the drug didn't help patients with glioblastoma live longer, they differed on a more subjective measure: quality of life (Gilbert et al. 2014; Chinot et al. 2014). However, the European Medicines Agency (EMA) Committee for Medicinal Products for Human Use (CHMP) recently reviewed all the documentation and decided not to approve its use in the treatment of glioblastoma. Avastin® has also been used with considerable success to treat the "wet" form of age-related macular degeneration (AMD). With wet AMD, abnormal blood vessels grow underneath the retina and cause the vessels to leak blood and fluid, which can scar the macula, resulting in vision loss.

3.5.2 Therapeutic Antibodies and Rheumatoid Arthritis

There are two major types of arthritis, rheumatoid arthritis (RA) and osteoarthritis. RA is a chronic long-term auto-immune disease which ultimately leads to destruction of the joints. RA is characterised by swelling of the joints, severe pain and inflammation. Osteoarthritis (OA) is the normal wear and tear of joints that accompanies the ageing process. An estimated 1.3 million people in the US alone have been diagnosed with Rheumatoid Arthritis that is nearly 1 % of the population. Over 70 % of those diagnosed are women, whose symptoms generally begin between the ages of 30 and 60. In contrast, symptoms do not usually occur until later in life for men. Because of the chronic nature of the disease, over time the constant inflammation of joints may lead to cartilage, tendon and ligament damage. RA can also affect organs and internal systems. The best means of avoiding disability and sending this disease into remission is early diagnosis and treatment.

TNF-α is a cytokine with a molecular weight of 17 kDa which was originally discovered in studies looking at its role in destruction of tumours and hence acquired its name. It is produced by many cells including macrophages and exists in both a soluble and also a membrane bound form. Over the last 15 years it has been shown to play a major role inflammation and has become a drug target for several major pharmaceutical companies (Valesini et al. 2007). Interestingly, TNF-α is not the only drug target identified in arthritis and several new products

are targeting interleukin-6 (IL-6) as well as other interleukins. In the next few sections we will look at some of the major success stories in this area most notably, Remicade®, Humira® and Enbrel®.

3.5.2.1 Remicade

Remicade® (Infliximab) is a an anti-TNF-α chimeric antibody (75 % human and 25 % mouse) that binds to soluble and cell-surface TNF-α (Calabrese 2003). Each infliximab molecule is capable of binding two TNF-α molecules, and up to three infliximab molecules can bind to each TNF-α homotrimer, thereby blocking all the receptor binding sites on TNF-α. It is produced in the murine myeloma cell line NS0 and marketed by Janssen Biotech which merged with Merck. Remicade® was originally approved by the FDA in 1998 and had total sales of nearly $7.5 billion in 2010. Remicade® was initially approved for treatment of rheumatoid arthritis and is now also approved for the treatment of Crohn's disease. Crohn's disease is inflammatory disorder of the bowel and other internal organs which can lead to ulceration of the bowel.

3.5.2.2 Humira

Humira® (Adalimumab) was initially discovered as a result of a collaboration between BASF Bioresearch Corporation and Cambridge Antibody Technology which commenced in 1993. Abbott Laboratories subsequently acquired the pharmaceutical arm of BASF Knoll and further developed, manufactured and marketed the Adalimumab. More recently Abbott Laboratories split into two independent companies, Abbot and Abbvie. As a result of this split Abbvie took over the further development and marketing of Humira® (which stands for Human Monoclonal Antibody in Rheumatoid Arthritis).

Humira® was first approved by the FDA for Rheumatoid Arthritis in 2002 and as of 2008 it has been approved by the FDA to treat rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, moderate to severe chronic psoriasis and juvenile idiopathic arthritis. Humira® is produced in CHO cells in media with no animal or human derived products. As Humira® weakens the immune system by blocking TNF-α, serious infections have occurred in patients taking Humira®. Fatal infections such as tuberculosis (TB) and other infections caused by viruses or bacteria have taken hold in the body in some patients. Humira® may also have adverse side effects such as hepatitis B, nervous system problems, blood problems, heart failure and immune reactions such as lupus-like syndrome or liver problem.

3.5.2.3 Enbrel

Enbrel® (Etanercept) was originally approved by the FDA for the treatment of rheumatoid arthritis in 1989. Enbrel® is manufactured by Pfizer in Europe and Amgen in the United States is produced in CHO cells. Enbrel® has also been approved to treat Crohn's disease. Enbrel is a fusion protein made from part of the soluble form of the TNF-α receptor fused to the Fc part of the human IgG chain. The fusion protein has a combined molecular weight of ~150 kDa. Enbrel® works to inhibit the action of TNF-α by binding to the circulating soluble form of TNF-α, leaving none available to bind to the TNF-α receptor. Enbrel® successfully decreases inflammation associated with rheumatoid arthritis and other inflammatory disorder in a large percentage of patients.

The fusion with the Fc portion of the IgG chain is a critical part of the downstream process as it allows one-step purification with Protein A. In addition, the presence of the IgG facilitates destruction of the complex by antibody-dependent cell cytoxicity by recruitment of effector cells (natural killer cells, macrophages) mediated by Fc part of antibody. Enbrel® is formulated in injection form in a pen format, thus allowing self-administration.

Conclusion

There is no doubt that mAbs in their many different formats have had a dramatic effect on the treatment of many common diseases. Their success in rheumatoid arthritis has been particularly impressive and has improved the quality of life for a large number of patients. However, more than 50 % of TNF inhibitor-treated patients do not achieve sufficient clinical benefit (Valesini et al. 2007). The management of the failure of TNF inhibitors can be distinguished into two major approaches: switch to another TNF inhibitor or switch to a different class of targeted agents (Blom et al. 2009).

Treatment with mAbs has at times resulted in serious side effects that at times have been life threatening (Rosenblum and Amital 2011). Many of the side effects occur as a result of suppression of the immune system and result in severe infections due to bacterial, mycobacterial, invasive fungal, viral, protozoal, or other opportunistic pathogens (Caramaschi et al. 2006). Among opportunistic infections tuberculosis, candidiasis, and pneumocystosis have been reported. In addition, there have also some reports of antidrug antibodies following treatment with several TNF-α antagonists (De Rycke et al. 2003; Bartelds et al. 2011).

The effectiveness of mAbs in treating many types of cancer most notably breast cancer has been extremely successful and ADCs in particular show significant potential for developing tumour specific therapies (Sapra and Shor 2013). With recent advances in genomics and proteomics we are now approaching an era where we expect to see the development of individualised

(continued)

or personalised medicine (Hamburg and Collins 2010) where the treatment will be targetted to the specific properties of an individual's tumour (Brenton et al. 2005). Advances in DNA sequencing has reduced the cost of sequencing the genome from $500,000 per genome in 2000 to $50,000 in 2010 and researchers estimate that the cost will be soon be only $1,000 (McDermott et al. 2011).

However, certain aspects of the pharmacokinetic and pharmacodynamic properties of these mAbs are still not fully understood. The variability in pharmacokinetics may be explained by inter individual variability with respect to genetics and clinical status. The half-life of IgG1, 2, and 4 in humans is approximately 21 days, whilst the half-lives of mAbs generally increase with the degree of humanization present: murine (1.5 days) < chimeric (10 days) < humanized (12–20 days) = or < fully human (15–20 days) (Colcher et al. 1998). The elimination of mAbs is a complex multifactorial process involving protein catabolism, target-mediated elimination, immunogenicity, proteolytic degradation, and glycosylation.

However, despite these uncertainties the future looks bright and new products continue to be developed and will continue to generate income for the pharmaceutical companies. Even so, many first-generation products have reached or are reaching the end of patent protection for example Herceptin®, Remicade® and Humira® and their market value renders them attractive biosimilar targets.

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Chapter 4
Bioreactors for Mammalian Cells

R. Pörtner

Abstract Cultivation of mammalian cells has become a routine technique. Nevertheless, design and layout of cell culture processes has to meet cell specific demands. Numerous bioreactors and cultivation systems for cell culture, either for production of biopharmaceuticals or for tissue engineering, have been developed. Due to the special characteristics of these cells specific solutions are required. Selection of a suitable type of cell culture bioreactor system and/or an appropriate operation mode (batch, fed-batch, and perfusion) is affected by technical, biological, economical and regulatory considerations. Obviously there is no single cultivation system suitable for all applications. Furthermore, to compare different bioreactor systems or operation modes experimentally for a certain application is time consuming and expensive. Therefore, selection of an appropriate bioreactor or cultivation system requires extensive knowledge and expertise. The following contribution gives an introduction to bioreactor systems and cultivation strategies applied.

Keywords Mammalian cells • Bioreactors • Process strategies • Tissue engineering

4.1 Introduction to Cultivation Systems for Mammalian Cells

Mammalian cell culture technology has become a major field in modern biotechnology, especially for the area of human-health. Well established products from mammalian cells comprise viral vaccines, monoclonal antibodies for diagnostic and therapy, as well as recombinant therapeutic proteins (Butler 2005; Ozturk 2006; Howaldt et al. 2011; Walsh 2012; Plotkin 2005; Rader 2008; Shukla and Thömmes 2010; Chon and Zarbis-Papastoitsis 2011; Modjtahedi et al. 2012; Ecker and Ransohoff 2014). Regenerative medicine, tissue engineering or gene therapy opens up challenging new areas (Naderi et al. 2011; Fisher and Mauck 2013;

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Harrison et al. 2014). All these applications relay on the cultivation of mammalian cells, either primary cells or established cell lines under controlled conditions. Due to the broad variety of cell types, purposes, scales etc. it is obvious that no universal cultivation system or bioreactor exists (Eibl et al. 2009; Pörtner et al. 2005).

The main challenges for techniques required for cultivation of mammalian cells are slow growth rates with doubling times between 18 and 28 h, low productivity atleast for non-optimized cell lines, a high sensitivity against shear stress due to the lack of a cell wall (Butler 2004). Furthermore, many cell lines can only grow when adhered to a surface, and a suitable surface for attachment must be provided for these cells to proliferate. Adherent cells cause severe problems in large scale production, as they require large quantities of surface. One breakthrough for the industrial application of mammalian cells were the invention of microcarriers (e.g. for vaccine production) to support growth of adherent cells. In modern industrial production mostly cells adapted to grow in suspension, e.g. cell lines derived from Baby Hamster Kidney cells (BHK) or Chinese Hamster Ovary cells (CHO), are used.

As of the origin from multi cellular organisms, mammalian cells still hold the genetic program of inducing apoptosis or "programmed cell death". This can limit culture productivity in biotechnological processes. Conditions detrimental to the cells, e.g. substrate or oxygen limitation or metabolite inhibition have to be omitted as these can induce apoptosis (Al-Rubeai and Singh 1998; Cotter and Al-Rubeai 1995; Singh et al. 1994).

Much effort has been put in the development of appropriate cell culture media to ensure growth and product formation. Media that used to contain up to 10 % serum were continuously improved, and the cultivation in defined serum-free and even chemically defined, protein-free media is now common for most relevant industrial cell lines.

Great progress was achieved in the last decades regarding the development of mammalian cell culture technology (Heath and Kiss 2007; Eibl et al. 2009; Zhou et al. 2008; Hacker et al. 2009; Kelley 2009; Li et al. 2010; Rodrigues et al. 2010; Langfield et al. 2011; Munro et al. 2011; Jesus and Wurm 2011; Kantardjieff and Zhou 2014). Stirred tank bioreactors providing low shear environment by especially designed agitation and aeration systems were developed. Cultivation systems for immobilised cells such as hollow-fibre, fluidized-bed and fixed-bed bioreactors are intended to protect the cells from stressful conditions. With the invention of single-use-bioreactors new culture concepts were made possible. Special cultivation systems for tissue engineering were designed. In the following the basic requirements and design concepts will be introduced, followed by a detailed discussion of the most common bioreactor concepts.

4.1.1 Requirements and Categorization of Cultivation Systems

The cultivation of mammalian cells can be performed for various purposes, with respect to production of biopharmaceuticals e.g. for

- Cell line development and selection
- cultivation of adherent or suspendable cells in incubator scale
- production of small amounts of antibody or recombinant protein
- determination of kinetic parameters
- medium development
- handling of a large number of cell lines p.a.
- process development/Scale-up
- production process for high product titers

with respect to Tissue Engineering e.g. for

- production of retroviral constructs for gentherapy
- propagation of stem cells
- cultivation of tissue cells
- cells for in vitro tests

It is obvious that different cultivation systems (compare Fig. 4.1) on different scales are required to address these purposes. Its type and configuration considerably influence cultivation results and as a consequence process efficiency (Wurm 2004, 2005). In general, a cell culture bioreactor has to meet the following demands (adapted from Eibl et al. 2009):

- A well controlled environment with respect to pH, temperature, dissolved oxygen, dissolved CO_2-concentration etc.
- Homogeneous and low–shear mixing and aeration
- Effective mass and heat transfer
- A surface for cell attachement in case of adherent cells
- Measurability of process variables and key parameters
- Scale-up capability
- Long-term stability and sterility
- Ease of handling
- Reasonable maintenance.

For production of biopharmaceuticals, the available cell culture systems used can be divided in non-agitated (multiwall plates, dishes and flasks, culture bags) and agitated systems (Eibl et al. 2009). The latter can be further distinguished due to the mode of agitation. This can be

- mechanical, either by external devices (shaker, roller unit, rocker unit) or by internal devices (rotating shaft stirrer, tumbling shaft stirrer, oscillating vibromixer)

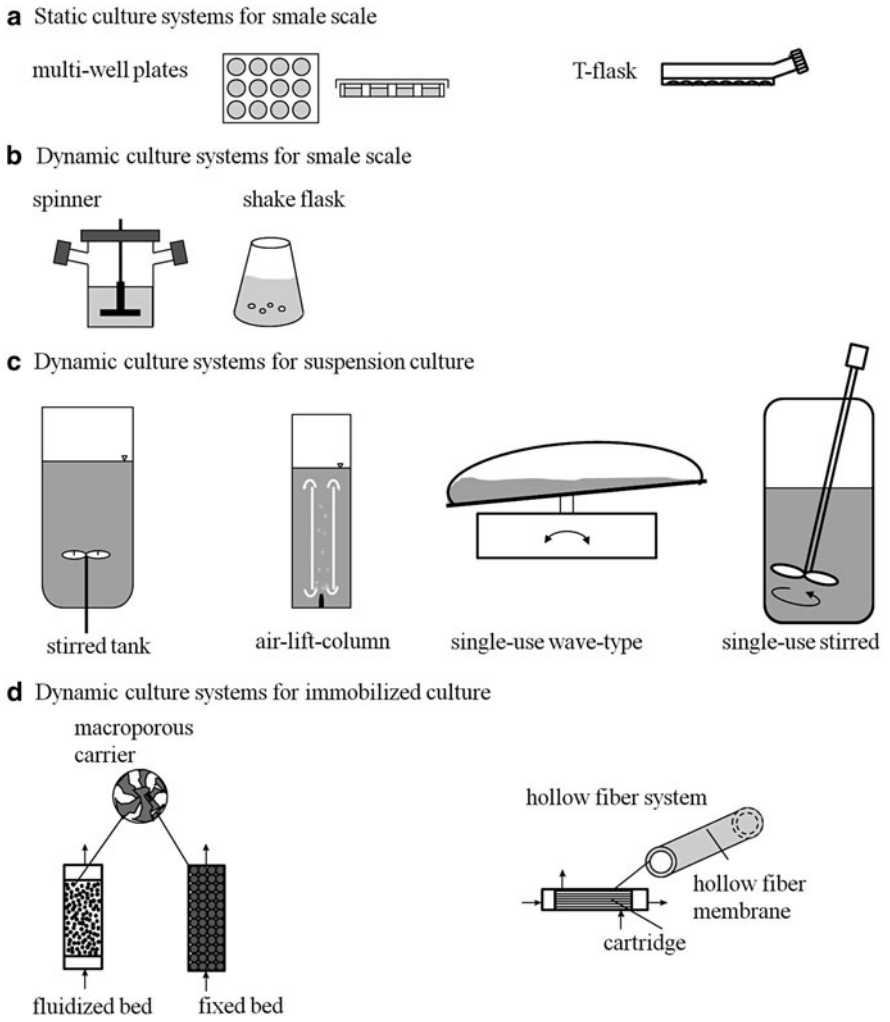

Fig. 4.1 Overview on typical cultivation systems and bioreactors for mammalian cells

- hydraulical (perfused hollow fibre bioreactor, fixed bed and fluidized bed bioreactors)
- pneumatical (bubble columns and air-lift-bioreactor)

Alternatively, bioreactors can be distinguished due to the applied mode of cultivation. Two main strategies were followed for increase of process intensity:

(i) Volumetric scale-up of lab-scale and pilot-scale-bioreactors at similar process intensity (cells/volume). Here systems originally developed for microbial fermentation (stirred tank, bubble column, air-lift-reactor) are applied, where cells are cultivated either in suspension or, if required, on microcarriers.

(ii) Increase of process intensity (cells/volume), as cells can grow at very high, tissue-like cell densities ($>10^8$ cells mL^{-1}). In this category high-density systems for immobilized culture (fixed bed, fluidized bed, hollow fibre reactor) have to be named, where cells are immobilized either in macroporous support materials or within a compartment created by membranes.

In Sect. 4.2 static and dynamic culture systems for small-scale are described, Sect. 4.3 is dedicated to dynamic bioreactors for suspension culture, Sect. 4.4 to dynamic bioreactors for immobilized cells.

For tissue engineering different culture concepts have been developed as well (Martin et al. 2004; Pörtner et al. 2005) (see Sect. 4.5).

4.1.2 Immobilization of Mammalian Cells

Biocatalysts (e.g. cells) are regarded as immobilized when they are restricted in their motility while their metabolic or catalytic activity is maintained (Lundgren and Blüml 1998). For mammalian cells different techniques for immobilizing mammalian cells have been proposed, including entrapping cells on a particle surface or in the interstices of a porous particle, encapsulation of cells within gels, or growth of cells within compartments formed by membranes (Lundgren and Blüml 1998; Butler 2004; Davis and Hanak 1997; Davis 2007). These techniques are widely applied, as they offer some solutions to problems inherent in mammalian cell culture (adapted from Czermak et al. 2009, modified):

- Growth of adherent cells in suspension type reactors.
- Protection against shear stress (Lüdemann et al. 1996).
- Growth of cells in tissue like cell densities ($>10^8$ cells mL^{-1}) allowing for smaller reactor volumes.
- Preliminary separation of (extracellular) products and cells, easing requirements for downstream.
- Growth of tissue cells in three-dimensional structure to mimic an organotypic behaviour
- Operation in perfusion mode

Most techniques applied for immobilization relay on non-porous (solid) or porous carriers. Solid carriers provide a smooth surface for cell attachment. A carrier is considered as macroporous, if the average pore size is between 30 and 400 µm allowing for cell growth within the carriers. The porosity of these carriers, defined as the ratio between volume of the pores and the total carrier volume (in percent) is normally between 60 % and 99 %. Macroporous carriers are suitable for immobilizing adherent as well as non-adherent cell lines. Furthermore they allow cells to grow in a three-dimensional, almost tissue-like structure at higher densities compared to solid or microporous carriers. The desired features of a

carrier can be defined according to Lundgren and Blüml (1998) as well as Pörtner and Märkl (1995):

- autoclavable
- available in large batches for industrial customers
- available with documents required for approval
- high batch-to-batch consistency
- suitable for a large number of cell lines (adherent and non-adherent cells)
- good long-term stability
- high surface-to-volume ratio (large multiplication steps)
- material of non-biological origin (minimal viral risks)
- macroporous for high cell density and shear force protection
- efficient diffusion from the medium into the center of the carriers
- non-toxic, non-immunogenic matrix
- size appropriate to reactor system
- simple immobilization and harvesting of cells
- mechanical stability
- uniform size distribution
- reusable
- possibility to count cells
- transferable between vessels (ease of scale-up)

Even if an "ideal" carrier does not exist, the above list gives some arguments when selecting a carrier or evaluating different suppliers. Depending on the intended culture system, the properties of the carriers, especially the size varies significantly (from 100 µm to 5 mm). In the following two categories of carriers will be distinguished, on the one hand microcarriers for use in suspension type bioreactors, on the other hand carriers for fixed bed and fluidized bed bioreactors.

Microcarriers are the most important technique for growth of adherent cells in suspension type bioreactors, mainly stirred tanks. The term "microcarrier" refers to small beads, either solid or macroporous, having a diameter of approx. 100–300 µm with a density slightly higher than the growth medium. The technique was first invented in the late 1960s by van Wezel (1967) and opened up new possibilities for a suspension-like culture of adherent cells in stirred tanks up to a scale of several thousand liters, especially for vaccine production. Microcarriers made of different materials (e.g. DEAE-Sephadex, DEAE-polyacrylamide, polyacrylamide, polystyrene, cellulose fibers, hollow glass, gelatin or gelatin-coated dextran beads) are available (van der Velden-de Groot 1995; Butler 2004). In microcarrier culture, cells grow as monolayers on the surface of solid spheres (Fig. 4.2a) or three-dimensional within the pores of macroporous structures. The carriers are usually suspended in culture medium by gentle stirring. Critical aspects for process design are the required amount of microcarrier, the inoculation density of cells per microcarrier, the seeding technique, harvest of cells and scale-up (Rodrigues et al. 2014; Handbook GE Healthcare).

Cultivation of cells in fixed bed and fluidized bed bioreactors (compare Sect. 4.4.1) requires carriers with high porosity, and large pore size among others.

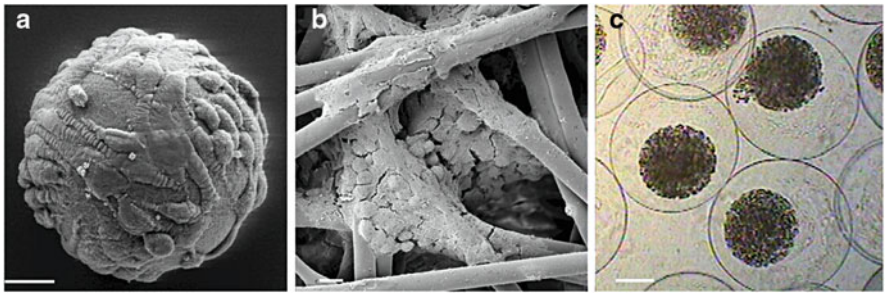

Fig. 4.2 Examples for immobilized cells (**a**) Chondrocytes grown on Cytodex 3 (bar 20 µm), (**b**) Hep-G2 cells grown on Fibra-Cel® (bar 20 µm), (**c**) encapsulated hMSC-TERT (bar approx. 1 cm, with courtesy of Prof. Czermak, TH Mittelhessen, Germany)

Therefore a large number of macroporous carriers and support materials for this application have been suggested, including glass (natron, borosilicate), cellulose, collagen, synthetic materials, e.g. polypropylene, polyurethane, polyethylene terephthalate (PET) or ceramic (Meuwly et al. 2007; Pörtner and Platas 2007). Experimental results prove that most of these carriers are suitable for a large number of cell types, either primary cells or established cell lines (e.g. hybridomas, CHO, hepathocytes). Modification of the surface is a critical parameter, as anchorage dependent cells, especially primary or non-established cell lines seem to be much more sensitive to the carrier surface than established cell lines. Therefore a careful selection of the carrier for a specific cell type is recommended.

In contrast to microcarriers, the market offers a limited number of carriers for fixed bed and fluidized bed cultures (Meuwly et al. 2007). The earlier macroporous carriers were mostly spherical, made of glass (SIRAN) and had usually a relatively low internal porosity. Alternatively carriers with a higher internal porosities ranging from 0.80 to 0.95 were developed. Nowadays carriers based on fiber architecture are well established and commercially available, e.g. Fibra-Cel® (Fig. 4.2b) consisting of polyester non-woven fibers and polypropylene mesh (Bohak et al. 1987; Meuwly et al. 2006) or BioNOC II™ consisting of PET (Drugmand et al. 2012a).

Mass transfer limitations due to diffusive transport limit the cell density within a carrier. Here especially oxygen supply is critical (Fassnacht and Pörtner 1999). From this point of view smaller carriers would support a higher volume specific cell density. Therefore for fluidized bed bioreactors a carrier size of 0.6–1 mm is an appropriate choice. But as in the case of fixed reactors smaller carriers create narrow free channels, which can be blocked by cells growing in these channels, here diameters of 3–5 mm for spherical carriers are recommended (Czermak et al. 2009). The above mentioned fiber carriers are quite flat (<1 mm) to prevent oxygen limitation. Beside the size of the carrier, the cell density within a carrier depends on parameters such as porosity, cell type etc.

Microencapsulation offers a further possibility for immobilization of mammalian cells, and to protect cells against stressful culture conditions (Huebner and

Buchholz 1999; Butler 2004; Hübner 2007; Weber et al. 2007). Techniques for encapsulation involve solid beads (Fig. 4.2c), coated beads, and membrane coated hollow spheres. Optimal size for beads is 300–500 μm to prevent mass transfer limitation. The beads containing the cells can be cultivated in suspension reactors similar to microcarriers, even under high impeller speed or bubble aeration with increased aeration rate. The fragile nature of the microcapsule is detrimental with respect to scale-up.

4.1.3 "Single Use"-Bioreactors

The single-use-concepts were reviewed extensively by Eibl et al. (2010) and Eibl and Eibl (2011a, b). In the following important variants will be highlighted. Originally only small scale equipment for lab-use (flasks, roller bottles etc.) or hollow-fibre bioreactors were offered for single use as plastic ware. As these devices cannot be sterilized by steam, they were pre-sterilised by radiation or treatment with ethylene oxide and disposed after use. The development of disposable or single-use bioreactors as an alternative to common, mostly reusable cultivation and bioreactor systems opened a new field for this technique. The idea was to address problems related to early process development such as flexibility, cost effectiveness, time to market as well as quality and regulatory issues. These systems are mostly based on bag-technology. The breakthrough of this challenging technology was paved by the wave-technology, disposable bags mounted on a rocking device. The success and growing acceptance of this technology encouraged several producers to offer bioreactors, mainly stirred bioreactors based on bag technology. Bag-volumes up to 2,000 L are in use. Advantages of single-use bioreactors can be seen in reduced cleaning procedures, lack of validation issues (cleaning, sterilization, etc.), lower investment costs, easier to adapt to changing process demands and less contamination risk. Nowadays single-use technology platforms are widely applied in production of biopharmaceuticals (e.g. monoclonal antibodies, viral vaccines, recombinant glycoproteins). New applications such as proliferation of stem cells have been performed successfully (Eibl and Eibl 2009b).

4.1.4 Process Strategies

As for all biotechnological processes, process strategies for operation of bioreactors can be classified in discontinuous modes (batch, repeated batch or fed-batch), continuous (chemostat, perfusion) or dialysis modes. Principles and balance equation can be found in Czermak et al. (2009); Abu-Absi et al. (2014). *Batch* and *fed-batch* dominate in industry although continuous cultures have been successfully implemented for commercial production of several products (Birch and Racher 2006).

A batch culture is characterised by growth of cells without further addition of substrate. During duration of the culture, the cells pass through the typical phases (lag-phase, exponential growth phase, stationary phase and death phase). Reduction in cell growth and induction of cell death due to substrate limitation or metabolite inhibition is mainly induced by apoptosis. During the cultivation additional compounds such as air or oxygen for aeration, acid or base for pH-control or anti foam agents to prevent foaming have to be added.

Batch processes are usually started with an initial cell density of approx. 1–2 10^5 cells/mL and operate for 1–2 weeks. After harvest the bioreactor has to be cleaned and refurbished before the bioreactor is re-started.

Batch cultures are regarded as simple, reliable and are often applied in lab as well as in industrial scale, e.g. for the first steps of a seed train (Abu-Absi et al. 2014). Nevertheless the productivity of batch cultures is limited by the initial concentration of substrates. On the one hand the solubility of certain medium compounds is limited. On the other hand too high substrate concentrations my induce substrate inhibition or a high cell specific substrate uptake rate leading to increased production of inhibiting metabolites. Furthermore a batch culture is running at a high volume specific productivity only for a short period of time when a high cell concentration is reached and substrates are not yet consumed completely. After harvest the bioreactor has to be cleaned and refurbished before the cycle is repeated (Krahe 2003).

Typical maximal cell concentrations for *batch* cultures are ranging from $1-3*10^6$ cells mL^{-1} for non optimized cell lines and medium up to 10^7 cells mL^{-1} or more for optimized cell lines and respective medium.

A first step to improve efficiency of a *batch* process can be seen in a so called "repeated batch". Here the main part of the cultivation broth is harvested along with the protein of interest at the end of exponential growth phase. The remaining part is used as inoculum for the next run. The culture system is filled with fresh medium and the process is newly started. This procedure can be repeated several times. In case of microcarrier culture the microcarriers are allowed to settle and fresh medium is added. Repeated batch processes are applied in industrial scale during seed train (Abu-Absi et al. 2014) or for production of fragile proteins, e.g. recombinant Factor VIII (Boedeker 2001).

Cell and product yield can be significantly improved by applying a *fed-batch* strategy, were nutrients are added after depletion according to an appropriate feeding strategy (Pörtner et al. 2004). Here the culture system is initially filled to 1/3 or 1/2 only and started as *batch*. As soon as substrates are consumed or have reached growth limiting values, nutrients such as glucose, amino acids and vitamins with trace elements are added mostly in concentrated form to avoid substantial dilution (Czermak et al. 2009; Abu-Absi et al. 2014). Non-nutrient salts are usually not concentrated to omit an increase in osmolality. *Batch* and *fed-batch* processes can be performed in small scale culture systems in flasks or bags, or on larger scale in suspension reactors.

The performance of a *fed-batch* process depends a lot on the appropriate design of the medium composition (reviewed by Abu-Absi et al. 2014) and the applied

feeding strategy (reviewed by Pörtner et al. 2004; Czermak et al. 2009). For optimization of both aspects such as nutrient consumption rates, metabolite production rates, metabolite accumulation, timing and duration of the feed, growth and product conditions have to be considered (Abu-Absi et al. 2014). Further challenges are:

- Exponential cell growth and a similar increase of demand for nutrients and oxygen
- Metabolic changes during cultivation, e.g. overflow metabolism at high substrate concentrations, and between following cultivations
- Highly concentrated feed medium bearing the risk of underfeeding or overfeeding (accumulation of inhibiting metabolites)
- A low number of on-line or at-line parameters is available during the cultivation making control of medium compounds difficult
- Control at low substrate concentrations desired, but risk of low cell growth or apoptosis

Simple control strategies comprise fixed feed rates, linear increase of feed rates or exponential increase of feed rates. As these strategies do not use any on line control, they require a fundamental knowledge on cell growth, and are suitable for established standard protocols (e.g. for production). Alternatively control strategies with on-line feedback from the cultivation can be applied:

- Feeding by measuring substrate consumption
- Control at desired substrate concentration (μ-stat)
- Proportional feeding based on base consumption for pH-control (lactate production)
- Proportional feeding based on on-line cell count (or turbidity)
- Proportional feeding based on oxygen uptake rate (OUR)

Feeding strategies that take into account the real-time state of the cell culture through feeding algorithms to control e.g. glucose and/or glutamine or oxygen uptake rate have been demonstrated to reduce by-product accumulation (reviewed by Abu-Absi et al. 2014).

More advanced strategies are based on mathematical models, such as

- "a priori"-calculation of feed rate based on kinetic model
- model-based, feedback, adaptive process control (Frahm et al. (2003), Pörtner et al. (2004))

As *fed-batch* strategies result in very high cell densities, accumulation of metabolites, mainly lactate can be detrimental to growth and productivity (Mulukutla et al. 2012; Le et al. 2012). A number of strategies for reduction of lactate especially for CHO-cells have been suggested, including substitution of glucose with galactose or mannose, use of chemically defined feed media, optimization of feed media by metabolic flux analysis, increase of copper concentration, limiting glucose levels to prevent overflow metabolism, use of pH to control lactate accumulation (reviewed by Abu-Absi et al. (2014)).

The main advantage of a *fed-batch* compared to a *batch* can be seen in a longer growth phase, higher cell concentrations (10^7–10^8 cells mL^{-1}) and higher product concentrations and a higher product yield. On the other hand a larger effort for equipment and control is required. Compared to perfusion culture, *fed-batch* strategies do not necessarily require cell lines of the same stability, result in a higher degree of lot-to-lot consistency and required equipment costs are lower (Lim et al. 2006). Furthermore, *fed-batch* strategies seem to have advantages with respect to environmental sustainability with a lower water and consumable usage profile compared to perfusion culture (Pollock et al. 2013).

In continuous chemostat culture, fresh medium is continuously pumped to the culture system and spend medium is removed. The volume of the culture broth remains constant. The spend medium (harvest) contains all medium compounds including the cells. After some time of cultivation all process parameters become constant (average values – steady state), if homogeneous mixing within the culture system can be assumed. During steady state the values for cell concentration as well as substrate and product (metabolite) concentration depends on the flow rates for medium supply and harvest, expressed by the dilution rate. With increasing dilution rate, first a constant or increasing cell concentration (depending on the death rate of the cells) can be observed with increasing dilution rate. But when the dilution rate approaches the maximal growth rate of the cells, the cells can not compensate for wash-out and the cell concentration decreases. Substrate concentration is very low at low dilution rates and increases close to the critical dilution rate. The course of the product concentration looks similar to the cell concentration.

Chemostat cultures without cell retention are a valuable tool for research (e.g. kinetic studies) (Frame and Hu 1991; Harigae et al 1994; Pörtner and Schäfer 1996), but not for production scale due to low cell and product concentration. Multi-stage chemostat cultures were suggested to improve the yield of this culture mode (van Lier et al. 1994).

Perfusion can overcome the drawbacks of a continuous chemostat culture to some extend by retaining the cells in the culture system and perfuse the system continuously with medium. This can be done by connecting cell retention devices (e.g. microfiltration, spin filters, centrifuges among others, compare Sect. 4.3.2.4) to a suspension bioreactor (stirred tanks, bubble columns and air-lift reactors) or by immobilizing the cells (e.g. fixed bed or fluidized bed bioreactor, hollow fiber systems) (Catapano et al. 2009). Similar to a chemostat, here fresh medium is pumped continuously to the culture system and spend medium (harvest containing the desired product) is removed with the same flow rate. But as the cells are retained in the bioreactor, significantly higher flow rates can be applied leading to higher cell concentrations within the bioreactor.

Advantages of perfusion compared to *batch*/*fed-batch* systems are the ability to grow cells to a very high density (up to 10^8 cells mL^{-1}) and to maintain the cell density for longer periods of time, steady-state conditions, low retention times of fragile products, the ease of handling media exchanges for the purpose of fresh feed and product harvest, the easy removal of metabolites and other inhibitors, and the prospect of easy scale up. When dealing with adherent cells, perfusion reactor

design becomes slightly simpler (e.g. fixed bed and fluidized bed) due to immobilization of cells within macroporous carriers. The main advantage of perfusion cultures can be seen in the reduced bioreactor size (approx. 1/10th of a suspension reactor without cell retention). Nevertheless there are some difficulties in the perfusion concept. Extra equipment such as the retention device itself, pumps for feeding, harvest and medium circulation, storage tanks for feed and harvest are required. Harvest product titers are typically lower compared to fed-batch cultures. The amount of media needed to complete a moderate to long term run can be excessively large. Process development and characterisation can be more complex. A further possible drawback of continuous cultivations is the possibility for variability over the time of the run, e.g. genetic instability over time, i.e. mutations that will decrease product formation. Besides these concerns there are a growing number of pharmaceutical products in the market produced successfully from perfusion systems (Kompala and Ozturk 2006; Bonham-Carter and Shevitz 2011; Vogel et al. 2012; Warikoo et al. 2012; Pollock et al. 2013).

Non-porous dialysis membranes allow for simultaneous enrichment of cells and high molecular weight compounds. Cells are retained within a culture device by this membrane. While low molecular weight compounds (substrates, metabolites) can pass through the membrane, high molecular weight compounds are retained. The molecular weight of the retained substance depends on the cut-off of the membrane (e.g. 10,000 DA). Dialysis processes can be run as *batch*, *fed-batch* or continuously (Comer et al. 1990; Pörtner and Märkl 1998).

4.1.5 Monitoring and Control

Non-optimal conditions within the bioreactor can result in a decreased product formation and a less efficient process. Monitoring and control of a number of parameters, either of a physical or chemical nature, is crucial for optimal performance of a bioprocess (Gnoth et al. 2008). The most common parameters to be monitored on-line and controlled during a bioprocess are agitation rate, temperature, pH, oxygen partial pressure pO_2 and carbon dioxide partial pressure pCO_2. In addition, at-line or off-line procedures provide data on the state of the cell culture as well as the physical environment of a reactor. As in industrial manufacturing often very large datasets are generated, and methods for data acquisition, aggregation analysis have to be implemented (Abu-Absi et al. 2014). In the following the main techniques are briefly discussed.

The temperature is very critical for mammalian cells and must be maintained at the desired set point (mostly 37 °C + −0.5 °C). A higher variation in temperature often leads to reduced growth and production rates or increased cell death (Hartnett 1994). To measure the temperature within the system, resistance temperature devices (RTD) are preferred (PT-100 or PT-1000) due to their high accuracy and reproducibility. Alternatively thermocouples were suggested, which are cheaper but also less accurate (Doyle and Griffiths 1998; Riley 2006). Depending on the

actual state of the system and the desired temperature value, heating or cooling of the system is induced.

The pH has to be maintained in a quite narrow range as well, usually 7.2 ± 0.1. The preferred pH electrodes are constructed from a special composition glass which senses the hydrogen ion concentration. The alkali metal ions of the glass and the hydrogen ions in solution undergo an ion exchange reaction generating a potential difference proportional to the pH. This potential is then compared to a constant potential built up at the reference electrode to determine the actual pH in the medium to be measured. Usually measuring and reference electrode are combined. Alternatively optodes are in use, especially for single-use bioreactors (Klimant et al. 1995; Wolfbeis 1991; Wittmann et al. 2003) (see below).

The pH can be controlled by varying the CO_2-concentration in the gas phase, if a medium containing sodium-bicarbonate is used. Alternatively or at very high cell concentrations, addition of liquid base (e.g. KOH or NaOH) or acid (e.g. HCl) is used to control the culture pH. In this case, the medium should have at least some buffer capacity to avoid oscillation around the set point. Problems of this method can be seen in increased osmolality, dilution of the culture broth or increased cell death at the point of addition due to insufficient mixing (Langheinrich and Nienow 1999).

Oxygen is consumed by the cells and has to be replaced by an appropriate aeration system (compare Sect. 4.3.2.1) to ensure an optimal oxygen partial pressure pO_2 (approx. 20–50 % of air saturation). The most commonly applied device to quantify the dissolved oxygen concentration in a cell culture medium is a so called polarographic Clark-electrode (Cammann et al. 2002; Chmiel 2011). Here, the oxygen diffuses from the culture medium across an oxygen-selective membrane and is then reduced at a negatively polarized platinum electrode. This cathode is connected to a reference silver anode by an electrolyte solution (KCl). Alternatively optodes are in use (Klimant et al. 1995; Wolfbeis 1991; Wittmann et al. 2003). Here pulsed monochromatic light is carried in an optic fiber to the oxygen sensor and excites the immobilised fluorophore (e.g. ruthenium complexes). The excited complex fluoresces and emits energy at a higher wavelength. The collision of an oxygen molecule with a fluorophore in its excited state leads to a non-radiative transfer of energy. This internal conversation decreases the fluorescence signal.

To maintain the desired set point, air or pure oxygen is sparged directly into the culture, depending on the required oxygen transfer rates and the achievable flow rates. To some extend the stirrer speed can be increased in case of stirred tank reactors, as far as cells are not damaged due to too high shear rates.

Furthermore the concentration of dissolved carbon dioxide within the cell culture medium is very important. Dissolved carbon dioxide can now be measured *in situ* utilizing a fiber optic chemical sensor (Wolfbeis 1991; Pattison et al. 2000; Ge et al. 2003; Riley 2006; Chu and Lo 2008).

Additionally to these well established devices, new on-line techniques have found their way from the lab scale to industrial application, e.g. in situ microscopy or impedance spectroscopy for cell concentration, IR-spectroscopy for metabolic

compounds (substrates, metabolites) (Justice et al. 2011; Abu-Absi et al. 2011; Hakemeyer et al. 2012, 2013).

4.1.6 Parameters for Characterization of Bioreactors

Numbers used to characterize culture systems and bioreactors, mainly those intended for suspended cells or cells grown on microcarriers, include

- Geometric dimensions such as the ratios between vessel height H and vessel diameter D (aspect ratio H/D), stirrer diameter d_R to vessel diameter D (d_R/D) in case of stirred tanks, among others
- Volumetric power input (P/V), ratio of power input P and volume V
- Power number (Newton number Ne) defined as

$$Ne = \frac{P}{n^3 d_R^5 \rho}$$

  for stirred bioreactors, with stirrer speed n and fluid density ρ

- Volumetric mass transfer coefficient (k_La) for oxygen or CO_2
- Mixing time (Θ_M)
- Reynolds-number defined as

$$Re = \frac{n d_R^2 \rho}{\eta}$$

  for stirred bioreactors with fluid viscosity η

- Impeller tip speed (u_{tip}), defined as

$$u_{tip} = n \pi d_R$$

- volumetric gas flow rate expressed as volume gas per volume reactor fluid and minute (vvm)
- Superficial gas velocity
- Maximal tolerated shear rate

These numbers can be used to compare process parameters in different bioreactor systems or for scale up from lab to production scale. Process parameters such as impeller speed or volumetric gas flow rate can be derived from these numbers. Usually they are not suitable for a deeper description of the fluid flow within the bioreactor. This can be accomplished by computational fluid dynamic (CFD)-

simulations (Zhang et al. 2005, 2010; Kelly 2008; Oncül et al. 2010; Gelves et al. 2013).

Methods for determination of some of the above mentioned parameters will be discussed in the following. For single use bioreactors it is referred to Eibl and Eibl (2011a).

The specific power input is one of the most accepted criteria for scale-up and process transfer between bioreactors. For stirred tank bioreactors it can be determined from the electric power consumed by the engine, but losses from friction in the bearing have to be taken into account. As these can be quite high especially for small and lab scale bioreactors, it might be useful to work with model systems equipped similar to the bioreactor itself. Alternatively the power input can be estimated from the dimensionless power number Ne. A number of correlations and data sheets can be found in the literature, often expressed as $Ne = f(Re)$ and curves for different stirrer and reactor design (Rushton et al. 1950a, b; Einsele 1978; Henzler 1982; Chudacek 1985; Markopoulos and Pantuflas 2001). These correlations have to be corrected in case of bubble aeration (e.g. the Froude Number Fr), multi stage impellers, varying dimensions of the vessel diameter, the liquid height, the lower-impeller distance to tank bottom, the propeller pitch, the impeller blade width and blade length, or varying number of impeller blades. An example for estimation of power input for lab scale reactors can be found in (Platas et al. 2012).

For determination of power input in shake flask two approaches have been suggested.

Büchs et al. (2000a, b) have analyzed power consumption in shake flasks and presented a correlation for a modified power number as a function of the Reynolds number at the thin liquid layer at the flask wall. In analogy to a stirrer blade inside a bioreactor, this equation considers the dimensionless parameter impeller blade width to the impeller diameter, which is changed for shake flasks by the relationship between the height of the liquid in the flask during rotation and the shake flask maximum inner diameter. Care has to be taken about "out-of-phase"-regimes, which are often found at low shaking frequencies, high viscosities, low filling volumes, short shaking diameters and high liquid viscosities. In a different approach, Kato et al. (1995) determined the power consumption in horizontally shaking cylindrical vessels by using the average energy consumption of the shaking machine for different flask and shaking diameters and shaking velocities at higher viscosity values. The experimental data on torque measurement were fitted to a Re and Fr dependent correlation. In contrast to Büchs et al., Kato et al. used the shaking diameter for calculation of the Reynolds number. Both approaches might lead to different results (Platas et al. 2013) and should be validated experimentally.

A very common method for measuring the k_La is the "dynamic method" based on the 2-film-theory (Czermak et al. 2009; Chmiel 2011). The main advantage of the dynamic method is the low cost of equipment needed. Care has to be taken especially for smaller reactor systems with huge head space. Here the head space should be flushed with air during measurement to guarantee the required air saturation in the gas phase. Alternatively the sulfide-method can be applied.

If a direct determination is not possible, correlations and diagrams for estimation of k_La can be used (Calderbank and Moo-Young 1961; Henzler and Kauling 1993; Lemoine and Morsi 2005). But as these correlations were usually not determined for conditions typical for cell culture reactors, care has to be taken and the values have to be verified experimentally (own data, not published).

For determination of mixing time Θ_M a colorimetric method employing Lugol's solution can be used for lab-scale bioreactor systems (Platas et al. 2012). This consists of elemental iodine and potassium iodide in a starch solution, and sodium thiosulfate as a titration agent. The disappearance of the intense blue color can be followed by a camera and further analyzed manually or with appropriate software. By this, the method allows for an easy visualization of the decolourization process as well as for identification of local death zones. Moreover the experimental determination of the mixing time with this method is not affected by the reaction time of the measuring probes. For experimental purposes, the mixing time is considered at 95–98 % of the final equilibrium concentration.

An equation for estimating the mixing time Θ_M was suggested by Nienow (1996)

$$\Theta_m = 5.9 \left(\frac{P}{V}\right)^{-1/3} \left(\frac{d_R}{D}\right)^{-1/3} D^{2/3}$$

with the mean power input per volume P/V, stirrer diameter d_R and vessel diameter D. This equation can be applied for both axial and radial flow impellers. It implies an increase in the mean power input per volume, if the mixing time is kept constant during scale-up. On the other hand the tolerable power input per volume can be restricted due to shear effects. As this equation was determined for larger reactor systems, it should be verified experimentally for lab scale bioreactors (own data, not published).

4.2 Small-Scale Culture Systems

Small-scale culture systems (e.g. microtiter plates, petri dishes, t-flasks, roller bottles, shake flasks and spinner) are characterized by simple design, and a low level of instrumentation and control. For this reason they usually require external equipment such as incubators and/or shakers to ensure an appropriate physical and chemical environment for the cells. Within an incubator optimal temperature, humidity and other conditions such as the carbon dioxide (CO_2) and oxygen content of the atmosphere are maintained at appropriate levels. For cultivation of mammalian cells the relative humidity is typically >80 % to prevent evaporation and a slightly acidic pH is achieved by maintaining a CO_2 level of 5 %. For use of dynamic culture systems (e.g. roller bottles, shake flask and spinner) incubators can be equipped with roller unit, shaker platforms or spinner unit.

Advantages of small-scale culture systems are simple handling, low costs, flexibility, and that most of them can be used for adherent and suspendable cells. Drawbacks are low cell density and product yield, small volume, and that most of these systems are not scalable.

The devices in this category are usually operated as *batch*, were the culture is either stopped at some defined point (duration, decrease of cell growth) or maintained by repeated medium exchange. Adherent growing cells can be harvested and subcultivated e.g. by trypsination.

4.2.1 Static Culture Systems

Originally culture flasks and plates were developed for growth of tissue clots or adherent cells, mostly primary cells under static conditions under a controlled atmosphere within an incubator. Nowadays they are used for cultivation of suspendable cells as well. For support of attachment of adherent cells flask with a flat bottom were designed. Adherent cells typically grow as monolayer. Above the cells a thin layer of medium is placed to provide nutrients and to allow for sufficient oxygen diffusion from the headspace above the medium. Flasks and plates are offered as plastic ware, mostly made of polystyrene or polypropylene. The bottom surface of the flask or plate can be modified using either corona discharge or gas-plasma to enhance growth of either adherent or suspendable cells. As all these materials are usually not sterilisable by steam, they are purchased pre-sterilized by γ-irradiation or ethylene oxide and are intended for single use only. Most of these culture systems are transparent and allow for a microscopic observation of the culture. Volumes range from tens of nanolitres (microtiter plates) to litres (multi-layer cell culture system).

A microtiter plate is a flat plate with multiple "wells" used as small test tubes. Typically they consist of 6, 24, 96, 384 or even 1,536 sample wells arranged in a 2:3 rectangular matrix. New developments go up to 3,456 or even 9,600 wells. Microtiter plates are offered for different applications beside cell culture, involving filtration, separation, optical detection, storage, reaction mixing. They fit in most plate readers, e.g. ELISA reader. For automated handling of microtiter plates in screening platforms special robots for liquid handling, plate transport, incubation and storage were developed.

T-flask consists of a rectangular canted neck body with an outlet port on one side. Typical size of the bottom is 25, 75, 150 or 225 cm^2. The port is closed by a solid cap, which has to be opened slightly during cultivation, or a cap equipped with a microfilter to allow for penetration of air from the incubator has to be used.

To support larger numbers of adherent growing cells, multitray cell culture systems consisting of a series of stacked trays (up to 40) and surface areas up to 24,000 cm^2 are in use, e.g. for mass production of stem cells or in human vaccine manufacturing (Eibl and Eibl 2009).

As usually cell and product concentrations in static flasks and plates are quite low, systems supporting higher cell and product concentrations e.g. by integrating dialysis membranes into T-flasks were developed. This semi-permeable membrane with a cut of 10 kDa allows a continuous diffusion of nutrients into the cell compartment with a concurrent removal of any inhibitory waste product, while cells and product accumulate (Baumann 2005).

4.2.2 Dynamic Culture Systems

Roller bottles are cylindrical containers originally invented for the culture of monolayers of adherent cells. Cells grow attached to the inner surface of the container, which is filled to approx. one third with medium. The container revolves slowly. By this the cells are exposed to medium in the lower part and to oxygen in the upper part. The gentle agitation prevents gradients from forming within the medium that may adversely affect growth. Roller bottles are made of similar material as T-flasks, are therefore offered pre-sterilised and intended for single-use, and are available typically with surface areas of 1,050 cm^2. Multiple roller bottle units require an automated multiple roller bottle platform. Roller bottles were invented originally to increase the surface area required for cultivation of large numbers of adherent cells and paved the way for large scale manufacturing of viral vaccines before introduction of the microcarrier technology. Modifications were developed to increase cell and product concentration. The miniPERM bioreactor for cultivation of suspendable cells is made of two connected modules separated by a dialysis membrane. One chamber serves as a culture chamber, the second one as medium reservoir (Falkenberg 1998).

Spinner flasks (approx. 100 ml up to 5 L) were developed for cultivation of suspendable cells or adherent cells grown on microcarriers. They are equipped with conical pendulum or paddle-type magnetic impellers and are intended for use in a humidified CO_2 incubator or, for larger volumes, in warm rooms. They are available as glass ware or plastics for single use. The flask is often equipped with too ports for inoculum, medium exchange or sampling. Impeller speed is kept low (approx. 30–100 rpm). Oxygen is supplied via the head space through slightly opened caps or caps with sterile filters. This simple concept allows for easy handling, but only low oxygen transfer rates (k_La approx. 0.1–4 h^{-1}) (Spier and Griffiths 1983). As an alternative concept with improved oxygen transfer, Heidemann et al. (1994) developed the “Superspinner”, a standard laboratory flask equipped with a microporous, hydrophobic polypropylene membrane tube for bubble-free aeration (see below). The coiled membrane tube is mounted on a pendulum impeller, driven by a magnetic impeller, and perfused with an air/CO_2-mixture from within the incubator.

Shake flask, traditionally used for cultivation of microbial cells, are nowadays well accepted for cultivation of suspendable cells or microcarrier culture for

adherent cells. They are available in a variety of sizes (up to several liter) and closure styles, and applied preferably in process development.

4.3 Bioreactors for Suspension Culture

Bioreactors for suspension culture belong to the group of dynamic systems (compare Sect. 4.1.1) and can be either mechanically driven (stirred tank bioreactor) or pneumatically driven (bubble column, air lift bioreactor). Whereas stirred bioreactors are used for cultivation of either suspendable cells or adherent cells grown on microcarriers, bubble column and air lift bioreactors are mostly used for cultivation of suspendable cells due to the impact of rising bubbles on cells grown on microcarriers. Suspension reactors are characterized by advantages such as: conventional reactor systems, know-how on design and sterile operation, good mass transfer, homogeneous mixing, sampling and determination of cell concentration possible, and high scale-up potential. Disadvantages are: oxygen supply difficult at high cell densities, cell damage by shear and/or foam (bubble aeration), relatively high demand for control (temp., oxygen, pH, flow rates), perfusion required for very high cell densities (Eibl et al. 2009). In the following first cell damage in suspension bioreactors is discussed, than design of suspension bioreactors.

4.3.1 Cell Damage in Stirred and Bubble Aerated Bioreactors

Mammalian cells react very sensitive to shear forces, as they are surrounded by a plasmatic membrane basically composed by phospholipids. This membrane builds a double layer, which can only withstand weak mechanical forces. In conventional bioreactor systems, aerated stirred tanks and bubble columns, adherent cells grown on microcarriers or suspended cells are exposed to variously intense hydrodynamic forces and these forces may induce various physical responses, including reduced growth, cell death or other metabolic reactions. A number of reviews have summarized the main fluid-mechanical and biological aspects of cell damage (Cherry 1993; Papoutsakis 1991; Chisti 2000, 2001). In the following the main effects in stirred and bubble aerated bioreactors will be discussed and consequences on design and operation of these types of bioreactors are elaborated.

In stirred reactors shear forces acting on cells are induced by the rotating stirrer and eddy breakdown in the turbulent flow. The intensity of these forces depend on the type and size of the impeller, the impeller speed and other design parameters such as height to diameter ratio, number of baffles or shape of the bottom. Obviously, cells grown on microcarriers experience more severe hydrodynamic forces than do freely suspended cells (for review see Chisti 2001). This is because in the mostly turbulent environment within highly agitated or aerated systems, the smallest length scale of fluid eddies (Kolmogoroff length) can easily approach the

dimensions of microcarriers, resulting in high local relative velocities between the solid and the liquid phases. Additionally, collision among microcarriers and between the impeller and microcarriers may damage attached cells.

The most important effects of bubbles on cells are break-up of bubbles at the surface and foam formation (Chisti 2000). Cell lines differ tremendously in their sensitivity to aeration. Small bubbles (e.g. <2 mm diameter) are more damaging than large bubbles (e.g. ~10 mm diameter). Cell damage is directly affected by the aeration rate. Sparging-associated damage may be enhanced by impeller agitation. Cell damage caused by bubbles and foam can be significantly reduced by adding shear-protective substances (e.g. Pluronic®–F68) and/or antifoam agents (e.g. antifoam C) (Ozturk 2014). The protective effect can be physical and/or physiological (biochemical) depending on the specific agent.

In a complex, mostly turbulent environment within a bioreactor the local shear rate varies within the vessel and it is more difficult to associate cell damage with the magnitude of the prevailing shear rate or the associated shear stress. Even more complex are the mechanisms of cell damage caused by gas bubbles in sparged bioreactors, bubble columns or air-lift-bioreactors. Furthermore cell damage increases significantly in serum- or protein-free medium. For design and operation of sparged and agitated bioreactors some general conclusions can be drawn (compare Chisti 2000; Fenge and Lüllau 2006; Ma et al. 2006, modified)

- The sole purpose of the impeller should be to suspend cells and to mix the fluid gently, not to disperse rising bubbles.
- The sparger should be located such that the rising bubbles do not interact with any impellers and the volumetric gas flow rate should be kept as low as possible (below 0.1 vvm).
- For air-lift and bubble column bioreactors the aspect ratio (ratio between height and diameter) should be ~14 for small scale and 6–7 for large scale.
- A suitable additive such as Pluronic®–F68 should be used whenever feasible.

Despite a number of concepts suggested in the literature to describe shear effects caused by fluid-mechanical forces or bubbles, none of these concept allow for a precise layout of large scale bioreactors (design, operating parameters such as aeration rate, power input or stirrer speed) without additional experiments. Furthermore, experimental findings on a small scale have to be validated in pilot scale before being transferred to the final industrial scale. Last but not least, the tolerance of suspended mammalian cells or cells grown on microcarriers to shear forces depends on cell line, medium, process, flow regime and bioreactor dependent. In the following consequences on design (e.g. type of stirrer or sparger) and operation (volume specific power input, mixing time, k_La, impeller tip speed, aeration rate etc.) will be given.

4.3.2 Design of Suspension Bioreactors

A bioreactor system for suspension culture consists typically of a bioreactor vessel which is equipped with a drive for the stirrer in case of stirred tank bioreactors, a measurement and control unit, a heating and cooling circuit, gas supply and removal, feed tanks, sampling and harvest systems, sterile couplers, fittings, piping etc. A practical approach for set-up and instrumentation of a lab scale bioreactor system can be found by Martens et al. (2014). For bioreactors on pilot and production scale, additionally safety installations (e.g. safety valves), a burst discs etc. might be required. Furthermore, larger bioreactors can be equipped with systems for "Cleaning in place" (CIP) and "Sterilization in place" (SIP). For continuous perfusion, systems for cell retention can be installed (Krahe 2003; Ozturk and Hu 2006; Ozturk 2014). Suspension bioreactors up to 25,000 L have been installed on production scale, here mostly stirred tank bioreactors (Farid 2007).

4.3.2.1 Stirred Tank Bioreactors

Vessel Design

As discussed above, cell damage due to shear stress caused by agitation and/or aeration has a strong impact on design and operation of stirred tank bioreactors. A stirred, aerated bioreactor consists basically of a cylindrical vessel, which is equipped with one or more impellers mounted on a rotating shaft driven by an external drive. Close to the bottom a sparger (if applied, see below) is located for supply of gas bubbles. Furthermore up to four buffles can be installed to prevent vortexing and to improve mixing. It must be ensured that baffles do not cause cell damage, e.g. in the case of microcarrier cultures. As this problem is more severe on lab scale, here often a curved bottom is used instead of a flat bottom as for microbial fermentation to improve homogeneous mixing.

Heating and cooling of the bioreactor can be accomplished by different means depending on the size of the reactor. Lab scale reactors can be tempered by a heating blanket or a heating/cooling jacket wrapped around the vessel, an electrical heater immersed in the medium, or by pumping a preheated liquid (usually tap water circulating in a loop) through a double jacket. The later is the most common version for larger reactors as well (Krahe 2003). Very large reactors can be equipped with internal heating or cooling coils to improve heat transfer.

With respect to the materials used for the vessel, top, fittings, impellers etc. a distinction must be made between different bioreactor scales on the one hand and steam sterilisable, re-usable and single-use, pre-sterilised bioreactors on the other hand. A well established material for vessels at lab scale up to volumes of approx. 50 L is glass, as glass is transparent, easy to clean, inert and steam sterilisable. During the last years stirred single-use bioreactors were invented consisting of

polycarbonate or polystyrene. Top, fittings, and impellers can be made out of austenitic steel or polyetheretherketone (PEEK) for steam sterilisable bioreactors, out of polycarbonate for single-use bioreactors.

On pilot or production scale steam sterilisable vessels are made of austenitic steel, commonly 316 or 316 L steel. The expensive 316 L is preferred for GMP-production due to the excellent electropolishing and welding characteristics (Krahe 2003). Single-use stirred bioreactors consists out of pre-sterilised bags made of polyethylene or ethyl vinyl acetate (EVA) films including the stirrer etc.. They are mounted inside a stainless container to shape and fix the bags, which incorporates heating blankets or double jackets for temperature control.

The ratio of vessel height H to diameter D can vary between 1 and 3 (Krahe 2003). For small scale and lab scale reactors often $H/D = 1$ is preferred. At larger scale H/D up to 3 is common to improve retention time of gas bubbles in the reactor and to enlarge the heat exchange capacity of the vessel wall. For production scale reactor the H/D-ratio can be even larger.

A detailed mechanistic view on mixing, homogenization of the culture broth and sufficient mass and heat transfer in stirred tank bioreactors for mammalian cell culture has been discussed by Sieblist et al. (2011a, b).

Impeller

A number of impellers have been suggested for cell culture so far for appropriate mixing of the culture broth (Czermak et al. 2009). To prevent cell damage by shear stress, the main purpose of the impeller(s) is to suspend cells or microcarriers and to mix the fluid gently (see above). For this, impellers having larger dimensions (diameter ≥ 0.5 of vessel diameter) and operating at lower stirrer speed as compared to microbial fermentation are preferred. The Rushton turbine, often used in microbial fermentation due to excellent mixing and bubble dispersion characteristics, is regarded as less suitable for cell culture as it requires rather high impeller speed for sufficient mixing and by this can damage shear sensitive cells. Nevertheless this type of stirrer has been successfully applied in lab and large scale reactors, often in multistage and/or in combination with other impellers. Alternatively larger impellers with an axial-flow characteristic are in use (marine, pitch-blade or 3-blade-segment impeller) (Ozturk 2014). As larger impellers can be operated at lower impeller speed to provide appropriate mixing, cell damage is significantly reduced. Again one or more impellers can be mounted on the shaft depending on the scale and H/D-ratio. Independent of the stirrer selection in the bottom position, an impeller creating an axial flow profile is often used on top of the bioreactor to facilitate good recirculation of bubbles in the bioreactor and good axial mixing (Varley and Birch 1999; Marks 2003). This is especially important to mix feeds and base commonly added on top of the liquid surface adequate (Ma et al. 2006; Reuss 1993). The rotating shaft can be driven from the bottom or from the top (compare Krahe 2003). Magnetic coupling is quite common for autoclavable bioreactors. Meanwhile even larger reactors can be equipped with this technique. Details

regarding choice of impeller, scale-up and further engineering aspects are given by (Ma et al. 2006; Nienow 1990).

Aeration Systems

Supply of oxygen is one of the most important factors in operating cell culture bioreactors as oxygen solubility in the medium is low and limitation will induce cell death in mammalian cells. Furthermore, removal of CO_2 to prevent a critical accumulation is essential. Variation of CO_2 concentrations is used to control the pH of the medium in the beginning of the culture, when using sodium bicarbonate as the pH buffer. In addition CO_2 is a potentially inhibitory byproduct in cell cultures, especially as it accumulates at high cell densities (Ma et al. 2006; Ozturk 1996). Both, appropriate oxygen supply and stripping of CO_2 are technically difficult, especially at very high cell densities (nowadays up 10^8 cells mL^{-1}) (Godoy-Silva et al. 2010). Techniques have to be applied that avoid shear effects on the cells, especially effects caused by bubbles or foaming, as discussed above.

The cell specific oxygen uptake rate OUR of mammalian cells is an the range of 0.5×10^{-10} to 8×10^{-10} mmol $cell^{-1}$ h^{-1} (Czermak et al. 2009), therefore a cell density of 10^7 cells mL^{-1} would require a volume specific oxygen transfer rate OTR of 0.5–8 mmol L^{-1} h^{-1}. The oxygen transfer rate OTR is given by:

$$\mathrm{OTR} = k_L a(c_L^* - c_L)$$

with k_La : mass transfer coefficient (s^{-1}), c_L: concentration of oxygen in solution (mmol L^{-1}), c_L^* : equilibrium solubility of oxygen – oxygen saturation (mmol L^{-1})

For aeration with air $c_L^* = 0.2$ mmol L^{-1}, for aeration with pure oxygen $c_L^* = 1$ mmol L^{-1}. If the oxygen concentration is controlled at 20 % of air saturation, $c_L = 0.04$ mmol L^{-1}. To support 10^7 cells mL^{-1} by aeration with air would require k_La values between 3 and 50 h^{-1}, aeration with pure oxygen k_La values between 0.5 and 8 h^{-1}.

Methods for oxygen supply in small and lab scale stirred bioreactors are e.g. surface aeration, bubble aeration (sparging), bubble-free membrane aeration. If required, the partial pressure of oxygen in the headspace or the supplied air can be increased. Because of high mass transfer rates and operational simplicity, bubble aeration is the preferred method in the large scale (Aunins and Henzler 1993; Griffiths 2000; Varley and Birch 1999; Godoy-Silva et al. 2010).

In surface aeration mass transfer occurs via the surface of the liquid. According to Henzler and Kauling (1993) k_La depends mainly on the agitation rate. As the later has to be kept low to prevent shear effects, oxygen transfer by surface aeration is low (k_La approx. 0.5 h^{-1}). Therefore surface aeration is sufficient only for low density cultures (2–5*10^6 cells mL^{-1}) in small vessels like T-flasks, roller bottles and bench scale vessels (<1 L), where a relatively large ratio of air-medium interface to volume exists (Ozturk 2014). An exception is the single-use wave

bioreactor, were an appropriate oxygen supply is induced by intensive wave motion on a rocking device (Singh 1999; Eibl and Eibl 2011a, b, see below).

Bubble aeration by direct sparging into the medium has long been regarded as detrimental to the cells due to cell damage caused by bubble disruption and foaming. Especially in small and lab scale stirred bioreactors these effects seemed to be crucial. But as alternative techniques such as surface aeration or bubble-free membrane aeration are not suitable for higher cell densities or large scale, great efforts to optimize bubble aeration systems have been made (Czermak et al. 2009; Ozturk 2014). The detrimental effects of bubbles or foam formation can be overcome by applying low volumetric gas flow rates (0.005–0.1 vvm), and by using serum- and protein-free medium and protecting agents such as Pluronic F68.

Macrospargers with a simple design (pipe, ring) work quite well, even if they generate quite large bubbles in the mm range, and therefore low k_La values (approx. 5–10 h^{-1}) (Czermak et al. 2009). As an increase of k_La by increasing the impeller speed would cause shear induced cell death, a higher mass transfer can be achieved by increasing the oxygen content in the gas. By this even high cell densities can be supplied sufficiently.

Alternatively microspargers (made of ceramic, sintered stainless steel or polymeric materials such as polyethylene) were invented (Varley and Birch 1999; Nehring et al. 2004). Due to the very small bubbles (several hundred μm range), k_La-values in the range or 10–30 h^{-1} are possible (Czermak et al. 2009). As these small bubbles would create more foam if operated continuously, here gas flow (air or oxygen) is often pulsed to prevent foam formation. For larger reactors this problem is less important, as small bubbles might be dissolved completely before reaching the surface. Microbubbles are not well suited for stripping of CO_2, as the small bubbles are saturated with CO_2 very fast. This problem can be overcome by combining a microsparger for oxygen supply and a macrosparger for CO_2 stripping (Ozturk 1996). Despite the advantages of microspargers compared to macrospargers, microspargers are rarely used beyond pilot-scale bioreactors however, due to cGMP-related constraints such as CIP validation, foaming issue, and low efficiency of CO_2-removal (Marks 2003; Mostafa and Gu 2003).

Alternatively bubble-free membrane aeration systems have been developed. Here the gas-liquid-interface is provided by open-pore membranes or diffusion membranes installed in the medium (Aunins and Henzler 1993; Henzler and Kauling 1993; Qi et al. 2003). For microporous membranes the medium is in direct contact with air in the micropores of the membrane (Lehmann et al. 1987; Schneider et al 1995). The air-liquid interface in the pores is controlled by pressure and hydrophobic force. For diffusion membranes oxygen diffuses first from the gas phase into the oxygen soluble membrane (very common: silicone rubber) and then into the culture medium (Frahm et al. 2007). For membrane aeration systems cell damage is usually less of a problem. But as large membrane areas are required (approx. 1–3 m of membrane tube per L reactor) and complex designs for installing the membrane tube in the reactor are required, scale-up is limited. Even if reactors up to approx. 200 L working volume have been successfully operated with

membrane aeration systems (Frahm et al. 2009), this technique is nowadays mainly found in small-scale culture systems (Superspinner, compare Sect. 4.2.2).

Performance Parameters

For design, operation and scale-up, appropriate values for parameters such as volumetric mass transfer coefficient (k_La), volumetric aeration rate, maximal tolerated shear rate, volumetric power input (P/V), impeller tip speed, mixing time (Θ_M) have to be found. These depend on physical system features, e.g. reactor volume, geometrical properties such as vessel height-to-diameter, impeller type and diameter, rotation speed of impeller, type and location of sparger, and on cell related parameters such as cell type (suspendable, adherent), type of medium, cell density etc..

A sufficient volumetric mass transfer coefficient (k_La) required for optimal supply of oxygen and CO_2 removal (see above) can be seen as a "minimal requirement". Higher values than required do not have an advantage, but are not detrimental, if too high impeller speed and shear forces are omitted. Up to lab and pilot scale it is usually no problem to guarantee appropriate k_La-values even for very high cell densities by using advanced aeration techniques including microsparger or by supplying gas with increased oxygen partial pressure. For large scale reactors this problem is more severe. Xing et al. (2009) reported on max. k_La-values of 3.4 h^{-1} for a 5,000 L bioreactor equipped with a macrosparger (pipe sparger). By this, cell densities of max. 7×10^6 cell mL^{-1} could be supported.

The volumetric gas flow rate is usually kept constant as they do not have a significant influence on the bulk mixing, if the impeller is not flooded, which is a rare case for cell culture. An appropriate value for the gas flow rate depends on foam formation etc..

The maximal tolerated shear rate is a "maximal requirement" as too high values are detrimental. It is still under discussion as how to predict a representative shear rate or shear stress in a stirred tank bioreactor. Yang et al. (2007) calculated the shear rate at impeller tip for a Rushton turbine for a fixed impeller speed of 68 min^{-1} on different scales. The shear rate increased from 225 s^{-1} at 3 L to 1,877 s^{-1} at 2,500 L. Within a control experiment at 3 L scale they confirmed that the cells survived at least a shear rate of 1,169 s^{-1}. Nevertheless, this example shows that it still difficult to use this parameter for reactor lay-out and scale-up (see below).

Volumetric power input (P/V), impeller tip speed or mixing time (Θ_M) usually have an optimal range depending on the scale. For the volumetric power input (P/V) and the impeller tip speed too low values result in insufficient mixing, too high values in too high shear forces.

With respect to the mean power input per volume Chisti (2000) suggested to keep this value below ~ 1.000 W m^{-3} to keep shear effects below critical levels. In view of recent references, these values seem to be quite high. Platas et al. reported on optimal values for different lab scale bioreactors in the range of 50–200 W m^{-3}

for the maximal growth rate. Appropriate values for large scale reactors seem to be more at the lower end of this range (Xing et al. 2009), values reported for single-use bioreactors as well (Catapano et al. 2009).

For the impeller tip speed a range of 1–2 m s^{-1} has been suggested in older publications (Fenge and Lüllau 2006). Even if some others postulate that a tip speed of about 2 m s^{-1} is needed to ensure sufficient homogeneity (Ma et al. 2006), recent publications indicate lower optimal tip speeds. Platas et al. (2012) found a maximal cell growth at impeller tip speed in the range of 0.8–1.2 m s^{-1} at least for lab scale reactors. According to Yang et al. (2007) higher values might be acceptable on larger scales.

For the mixing time too high values indicate an insufficient mixing, too low values bear the risk of too hear shear forces. Platas et al. found a maximal cell growth at mixing times in the range of 8–12 s at least for lab scale reactors. On larger scales higher values (around 100 s) have to be expected (Xing et al. 2009).

4.3.2.2 Bubble Columns and Air-Lift Bioreactors

Bubble columns and air-lift reactors present an alternative to stirred reactors, as aeration and mixing is achieved by gas sparging without mechanical agitation (Chmiel 2011). These reactors are structurally very simple; advantages compared to stirred reactors include low capital and energy cost, lack of moving parts, and satisfactory heart- and mass transfer performance, ease of scale-up, low shear characteristics etc..

Bubble columns consists of a cylindrical vessel with height much larger than the diameter, were gas is injected by a sparger located close to the bottom of the column. They typically have no further internal structures. Hydrodynamics and mass-transfer characteristics depend on gas flow rate, sparger design, column diameter and medium properties (e.g. viscosity). Examples for large scale cultivation of mammalian cells have not been reported mainly due to the poor mixing in bubble columns (Varley and Birch 1999).

In air-lift reactors the reactor is subdivided in two connected compartments to improve mixing in aerated columns without mechanical agitation. Gas is sparged in one part only, the “riser”, creating an up-flow of gas/liquid. In the second part, the “downcomer”, liquid is flowing downward due to the density gradient between riser and downcomer. With respect to geometrical configuration, internal or external loop design can be distinguished. In vessels with an internal loop, riser and downcomer sections are split either by a cylindrical draft tube or a vertical baffle. In external loop reactors, riser and downcomer are two separate cylindrical columns. For cultivation of mammalian cells reactor scales up to 5,000 L have been reported (Varley and Birch 1999, http://www.lonza.com/custom-manufacturing/capabilities-overview/our), almost solely internal loop reactors. A single-use air-lift concept up to 50 L is offered as well (http://www.biosciencetechnology.com/product-releases/2013/06/airlift-bioreactor-simplifies-cell-culture-fermentation#.UuAfoYa1JD8).

Air-lift reactors usually provide better mixing than bubble columns, the operation range is much broader. Hydrodynamic behaviour of these reactors depend to some extend on the geometry as well as on gas flow rate, gas hold up and circulation velocity, with the gas flow rate as the only independently controllable variable. For further review of the state-of-the-art compare Varley and Birch (1999). Design criteria are the aspect ratios (height to diameter, approx. 6:1 and 12:1), similar cross-sectional area of riser and downcomer, superficial gas velocities (0.001–0.01 m s^{-1}) etc.

Scale-up of air-lift reactors for cultivation of mammalian cells is still a difficult task, despite extensive studies on hydrodynamics and scale-up in general. This is mainly due to the fact that superficial flow velocities are in most cases well above those tolerable in air-lift reactors for mammalian cell culture due to the high shear sensitivity of the cells. Strategies to scale-up air-lift reactors are generally based on geometric similarity, constant k_La, and either constant superficial gas velocity, constant gas flow rate per unit volume of liquid or constant mixing time (Varley and Birch 1999). Similar to stirred tank reactors, it is not possible to keep all these parameters constant at the same time. As for large scale mammalian cell culture it is very important to maintain mixing times at appropriate levels, Varley and Birch (1999) recommend keeping the mixing times as constant as possible during scale-up.

Even if air-lift reactors are not that widespread as stirred tank reactors, there is a number of successful use of air-lift reactors for mammalian and insect cells (reviewed by Varley and Birch 1999; Fenge and Lüllau 2006). Therefore it can be concluded, that air-lift reactors are a powerful alternative to stirred tank reactors on large scale, provided that sufficient scale-up strategies are available.

4.3.2.3 Wave Mixed Bioreactors

Wave mixed bioreactors can be seen as the initiator for a broad acceptance of single use technology (compare Sect. 4.1.3). The bag reactor intended for suspension culture of cells or cells grown on microcarriers is agitated by a rocking motion and therefore does not need any mechanical agitators inside the single-use bag. The bag is filled partially and placed on a rocker platform, which moves periodically up and down resulting in a one-dimensional oscillatory motion. By this a wave inside the bag is induced. Characteristics of the wave depend on the bag shape/geometry (differs between scales), the rocking angle, the rocking rate, the filling volume and the fluid properties. Oxygen is supplied from the head space via the liquid surface. Due to the intensive motion of the liquid surface appropriate mass transfer coefficients in the range of 10–20 h^{-1} can be realized. Volumes range from 0.1 L up to 500 L working volume.

Alternative concepts for induction of motion are in use, e.g. single-use bioreactors inducing a two-dimensional motion by combining a vertical rocking motion with a horizontal translation, or even a bi-axial translation resulting in a

three-dimensional motion to give the flow a tumbling characteristic. For more details and references see Eibl and Eibl (2011a).

4.3.2.4 Devices for Cell and Product Retention

Even if for suspension reactors *batch, repeated batch* and *fed-batch* are still the preferred mode of operation on industrial scale, a number of continuous perfusion techniques have been successfully applied to increase cell and product concentration as well as time space-yield of the process. An overview on industrial application of perfusion cultures for production of monoclonal antibodies and recombinant proteins is given by Pollock et al. (2013). The main goal of perfusion culture is the long-term continuous cultivation up to several months at very high cell density and high viability, preferably under physiological steady-state conditions for the cells. Due to high volumetric perfusion rates (1–10 volumes per reactor volume per day) the residence time of the product is low, an advantage for fragile, glycosylated proteins (Voisard et al. 2003). The basic principles will be summarized in the following.

Basically, perfusion refers to the continuous exchange of spent medium from the culture broth while retaining the cells in the bioreactor. Retention devices can be either internal or external. External devices allow for an easier exchange of the device upon failure and are advantageous with respect to scale-up, as in some cases this can be done by applying parallel units. The main draw-back of these techniques is the need to use pumps for recycling the cells, which might cause mechanical damage to the cells (especially for fragile cells, cell aggregates or microcarrier cultures) and might hold the risk for contamination.

Retention of cells due to their size can be performed by means of filtration or dialysis. Filtration refers to mechanical separation of solid particles from a fluid by a physical barrier; dialysis is based on diffusion of solutes across a semipermeable membrane. With respect to filtration, the main challenge is to prevent fouling and clogging. Due to the intended very long run times of cell culture processes, solving this problem is essential and decides on acceptance for industrial purposes. Well established in industry are the ATF-technology (Clincke et al. 2013a, b) and spin filters (also called rotor filter or rotating sieve) (Fenge and Lüllau 2006).

Filtration techniques are mainly intended to retain the cells. Products, even high molecular weight proteins, are usually not enriched. Dialysis, a membrane technique as well, allows for simultaneous enrichment of cells and high molecular products. Since the non-porous membrane selectively allows low molecular weight molecules to pass while retaining those with a higher molecular weight and cells, dialysis can effectively be used as a separation process based on size exclusion ("cut-off") (compare Pörtner and Märkl 1998; Frahm et al. 2003). Size of reactor systems applying dialysis technique varies from several mL up to approx. 2 m^3.

Cell retention by density is hampered by the very small density difference between cells and culture medium, as mentioned above. The most relevant techniques are gravity settlers, centrifuges, acoustic filters, and hydrocyclones.

Gravity settlers are technically quite simple and robust, as they do not have any moving parts to damage cells (Fenge and Lüllau 2006; Voisard et al. 2003). The low gravitational retention efficiency can be increased by reducing the distance for cell sedimentation, e.g. by passing the cell suspension through inclined parallel plates in a laminar regime. Cells sediment, settle on the lower plate, and slide down. By this they generate a countercurrent flow enhancing the settling efficiency (Boycott effect). The main drawback, oxygen limitation within the non-aerated settler due to long residence times (approx. 1–2 h), can be overcome by decreasing the temperature within an external retention loop to room temperature or even lower to reduce the metabolic activity of the cells. Settlers up to 3,000 L scale are in use (Pohlscheidt et al. 2013).

In centrifuges a centripetal acceleration is used to separate substances of greater and lesser density. By this the separation efficiency is significantly improved compared to gravitational settling. A number of centrifuges for perfusion cultures have been suggested (reviewed by Fenge and Lüllau 2006; Voisard et al. 2003). The main advantages of centrifuges are (i) no clogging or fouling, (ii) a separation rate controlled by g-force and feed flow rate, and (iii) separation of viable and dead cells by adapting g-force. Critical for industrial application are insufficient robustness and reliability.

In acoustic filters an ultrasonic separation of cells and medium is achieved (Baptista et al. 2013). Basically the particle size is increased by exposing the cell suspension to an acoustic resonance field, where three forces act on the cells: (i) the primary radiation force due to the compressibility and density difference between cells and medium driving the cells toward the antinodes of the resonance field, (ii) the secondary radiation force due to interaction between the cells inducing cell aggregation, and (iii) the Bernoulli force driving cell aggregates to the local maxima of the acoustic velocity amplitude within the velocity antinodes planes. Critical aspects of this technique are a narrow range of operation parameters for optimal cell retention, harvest limited by cell concentration and input power, and difficult scale-up.

Hydrocyclones (Pinto et al. 2008) apply centrifugal forces by injecting a cell suspension tangential to the wall with typical pressure drops of 0.5–4 bar. Concentrated cell suspension exits in the underflow, clarified medium exits in the overflow of the device. For perfusion culture of mammalian cells hydrocyclones are especially attractive because of the small volume, a high separation efficiency, and low shear forces. Critical parameters are scale-up, operation parameters, and lack of available data for design.

For evaluation of cell retention techniques first of all aspects such as the retention capacity and length of operation time in perfusion mode have to be considered. Furthermore simplicity of operation, robustness, scalability, and costs are relevant. The potential of available cell retention techniques for use in large-scale manufacturing processes was discussed at length by Voisard et al. (2003) and Catapano et al. (2009). The retention techniques available today are well established and the number of successful applications on industrial scale is increasing. Nevertheless it seems that the industry still hesitates to apply continuous

perfusion techniques instead of simpler *batch* and *fed-batch* strategies. This is partly due to the fact that the ideal cell retention device does not exist and therefore prediction of performance of a respective retention device for a specific cell line is difficult. As a consequence perfusion culture is still regarded as a technology associated with uncertainty and risks for manufacturing operation. Perfusion cultures are especially suited for unstable, non-growth associated recombinant products.

4.3.3 Scale-Up Considerations

Appropriate design and lay-out of a bioreactor system for production scale is a key issue for successful production of biopharmaceuticals. Usually a new cultivation process is investigated and optimized on a laboratory scale. These data are used to scale-up the process to the final scale. Factors which need to be considered include the size of the production vessel, the required operation parameters such as gas flow rate and the stirrer speed (for stirred tank reactors), required scale-up steps (Catapano et al. 2009). Design and lay-out of operation parameters depend significantly on the scale of the reactor, among others. Whereas cultivations on lab-scale in different types of reactors can be compared by parameters such as volumetric power input, impeller tip speed or mixing time sufficiently (Platas et al. 2012), scale-up is still a problem. Different strategies are used to scale-up a process including specific power input, impeller tip speed, impeller shear rate, specific impeller pumping rate, mixing time (Varley and Birch 1999; Ma et al. 2006; Li et al. 2010). The constant specific power input seems to be widely used as scaling criterion in mammalian cell cultures (Langheinrich and Nienow 1999). It is not possible to maintain all variables constant simultaneously due to restriction in design and configuration of different bioreactors, as shown by Yang e al. (2007). When scaling up a cell culture process from 3- to 2,500-L, constant mixing time resulted in atypical and unpractical high agitation speed (190 rpm), constant tip speed led to atypical and unpractical low impeller speed (9 rpm) in 2,500-L bioreactor. Thus, a compromise had to be made between these extremes. Finally an impeller speed of 65 rpm was used at the 2,500-L scale for the balance of tip speed and mixing time. As it can hardly be tested on the final scale, if the determined parameters are appropriate, control experiments on lab-scale are recommended.

For the production of biopharmaceuticals on large scale a seed train is required to generate an adequate number of cells for inoculation of the production bioreactor (Ozturk 2014). A production process starts with a frozen vial of cells, usually from a cell bank (Birch and Racher 2006). Ozturk (2014) distinguished three stages: seed train expansion, inoculation train and production bioreactor. For seed train expansion culture systems with increasing size and changing configuration are used, e.g. T-flasks, shake flasks, roller bottles, spinner flask, small scale stirred reactors, wave bioreactors. During inoculation train, the cell density is further increased

within a number of bioreactors within increasing volume. The volumes and numbers of the culture systems depend on the required cell number, the minimal required cell density after inoculation and the maximal cell density at the end of exponential growth phase, expressed by the split ratio. A typical seed train starting at lab-scale might be frozen vial from – flasks/roller bottles/bags/ – 20 L – 80 L – 400 L – 2,000 L – 10,000 L. The time required from the frozen vial to the final production scale can be in the range of 3–4 weeks. Development and operation of a seed train is time- and cost-intensive but offers potential for optimization. Frahm (2014) described a method and a protocol for a model based design and optimization of a seed train.

4.4 Bioreactors for Immobilized Cells

4.4.1 Fixed Bed and Fluidized Bed Bioreactors

Fixed bed and fluidized bed bioreactors have gained growing attention in the cultivation of mammalian cells since they allow cultivation of immobilized cells within macroporous carriers (compare Sect. 4.1.2) at a very high cell density. A fixed-bed bioreactor (also referred to as "packed bed bioreactor") consists of a vessel filled or packed with carrier material used as support for immobilization of cells. Whereas in fixed bed bioreactors the stationary bed of carriers is not agitated, in fluidized bed bioreactors the carriers are kept floating ("fluidization"). Both types of bioreactors have shown their potential in the establishment of optimum culture conditions with a wide versatility for many cell culture purposes (reviewed by Fenge and Lüllau 2006; Meuwly et al. 2007; Pörtner et al. 2007; Catapano et al. 2009). Compared to suspension reactors fixed bed and fluidized bed reactors have the following advantages: high volume specific cell density and productivity, low shear rates, simple medium exchange and cell/product separation, productivity on a high level during long-term cultures. Disadvantages are: non homogeneous cell distribution, determination of cell concentration and cell harvest are difficult. In the following some general engineering principles of fixed bed and fluidized bed bioreactors applied for mammalian cell culture will be introduced and examples for cultivation of different types of mammalian cells will be given.

4.4.1.1 Fixed Bed Bioreactors

A number of different design concepts for fixed bed bioreactor systems have been proposed in the literature (Meuwly et al. 2007; Fenge and Lüllau 2006; Pörtner and Platas 2007; Havelange et al. 2012). Only a few systems have been commercialised. The general problem for design and operation of fixed bed bioreactors is to fulfil the

certain, sometimes competing process requirements such as meeting the high demand of oxygen required by the cells growing in a very high cell density, appropriate supply of nutrients and preventing cell damage due to too high shear rates. Almost all fixed-bed bioreactor systems consist basically of a column filled or packed with carrier material used as support, where the cells grow immobilized, and a medium reservoir for conditioning of the culture medium (Meuwly et al. 2007; Catapano et al. 2009). The medium is recirculated in a loop through the fixed bed and back to the reservoir for conditioning, especially for oxygen enrichment, while the cells (adherent or non-adherent) are retained in the fixed-bed. Oxygen supply is often done by simple bubble aeration, as the cells do not get in contact with the bubbles and therefore cell damage due to bubbles are not an issue. Drugmand et al. (2012a, b) suggest a "waterfall"-like oxygenation. In the conditioning reservoir the exhausted, product-containing medium is exchanged batch wise or continuously. Usually further control elements such as sensors for pO_2, pH and temperature are installed in the reservoir as well as for process control. For monitoring cell growth within the bed the use of dielectric spectroscopy has been suggested (Noll and Wandrey 1997; Ducommun et al. 2001; Drugmand et al. 2012a, b).

With respect to scale-up the main problem is the appropriate supply of oxygen. In an axial stream of the medium through the fixed bed (also referred to as "axial-flow") without any further aeration the concentration of oxygen in the medium decreases from the bottom of the bed to the top. The degree of oxygen depletion depends on the number of immobilised cells, the cell specific oxygen consumption rate, the mean flow velocity through the bed (approx. 1 mm s^{-1}, Meuwly et al. 2007) and the height of the bed. The height of an axial-flow fixed bed should be in the range of approx. 10 cm to prevent oxygen limitation in the upper zones of the bed.

A well established concept for scale up of the fixed bed volume is a radial-flow fixed bed (reviewed by Meuwly et al. 2007; Catapano et al. 2009). The medium is pumped from the medium reservoir either through the centre of the cylindrical bed along the radius or vice versa. As the length for oxygen depletion depends on the radius of the bed, the height of the bed can be enlarged without the risk of insufficient oxygen supply. Reactor volumes up to 5 L fixed bed volume have been applied successfully (Pörtner et al. (2007)).

An alternative concept for scale-up was realized in the iCELLis™ fixed bed bioreactor. The reactor is based on an agitation system powered by a proprietary centrifugal-based flow impeller, a waterfall oxygenation of the culture medium and a packed-bed made up of a macroporous carrier (Drugmand et al. 2012a, b). For scale-up not the height, but mainly the diameter is increased. Fixed bed volumes up to 25 L are offered (ATMI).

Due to a low shear stress, immobilization favors the application of serum- or protein-free medium; it allows an easier medium exchange and reduces the steps of down-stream processing. Of special importance is the improved culture stability through immobilization (reviewed by Catapano et al. 2009). With a simple, and easy-to-handle bioreactor design, fixed bed bioreactor systems enable a high volume specific cell density and productivity and a versatile range for production of

biopharmaceuticals over long periods of time (up to several months) (Meuwly et al. 2007; Fenge and Lüllau 2006). Whereas the earlier studies focused on production of biopharmaceuticals as an alternative to suspension bioreactors or hollow fiber systems, new applications can be found in other areas including gene and cell therapy or tissue engineering. Fixed bed bioreactors have been successfully applied for cultivation of hybridoma, Transfectoma, BHK, CHO, CHO, VERO for vaccine production, L293, HeLa, NIH3T3, insect cells, packaging cell lines for production of viral constructs for gene therapy, immortalized and primary hepathocytes, human kidney cells or human melanoma cells, among others (Catapano et al. 2009).

4.4.1.2 Fluidized Bed Bioreactors

Design variants for fluidized bed reactors and applications have been reviewed by Fenge and Lüllau (2006) and Catapano et al. (2009). The general problem for design and operation of a fluidized bed, similar as for fixed bed bioreactors, is to fulfil the certain, sometimes competing process requirements such as meeting the high demand of oxygen required by the cells growing in a very high cell density, appropriate supply of nutrients and maintaining a fluidized stage of the carriers (gravity of the carrier, fluidization velocity). Again, almost all design concepts are based on a loop for medium recirculation, either external or internal. Within this loop oxygen is supplied to achieve a certain oxygen concentration at the entrance of the bed, fresh medium is fed and spend medium harvested. Usually further control elements such as sensors for pO_2, pH and temperature are installed in the loop for process control. In case of an external loop the medium has to be pumped with a high flow rate, which can expose a shear-stress on circulating cells. Furthermore sterilization problems might occur in large scale. A further problem with respect to scale-up of fluidized bed bioreactors is as well the appropriate supply of oxygen. In an up-flow stream of the medium without any further aeration the concentration of oxygen in the medium decreases from the bottom of the bed to the top. Even if the flow velocity in fluidized bed bioreactors is usually higher compared to fixed bed bioreactors, the maximal flow velocity is limited by the gravity of the carrier and therefore the bioreactor height and the scalability is limited. This problem of an oxygen gradient along the height of the bioreactor can be overcome to some extend by integrating a membrane module directly into the fluidized bed (Born et al. 1995). But due to the huge membrane area required for oxygen supply, only small scale systems in the 1 L range have been commercialized (Catapano et al. 2009).

An alternative fluidized bed concept with an internal medium recirculation was developed by the group of Katinger and Blüml (Lundgren and Blüml 1998). The concept comprises two chambers divided by a distributor plate. An internal draft tube for medium recirculation runs through both chambers. Liquid agitated by an impeller in the lower chamber is conveyed via the distributor plate into the upper chamber. Settled microcarriers are fluidized by the hydrodynamic pressure. The bed expands depending on the impeller speed. Culture medium circulates back down

through the draft tube into the lower chamber. Oxygen bubbles are sparged into the culture medium in the carrier-free zone in the upper bioreactor chamber. Coalescent bubbles immediately rise to the surface and only the suspended liquid saturated with microbubbles flows down to the impeller. Fluidized bed volumes between 0.1 and 40 L have been reported (Lundgren and Blüml 1998).

4.4.2 "Hollow-Fiber"-Bioreactors

Hollow fiber reactors consist basically of a bunch of hollow fibers (e.g. cellulose) within a cartridge (Davis 2007). Cells are immobilized in the extracapillary space of the fibers. The fiber cartridge is connected to a medium reservoir, a gas exchanger and a circulation pump. Within this loop probes for monitoring of pH, oxygen or temperature can be installed. The whole set-up can be placed in an incubator. Hollow fiber systems are usually supplied "ready-to-use" for single-use. Therefore the systems are relatively easy and rapid to install and set-up, even at production scale (Stacey and Davis 2007).

Medium is pumped from the reservoir first through a gas exchange cartridge for oxygen supply, then through the intracapillary space, and finally back to the reservoir. As the fibers, typically ultrafiltration membranes with a cut-off about 10 kDa, are permanently perfused with medium enriched with oxygen, low molecular nutrients as well as oxygen pass through the fiber walls into the extracapillary space to the cells manly by diffusion, supplemented by Starling flow (Davis 2007). Low molecular metabolites leave the culture chamber the same way. High molecular compounds (e.g. growth factors or serum) have to be supplied by the medium in the extracapillary space directly to the cells. Usually the requirements for these compounds are lower compared to suspension culture. As the extracapillary space with the cells is not perfused, the cells are not exposed to high shear stress.

Hollow fiber systems are intended to run for long time, up to several months. The medium in the reservoir bottle is changed every 1–2 days depending on the nutrient uptake of the cells. In the extracapillary space the medium is usually not changed at the start of the culture. Later on medium is changed every few days to replenish the high molecular weight growth factors and to harvest high molecular products (e.g. monoclonal antibodies) in very high concentrations (Gramer 1998; Davis and Hanak 1997).

Cell concentrations in the extracapillary space are in the range of 10^8 cells mL^{-1}. Hollow fiber systems are well suited for suspendable cells, but less for adherent cells as the common membrane materials do not support cell attachment. As the cells are entrapped within the fiber cartridge, sampling and determination of cell number and cell viability is difficult.

Increase of the culture volume can hardly be achieved by increasing the length of the cartilage, as this would result in gradients of oxygen, nutrients and toxic metabolites and lead to non-uniform colonization of the extracellular space. To some extend these effects can be minimized by a reversal of the direction of

medium flow in the capillaries or by inducing a mass flow of medium across the fiber walls by an intermittent pressure pulse from the extracapillary space to the intracapillary space. Increasing the diameter is not an option as well due to manufacturing problems. Therefore for increase in scale mostly several hollow fiber cartridges are run in parallel (Davis 2007). Due to the limited scalability, hollow fibers are well suited for production of small quantities of product but less for products required in kg quantities per year. Therefore they are used for production of biopharmaceuticals required in smaller quantities or diagnostic products (Stacey and Davis 2007; Vermasvuori and Hurme 2011). Other successful application can be found in tissue engineering and regenerative medicine (see Sect. 4.5).

Design and lay-out, especially prediction of cell performance within a hollow fiber cartridge is still difficult, as data on cell growth and productivity gained from T-flask or suspension reactors do not correlate with data in high-density hollow fiber cultures. Therefore different approaches have been suggested to scale-down hollow fiber systems for process development (Gramer and Poeschl 1998; Davis 2007).

In summary, advantages can be seen in very high cell densities, high product concentration, lower serum demands (if required), long-term stability, easy handling and low cost, and a higher ratio of product to medium-derived impurities. Disadvantages are: limited scale-up, mass transfer problems/concentration gradients, difficult to determine cell number, proteolytic activity on the product.

4.5 Bioreactor Concepts for Tissue Engineering

The goal of Tissue Engineering is the generation of artificial 3-dimensional (3D) tissues from human or mammalian cells. These tissue constructs should have organ-specific properties with respect to biochemical activity, microstructure, mechanical integrity and bio stability. Applications can be seen in regenerative medicine to restore, maintain or improve tissue function (Meyer 2009; Atala 2011), in substance testing or drug screening (Marx and Sandig 2007) or as disease or tumor models (Stolpe and Toonder 2013; Young 2013).

For generation of 3D-tissue, bioreactor systems are crucial (Pörtner et al. 2005; Wendt et al. 2009; Eibl and Eibl 2009a, b). They allow for a perfused three-dimensional structure, enable reproducible and controlled changes in specific environmental factors, and can provide the technical means to perform controlled studies aimed at understanding specific biological, chemical or physical effects. For later clinical or routine applications, bioreactors allow for a safe and reproducible production of tissue constructs. Therefore bioreactor systems are seen as an advantageous method in terms of low contamination risk, ease of handling and scalability compared to flask culture. With respect to drug screening, bioreactors are capable of efficient, reproducible handling of a large quantity of tissue constructs in parallel (high throughput screening). Despite these promising prospects, to date the goals and expectations of bioreactor development have been fulfilled only to some extent.

Design of bioreactors for three-dimensional tissue constructs is very complex and still at an early stage of development, especially for use in drug screening. In the following important aspects for bioreactor design are summarized and an overview of existing concepts is provided.

With respect to tissue engineering, bioreactors are used for different purposes, e.g. cell proliferation including mass production of stem cells, generation of 3D tissue constructs for clinical application, direct organ support devices or substance testing ("organs-on-a chip"). Besides common requirements such as control of environmental conditions (oxygen tension, pH, temperature, shear stress, aseptic operation), a bioreactor system for Tissue Engineering should allow for automated processing steps. This is essential not only for controlled, reproducible, statistically relevant basic studies, but also for the future routine manufacturing of tissues for clinical application or drug screening. Furthermore, specific key criteria for 3D tissue constructs have to be met. Most tissue culture concepts are based on scaffolds loaded with cells. For this, proliferation of cells, seeding of cells on macroporous scaffolds, nutrient (particularly oxygen) supply within the resulting tissue etc. are important aspects (Wendt et al. 2009). There are numerous studies showing improved tissue properties after mechanical stimulation during tissues culture. Therefore bioreactors applying mechanical or hydrodynamical stimulation have been developed (Pörtner et al. 2009, 2013).

An overview of culture systems and bioreactors used for the main purposes required for the engineering of 3D tissue constructs including cell maintenance, proliferation and tissue formation can be found in Kasper et al. (2009, 2010, 2012). T-flasks, Petri dishes, multiwell plates etc., developed for monolayer culture of adherent cells, are usually used for cell maintenance and proliferation. These systems allow for sterile handling procedures and are easy to use, disposable and low-cost. As they are usually applied within an incubator, environmental parameters including pH, pO2 can hardly be controlled. Furthermore, they require individual handling steps, e.g. for cell seeding, medium exchange or sampling. For large quantities of cells/constructs manual handling has to be replaced by sophisticated robotics to guarantee reproducible results. A further drawback is the limited increase in cell number (approximately 10–20 times during cultivation). If large numbers of cells are required (e.g. mass production of stem cells), several enzymatic subcultivation steps have to be performed, accompanied by an increase in passage number and cell de-differentiation. Alternatively small well-mixed bioreactors (for example, shake flasks, stirred vessels) have been suggested for cell proliferation. Here the mostly adherent cells are grown on microcarriers (see above).

Alternatively, 3D tissue cultures can be grown in fixed bed bioreactors or membrane based bioreactors. As shown above, in fixed bed reactors the cells are immobilized in macroporous carriers or in networks of fibres which are arranged in a column. With respect to tissue engineering, they have been investigated for several applications including the cultivation of liver cells as an extracorporal liver device, proliferation of stem cells, cultivation of cardiovascular cells and

cartilage cells or as an in vitro human placenta model (Court et al. 2003; Kasper et al. 2009, 2010; Weber et al. 2010).

In membrane bioreactors, especially hollow-fibre reactors, cells are cultivated at tissue-like densities in a compartment which contains one or several types of membranes for nutrient and oxygen supply and removal of toxic metabolites. Several examples of modified membrane bioreactors exist for the 3D culture of tissue cells including hepatocytes, skin cells or other human cells (Kasper et al. 2009, 2010).

Most of the culture systems and bioreactors discussed so far, which are all based on systems developed for the cultivation of mammalian cells and adapted to the engineering of 3D tissue constructs, can hardly be used in the generation of implantable tissue constructs (Pörtner et al. 2005). Each type of tissue intended for implantation (for example skin, heart valve, blood vessel, and cartilage) requires a different geometric structure and a specific bioreactor design. Several concepts have been suggested, where tissue-specific inserts for various types of tissue (such as cartilage, skin and bone) can be applied (Kasper et al. 2009, 2010). These can be cultivated with manual medium exchange (e.g. the prominent rotating-wall vessel) or with permanent perfusion (e.g. flow-chambers). In addition to these examples of multipurpose bioreactors, numerous tissue-specific culture systems have been suggested and reviewed with respect to specific tissues. Most of them are custom-made; only very few have been commercialized.

"Organs-on-a-Chip" are intended to simulates the activities, mechanics and physiological response of entire organs and organ systems. Applications can be seen in substance testing or drug screening or as disease or tumor models. Several organs have been simulated by microfluidic devices, e.g. heart, lung, kidney, artery, bone, cartilage, skin and more, either as single organ or several organs combined (Park and Shuler 2003; Huh et al. 2012, 2013; Wagner et al. 2013). Most are designed as 3D cell-culture models, as it is assumed that they exceed 2D culture systems by promoting higher levels of cell differentiation and tissue organization (Huh et al. 2011). The application of microfluidics enables the efficient transport and distribution of nutrients and other soluble cues throughout the viable 3D tissue constructs. The existing devices for "organs-on-chip" vary in design and approach. For routine applications, further standardization, validation and optimization is required.

4.6 Considerations on Bioreactor Selection and Process Design

Design of a process has to consider manifold cell characteristics, purpose of cultivation or scale of the required bioreactor (compare Sect. 4.1). Selection of a suitable type of cell culture bioreactor system and/or an appropriate operation mode (*batch, fed-batch*, and perfusion) is affected by technical, biological, economical

Table 4.1 Criteria relevant for selection of cultivation systems for mammalian cells

Characteristics	Criteria
Cells	Morphology, shear sensitivity, doubling time, adherent or growth in suspension, process parameters (pH, temp., oxygen, CO_2), genetic stability, medium
Product	Stability, quantity, production kinetics
Process	Automation, scale, operation mode (*batch*, *fed-batch*, perfusion), cleaning, inoculum
Administrative	Regulatory affairs and GMP requirements

and regulatory considerations. To some extend guidelines given in the literature can be helpful, but these are mostly qualitatively. Only very few reports compare different reactors system, and in many cases these are intended to promote a certain type of (new) bioreactor (Varley and Birch 1999; Fenge and Lüllau 2006). Important criteria relevant for selection of a cultivation system for mammalian cells are given in Table 4.1 (Catapano et al. 2009).

To start a selection process, it is helpful to address some questions defining the requirement specifications. The factors summarized in Table 4.1 shall give some basic ideas in this respect, but is by far not complete. For further details refer to Fenge and Lüllau (2006) as well as Varley and Birch (1999).

Beside selection of a suitable cultivation system or bioreactor, the process itself has to be optimized. Here strategies such as "Design of experiment", metabolic flux analysis or biomechatronic design can be applied (Chun et al. 2003; Goudar et al. 2006; Mandenius and Björkman 2012). For experimental evaluation of multiple factors screening platforms are in use for high-throughput process optimization (Jordan and Stettler 2014). For optimization of processes established in large scale, scale down models are suggested to mimic and optimize the performance of the large scale process (Warr 2014). Manufacturing platform technologies may help to simplify design of processes with similar characteristics (Vogel et al. 2012).

When it comes to cGMP production of biopharmaceuticals or tissue engineering products, intensive characterisation of the manufacturing process is required (Abu-Absi et al. 2010; del Val et al. 2010; Horvath et al. 2010; Chen 2014; Marasco et al. 2014). For cell culture manufacturing processes, "Quality by design" (PbD) principles have been developed to meet the respective guidelines (Marasco et al. 2014). QbD principles are applied during the whole lifecycle of a product from process design, process definition, and process characterization to process validation and continued process verification to assure the product quality within a registered design space. QbD principles include design and understanding of product characteristics, their linkage to patient safety and clinical efficacy, characterisation of interaction between critical product quality attributes and the manufacturing process, and control strategy for the later.

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Chapter 5
Mass Transfer and Mixing Across the Scales in Animal Cell Culture

Alvin W. Nienow

Abstract As in all aerobic bioprocesses, the oxygen transfer rate is a critical parameter that needs to be met for the satisfactory cultivation of animal cells. Oxygen in solution has to be continuously provided because of its low solubility in aqueous solution which is continually being utilised by the growing cells (at the current time, reaching a cell density of ~10^7 cells mL^{-1} in bioreactors up to 25 m^3). Such a process requires a certain specific power input (or mean specific energy dissipation rate) to be used, which also has to provide a satisfactory level of other mixing parameters. However, though for animal cells, the specific power required is relatively low (typically < ~0.05 W/kg), because of the lack of a cell wall, there is still a perception that 'shear damage' may occur. Another aspect of oxygen mass transfer is the need to provide a continual inflow of oxygen into the bioreactor, typically by sparging (rates < ~0.01 vvm). However, especially at large scale, sparging may lead to excessive foaming requiring the use of antifoam to control it; and bursting bubbles damage cells unless protective agents are used. Both these additives negatively affect the rate of mass transfer. Another critical aspect directly linked to oxygen transfer is the molar equivalent production of carbon dioxide by the cells. Stripping of CO_2 is essential to prevent physiologically damaging levels of pCO_2 being produced as well as issues associated with pH and osmolality. Essentially, oxygen transfer, carbon dioxide stripping and mixing parameters are all intimately connected and need to be considered in an integrated way. In this chapter, oxygen mass transfer and carbon dioxide stripping theory and practice are considered in detail including non-stirred and single use bioreactors. In addition, other mixing parameters such as blending, heat transfer and scale-up/scale-down in stirred bioreactors are briefly considered as these are used commercially from the 15 mL ambr™ to the 25 m^3 scale. Because the perceived 'shear-sensitivity' of animal cells to stirring and bubbling has impacted on how aeration and stripping have been addressed in practice, these topics too are worthy of consideration in any integrated approach. Finally, some recommendations regarding sparger selection and impeller choice are also made.

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5.1 Introduction

Stirred bioreactors have become the bioreactor of choice for most animal cell culture processes and with the growing popularity of the microbioreactor, ambr™ (Fig. 5.1 (TAP Biosystems, UK)) for clone selection and process optimization, etc., at all scales for both sterilisable and single use bioreactors. Though other single use bioreactors have also entered the field, particularly the wave-type, many of the principles by which cells are grown successfully in them are the same. Therefore this chapter will concentrate on mass transfer and mixing in stirred bioreactors; that is bioreactors in which rotating stirrers are present as the main energy source for achieving all the different processes that constitute mixing. Mixing involves a range of physical phenomena that are dependent on the rate of energy input by the rotating impellers such as homogenizing (blending) the liquid phase, achieving adequate heat transfer and dispersing sparged air into smaller bubbles thereby ensuring adequate oxygen transfer into the media and CO_2 stripping from it. Typically with aerobic processes, it is the rate of oxygen transfer that sets the specific power input (equivalent to the mean specific energy input rate), which is then dissipated in the bioreactor. Therefore, it is a major determinant of all the mixing processes occurring in it.

The special feature of animal cell culture because the cells lack a wall is the perception that this specific energy dissipation rate may damage the cells by

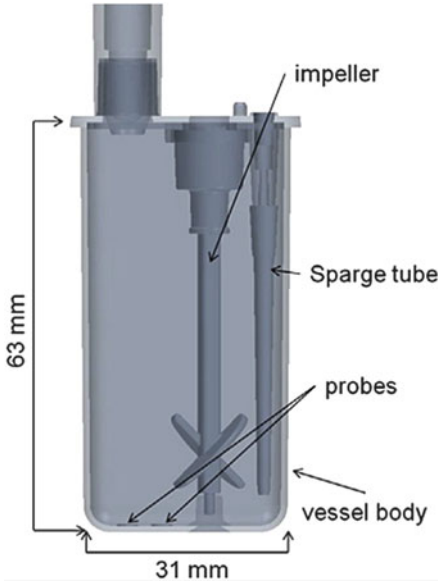

Fig. 5.1 15 mL ambr™ stirred bioreactor (depth 18 mm) (Adapted from Nienow et al. 2013a)

Table 5.1 Physical phenomena in stirred bioreactors for animal cell culture important for analyzing mass transfer and mixing across the scales

(a) Reynolds number, $\rho ND^2/\mu$ to establish flow regime
(b) Mass transfer coefficients, k_La
(c) Unaerated power draw, P (W) [or mean specific energy dissipation rate, $\overline{\varepsilon}_T$ (W/kg) or specific power, $P/\rho V$ (W/kg)]
(d) Aerated power draw, P_g (W) or specific power, $P_g/\rho V$ (W/kg)
(e) Local specific energy dissipation rate, ε_T (W/kg) including the maximum near the impeller $(\varepsilon_T)_{\max}$ (W/kg)
(f) Liquid-phase mixing time, θ_m (s)
(g) Gas-phase mixing (plug flow or back-mixed)
(h) Flooding–loading transition

inhibiting growth and/or productivity or changing the quality of the product. An integrated approach to mass transfer and mixing in cell culture must also include air sparging and dispersion which is essential for meeting the oxygen demand at the high cell densities now being produced (10^7 cells mL^{-1} or more); and again the possibility of damage to cells from sparging (see Table 5.1).

Since mixing and mass transfer are physical phenomena, they can initially be studied without the complexity of actually growing cells. The type of information needed is also listed in Table 5.1 and it is important to know how changes of scale affect these parameters too. For animal cell culture, since the oxygen demand is relatively low compared to bacterial fermentations even at the current cell densities being achieved, the rate of specific energy input and dissipation is also low. An important fluid dynamic consideration which is critical for all the parameters in Table 5.1 is the Reynolds number, Re, defined as

$$Re = \rho ND^2/\mu \tag{5.1}$$

For $Re > \sim 10^4$, the flow in a bioreactor is turbulent. Therefore, as shown below, in most bioreactors for growing animal cells, where the physical properties of the media are similar to water, from the bench scale upwards in scale, the flow is turbulent. Thus, the phenomena in Table 5.1 need to be obtained under these conditions. It also important to know how the cells respond to turbulent flow under the agitation and sparging conditions typical of bioreactors (Nienow 2014).

Since this chapter is particularly concerned with mass transfer, it will be considered in detail first. However, since mass transfer depends on the mean specific energy dissipation and sparge rate, both of which affect the various mixing topics in Table 5.1 and give rise to concern regarding hydrodynamic stress on cells, these aspects are also considered somewhat briefly. Finally, since many of the data obtained at smaller less expensive scales of operation are then used in the design and operation of commercial cell culture bioreactors, consideration of scale-up/scale down is included again briefly for completeness.

5.2 Oxygen Mass Transfer

To meet the oxygen demand of the cells (*OD*), oxygen has to be continuously supplied because of its limited solubility in the media. In the case of animal cells, the specific oxygen demand (*SOD*), which is independent of scale, is low (typical values of about 10^{-16} to 10^{-17} mol s^{-1} $cell^{-1}$ (Nienow et al. 1996; Xing et al. 2009) for CHO cells) and though cell density, *X*, has steadily increased over the years to about 10^7 cells mL^{-1}, relative to many other organisms, the overall $OD = (SOD.X)$ is also low. Provided the cell density achieved is the same across the scales, then so is the *OD*. This low *OD* means that the oxygen transfer rate required is low and therefore because it is linked to the specific power input from the agitator when aerated, $P_g/\rho V$, is also typically low ($< \sim 0.075$ W kg^{-1}) as is the sparge rate ($< \sim 0.01$ vvm) (Nienow 2006). These factors impact on the way the basic mass transfer model is modified in order to calculate the mass transfer coefficient from the basic mass transfer equation which will now be addressed.

5.2.1 Basic Oxygen Mass Transfer Concepts and Equations

Figure 5.2 shows the steps by which oxygen passes from a bubble at a partial pressure p_g (partial pressure of oxygen in air, oxygen-enriched air or pure oxygen also allowing for back pressure) to the animal cell. The steps consist of transport through the gas film inside the bubble, across the bubble-liquid interface, through the liquid film around the bubble, across the well-mixed bulk liquid (media plus product in solution) through the liquid film around the cell where it is utilized within the cell. Each step offers a resistance to oxygen transfer; and each step itself is dependent on the mass transfer coefficient for that step, the area available over which mass transfer can occur and the relevant driving force. For very small entities

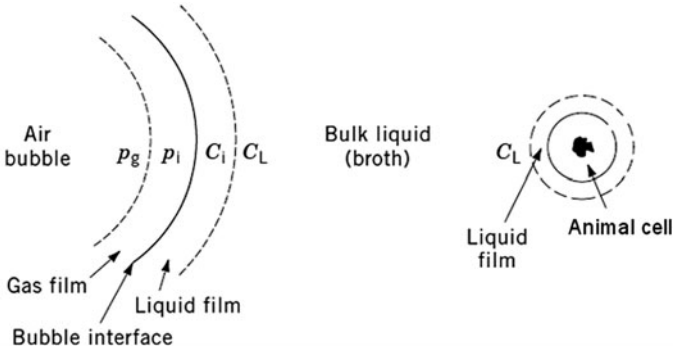

Fig. 5.2 Oxygen mass transfer steps

like animal cells, the mass transfer coefficient is very high (Nienow 1997a) and the specific surface area, a_P (both α 1/particle size) is very large so that step is very fast.

At the gas liquid interface, the area is the same for both the gas film and the liquid film but the mass transfer coefficient increases with an increase in the diffusion coefficient. Since the latter is much higher for the gas phase than for the liquid, the rate limiting step is in the liquid film. In addition, as a result, $p_g \approx p_i$. In addition for all mass transfer processes, it is assumed that equilibrium exists at the interface between the two phases. This assumption implies that, at the interface, the concentration of the gas in the liquid, C_i, is equal to its solubility at its partial pressure in the gas phase, p_i. Since, for sparingly soluble gases such as oxygen, there is a direct proportionality between the two,

$$p_i = HC_i \tag{5.2}$$

where H is the Henry's law constant.

The rate of mass transfer, J, at the gas liquid-interface is then assumed to be proportional to the concentration differences existing within each phase, the surface area between the phases, A, and a coefficient (the gas or liquid film mass transfer coefficient, k_g or k_L, respectively) which relates the three. Thus

$$J = k_g A\left(p_g - p_i\right) = k_L A(C_i - C_L) \tag{5.3}$$

and

$$C_i = p_i/H = p_g/H = C_g^* \tag{5.4}$$

where $C_g{}^*$ is the solubility of oxygen in the media that is in equilibrium with the gas phase partial pressure of oxygen. Thus the aeration rate per unit volume of bioreactor, N, is given by

$$N = J/V = k_L(A/V)\left(C_g^* - C_L\right) = k_L a\left(C_g^* - C_L\right) \tag{5.5}$$

where a is the specific area of bubbles Thus, for satisfactory operation, the maximum oxygen demand of the cells (OD_{max}) must be met and it is related to the maximum cell concentration (X_{max}) by

$$OD_{\max} = SOD.\mathrm{X}_{\max} \tag{5.6}$$

Thus, for stable operation, OD (or Oxygen Uptake Rate, OUR) needs to be met by the oxygen transfer rate, OTR. Thus,

$$OD_{max} = OUR_{max} = OTR = k_L a\left(C_g{}^* - C_L\right) = k_L a.\Delta C_L \tag{5.7}$$

For satisfactory operation, $C_L > (C_L)_{crit}$ where $(C_L)_{crit}$ is the critical oxygen concentration below which the performance of the bioreactor begins to deteriorate

whilst above it, the performance is zero order with respect to dissolve oxygen concentration, i. e. independent of it. It may in some cases, need to be below some upper level that inhibits growth. Neither the upper or lower levels have been well established for animal cells though values between 5 % and 95 % of saturation with respect to oxygen in air (between 5 % and 95 % dO_2) have been reported not to have any impact on cell growth (Nienow 2006). Nevertheless, it is more common to try to control dO_2 in the range 30–50 % of saturation.

C_g^* depends on the partial pressure of oxygen in the gas phase, p_g and this can be related to the total pressure, P_g from Dalton's law of partial pressures

$$p_g = P_g y \tag{5.8}$$

where y is the mole (volume) fraction of oxygen in the gas phase and P_g is the total pressure, i. e. back pressure plus static head. C_g* is also a function of the liquid composition (and is lower in solutions containing electrolytes than in pure water) and reduces with increasing temperature. Thus the driving force for mass transfer can be increased by enhancing C_g*; and lowering C_L provided it is $> C_{crit}$. However, in a large scale bioreactors (which may be up to 25 m^3) where circulation times of the liquid are sufficient for significant oxygen depletion by the respiring cells (Sweere et al. 1987), the average value may need to be kept well above C_{crit} so that local values below it can be avoided. Hence, the choice of values in the range 30–50 %.

Nevertheless, in a stirred bioreactor, the liquid is generally considered well-mixed, i. e. C_L is spatially constant. This is a reasonable assumption for the liquid phase in animal cell culture except at the larger scales. The measurement of the local concentration is done by a polarographic electrode and the reading obtained is dependent on the velocity over the probe. Since the velocity varies so much, establishing the exact concentration field is generally not possible. Assumptions also have to be made regarding the mixing of the gas phase. It may be well mixed so that

$$p_g = constant = \left(p_g\right)_{out} \tag{5.9}$$

where $(p_g)_{out}$ is the partial pressure of oxygen in the exit gas and

$$C_g^* = \left(p_g\right)_{out}/H \tag{5.10}$$

On the other hand, for ease of determining k_La from experimental data, it is often assumed that the gas is well mixed and $p_g = (p_g)_{in}$, i.e. no oxygen is utilized. This is the so-called no-depletion model. In this case,

$$C_g^* = \left(p_g\right)_{in}/H \tag{5.11}$$

For larger-scale bioreactors (Pedersen 1997), the gas phase is generally considered as being in plug flow, so that a log mean value of driving force is obtained, ΔC_{log}:

$$\Delta C_{ln} = \Delta C_{in} - \Delta C_{out}/\ln(\Delta C_{in}/\Delta C_{out}) \tag{5.12}$$

where

$$\Delta C_{in} = \left(p_g\right)_{in}/H - C_L \tag{5.13}$$

and

$$\Delta C_{out} = \left(p_g\right)_{out}/H - C_L \tag{5.14}$$

The assumption made is not very important in cell culture except at large scale and high cell densities because the amount of oxygen removed compared to that introduced is quite small. On the other hand it is very important in high oxygen demanding bacterial and mycelial fermentations.

5.2.2 *The Volumetric Mass-Transfer Coefficient, k_La*

In bioreactors with pipe or ring spargers, the bubble size generated which controls a and the mass transfer coefficient, k_L both are largely dominated by bubble break-up in the impeller region. Because of this close connection with agitation and the difficulty of linking either to fundamental fluid dynamic phenomena, there is little to be gained by separating them in this article. However, both are dependent on agitation intensity usually best considered as specific energy dissipation rate, ε_T and significantly, liquid composition. In general, both solutions and suspensions reduce the mobility of the bubble interface thereby reducing the ease with which oxygen can pass across the interface (Davies 1972; Prins and van't Riet 1987; Sieblist et al. 2013) and, consequently, the value of k_L. However, electrolytes and alcohols also reduce the mean bubble size, usually denoted as d_{32}, the Sauter mean diameter. The latter is the mean bubble size giving the same volume/surface area ratio as the whole gas hold-up in the bioreactor, ε_G given by

$$\varepsilon_G = (H_G - H)/H_G \tag{5.15}$$

where H_G is the height of liquid in the bioreactor during aeration and H is the height without aeration. Thus, the bubble size, hold-up and the specific interfacial area between the two phases, a, are related by

$$d_{32} = 6\varepsilon_G / a \tag{5.16}$$

Clearly, smaller bubbles individually have a higher specific surface area but they also tend to circulate more with the liquid motion thus giving a higher hold up with both factors increasing a and thus $k_L a$.

These two opposing effects (the additive lowering k_L but enhancing a) make the prediction of $k_L a$ very difficult. Thus, in the media for animal cell culture, Pluronic F68 has to be present if sparged aeration is employed in order to prevent fluid dynamic stress from bursting bubbles from damaging the cells (Nienow 2006); and with pipe or ring spargers, it lowers k_L by a factor of 2–3 (Sieblist et al. 2013). It also caused a reduction in $k_L a$ with sinter-type (Sieblist et al. 2013). Antifoam has a similar effect in media (Lavery and Nienow 1987; Morao et al. 1999) and during actual cultivation when it is often essential in order to prevent excessive foaming (Mostafa and Gu 2003).

On the other hand, electrolytes and alcohols both lead to significant reductions in bubble size. In some ways, this similarity is surprising since some theoretical correlations suggest that bubble size is reduced by reducing surface tension. However, this theoretical approach assumes that the bubble size is dependent only on break-up whilst in fact the equilibrium size depends on the competing mechanisms of break-up and coalescence. Thus, even though the surface tension of salt solutions is slightly higher than water and the presence of alcohols lowers it by a factor of about 2, in both cases, bubble size is smaller than in water. It is the way the electrolyte ions or the hydrophilic part of the alcohol align themselves at the bubble-solute interface which causes electrostatic surface charges and these in turn hinder coalescence. In that case, the combined effect is an increase in $k_L a$ (Machon et al. 1997; Hu et al. 2007).

The main reason for this increase is because when the bubbles are smaller, bubbles circulate more easily which leads to a larger hold-up, ε_G with increasing agitation intensity and hence specific surface area, a (Eq. 5.15). Since agitation only has a weak impact on bubble size in sparged systems (Takahashi and Nienow 1991) and there is a strong physical linkage between bubble size and hold up, ε_G and $k_L a$ are often related to agitation and aeration rate in similar ways. However, exceptions to this interrelationship have been reported (Martin et al. 1994) and therefore it should be treated with care. For example, if a certain agitator produces a higher hold-up without a commensurate increase in $k_L a$, it represents a reduction in performance because productivity is linked to the volume of broth in the fermenter.

Finally, it should be note that $k_L a$ is temperature dependent, the relationship being:

$$(k_L a)_T = (k_L a)_{20} \alpha^{(t-20)} \tag{5.17}$$

where $(k_L a)_{20}$ is the value at 20 °C, $\alpha \approx 1.022$, and t is the temperature, °C; i.e. $k_L a$ increases by ~ 2.5 %/C.

5.2.3 The Measurement of k_La

There are two main methods for measuring k_La, the unsteady-state and the steady-state method. Each has different advantages and weaknesses and both may give inaccurate results if the polarographic probe or fluorescent optical patch is not in a region of high liquid velocity so that the local dO_2 at the surface of the probe is not depleted. If the velocity is low, erroneous low dO_2 readings may be obtained.

5.2.3.1 The Unsteady-State (Dynamic) Method

The unsteady-state (also known as dynamic) technique is the most common because at first sight it appears very simple. Basically, the level of dissolved oxygen is first reduced to zero (or close to it), either by bubbling through nitrogen or by adding sodium sulfite (van't Riet 1979). Then, after switching to air sparging, the increase in dissolved oxygen concentration as a function of time is followed using a polarographic oxygen electrode or more recently for animal cell culture, a fluorescent dissolved oxygen probe of the type made by Precision Sensing GmbH, Germany (Nienow et al. 2013a; Sieblist et al. 2013).

However, potentially, it is very difficult to get accurate k_La data using the dynamic technique. Firstly, one of the assumptions set out earlier about the extent of gas-phase mixing has to be made and the choice can be very significant, giving when k_La values are high values differing by an order of magnitude for the same raw data depending on the assumption made (Linek et al. 1987; Nienow 2003a). Secondly, the electrode or patch response time also has to be relatively fast so that

$$\tau_p << 1/5k_La \tag{5.18}$$

(van't Riet 1979) if a correction for the true dO_2 compared to that on the probe is to be neglected. At high k_La as found in many bacterial fermentations, correction is nearly always required. Finally, the assumption that the liquid phase is well mixed (i.e. the dO_2 is the same at all locations) is often not satisfied especially at large scale and high k_La values. In fact, certain papers have ignored this requirement and where oxygen electrodes have given different reading at various locations have used the data to give local k_La values (Lu et al. 2002). Such values are completely erroneous, of course (Nienow 2003b). To get local k_La values experimentally, the liquid phase mixing itself has to be modeled by subdividing it into one or more well-mixed regions associated with different impellers and the exchange flow rate between them must be estimated (Vasconcelos et al. 1997).

Fortunately, these problems associated with the unsteady, dynamic technique are greatly reduced for animal cell culture. The maximum k_La values required to satisfy the oxygen demand of cell cultures up to 10^7 cells/ml is about 15 h^{-1} and even though the sparge rate is low (~0.01 vvm), the depletion of the gas phase is quite small, so the no depletion model can be used with a satisfactory degree of accuracy.

In addition, the response time of electrodes or patches is $<< 1/5k_La$ and so it can be neglected. Therefore, the basic equation that needs to be solved is

$$OTR = dc_L dt = -k_L a.\left(C_g^* - C_L\right) \tag{5.19}$$

The no-depletion model allows this to be simply integrated because C_g^* can be assumed constant in equilibrium with the inlet gas composition at all times. This gives

$$k_L a(t - t_0) = \ln\left(C_g^* - C_L / C_g^* - C_{L,0}\right) \tag{5.20}$$

where the subscript 0 refers to the time at which the initial liquid phase dO_2 for the experiment was measured or utilized for the calculation of k_La. Expressing this equation in terms of the % dissolved oxygen, $X\%$, concentration from the measurement gives

$$k_L a = \ln(100 - X/100 - X_0)/\Delta t \tag{5.21}$$

k_La is then usually obtained by expressing the results on a semi-log plot using data from around 20 % to 80 % dO_2. If the dO_2 is reduced to zero, the equation can be rearranged to give

$$X = 100 - \exp[-t/(1/k_L a)] \tag{5.22}$$

From here it can be seen that when $t = 1/k_La$, $X = 63$ %. This method gives a very quick way of obtaining a ball-park figure for k_La.

As already pointed out, k_La is very sensitive to the precise composition of the liquid phase. Thus, the k_La of distilled water after reducing dO_2 to zero by nitrogen degassing is significantly less than that obtained if sulfite deoxygenation is used. This difference occurs because the sulfite acts as a coalescence inhibitor, greatly reducing the bubble size and enhancing hold-up, and therefore increasing a and k_La. Even the k_La in an actual cell culture is generally not identical to that obtained in any cocktail of chemicals which aim to mimic it such as the actual culture media including Pluronic F68 plus antifoam. The latter is commonly used and is the best approximation available. However, the dynamic technique can be adapted to work on an actual cultivation (Bandyopadhyoy et al. 1967; Langheinrich et al. 2002) which represents a significant advantage for the dynamic method.

5.2.3.2 The Steady State Technique

In the steady state method, Eq. 5.5 is utilized. One method for obtaining the *OUR* is to use a real culture and base it on the difference between the air concentration in the inlet air and that in the exit; and on the airflow rate. Provided the fall of oxygen

concentration is sufficient, this approach also enables the change of k_La with time and agitation conditions during the fermentation to be monitored (Albaek et al. 2011). However, for animal cell culture, the difference in oxygen concentration between inlet and outlet is usually insufficient to give accurate results. Another approach is to use a suitable mimic media plus catalase as a catalyst to break down hydrogen peroxide which is pumped into the bioreactor. Thus oxygen is generated in solution and oxygen is in fact stripped from the fluid (Cooke et al. 1991; Gezork et al. 2001). For both techniques assumptions have to be made about Henry's law constant, H which is required in order to obtain C_g^*; H, like k_La, is also rather sensitive to composition. To the author's knowledge, the hydrogen peroxide technique has not been used for cell culture.

5.2.4 Correlations for Calculating k_La

5.2.4.1 Stirred Bioreactors

There are a very large number of correlations available in the literature for estimating k_La for sparged, stirred systems but the two equations developed from a survey by Van't Riet (1979) are still commonly used as bench marks. The equations are of the form,

$$k_La = A(\bar{\varepsilon}_T)_g^{\alpha}(v_S)^{\beta} \tag{5.23}$$

where $(\bar{\varepsilon}_T)_g$ is the mean specific energy dissipation rate, W/kg, (though it is also often expressed as kW/m^3, the two being numerically equal in media of density = 1,000 kg/m^3) from agitation and sparging and v_S is the superficial gas velocity (numerically equal to the volumetric sparged flow rate, Q_G (m^3/s) divided by the cross-sectional area of the bioreactor). Critically, since this is a dimensional equation, the numerical value of A is dependent on the units used to express the parameters in Eq. 5.23. In addition, as already noted, since k_La is very sensitive to composition, A also depends on the properties of the fluid present in the bioreactor. With very viscous broths as found in mycelial fermentations for example, k_La falls significantly and a viscosity term with a negative exponent is included in Eq. 5.23. For the small variations in viscosity associated with media composition, anti-foam and Pluronic F68, any impact on k_La is taken into account in the constant A.

Van't Riet (1979) found that he could correlate a large amount of literature data (itself based on the 'no-depletion' assumption) in two equations of the form shown in Eq. 5.23. The first, for coalescing systems (mainly based on literature studies with water) gave $A = 2.6 \times 10^{-2}$, $\alpha = 0.4$ and $\beta = 0.5$ when the units of k_La are s^{-1}, of $(\bar{\varepsilon}_T)_g$, W/m^3 and of v_S, m/s. For non-coalescing systems (mainly based on electrolyte solutions) which have smaller bubbles, generally leading to higher k_La values compared to water, the constants $A = 2 \times 10^{-3}$, $\alpha = 0.7$ and $\beta = 0.2$. The

dimensional nature of this equation makes it somewhat unsatisfactory especially the impact of the units used for the various parameters but the most important points are that it was found to apply regardless of the impeller type and independent of scale.

The applicability of an equation of the form of Eq. 5.23 for stirred animal cell culture bioreactors was shown by Hoeks et al. (2004) for k_La in culture media across the scales for 0.012, 0.5 and 20 m^3 bioreactors (Fig. 5.3). Langheinrich et al. (2002) showed similar k_La values at the same $(\bar{\varepsilon}_T)_g$ and superficial air velocity during actual cell culture in 8 and 0.1 m^3 bioreactors. In addition, in water, good agreement (with the appropriate constants) with the Van't Riet correlation for two different impeller types, even though the values of $(\bar{\varepsilon}_T)_g$ and v_S used to give the original equation were an order of magnitude or more greater than in animal cell culture. In order to do so, Langheinrich et al. (2002) found that, because of the low specific energy dissipation rates associated with the agitation, inclusion of the specific energy dissipation rate from sparging ($=gv_S$ in W/kg) was particularly important. Lavery and Nienow (1987) also found the Van't Riet equation for water fitted their bench scale water data well.

A recent correlation for large scale k_La values measured by the dynamic technique was given by Xing et al. (2009). They mimicked a media (which included sodium chloride, sodium bicarbonate and 1 g/L Pluronic F68) used by Bristol-Myers Squibb for Chinese hamster ovary (CHO) cell culture at 3–4.4 m^3 volume with a pipe sparger in a 5 m^3 bioreactor. The equation was

$$k_La = 0.075\left(P_g/V\right)^{0,47} v_S^{0.8} \tag{5.24}$$

where k_La is in s^{-1}, P_g/V in W m^{-3} and v_S in m s^{-1} Since Eq. 5.23 was obtained under conditions that closely mimic those used during large-scale culture, it may be a good one to use (with caution) for such cases. On the other hand, the air flow rate used was surprisingly low (0.0013 vvm) which may explain why the exponent on v_S was so high. It is also interesting to note that P_g/V was rather high (up to 125 W m^{-3}) and the maximum k_La, rather low (<3.5 h^{-1}).

At the other size extreme, Nienow et al. (2013a) have measured k_La, again using the dynamic method, in medium plus Pluronic F68 and antifoam (as well as water)

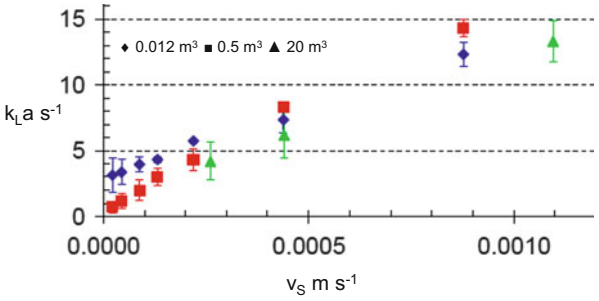

Fig. 5.3 k_La as a function of superficial gas velocity in cell culture bioreactors at three scales at constant mean specific energy dissipation rates (Adapted from Hoeks et al. 2004)

in the 15 mL ambr^{TM} bioreactor (see Fig. 5.1). They found the values for both compositions surprisingly similar and for the mimicking mixture

$$k_La = 1.74\left(P_g/V\right)\left(Q_G\right)^{0.15} \tag{5.25}$$

where k_La is in h^{-1}, P_g/V is in W m^{-3} and Q_G is in mL min^{-1}. Here, the vvm was very high (0.07 vvm) and the exponent on flow rate, low. However, because of the small scale, v_S was very low and therefore a high P/V (up to ~500 W m^{-3}) was required to get k_La values up to 10 h^{-1}. These large variations in the flow rate expressed in different ways and in consequence specific power input/energy dissipation rate are a good example of the changes which take place when the variation of scales is very significant. Nevertheless, all the k_La values in the ambr^{TM} were close to those predicted from the correlation of Van't Riet for coalescing (water) conditions, probably because the increase expected due to the presence of salts in the media are balanced by the reduction associated with antifoam and Pluronic. The use of the ambr^{TM} in scale down studies is discussed again later in Sect. 5.9.

These correlations are not generally applicable when using sparging devices which control the bubble sizes, often called fritted spargers (Sieblist et al. 2010). Frits can be composed of Teflon, stainless steel, glass and other materials. Most often they are formed from small solid particles of the order of tens to a few hundred microns (often spheres) that are pressed into a shape and then sintered to form a stable porous wall. Usually, the sintered frits are closed tube elements or flat membranes characterized by their mean pore size, which depends on the size of the solid particles used to build the frit and the associated sintering process. As the solid material in the frit cannot be arranged regularly, the pore size distribution is rather broad so that the bubble sizes produced by them and therefore their mass transfer performance is somewhat variable from frit to frit of the same nominal size (Sieblist et al. 2010).

Mustafa and Gu (2003) using sparge stones found k_La values with a 10 μm sparge stone in a 1 m^3 bioreactor with antifoam were 5–10 h^{-1}, approximately double that with an open pipe (but without antifoam), whilst at the 1.5 L scale, the sparge stone gave k_La values without antifoam up to 180 h^{-1} when the open gave values up to ~7.5 h^{-1}. Because the bubble size is controlled by the sparger, they found little increase in k_La with increasing impeller speed but an approximately proportional increase with increasing air flow rate. Sieblest et al. (2013) using sintered metal spargers measured bubble size of about 7 mm with a ring sparger and about 3 mm with a 47–100 μm metal sparger whilst photos of bubbles from a 25–47 μm one showed that they were very much smaller.

Clearly, the functionality found with such spargers is very different to those with open pipe and ring spargers and very dependent on the pore size. The small bubbles also are prone to cause foaming and Mustafa and Gu (2003) had to use antifoam at the 1 m^3 scale to prevent it at 0.002 vvm whilst foaming did not occur at 0.01 vvm with an open pipe sparger. Overall, the use of sintered spargers or sparge stones at the small scale leads to high k_La values at very low gas flow rates and agitation

intensities. As reported by Xing et al. (2009), this can lead to unsatisfactory designs at the large scale as such spargers lead to foaming; and inadequate agitation intensities with other sparger types lead to insufficient oxygen transfer. As discussed below, CO_2 stripping is also a potential issue.

For stirred single-use bioreactors (SUBs) with open pipe or ring spargers, the same type of functionality as that illustrated by the Van't Riet equation applies. Energy needs to be dissipated and air needs to be sparged if satisfactory mass transfer is to be achieved. The real question is, are these parameters large enough to achieve the k_La required for the cell density that could potentially be achieved? If yes, then the SUB will be satisfactory from an oxygen transfer perspective. If no, then the cell density achieved will be less than the maximum achievable and the performance less than optimum. If the oxygen demand is met by using a porous sparger, then the other issues arise.

5.2.4.2 Headspace Aeration and Shaken Bioreactors

If energy is imparted by rocking, rotation or shaking, then aeration occurs through the top surface as it does with headspace aeration in stirred bioreactors. For stirred bioreactors, $k_La \propto N^{0.6} \propto (\bar{\varepsilon}_T)^{0.2}$; and it is also improved by placing the impeller close to the top surface. The use of dual impellers is therefore helpful and effective (Lavery and Nienow 1987). Nevertheless, the headspace k_La is only about 50 % of the sparged one at the bench scale. Similarly with the use of rocking or shaking motion, if it is vigorous enough (the energy input is sufficient to provide high enough velocities at the gas liquid interface or ingest enough bubbles by the action of waves), the k_La will be adequate (Oosterhuis et al. 2013) (which it is at the shake flask (Anderlei and Büchs 2001), spin tube (de Jesus et al. 2004) and microwell scale (Hermann et al. 2003; Betts et al. 2014) In general, unless the horizontal area is increased relative to the depth of the bioreactor when using headspace aeration, the specific area available for mass transfer falls with increasing scale ($\propto$ (1/ linear dimension for geometrically similar configurations)). Hence, k_La should also fall, which makes the claimed performance for some large scale orbitally shaken bioreactors hard to understand (De Jesus and Wurm 2013). This change with scale increases the need to enrich the air with oxygen to increase the driving force and run the risk of pCO_2 build up as discussed below. The rate of airflow across the upper surface also has an impact but it does not represent a way of using air efficiently compared to bubbling aeration when agitating.

5.2.4.3 Bubble Columns

In the 1980s, it was considered bubble columns and airlift bioreactors were less likely than stirred ones to damage animal cells during culture (Varley and Birch 1999). However, once it became apparent that that bursting bubbles represented a

serious threat to cell viability, their use rapidly declined; even after the effectiveness of Pluronic F68 as protective agent became apparent. Nevertheless, they do offer a relatively simple way of providing oxygen transfer at rates which enable cells to grow and especially at the small scale, the use of Pluronic F68 should protect the cells.

For open pipe and ring spargers in water for a wide range of bubble column sizes based on the literature and their own work, Heinen and van't Riet (1982) found

$$k_L a = 0.32 v_S^{0,7} \tag{5.26}$$

which, when compared on a specific power basis, showed similar $k_L a$ values to those in agitated systems. Doig et al. (2005) worked with small bubble columns of 10 and 40 mm diameter with an aspect ratio of 2 and found a similar relationship between $k_L a$ and superficial gas velocity to that in Eq. 5.26. Specific power inputs up to 0.4 W/kg were generated leading to $k_L a$ values up to 300 s^{-1}. Clearly, bubble columns are capable of meeting the oxygen demands of cell culture, though the question of cell shear sensitivity to bubbling at scale still persists.

5.3 Carbon Dioxide Stripping

5.3.1 The 'Apparent' Mass Transfer Coefficient Issue

In principal, the basic equations for aeration (oxygen mass transfer) apply to carbon dioxide stripping (CO_2 mass transfer). However, in detail, they are very different. Firstly, the solubility of CO_2 at 25 °C and 1 bar is 33 mmol/L, more than two orders of magnitude greater than that of oxygen in air (0.2 mmol/L), i.e. Henry's Law constant is much smaller. Thus the application of the basic theory is different since that for oxygen is for a sparingly soluble gas whilst CO_2 is extremely soluble. As a result, when a bubble of air is passed through media depleted of oxygen but saturated with CO_2, it quickly fills (saturates) with the latter because of its high solubility so that the driving force at the start, very quickly becomes zero and no further CO_2 can be transferred. For oxygen, the concentration falls only slowly and it continues to supply O_2 as it rises as illustrated in Fig. 5.4.

The other parameter that determines the rate of mass transfer is k_L. Basic mass transfer theories suggest that $k_L \propto D_L^a$ where D_L is the mass diffusivity of the transferring species and $a = 1$ for the film theory of mass transfer and 0.5 for the penetration theory. Thus, since D_L is 1.92×10^{-9} m^2/s for CO_2 in water at 25 °C and 2.1×10^{-9} m^2/s for O_2, the difference should only be 5–10 %. This estimate of k_L for CO_2 based on k_L for O_2 is important because measuring $k_L a$ for CO_2 is difficult. Indeed, it is often done incorrectly using the unsteady state technique assuming that the gas composition does not change as it rises through a CO_2-saturated aqueous solution. i. e. equivalent to the no-depletion model for oxygen

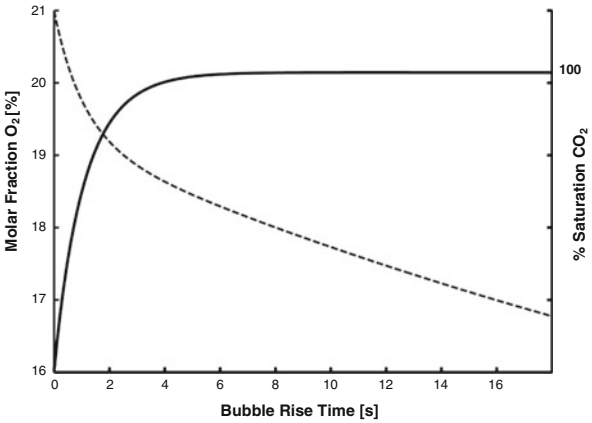

Fig. 5.4 The change of oxygen and carbon dioxide concentration in a bubble of air as it rises through water saturated with CO_2 and stripped of O_2 due to the different solubility of the two gases (Adapted from Sieblist et al. 2011a)

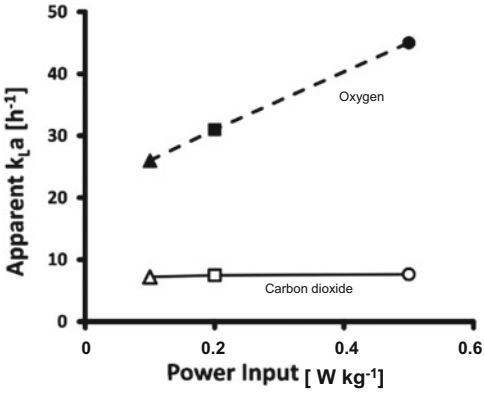

Fig. 5.5 'Apparent' k_La as a function of the specific power input at a constant aeration rate for oxygen and carbon dioxide transfer (Adapted from Sieblist et al. 2011a)

transfer. However, as indicated above, the bubbles rapidly fill with CO_2 and the driving force for CO_2 mass transfer falls to zero. Since enhanced agitation intensity only leads to smaller bubbles and higher hold up, hence a longer residence time for bubbles in the vessel, agitation intensity has a minimal effect if any on the value of k_La (an 'apparent' k_La) when measured this way. On the other hand, with increasing airflow, the bubbles even if they eventually become saturated remove more CO_2 so that the apparent k_La is enhanced. This relative impact of agitation and sparge rate on 'apparent' k_La is shown in Fig. 5.5.

5.3.2 *CO_2 Evolution Rate, CER, and Control of pCO_2*

At the initial stages of cultivation, it is necessary to control pH by sparging CO_2 in with the air, essentially along the lines first proposed by Telling and Stone (1964). Because of its high solubility, this mass transfer step is easily accomplished.

However, later in the culture, the CO_2 in solution can easily increase excessively. The respiratory quotient, RQ (mol CO_2 produced per mol O_2 consumed) for animal cell culture is approximately 1 (Ozturk 1996; de Zengotita et al. 2002). Thus for every mol of oxygen transferred into the media that is utilized by the cells, no matter whether this is achieved by higher k_La or higher driving force, a mol of CO_2 is produced. However, if the oxygen transfer is achieved at low sparge rates, for the reasons discussed above, CO_2 will not be stripped out of the media. Even so, because of the perceived 'shear' sensitivity of animal cells (discussed further below), low air flow rates are often used and oxygen transfer rates are maintained high by using a high oxygen driving force (oxygen enriched air or enhanced back pressure) or high k_La (using a fritted sparger to give small bubbles of high specific area, a).

High levels of pCO_2 of 150–200 mmHg levels have been reported in 1,800–2,500 L bioreactors and in high cell density perfusion bioreactors (Goudar et al. 2007) and detrimental effects have been reported at these levels for a number of systems; hybridoma, NSO and BHK cells (de Zengotita et al. 2002), CHO (Mostafa and Gu 2003) and insect cells (Garnier et al. 1996). De Zengotita et al. (2002) also showed that the cells they studied grew well in the range ~ 35 to ~ 80 mmHg pCO_2, comparable to the physiological range of 31–54 mmHg pCO_2.

The level of pCO_2 and the ability to strip it out is closely connected to the sparge rate in the following way. Assuming that the respiratory quotient, $RQ = 1$ and that the k_La for CO_2 and O_2 are the same, then

$$OUR = k_La\Delta C_{O2} = CER = k_La\Delta C_{CO2} \tag{5.27}$$

so that

$$\Delta C_{O2} = \Delta C_{CO2} \tag{5.28}$$

Assuming the dissolved oxygen is controlled at 40 % of saturation, then since in air there is only 21 vol.% oxygen, the partial pressure of oxygen is $760 \times 0.21 \times 0.4 = \sim 60$ mmHg. Thus $\Delta C_{O2} = \sim 100$ mmHg. Therefore, ΔC_{CO2} at the exit would also have to be ~ 100 mmHg. However, as shown above for many cells, the toxic level for CO_2 in solution is about 15–20 % of saturation, i. e. ~ 110–150 mmHg, so that C_L for CO_2 must be less than such values and the concentration of CO_2 in the exit gas < ~10–50 mmHg, i. e. <~1.5–6.5 vol.% CO_2.

How is this to be achieved? A mass balance on CO_2 with CER in mol/m^3 air s gives

$$CER = (Q_G/V)(P/RT)\left(y_{CO2,\ out} - y_{CO2,\ in}\right) \tag{5.29}$$

Since $y_{CO2,\ in}$ is essentially zero (0.04 % in air), then

$$CER = (Q_G/V)(P/RT)y_{CO2,\ out} \tag{5.30}$$

Similarly, a mass balance for OUR gives

$$OUR = (Q_G/V)(P/RT)\left(y_{O2,\,in} - y_{O2\,,\,out}\right) \tag{5.31}$$

or

$$OUR = (Q_G/V)(P/RT)\left(0.21 - y_{O2\,,\,out}\right) \tag{5.32}$$

if only air is sparged. However, since $CER = OUR$, then

$$y_{CO2,out} = 0.21 - y_{O2,out} = \Delta y_{O2} < \sim 0.065 \tag{5.33}$$

in order to keep the pCO_2 low enough to avoid a deterioration in the culture. Since for the same cell density on scale-up, oxygen demand (and therefore OUR) remains the same, from Eqs. 5.31 and 5.32, to hold the mass balances the same, Q_G/V should be held constant, i. e. constant *vvm* scale-up ensures that if the pCO_2 is acceptable at the small scale, it will remain so at large.

Since constant *vvm* scale up leads to a linear increase in superficial velocity, v_S, with scale and in industry (Sieblist et al. 2011a), the main concern is the OUR, then because of the perception of the 'shear' sensitivity, such an approach is often not followed. There is also a concern for foaming. Because of that perception, scale-up is done at less than constant *vvm* but since $CER = OUR$ (Eq. 5.27), then

$$y_{CO2\,,\,out} \propto V/Q_G \propto (1/vvm) \tag{5.34}$$

leading to higher pCO_2 levels at the larger scales, which, depending on the flow rate chosen, may lead to a poorer culture performance. Alternatively, the required OUR is obtained by increasing the oxygen concentration to increase the driving force for oxygen-mass transfer. However, from Eq. 5.31, if $y_{O2,\,in}$ is increased by using oxygen, oxygen enriched air or back pressure whilst OUR remains the same to meet a particular oxygen demand, then Q_G/V is reduced. Thus from Eqs. 5.27 and 5.30, pCO_2 increases.

Of course, the pCO_2 at the small scale may be much lower than that at which problems occur, especially if a high *vvm* gas flow rate is used as in the ambr™ as discussed in Sect. 5.9. In that case constant vvm scale-up may not be required. For such a situation, Sieblist et al. (2011a) suggest that a practical experimental approach to the optimization is to first enhance the gas throughput to a value where the pCO_2 concentration is kept within the desired interval (preferably established at the small scale) and then, secondly, to increase the impeller speed (and hence power input) so that in accordance with Eq. 5.23 for the particular media, the k_La is sufficient to cope with the oxygen transfer demands. As discussed below, such an increase in speed is unlikely to damage the cells.

5.3.3 pH and Osmolality

Once dissolved in an aqueous system such as cell culture media, CO_2 can react with different ions in the media, thus giving rise to the lowering of pH which is also detrimental to cell culture. In order to maintain the pH in the desired range, alkaline salts must be added which increases the osmolality and can lead to growth inhibition (de Zengotita et al. 2002; Mostafa and Gu 2003). Thus, if CO_2 mass transfer (stripping) is inadequate, problems may arise from high pCO_2, pH control issues and high osmolality. It is a critical aspect of large scale, high density animal cell culture (Xing et al. 2009; Nienow 2010).

5.4 Heat Transfer

Even at the high cell densities now being achieved, the actual oxygen uptake rate (OUR) is low compared to other types of fermentation. Since metabolic heat release, Q_H (W m^{-3}) is proportional to the OUR (mol O_2 m^{-3} s^{-1}) (Van't Riet and Tramper 1991), i. e.,

$$Q_H = \sim 4.6 \times 10^5 OUR \tag{5.35}$$

Q_H for animal cell culture is also relatively low. Therefore, though heat release scales with the volume of the reactor, i.e., T^3 whilst cooling surface area scales with T^2, simple cooling jackets are sufficient for good temperature control even at the commercial scale. Typical operating temperatures are cell line specific and for mammalian cells vary between about 36 °C to 38 °C whilst for insect cells, it lies between 25 °C and 30 °C. The agitation intensity itself has little impact on the overall heat transfer coefficient.

5.5 Homogeneity Issues

Even though the agitation intensity required to produce the required OTR is relatively gentle (typically < 0.05 W/kg), the flow in animal cell culture bioreactors from the bench scale and up is turbulent, $Re > \sim 10^4$. Under these conditions, the time required to homogenize the contents of a bioreactor, the mixing time, θ_m is given by

$$\theta_m = 5.9(\bar{\varepsilon}_T)_g^{-1/3}(D/T)^{-1/3}T^{2/3} \tag{5.36}$$

for bioreactors for $H = T$ with a single impeller independent of whether it is an axial or radial flow type (Ruszkowski 1994; Nienow 1997a,b). Equation 5.35 has also

recently been shown to fit the mixing time measured at the bench scale using 'Elephant Ear' and other axial flow impellers (Simmons et al. 2007). Thus if a similar $(\overline{\varepsilon}_T)_g$ is used at different scales in order to achieve the required k_La values as indicated by Eq. 5.23, mixing time increases with (scale)$^{2/3}$ if geometric similarity is maintained. This increase enhances the inhomogeneity both spatially and temporally on the large scale with respect to the bench in relation to dO_2, pH and nutrients in fed batch cultures.

For triple impellers in a vessel with an aspect ratio of 3, the equation for mixing time obtained by Cooke et al. (1988) was

$$(\theta_m)_{radial} = 3.3N^{-1}Po_g^{-1/3}(H/D)^{2.43} \tag{5.37}$$

for radial flow impellers spaced T apart with the submergence of the upper impeller, ~ 2/3 T. Equation 5.37 can be rearranged to show that with dual impellers too

$$(\theta_m)_{radial} \propto (\overline{\varepsilon}_T)_g^{-1/3}T^{2/3} \tag{5.38}$$

i.e. the same increase with scale as for single impellers. For a radial impeller as the lower impeller and one or two down-pumping axial flow impellers, $D/T = 0.5$ above, Cooke et al. (1988) found the constant 3.3 in Eq. 5.37 was reduced by approximately 50 %. Subsequently, Eq. 5.37 was found to fit data well in vessels of aspect ratio 2 with dual, radial flow impellers (Cronin et al. 1994), and suitably modified to a constant of ~ 1.5, for dual axial flow, high solidity ratio impellers whether up- or down-pumping (Hari-Prajitno et al. 1998). Similar improvements in homogenization have been reported for axial flow impellers and radial-axial combinations (Vrabel et al. 2000; Siebliest et al. 2011b).

The impact of the increased inhomogeneity on increasing scale has been shown to lead to detrimental performance with respect to dO_2 and nutrients (Enfors et al. 2001). The impact also depends on the size of the deviation from the desired controlled value following the addition of pH control chemicals (Osman et al. 2002) and nutrients as well as its duration as indicted by the mixing time. Such excursions can be minimized by sub-surface addition close to the impeller (Langheinrich and Nienow 1999) where the maximum local specific energy dissipation rate, $(\varepsilon_T)_{\max}$, is found and leads to a rapid local dispersion of the additive (Nienow 2010).

5.6 Choice of Agitation Conditions and Agitator

5.6.1 Mean Specific Energy Dissipation Rate, $\overline{\varepsilon}_T$

In order to meet the *OUR* required by achieving an adequate k_La value, then a sufficiently high energy dissipation rate, $\overline{\varepsilon}_T$ has to be imposed by the impeller as shown by Eqs. 5.23, 5.24 and 5.25. The power imposed by an impeller is given by

$$P = Po\rho N^3 D^5 \tag{5.39}$$

where Po is the power number, a characteristic of each impeller type. The mean specific energy dissipation rate (or specific power) is given by

$$\overline{\varepsilon}_T = P/\rho V \tag{5.40}$$

For the turbulent regime, $Re > \sim 2 \times 10^4$, the power number is constant with values of about 5 for Rushton turbines with lower values for axial flow impellers such as 'Elephant Ear' impellers (~1.7 (Simmons et al. 2007)) and Lightnin' A315 hydrofoils (~0.85 (Nienow 2010)). Po can be determined from shaft torque experimentally (Kuboi et al. 1983) or predicted by computational fluid dynamics (CFD) (Nienow et al. 2013a; Werner et al. 2014); or with care, from electric power draw (Nienow et al. 2013a).

In general, when air is sparged into a bioreactor, the level of agitation is able to disperse it and the specific power from agitation falls to an extent which depends on the agitator chosen. Eventually, at a sufficiently high flow rate (which may be as high as 2 vvm), air dispersion by the impeller ceases, at which point the impeller is said to flood. However, in cell culture, at the agitation intensity required to give the desired specific power input to achieve adequate mass transfer, most of the dispersion is provided by the sparger and the impeller has little or no additional effect. Also, the air flow rate is typically around 0.01 *vvm* and air passes through the impeller region without significantly affecting the flow pattern close to the agitator. As a result, $P_g \approx P$, $Po_g \approx Po$ and so the mean specific energy dissipation rate under aerated conditions from the impeller during cell culture is essentially equal to $\overline{\varepsilon}_T$ (Nienow 2006).

Since Eqs. 5.23–5.25 are independent of impeller type, the implications of this analysis is that for air dispersion and mass transfer, agitator choice is not important.

5.6.2 Hydromechanical Stress Issues Due to Agitation

Clearly, obtaining sufficiently high k_La values does not impose a problem from the agitation perspective. It is achieved in high cell density fed batch bacterial fermentations where k_La values up to 800 h^{-1} are required whilst in animal cell culture

they are of the order of 10–15 h^{-1}. The issue is does this agitation intensity lead to deterioration in culture performance or product quality since, because of the lack of a cell wall, animal cells are perceived to be so-called 'shear-sensitive'? This concept has also led to the development of 'low shear' impellers, ones that are marketed as less likely to damage cells.

However, the modern theory of cell damage (Hewitt and Nienow 2007), as with dispersed entities, shows that damage does not occur provided the entity (here the cell) is smaller than the Kolmogorov scale of turbulence, λ_K (Nienow 2010). The Kolmogorov scale can is determined from

$$\lambda_K = \left(\nu^3/(\varepsilon_T)_{\max}\right)^{1/4} \tag{5.41}$$

where ν is the kinematic viscosity. It has also been pointed out (Nienow 2010), that for many impellers including the so-called low-shear 'Elephant Ears' type (Zhu et al. 2007), the ratio of $(\varepsilon_T)_{\max}/\overline{\varepsilon}_T$ is very similar at the same D/T ratio. Since $\overline{\varepsilon}_T$ is fixed by the k_La required whatever the impeller type, so $(\varepsilon_T)_{\max}$ is also independent of impeller type. Thus, the concept of low and high shear impellers is invalid.

Another concern for damage is related to tip speed. Here there is an anomaly. When impellers are called 'high shear', they have high power numbers; conversely, 'low shear' have low power numbers. Hence, from Eqs. 5.39 and 5.40, to impart the $\overline{\varepsilon}_T$ value required for mass transfer, 'low shear' impellers have to rotate at a higher tip speed than high shear. Thus, based on tip speed, 'low shear' impellers are more likely to damage cells than 'high shear'. The concepts of high and low shear impellers and tip speed as a determinant of 'shear damage' are misleading.

Finally, some practitioners consider baffles cause regions of 'high shear'. However, measurements of local ε_T indicate the value decreases with the distance from the impeller (Wernersson and Tragardh 1999), without any local increase near the baffles (Geisler et al. 1994). Thus, given the advantages of using baffles for improved homogenization and for ensuring an accurate the prediction of the requisite $\overline{\varepsilon}_T$ (Nienow 2010), bioreactors with baffles are preferred, especially with increasing scale.

Recently, three different CHO cell lines have been cultivated at $\overline{\varepsilon}_T$ values up to 1 W/kg in turbulent, baffled, bioreactors in work undertaken in Genentech (Nienow et al. 2013b) with dual Rushton turbines and in Novartis (Sieck et al. 2013) with dual 'Elephant Ear' impellers. For the three CHO cell lines investigated, the cell density and viability at the bench scale was the same at 1 W/kg as they were in runs undertaken at 0.01–0.02 W/kg as used at the commercial scale. Over the same range of $\overline{\varepsilon}_T$, the antibody quality was also the same and the amount produced was almost unchanged. This insensitivity of cell growth to high agitation intensity fits in well with an earlier review (Nienow 2006) covering TB/C3 mouse hybridomas, EBNA cells, HPV cells, a CHO320 cell line as well as insect cells.

Clearly, it is never possible to prove that all animal cells are equally insensitive to high agitation intensity. Thus, there is always a need to check that the mean specific energy dissipation rate required to achieve satisfactory mass transfer will

not damage the cells on scale up. Given that the flow at the bench and in commercial bioreactors is turbulent, it is also recommended to make that assessment under turbulent flow conditions (Nienow et al. 2013b; Sieck et al. 2013). To allow for the uncertainties concerning large scale turbulent flow structures, each practitioner can decide for themselves what higher value to use. However, Nienow (2014) recommends using an agitator speed at the bench scale ~ 2 times higher than that required to meet the mass transfer needs, equivalent to ~ 10 times increase in $\overline{\varepsilon}_T$.

5.7 Hydromechanical Stress from Sparging

Air sparging contributes to the enhancement of k_La through the value of v_S chosen in accordance with Eqs. 5.23, 5.24, and 5.25 and also because it adds to $(\overline{\varepsilon}_T)_g$

$$(\overline{\varepsilon}_T)_g = (\overline{\varepsilon}_T) + v_S g \tag{5.42}$$

where g is the gravitational constant and $v_S g$, the mean specific energy dissipation rate from sparging. In addition, increased sparging rates increase the driving force for oxygen uptake and, as explained above, particularly for CO_2 stripping in order to prevent a build up of pCO_2 at the high cell densities produced at the end of cultivation. As a result of the latter, scale-up at approximately constant *vvm* (typically about 0.01 *vvm*) is beneficial which leads to a v_S value which increases in proportion to linear scale for geometrically-similar bioreactors.

It has been known for a long time that sparging aeration can destroy animal cells (Handa-Corrigan et al. 1989). This action arises because these cells attach to bubbles (Chalmers and Bavarian 1991) and as they burst at the upper surface of the media, the local stresses on the cells due to bursting (Boulton-Stone and Blake 1993) are many orders of magnitude greater than those due to agitation (Nienow 2006). However, though somehow forgotten, Kilburn and Webb (1968) showed the use of the surfactant, Pluronic F68 at concentrations greater than about 0.5 g L^{-1} acted as a protective agent, due, it was later established, by preventing cell attachment (Chalmers and Bavarian 1991). The use of Pluronic F68 is now ubiquitous to cell culture and generally comes with the media formulation (though occasionally 'rogue' poor cultivations have been ascribed to poor quality surfactant).

Nevertheless, the perception of damage due to bursting bubbles still exists and is one of the reasons for using enriched air or oxygen and sparge stones for enhancing mass transfer. However, as reported by Mostafa and Gu (2003), to avoid CO_2 build up using a sparge stone at 0.002 *vvm* in a 1 m^3 bioreactor, an increase in volumetric air flow rate by a factor of 5 using an open pipe sparger did not damage cells but in fact led to a much improved performance including less foaming. In fact, to the author's knowledge, there have not been any recent reports in the peer reviewed literature of damage due to bursting bubbles.

However, a problem has been reported by Zhu et al. (2008) at the 10 m^3 scale associated with excessively high exit velocities from the holes in a ring sparger. They found that on scale-up at about constant *vvm* leading to increasing superficial gas velocity, when the local velocity through the sparger holes was > ~30 m/s, there was a significant decline in NS0 culture performance. By redesigning the ring sparger to give, at the same *vvm*, hole velocities of ~ 23 m s^{-1}, similar cell density and viability and antibody production were achieved at the 10 m^3 scale and at the 0.6 m^3. As an initial design consideration, it would seem sensible to maintain sparger exit velocities below ~ 25 m s^{-1} (Zhu et al. 2008).

5.8 Agitator and Sparger Choice

Since all impellers are equally efficient at achieving mass transfer and have similar $(\varepsilon_T)_{max}/\overline{\varepsilon}_T$, the choice does not need to be made in relation to obtaining the desired k_La or avoiding 'shear' damage. However, it is clear that on scale-up, mixing time is increased but this tendency is mitigated by using axial flow impellers ($D = 0.4$–$0.5\,T$) and relatively low aspect ratio vessels. However, it has been shown that if cell damage does occur due to bursting bubbles, increasing the AR of a bioreactor reduces the proportion of the media in which it takes place (Nienow 2006). If a higher AR is chosen, the use of a second agitator becomes increasingly important at the commercial scale to minimize the increase in inhomogeneities. Thus, aspect ratios up to about 1.2 and dual, wide-blade, high-solidity axial flow impellers with subsurface feeding near the impellers should be selected. Indeed, though air dispersion is not a major consideration as discussed above, with constant *vvm* scale-up, air dispersion tends to become poorer and up-pumping impellers are better than down pumping in this respect and are thus recommended (Nienow 2010). Open pipe or ring spargers should be used to prevent excessive foaming and pCO_2 and osmolality issues, with exit gas velocities from the sparger being held at < ~25 m s^{-1}. It is perhaps also worth emphasizing that the sparger should be placed beneath the impeller for the best mass transfer performance.

Finally, in order to follow the protocol recommended above for pCO_2 and dO_2 control, variable speed agitation and sparge rate should be available, capable of giving sufficiently high mean specific energy dissipation rates for oxygen transfer; and air flow for carbon dioxide stripping.

5.9 Scale-up and Ultra Scale-Down Issues

Many of the issues associated with scale-up and scale-down have been addressed already in this chapter. However, recently, there has been a growing interest in increasing the number of clones tested in a selection programme and in order to

handle the numbers, to do so at ever smaller scales (Bareither and Pollard 2011; Pollard 2014). The issue then becomes, does the best culture at this small scale lead to a clone selection which remains the best on scale-up? In addition, in order to handle the number of experiments that it would be desirable to undertake, ideally it requires robotic configurations to be used. Traditionally, such tests have been carried out in shake flasks, but they have a number of issues. They are difficult to control and make measurement in; and they are manually intensive. Also, it has been found that the clones which perform best in the shake flask often do not perform best in a bench scale bioreactor, which is the next step up in scale (Nienow et al. 2013a).

Though many devices have been developed (Bareither and Pollard 2011), the 15 mL ambr™ from TAP Biosystems (Fig. 5.1) seems to have become the most popular, particularly as, like larger scale systems, it uses a variable speed impeller as its energy source and offers fully automated control of pH and dO_2 with the possibility of fed-batch operation. The ambr™ is able to run 24 or 48 parallel cultures at the same time and handle all the steps required for the ~ 14 day process robotically. It has been shown to give very similar cell culture performances (cell density, culture time, productivity and product quality) consistently over all the bioreactors in the ambr system; and equivalent to those in stirred bench scale bioreactors at Genentech (Hsu et al. 2012) and at Merck (Moses et al. 2012), with many other companies reporting similar results at a number of conferences. Interestingly, these publications also showed the cell density achieved and productivity were different (higher!) in shake flasks, though the product quality was the same in all three systems.

Interestingly, the physical mixing and mass transfer characteristics have only recently been reported (Nienow et al. 2013a). They showed that there were a number of physical aspects which are quite different in the anbr™ compared to larger scales. Firstly, the Reynolds number attainable at 1,500 rpm is in the transitional flow regime (~2,500) whilst in other stirred bioreactors from the bench scale up, the flow is turbulent. Also because of the small size of the bioreactor, the air flow, expressed as v_S ($\sim 5 \times 10^{-5}$ m s^{-1}), is very low. Thus, in order to meet the required *OTR* and give k_La values of up to about 10 h^{-1}, a specific power of up to ~ 0.4–0.5 W kg^{-1} is required. On the other hand, when expressed as *vvm*, the air (or oxygen) flow is high (up to 0.07 *vvm*). Thus, CO_2 stripping is effective. Finally, the bulk mixing time is enhanced in the transitional region to give times similar to those obtained at the bench scale.

The reasons for which the ambr™ gives results similar to bench scale stirred bioreactors has not been established. It may well be that at the scale of the cell, which is still below λ_K, the mass transfer to and from the cell is purely diffusive. Therefore, good control of dO_2 and pH as found at the bench scale with similar mixing times ensures the cells are exposed to similar environments; and the high *vvm* ensures low pCO_2. Thus, equivalent performances are obtained.

Conclusions

The fundamental theory of mass transfer has been developed and simplified to show how oxygen transfer rates depend on the concentration driving force on the one hand and the mass transfer coefficient, k_La, on the other. The former for the most common type of bioreactor, the stirred vessel from the small 15 mL ambr^{TM} to the largest commercial scales of about 25 m^3, is determined by the rate of gas sparging, the gas composition, back and static pressure. The latter, kLa, depends on the specific power input (mean specific energy dissipation rate) from the impeller and the sparged gas; and the superficial gas velocity (the volumetric flow rate divided by the cross-sectional area of the bioreactor) through the bioreactor. For oxygen, which is sparingly soluble in water, the theory can be used to develop simple experimental techniques for measuring k_La which can be further simplified because the specific oxygen demand of animal cells is low as is the current cell density achievable ($>10^7$ cells mL^{-1}) compared to the oxygen uptake rate with bacterial and mycelial fermentations. This ease of measurement is important because the absolute value of k_La is very sensitive to the presence of salts (which enhance it relative to water) and antifoams and protective agents such as Pluronic F68 (which reduce it). As a result, accurate correlations from which k_La can be predicted are not available. At present, the best appears to be that published by Van't Riet (1979) for coalescing systems.

The next mass transfer issue addressed is that of stripping carbon dioxide from the media. Stripping is necessary because for each mol of oxygen utilized by the cells, approximately a mol of CO_2 is produce. However, unlike oxygen, CO_2 is very soluble in aqueous systems and this causes significant difficulties when trying to measure k_La for CO_2. As a result, incorrect values are often quoted. It is shown that because of the similar values of diffusivity, the k_La for both gases must be very similar. On the basis of this similarity, it is shown that for effective stripping, sparging at approximately constant *vvm* across the scales is required.

Achieving effective oxygen transfer and CO_2 stripping is made more difficult from the perceived sensitivity of animal cells to hydromechanical stress from agitation and bursting bubbles. As a result, oxygen transfer is achieved at agitation intensities and sparge rates that are too low by using sparge stones to enhance interfacial area and enriched air or pure oxygen to increase driving force. In turn, this perception has led to problems of pCO_2 build up on scale-up which in turn can cause pH and osmolality issues. Therefore, 'shear sensitivity' in relation to mass transfer is discussed and an operating protocol is proposed to overcome high pCO_2 occurring. This protocol involves increasing the air sparge rate through ring or pipe spargers to strip CO_2 followed by enhancing agitator speed to give the required dO_2. Particularly notable are recent studies which show mean specific energy

(continued)

dissipation rates in stirred bioreactors including the ambr™ up to 0.5 W/kg per impeller have not led to any deterioration in cell growth or product titer or quality.

Since the mean specific energy dissipation rate required for mass transfer normally sets the level used in practice, the other important mixing parameters which are all determined by it are briefly discussed too along with related issues in non-sparged, non-agitated bioreactors.

Nomenclature

a	Specific area of bubbles, m^{-1}
A	Dimensional constant in Eq. 5.23
AR	Aspect ratio, H/T
C_L	Concentration in the liquid phase, mol m^{-3}
d_{32}	Sauter mean diameter, m
D	Agitator diameter, m
D_L	Diffusion coefficient, m^2 s^{-1}
g	Gravitational constant, 9.81 m s^{-2}
H	Bioreactor fill level, m; or Henry's law constant, atm. m^3 mol^{-1}
H_G	Height of aerated liquid, m
J	Rate of mass transfer, mol s^{-1}
k_g	Gas film mass transfer coefficient, mol s^{-1} m^{-2} atm^{-1}
k_L	Liquid film mass transfer coefficient, m s^{-1}
k_La	Specific mass transfer coefficient, s^{-1} or h^{-1}
M	Mass of media, kg
N	Agitator speed, s^{-1} or rpm; or specific rate of mass transfer, mol m^{-3} s^{-1}
OD	Oxygen demand, mol m^{-3} s^{-1}
OTR	Oxygen transfer rate, mol m^{-3} s^{-1}
OUR	Oxygen uptake rate, mol m^{-3} s^{-1}
p	Partial pressure, atm
P	Power, W; or total pressure, atm
P_g	Total pressure, atm
Po	Power number, dimensionless
Q_G	Air flow rate, m^3 s^{-1}
Q_H	Heat evolution rate, W m^{-3}
R	Gas constant, m^3 atm K^{-1} mol^{-1}
Re	Reynolds number, dimensionless
SOD	Specific oxygen demand, mol s^{-1} $cell^{-1}$
t	Temperature, °C; or time, s
T	Bioreactor diameter, m; or absolute temperature, K
v_S	Superficial gas velocity, m s^{-1}

VVM Specific volumetric flow rate, (min^{-1})
V Volume of media, m^3
y Mol fraction, dimensionless
X Cell density, cells m^{-3}; or % dO_2
Greek Letters
α,β Exponents
ΔC Concentration driving force, mol m^{-3}
ε_G Hold-up, dimensionless
ε_T Local specific energy dissipation rate, W kg^{-1}
$\overline{\varepsilon}_T$ Mean specific energy dissipation rate, W kg^{-1}
λ_K Kolmogoroff turbulence scale, m
μ Viscosity, Pa s
ν Kinematic viscosity, m^2 s^{-1}
ρ Liquid density, kg m^{-3}
τ_p Time constant of the oxygen probe, s
θ_m Mixing time, s
Subscripts
CO_2 Carbon dioxide
$Crit$ Critical oxygen concentration
I At the interface
In Entering at the sparger
G When air is sparged; or in the gas phase
Max Maximum
Out At the exit
O_2 Oxygen
Superscript
* At equilibrium

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Chapter 6
Hydrodynamic Damage to Animal Cells

Jeffrey J. Chalmers and Ningning Ma

Abstract Large scale, animal cell culture in stirred tank bioreactors, is responsible for greater than $100 billion in sales of human biopharmaceuticals. This success was achieved in spite of the concern of the "shear sensitivity" of animal cells. In this contribution, a summary of the current state of this "shear sensitivity" concern will be discussed, demonstrating that it is not in general a problem with current bioprocess. Example of what is considered the current limits above which effects of hydrodynamic and interfacial concerns begin to negatively impact bioprocesses will also be presented.

Keywords Shear stress • Hydrodynamic forces • Chinese hamster ovary cells • Glycosylation • Cell culture • Scale-up

6.1 Introduction

The innate ability of animal cells to properly fold, post-translationally process, and secrete proteins has made them the preferable host for producing a large number of biological therapeutic and diagnostic products. These properties, combined with the high current productivities (compared to what was achievable 30 year ago), has led to a commercial, world market greater than 100 billion US dollars from more than 150 different products, which include viral vaccines, monoclonal antibodies (MAbs), hormones, enzymes, growth and blood factors. Although the industrial exploitation of animal cell cultures started over five decades ago, (an early example is the production of the Salk polio virus vaccine in primary monkey kidney cells (Griffiths (2000)), it has been during the last 30 year period that there has been a rapid increase in the number of FDA approved products produced in mammalian

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cell culture, starting in 1986 with the production of recombinant tissue plasminogen activator (tPA).

Despite reports showing that animal cell culture has been conducted for over a 100 years, (Harrison 1907) no rigorous methodology for scale-up of animal cell culture exists. Diverse culture methodologies, typically with staring with small scale static cultures (T-flask), to roller bottles, cultures on either microcarriers, or freely suspended cells in batch, fed batch, or perfusion systems exist. While freely suspended, fed-batch processes have emerged as the predominate approach, much of this evolution has been guided by the particularities of the process, the cell line, and most importantly, expertise of specific organizations. Despite examples of large scale (1,000–2,000 l) airlift bioreactors (Birch et al. 1985; Varley and Birch 1999; Hesse et al. 2003) virtually all of the licensed processes for recombinant proteins, antibodies and vaccines use either freely suspended or microcarriers cultures in traditional stirred tank bioreactors with reported capacities up to 20,000 L.

Despite the concern of the "shear sensitivity" of animal cells, traditional stirred tank bioreactors, *STB,* is the preferred method of culture for a number of reasons, not the least of which is the vast empirical knowledge accumulated for the design, scale-up and operation of *STB*, in the chemical and biochemical industries over the last century. Further *STB* are versatile and relatively simple to operate. This chapter will discuss what is current know with respect to hydrodynamic forces and animal cells of industrial importance. For a discussion of the engineering considerations of animal cell bioreactors, see Chap. 5 of this volume.

6.2 Hydrodynamic Forces Acting on Cells

The perceived "shear sensitivity" of animal cells has been, and continues to be, a source of significant concern and confusion. Given the original mechanical and nutrient support of vascularized tissue, this concern is not unfounded. The relative large size of animal cells, compared to microorganisms further contributes to this concern. Finally, as will be discussed below, hydrodynamic forces, created as a result of mixing, in some conditions can remove animal cells attached to microcarriers.

At the most fundamental level, "shear stress" is only one of two types of hydrodynamic stress, the other being normal stress. Both stresses are mathematically defined as a viscosity multiplied by a velocity gradient; when the gradient is perpendicular to the flow it creates a shear stress, (i.e. du_x/dy), when the gradient is in the direction of the flow it creates a normal stress (i.e. du_x/dx). To add further complexity, in non-simple laminar flow, as well as in turbulent flow, the local flow rate is changing with time.

Beyond the biotechnology community, the very large field of chemical mixing is also interested in these forces; in most cases it is desired to maximizing these forces with the least energy input. Consequently, a great deal of research, both basic as well as highly practical, has been conducted to attempt to understand and optimize

mixing. Unfortunately, while ideally it is desirable to quantify and design mixing systems from first principles, it is still not currently possible/practical. This limitation has led to a number of semi-empirical equations and parameters to characterize and estimate global and local fluid conditions in *STB*.

Energy Dissipation Rate, EDR One of these characterization parameters, used in the mixing community for over 60 years, is the energy dissipation rate (Kresta 1998). Further, *EDR* was proposed by Blustein and Mackros (1969) to characterize cell damage to blood cells. *EDR* can be expressed mathematically for a incompressible Newtonian fluid as:

$$\varepsilon = \tau : \nabla U = \mu\left[\nabla U + (\nabla U)^T\right] : \nabla U = \mu \sum_i \sum_j \left[\nabla U + (\nabla U)^T\right]_{ij} \nabla U_{ji} \quad (6.1)$$

where ε is EDR (W/m^3), τ is the stress tensor (N/m^2), μ is the viscosity (Pa · s), U is the velocity vector, ∇U is velocity gradient vector, and ∇U^T is the transpose of ∇U. ∇U can be further defined as:

$$\nabla U = \begin{bmatrix} \frac{\partial U_x}{\partial x} & \frac{\partial U_y}{\partial x} & \frac{\partial U_z}{\partial x} \\ \frac{\partial U_x}{\partial y} & \frac{\partial U_y}{\partial y} & \frac{\partial U_z}{\partial y} \\ \frac{\partial U_x}{\partial z} & \frac{\partial U_y}{\partial z} & \frac{\partial U_z}{\partial z} \end{bmatrix} \quad (6.2)$$

When the flow conditions, and system geometries is sufficiently understood/defined, it is possible to obtain analytical solutions to Eq. 6.1 and determine both the *EDR* and shear and normal stresses that a cell experiences can be known. Mollet et al. (2004, 2007) provides solutions for a number of these situations.

Alternatively, for complex flow, and turbulent, or near-turbulent conditions, such as in a typical *STB*, as stated above, first principles solutions are not possible. In these cases, well known correlations, which have substantial experimental validation, can be used. For example, the mean specific energy dissipation rate, ε_{mean}, in a *STB* can be estimated from:

$$\varepsilon_{mean} = P/M = Po\rho N^3 D^5/M \quad (6.3)$$

where P/M is the specific power, M is the mass of the fluid, Po is the power number for the specifically used impeller, D is the impeller diameter, and ρ is the density of the fluid.

A number of studies, including the extensive studies by Zhou and Kresta (1996a, b) have experimentally demonstrated that the maximum *EDR* in a mixing vessel (*STB*) can range from 10 to 200 times the mean value:

$$\varepsilon_{\max} = \phi \varepsilon_{mean} \tag{6.4}$$

Where φ can vary from 10 to 200. As will be discussed below, significant work has been, and continues be conducted to relationship between *EDR* and lethal and non-lethal effects on cells

Kolmogorov Microscale of Turbulence, λ An alternative/complementary parameter to using *EDR* to characterize the hydrodynamic conditions in which an animal cell is subjected, is the application of the Kolmogorov's theory of turbulence to cell culture (Kolmogorov 1941a, b) in *STB*s. Out of this theory, which assumes that the turbulence is isotropic (uniform in all directions) a relationship/term is defined, referred to as the Kolmogorov microscale, λ:

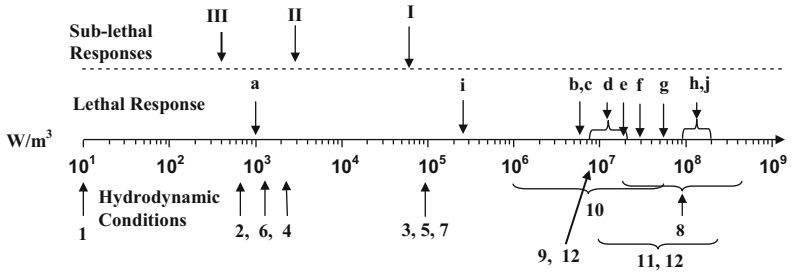
$$\lambda = \left(\frac{\nu^3}{\varepsilon}\right)^{1/4} \tag{6.5}$$

where ν is kinematic viscosity of the liquid (m^2/s). Independently, Cherry and Papoutsakis, (1986, 1988, 1989) and Croughan et al. (1987, 1989) suggested that when the value of λ is the same size as a single suspended cell, or a microcarrier with cells attached, significant cell death occurs. However, as with *EDR* discussed above, accurate, local estimates of *EDR*, ε, is needed.

6.3 Experimental Studies Attempting to Quantify Cell Damage

Over the last 50 years, a large number of studies have been conducted in an attempt to quantify the damage, or lack thereof, of hydrodynamic forces on animal cells. These studies can be simplistically divided into two categories: (1) studies that put suspended animal cells in a variety of devices in which hydrodynamic forces can be accurately determined on a scale compatible with a cell (i.e. rheological instruments), or (2) studies in which attempts were made to determine/quantify the hydrodynamic forces in typical bioprocess equipment (i.e. spinner flask, bioreactor, centrifuge, etc.) and subsequently relate the cell cultures performance to the conditions in the equipment.

As in any comprehensive comparison of studies from many laboratories over many decades, challenges exist in providing a basis by which this comparison can be made quantitatively. Never-the-less, Fig. 6.1 attempts to create such a comparison of some of the more relevant studies using *EDR* as point of comparison. While at the most fundamental level, *EDR* does not take into consideration all of the complexities of the flow, especially in turbulent systems, it does provide a "high-level" comparison. Figure 6.1 uses a linear line to represent the scalar value of *EDR*, with specific arrows representing specific *EDR* values grouped into (1) reported



Sub-lethal Physiological Responses Critical to Bioprocesses

Symbol	Cell	Mode of growth	Mode of test	Response	Reference
I	CHO	Suspended	10 days repetitive exposure	Recombinant protein Glycosylation profile change	Godoy-Silva et al. 2009b
II	LnCap	Attached	Time exposure to well defined shear stress	Membrane integrity failure, change in receptor number	McCoy et al. 2010
III	CHO	suspended	2 L Applicon bioreactor	Recombinant protein rate of production and glycosylation effect	Sieck et al. 2013

Lethal Responses (necrosis including LDH release)

Symbol	Cell	Mode of growth	Reference
a	CHO-K1	Anchorage for growth and test	Gregoriades et al. (2000)
b	PER.C6	Suspended (naïve or adenovirus infected)	Ma (2002)
c	CHO (GS)	Suspended	Godoy-Silva et al. (2009b)
d	Hybridoma	Suspended	Thomas et al. (1994); Zhang and Thomas (1993)
e	MCF-7	Suspended	Ma et al. (2002)
f	Mouse myeloma	Suspended	McQueen and Bailey (1989)
g	Hela S3, mouse L929	Suspended	Augenstein et al. (1971)
h	CHO-K1, Hybridoma HB-24	Suspended	Ma et al. (2002)
i	CHO-K1, apoptosis	Anchorage for growth, suspended during test	Mollet et al. (2007)
j	CHO-K1	Suspended (wild type and bcl2 transfected)	Mollet et al. (2007)

Hydrodynamic Conditions

Symbol	Process	Description	Reference
1	Agitation	Volume average energy dissipation rate in typical animal cell culture bioreactors	Varley and Birch (1999)
2	Agitation	Volume average energy dissipation rate in a 10L mixing vessel (Rushton Turbine impeller, 700 RPM)	Zhou and Kresta (1996a)
3	Agitation	Maximum local energy dissipation rate in a 10L mixing vessel (Rushton Turbine impeller, 700 RPM)	Zhou and Kresta (1996a)
4	Agitation	Volume average energy dissipation rate in a 22,000L fermentor (Rushton Turbine impeller, 140 RPM)	Wernersson and Trägårdh (1999)
5	Agitation	Maximum local energy dissipation rate in a 22,000L fermentor (Rushton Turbine impeller, 140 RPM)	Wernersson and Trägårdh (1999)
6	Agitation	Maximum local energy dissipation rate in a spinner vessel	Venkat et al. (1996)
7	Bubble rupture	Pure water (bubble diameter: 6.32 mm)	Garcia-Briones et al. (1994)
8	Bubble rupture	Pure water (bubble diameter: 1.7 mm)	Boulton-Stone and Blake (1993) Garcia-Briones et al. (1994)
9	Membrane filtration	CHO suspension pumped through Millipore Membrane and capillary tubes	Vickroy et al. (2007)
10	FACS	CHO cell damage sorted through a FACS	Mollet et al. (2008)
11	centrifugation	Bowl and disk centrifuge	Boychyn et al. 2001; Neal et al. 2003; Hutchinson et al. 2006
12	Capillary	Scale-down of industrial continuous centrifuge	Westoby et al. 2011

Fig. 6.1 Comparison of some of the more relevant studies using *EDR* as point of comparison. Above the logarithmic line EDR (w/m3) a number of sub-lethal and lethal effects of hydrodynamic forces on cells is presented while below the line various levels of EDR, created in various types of bioprocess equipment is presented

non-lethal effects on cells (arrows above the line), (2) lethal effects on cells, (arrows above the line) and (3) estimates of *EDR* values in a variety of situations/equipment (arrows below the line).

A number of significant points should be noted in Fig. 6.1. First, the mean and maximum EDR values (10^1–10^3 W/m^3) in typically operated *STB* are ***orders of magnitude*** lower than what has been shown by many studies (10^7–10^8) to kill suspended animal cells. Second, animal cells attached to microcarriers are damaged/removed from microcarriers at *EDR* values that are in the range of typically operated *STB*. While Fig. 6.1 indicates that typical *STB* operated at levels that are orders of magnitude lower than what has been shown to damage cells, that is not case for many post STB bioprocessing equipment, such as membrane filtration and centrifugation systems. Figure 6.1 also includes reported non-lethal effects of *EDR* on cells which will be discussed further below.

6.4 Cell Damage from Sparging

Sparing related cell damage has long been a threat to suspended cell culture and process scale-up. It has been well accepted that cell-bubble interactions, rather than agitation, play a major role in physical cell damage in bioreactors (Oh et al. 1989; Kunas and Papoutsakis 1990; Chalmers and Bavarian 1991; Trinh et al. 1994; Nienow 1996; Michaels et al. 1991; Ma et al. 2002). Potential cell damage zones in sparged bioreactors include the bubble formation, rising, and disengaging regions. The impeller region is also a possible damage zone should bubble coalesce or breakup occur as a result of agitation.

Various theoretical and experimental studies have demonstrated that the bubble disengagement region can be a major source of cell damage (Handa et al. 1987; Bavarian et al. 1991; Chalmers and Bavarian 1991; Orton and Wang 1991; Trinh et al. 1994). When a bubble sitting at the top media-air interface ruptures, a highly energetic event occurs, resulting in a rapid upward and downward jet of fluid.

A combination of high speed video photograph and computer simulations elucidated the rupture process (Boulton-Stone and Blake (1993); Garcia-Briones et al. (1994)). After a bubble rises above the liquid surface, the upper liquid film thins until a hole forms in the dome, surface tension subsequently pulls the film away from the hole, resulting in a toroidal ring retreating rapidly toward the bulk liquid, and down into the bubble cavity within the liquid. The collision of this flow of liquid results in an upward and a downward jet of fluid (Fig. 6.2a). The most intensive energy dissipation occurs during the collision at the bottom of the bubbles (Fig. 6.2b). Simulation also indicated that smaller bubbles are more damaging as the EDR associated with the bubble rupture increases with decreasing bubble size. The maximum local *EDR* during bubble rupture is several orders of magnitude higher than that generated by agitation (Fig. 6.1). As an alternative to the computer simulation approach, Christi (2000) using the Kolmogoroff microscale/eddy length correlation, also argued that rupturing bubbles are sufficient to damage cells.

Experimentally, Trinh et al. (1994) quantitatively studied the detrimental effect of bubble rupture on insect cells using a specially designed bubble column which allowed a large number of 3.5 mm bubbles to be generated and ruptured, with and

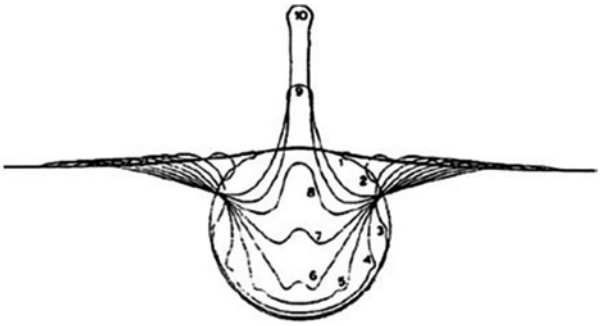


Fig. 6.2 Bubble rupture process (**a**) (Macintyre 1972) and local energy dissipation distribution when retreating film collides at the bottom of the bubble (Boulton-Stone and Blake 1993)

without the presence of the commonly used protective additive, Pluronic F-68. On average, 1,050 cells were killed by each bubble rupture; conversely, when 0.1 % Pluronic F-68 was present, no statistically significant cell death could be detected. To further confirm the damage mechanism, Trinh et al. also collected samples of the upward jet (flying liquid drops) from some of the rupturing bubbles. While virtually all of the cells in the bulk were viably, nearly all of the cells in the upward jet, which were at a concentration over twice that in the bulk, were dead.

A number of additives have been advocated to reduce cell damage in sparged and agitated animal cell cultures. The removal of serum (as a result of regulatory issues), which has some protective effect, further necessitated the use of protective additives. Additives that have been evaluated include: Pluronic F-68, polyvinyl alcohol (PVA), polyethylene glycol (PEG), dextran, and methylcellulose (Murhammer and Goochee 1990; Goldblum et al. 1990; Michaels et al 1991; Michaels et al. 1992; van der Pol et al. 1995; Hu et al. 2008). Of all of these, Pluronic F-68 was demonstrated to be the most effective, and has been widely used in virtually all large-scale, mammalian cell culture media. PF-68 is a nonionic surfactant, belonging to a family of triblock copolymers consisting of a hydrophobic center, poly(propylene oxide), and two hydrophilic tails composed of poly (ethylene oxide). The average molecular weight is 8,400. The protective effect of PF-68 is, to some extent, concentration dependent. The typical concentration ranges used is 0.3–3 g/L (Chisti 2000; Murhammer and Goochee 1990).

Three protective mechanisms for PF-68 and other protective additives had been proposed: (1) nutritive effects, (2) the physical strengthening of the cell membranes, and (3) the suppression of bubble-cell attachments. Because experimental results demonstrated that the time required to achieve measurable protective effects was short after the addition of the supplements, it has been generally assumed that the primary mechanism of protection was not nutritive (Kunas and Papoutsakis 1990; Goldblum et al. 1990; Michaels et al. 1991). Three independent laboratories have over the years demonstrated that physical properties/strength of the cell increased when PF-68 was present: Goldblum et al. (1990) in constant shear stress instrument, Ramirez and Mutharasan (1990) demonstrated decreased plasma membrane fluidity, and Zhang et al. (1992) demonstrated increased membrane bursting

tension of hybridoma cells when PF-68 was present. However, Zhang et al. (1992) recognized that the strengthened membrane alone was not enough for the protection observed in bioreactors.

PF-68 is a surfactant, one of a large family of surfactants made by BASF, all of which have clear surface tension/surface active effects (Mizrahi (1983) and Jordan et al. (1994)). Chattopadhyay et al. (1995) and Micheals et al. (1995) investigating the relationship of these surface active compounds and cell protection found that the most effective (with respect to cell protection) surface active, protective compounds not only significantly reduce air-medium interfacial tension, but also achieve it rapidly. Therefore, they suggested that the lowering of dynamic surface tension was more important than lowering the equilibrium surface tension. As PF-68 lowers dynamic surface tension, it could out compete cells absorbing to the air-liquid interface, hence preventing cell-bubble attachment. As a result, when a bubble ruptures, there is no or only limited number of cells in the impact region. Chalmers and Bavarian (1991) visually demonstrated that a large number of cells are present on the bubble surface when no PF-68 was present (Fig. 6.3a); conversely, the presence of 1 g/L PF-68 prevented nearly all of the cells from being attached to the bubble film (Fig. 6.3b)tached attached. Ma et al. (2004) collected foam from a bubble column and compared cell concentration in the foam liquid to that in the main liquid. They found that the ratio of cells in the foam liquid to that in the main body (defined as enrichment factor) could be 10 times higher when no PF-68 was presented (Fig. 6.4). The enrichment factor decreased with rising PF-68 concentration, reaching 1 at 0.1 g/L PF-68 and stabilized at 0.5 after PF-68 concentration reached 0.3 g/L. An enrichment factor of 0.5 at high PF-68 concentrations indicates that although PF-68 could significantly reduce cell-bubble attachment, there are still cells trapped in the foam. This is not surprising as the bubble film is thicker than cells' diameter, so that cells could be floating in the foam liquid without attaching to the air-liquid interface. As foam is always present on top of sparged cell culture, PF-68 could not completely removed cell from the vicinity of bursting bubbles. At a cell density of 10^7 cells/mL and PF-68 concentration of 1 g/L, there was about 10^3 cells around each bubble (Ma et al. 2004; Fig. 6.5).

With a molecular weight on the order of 8,400, the polymer PF-68 has the potential to interfere with downstream filters and purification resins. A potential

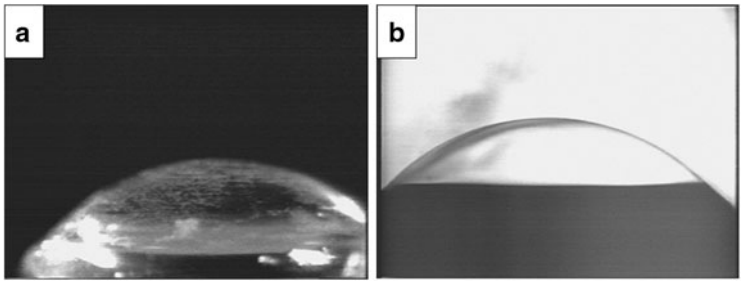


Fig. 6.3 Air bubble without (**a**) or with 1 g/L PF-68(**b**)

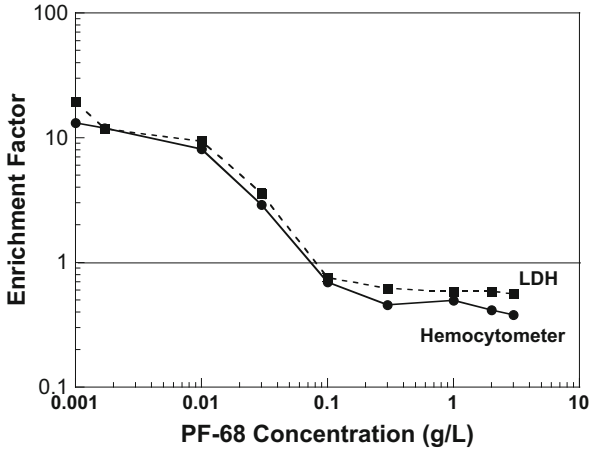


Fig. 6.4 The effect of PF-68 concentration on the enrichment factor, defined as the ratio of cell concentration in the film liquid and bulk liquid. The *dotted line* was determined by the concentration of lactate dehydrogenase and the *solid line* was determined by cell concentration (Ma et al. 2004)

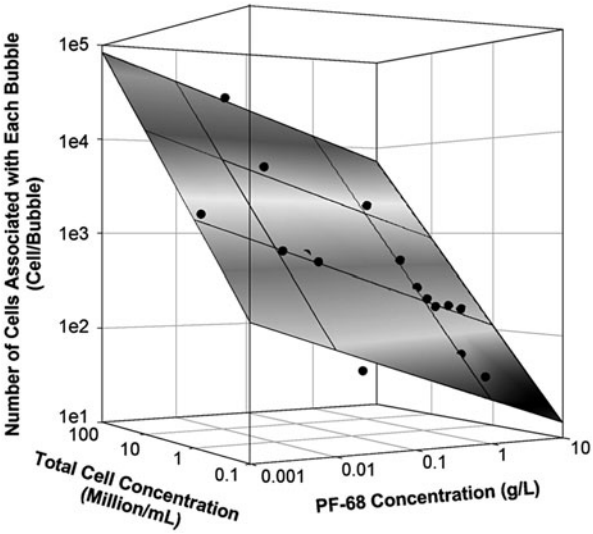


Fig. 6.5 A three-dimensional plot of the number of cells associated with each bubble as a function of cell concentration (cells/mL) and PF-68 concentration. The *dots* indicate experimental data (Ma et al. 2004)

alternative is a small molecule surfactants that could be more easily removed during down-stream purification process. Hu et al. (2008) screened various small-molecule ionic or non-ionic surfactants. Of a number of similarly structured molecules, n-Nonyl-b-D-Maltopyranoside showed the best properties of rapidly lowering the dynamic surface tension and low cytotoxicity. While a potential candidate to augment and or replace the protective effect of PF-68, a thorough evaluation of its impact on cell metabolism, product quality, and potentially patients is still needed.

In addition to the bubble rupture zone, and or the foam layer, cell damage at bubble formation site (sparger) has been reported (Murhammer and Goochee 1990;

Zhu et al. 2008). Zhu et al. (2008) found damage of NS0 cells at the sparger site at high linear air flow rates of 30 m/s, and the damage followed first-order kinetics. A similar phenomenon had been reported for microalgae culture where the critical gas-entrance velocity for cell damage was around 30–50 m/s (Barbosa et al. 2004). None of the additives was reported to be able to alleviate this damage. However, increasing the bore and size could minimize the sparger site cell damage. Extending these observations at gas spargers, Liu et al. (2014) has developed a new hydrodynamic correlation, stress induced turbulent energy production, *STEP*, which they suggest correlates well with experimental data indicating that increased gas flow rate at spargers increases cell damage. Unlike *EDR,* the authors suggest that *STEP* is a more complete and representative of the actual process by considering energy transfers associated with the two phase flow aspect of the bubble generation. Unlike EDR, or other purely hydrodynamic calculations which indicated that the highest EDR is away from the bubble generation area, *STEP* calculations are highest right at the bubble generation site, and *STEP* values increase as gas entrance velocities increase. It should also be noted that *STEP* values are vector with units the same as *EDR*.

6.5 Experimental Sublethal Effect of Hydrodynamic Stress

Compared to other forms of stimulation/assault, investigations on the physiological impacts of hydrodynamic forces on mammalian cells are limited. Further, the majority of studies on the effect of hydrodynamic and or physical forces on cells are on anchorage dependent cells of medical interest, not bioprocess interest/applications.

Nevertheless, these limited number of studies have revealed that it requires much lower hydrodynamic forces to elicit sublethal effects (in all but a few studies) than to cause catastrophic cell death. As summarized above, *EDR* values of 1×10^3–1×10^5 W/m^3, which correspond to shear stresses on the order of 1.0–10 N/m^2, can illicit non-lethal effects on suspended CHO cells. This is significantly lower than the level required to lyse similar cell lines. Using CHO cells grown attached to surface, Ranjan et al. (1995) reported that CHO cells up-regulated transcriptional activator c-fos when subjected to 2.5 N/m^2 (6.2×10^3 W/m^3) shear force for 1 h. Al-Rubeai et al. (1995) reported that hybridoma cells underwent apoptosis at *EDR* of 1.87×10^3 W/m^3. Motobu et al. (1998) observed that after 24 h exposure to 0.02 and 0.082 N/m^2 shear stress (*EDR* of 0.4 and 6.5 W/m^3), nonconfluent CHO cells were arrested at G_0/G_1 phase and mRNA level for the recombinant product was increased; conversely, confluent CHO cells were found not sensitive to the shear forces tested. Keane et al. (2003) found that long-term exposure of attached CHO cells to high level shear stress led to reduced productivity and altered glucose metabolism. Compared to cells exposed to low shear stress of 0.005 N/m^2 (0.02 W/m^3), cells exposed to high shear stress of 0.80 N/m^2

(6.4×10^2 W/m^3) for 32 h showed 51 % lower recombinant protein production, 42 % higher glucose uptake, and 50 % lower lactate production.

In addition to the single pass studies conducted in which levels of *EDR* were used which will disrupt cells (Fig. 6.1), using this same specifically designed flow contraction device, Godoy-Silva et al. (2009a) placed the system in a recycle stream with a typical bench scale bioreactor. The repetitive exposure, and significantly lower levels of *EDR*, was an initial attempt to simulate the fluid dynamic environment in large-scale bioreactors where cells circulate between high shear and low shear regions.

With this repetitive, recycle system, the potential, physiological impact of hydrodynamic stress on CHO and NS0 cells was considered, using variables of cell growth rate, product productivity, metabolism, and mAb product quality. At a EDR of 6.0×104 W/m^3, which is approximately two orders of magnitude lower than the single pass *EDR* where physical cell damage occurs, no significant physiological effects were observed; however, it was observed that mAb glycosylation pattern was shifted. The shift led to reduced G0 population, with a corresponding increase in G1 and G2 populations of the N-linked glycan on Fc. This shift of glycosylation pattern was confirmed with a second CHO cell line expressing a different mAb product. Conversely, a low EDR of 0.9×10^2 W/m^3 had no effect on the glycosylation pattern (Fig. 6.6). Sieck et al. (2013) observed a similar glycosylation pattern shift estimated to be a EDR value of 4×10^2 W/m^3 for a CHO cell line in an agitated bench-scale bioreactor where hydrodynamic stress was generated by the impellers. Seick et al. (2013) also observed reduced cell specific productivity at constant or periodic exposure to EDR of 4×10^2 W/m^3

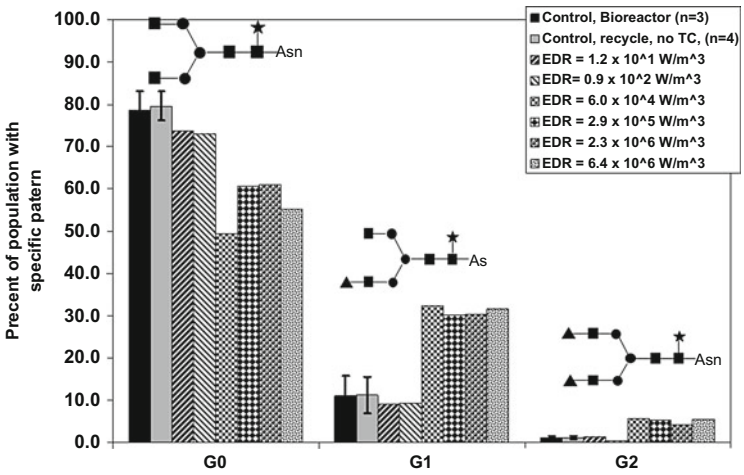


Fig. 6.6 Comparison of glycosylation profiles of mAbs produced in 2 L bioreactors under different level of hydrodynamic stress. Two sets of control results are included. One is the average of the four recirculating control bioreactors and the other one is the average of three historical non-recirculating bioreactors. The error bar indicates one standard deviation. (▲) galactose, (■) N-acetylglucosamine, (●) mannose, and (⋆) fucose

although neither cell growth nor glucose/glutamine metabolism was affected. Contrary to these detectable effects, Nienow et al. (2013) using similar systems to the study of Godoy-Silva et al., found no change in glycosylation for two CHO cell lines at EDR similar to that of Godoy-Silva et al. Further, in the Godoy-Silva et al. (2009b) study, it was reported that the NS0 cell line showed no change in glycosylation under high EDR. This contradiction appears to follow one of the common "rules of thumb" that specific effects can be cell line/clone dependent. However, all of these non-lethal effects are still above typical operating conditions in commercial STB and at this point have more "academic" than "industrial" significance.

Conclusion and Future Directions

We suggest that we have highlighted that traditional, animal cells used for recombinant protein production are robust in typical bioprocessing, with the only current possible areas of concerns occurring in some downstream processing equipment. That being said, the field has "selected" cell lines/types that have proven to be robust, and extending this to other cell lines is not as certain. Initial studies of several human blood cancer cell lines demonstrated surprising more sensitivity (several orders of magnitude) than typical CHO lines.

However, in one assumes that the rate of increase in the routinely achievable, final cell concentrations and product titers continues, it is highly likely that in the near future conditions in bioreactors will necessitate significantly higher levels of mixing and improved cell protective additives. At that time, the field could begin to push the limits of hydrodynamic conditions that can cause cell damage and a renewed interest in better understanding these effects, and prevent undesirable ones, will be needed.

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Chapter 7
Monitoring of Cell Culture

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Abstract Cell culture monitoring is essential for the continuous characterisation of cultivated cells. This helps us to understand each cell line's growth and metabolism properties and to establish and ensure reproducible cell cultivations. Monitoring not only comprises biological parameters, like cell count and viability, but also physiological parameters, like substrate, metabolite and possibly product concentrations. Furthermore, a large set of physicochemical parameters such as pH, temperature and osmolarity is routinely monitored in cell culture applications. A multiplicity of monitoring technologies exists. Often, there are several methods for one monitoring parameter to choose from and the choice depends on application and process needs. Moreover, new methods are developed at ever increasing speed. Today, cell culture processes and regulatory agencies demand detailed process understanding, which extensive monitoring is a prerequisite for. In this chapter, the current state of monitoring technologies and applications is reviewed. Particular emphasis is placed on biological parameters, i.e. cell density and viability as well as substrates, metabolites, and product concentration but also on cell stress and apoptosis. Furthermore, promising exploratory technologies are surveyed. Lastly, the chapter is meant to bridge the gap between existing technology-driven and more biology-oriented publications in this field.

Keywords Cell culture monitoring • Cell count and viability • Substrates, metabolites and product monitoring • Cell stress and apoptosis

7.1 Introduction

Culture monitoring is of utmost importance for all applications of cell culture technology, be it large scale recombinant protein production or laboratory scale cell culture in biomedical research. For each application, suitable monitoring

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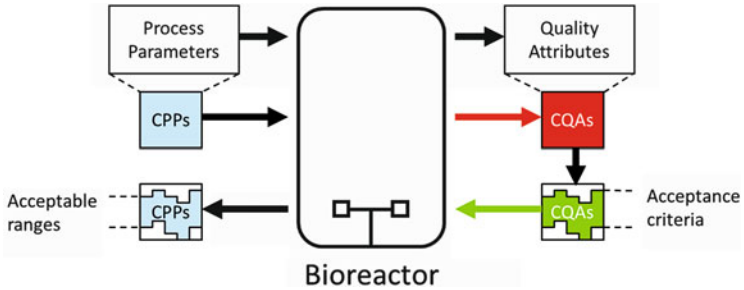

Fig. 7.1 Interplay of process parameters and quality attributes in quality by design. *CPP* critical process parameter, *CQA* critical quality attribute

parameters have to be defined. The monitoring – and control – of for example dissolved oxygen (DO) is important for industrial scale bioreactors but not so much for adherent T-flask cell culture in the laboratory. Additional challenges arise from different culture scales and the (un)availability of monitoring technologies in general. As much as e.g. the use of Clark electrodes is established for use in bioreactors, different technologies have to be developed to establish oxygen monitoring in small vessels or microtiter plates. Particularly in the area of biopharmaceutical process development, a shift from looking at the cultured cell as a "black box" to a deeper process understanding including the cells themselves requires enhanced monitoring means, e.g. detailed metabolite or apoptosis analysis. Even further challenges are caused by the advent of disposable, single use culture vessels necessitating the development of disposable and affordable sensors.

Furthermore, the "Process analytical Technology" (PAT) initiative of the FDA, the US Food and Drug Administration – compare Chap. 21 of this volume or e.g. FDA (2004) – demands process understanding and control based on monitoring raw materials and quality attributes, at best in real-time, necessitating a paradigm shift in process monitoring and sensor technology (Read et al. 2009).

Additionally, potent monitoring is fundamental in the context of Quality by design (QbD; International Conference on Harmonisation 2005, 2008, 2009; Rathore 2009; compare Fig. 7.1). In QbD, among all process parameters, the identification of the critical process parameters (CPPs) that influence the critical quality attributes (CQAs) is key. With knowledge about the acceptance criteria of the CQAs, the acceptable ranges of the CPPs can be set. To that end, the success of the QbD approach is based on several pillars like the use of qualified scale down models, application of risk assessment methodologies and sound experimental planning based on design of experiments. On top of these, another very important pillar of QbD is represented by the analytical capabilities to measure process parameters and quality attributes satisfactorily in a validated environment.

Furthermore, The European Medicines Agency (EMA), in a very recent guideline on process validation, stresses the importance of in-line, online and at-line monitoring for continuous process verification (European Medicines Agency 2014).

Taken together, culture monitoring is a challenging and rapidly evolving field and crucial for all aspects of cell culture technology.

7.2 Monitoring Principles

There are two main ways to obtain monitoring data of process parameters. Either, a sample of the cell culture is taken and the cells or the cell free supernatant of the sample is subsequently analysed. Here, great care has to be taken specifically for two reasons: Firstly, to take the sample the sterile barrier of the culture vessel has to be breached. Since microbial or – less often – viral contamination is a major risk of all cell culture operations, suitable sampling devices maintaining sterility need to be in place. These range from properly operated laminar flow hoods if a culture vessel has to be physically opened over steam sterilised sampling containers connected to a bioreactor to disposable sampling devices (as e.g. the Clave connector, ICU Medical, San Clemente, USA). Secondly, the sample has to be correctly treated in order to not obscure the original conditions as they were inside the culture vessel. For example, if the monitoring parameter is pH or DO, care has to be taken to prevent gassing out of CO_2 or O_2 between taking the sample and analysing it.

Alternatively, process parameters can be monitored without taking samples. An advantage of this method is that no sterile barrier has to be breached to obtain monitoring data. Also, this method has the advantage of speed – no time is lost through sampling, sample preparation and external analysis. Especially physical parameters like pH, DO and temperature are often measured in such a setting, providing a constant stream of measurement data and thereby the basis of control of the parameters. On the other hand, such direct monitoring requires capable sensors, which means they have to be operational inside a cultivation vessel (e.g. dye based cell monitoring methods and devices usually are not so) and also they must withstand cleaning and sterilisation operations (CIP / SIP) of the vessel.

Combinations of these two methods exist, e.g. technical solutions for sample taking by a continuously installed auto-sampler device and automated further analysis of the sample.

In general, monitoring methods are categorized as off-line, at-line, online and in-line/*in situ* (compare Fig. 7.2). In off-line analysis, samples are removed from the cultivation vessel and transferred to a different location (e.g. to a quality control laboratory) for analysis. In at-line monitoring, samples are removed and subsequently analysed with a short time lag and in close proximity to the cultivation

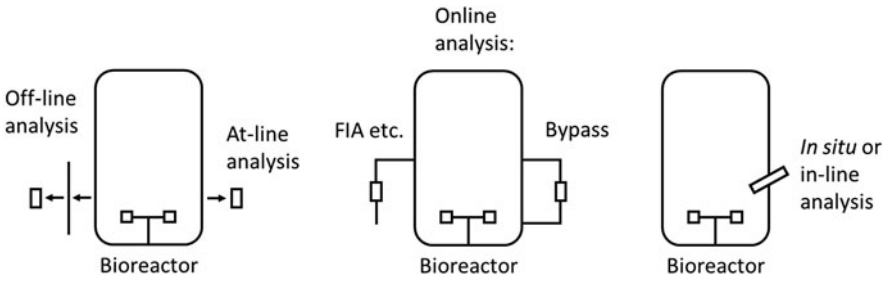

Fig. 7.2 Monitoring methods. *FIA* flow injection analysis

vessel. Online monitoring is characterised by either continuous removal of process liquids, guiding it past the sensor and feeding the (hopefully) unaltered liquid back into the cultivation vessel (bypass configuration) or by automated removal of a sample, preparation of the sample (e.g. dilution, addition of dyes), subsequent analysis and discarding of the sample. A prominent example of the latter is FIA (flow injection analysis). In-line or *in situ* analysis involves sensors that are directly installed in the cultivation vessel. Classic examples are pH, DO, conductivity or temperature probes, but more advanced solutions like Infrared (IR) and Raman spectroscopy sensors or *in situ* microscopy for cell monitoring exist (see below).

Deeper process understanding and advanced process optimisation often necessitating real time monitoring and control as well as PAT and QbD often favour a direct (at best *in situ*) monitoring of process parameters. This is due to above given advantages i.e. mainly measurement frequency and closed control loops. On the other hand, this is sometimes not possible due to the unavailability of suitable technology or due to economic considerations. For example, as much as it might be advisable to constantly monitor apoptosis in certain cell cultures, it is technically very demanding to connect flow cytometry devices to bioreactors, even if "only" in an online setting, and probably would be beyond process economics if done so at each bioreactor. Also, the nature of the bioprocess itself might influence the type of monitoring device chosen. For example, due to the long doubling times of mammalian cells (hours to days), it might be much more acceptable to monitor certain parameters off-line here than in the case of microbial processes with doubling times of 30 min.

Whether installed inside the cultivation vessel or used outside of it, monitoring devices must fulfil certain requirements to be usable in a laboratory and even more so in the tightly regulated area of industrial cell culture technology. These characteristics include adequate sensitivity and specificity, (mechanical) robustness and ease of use, suitability for cleaning and sterilising, and ease of validation.

Monitoring of process characteristics is sometimes done without the intention of control of the parameter. Sampling of cell culture vessels for microbial sterility tests would be an example for this situation. In the majority of cases, monitoring of a process parameter is connected to control of the parameter. Monitoring and control are paramount to enable robust performance of cell culture processes in a laboratory setting and even more so in the area of large scale application and commercial manufacturing. In general, such monitoring and control ensures maintenance of physicochemical conditions of importance for cell cultivation (e.g. temperature, pH value, DO and DCO_2 level) as well as biological/physiological parameters as e.g. glucose or glutamine content. A (not exhaustive) list of monitoring parameters can be found in Table 7.1. It is obvious that for some of these parameters a continuous flow of monitoring data is of key importance for control. In such cases, monitoring is preferably performed *in situ*. DO, pH, temperature and stirrer-speed in a bioreactor are classic examples. Other parameters are controlled less rigidly, e.g. the glucose content of a bioreactor might be adjusted by manual feed after daily sampling and off-line analysis.

Table 7.1 Typical process parameters and quality attributes for animal cell cultures. Additional parameters might be of value for different organisms or applications

Parameter	Typical monitoring device	Typical monitoring method
Physicochemical parameters		
Temperature	Resistance thermometer (PT 100 probe)	*In situ*
pH value	Electrochemical Ingold pH glass electrode / optical sensor	*In situ*
Dissolved Oxygen (DO)	Amperometric Clark electrode/optical sensor	*In situ*
Pressure	Membrane sensor	*In situ*
Stirrer speed	Revolution counter	*In situ* (on stirrer motor)
Foam	Conductivity probe	*In situ*
Fluid addition/ weight	Balance/load cell	*In situ* (on vessel)
Gas addition	Mass flow meters	*In situ* (gas lines)
DCO_2	DCO_2 probe (pH sensor in saturated bicarbonate)	*In situ*
Redox potential	Redox electrode	*In situ*
Osmolarity	Freezing point depression meter	Off-line
Physiological/biological quality attributes		
Biomass	Light scattering/absorbance Probes	*In situ*
Cell volume	Packed cell volume (PCV) tube	Off-line
Cell number and viability	Dye based and dye free methods	Off-line, on-line or *in situ*
Substrates and metabolites	Enzymatic, spectroscopic and other methods	Off-line or *in situ*
Lactate dehydrogenase activity	Enzymatic/photometric methods	Off-line
Product concentration	HPLC, photometric, spectroscopic and other methods	Off-line or *in situ*
Product quality	Various methods, e.g. SEC, IEC, LCMS, HPLC	Off-line
Apoptosis	Flow cytometry	Off-line
Metabolic state	Mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR)	Off-line
Transcriptomic state	Microarrays, RNA-seq (next generation sequencers)	Off-line

When choosing monitoring parameters and equipment, along with the (understandable) intention to gain as much information about a cultivation process as possible it is important to consider additional aspects. One of those is cost: Particularly in the academic sector, money might be a limiting factor. As much as it might be desirable to closely monitor apoptosis, the cost of flow cytometry reagents or the cytometer itself can restrict these efforts; the same holds true for monitoring key substrates and metabolites like glucose, glutamine, lactate and

ammonia, analysed either by bench top chemistry or automated analysers. In commercial manufacturing, measurements that are cost and time intensive have to be restricted to those parameters that are of key importance for product quality and quantity. A second aspect is the developmental stage of the process: During process development or in R&D in general, emphasis is on gaining a deep culture and process understanding and on the identification of critical process parameters. Here, many different methods and parameters might be assessed for process and product characterisation. In preparation for routine production, the number of parameters to be monitored has to be reduced to those of critical importance to control the process within predefined operational limits on the basis of the information gained in earlier stages of development.

7.3 Historical Perspective; Scope of This Article

Cultivation and monitoring of animal cells is a young discipline and routinely performed for not more than 60 years, starting with the advent of continuous cell lines in the 1950s. Industrial scale cell cultures are even younger, the companies Celltech and Genentech pioneering large scale (1,000–10,000 L) stirred tank cultivation processes in the 1980s. In monitoring those processes, emphasis was initially on basic physicochemical parameters, cell number, viability and product titer. From the 1990s onwards, physiological parameters like specific uptake (substrates) or production (byproducts and products) rates as well as metabolites were increasingly getting into focus (Konstantinov 1996) along with a more detailed "monitoring view" into cell cultivations. Quicker methods for metabolite monitoring, automated cell counters and the use of flow cytometry (not only in small scale culture and clone screening but also in industrial scale cultures and their scale-up; e.g. Sitton and Srienc 2008) developed the field further. These advanced monitoring approaches by now are extended amongst others towards UV- NIR- and fluorescence-measurements (Teixeira et al. 2009a), the latter covering a wide range of excitation and emission wavelengths in experimental 2D settings (Teixeira et al. 2009b). Not all experimental monitoring approaches proved at this stage suitable for routine application; e.g. are NADP(H) (Reardon et al. 1987) or Redox potential (Dahod 1982) not employed often as parameters in routine cell cultivation monitoring.

The monitoring of "classic" parameters like pH and DO saw technical advances, too; in these cases through optical methods (Hansen and Miró 2007; Naciri et al. 2008; Lam and Kostov 2010; Teixeira et al. 2009b) that in some applications proved advantageous over established electrochemical probes, particularly in the emerging field of single use bioreactors (Rao et al. 2009). Parallel developments can be observed in the area of microbial bioprocesses (e.g. Clementschitsch and

Bayer 2006). Additionally, there is an increasing body of data on monitoring pH, DO etc. in very small (mL scale) cultivation volumes which is important, among other things, for higher throughput clone screening approaches (Deshpande et al. 2004; Puskeiler et al. 2005).

It is beyond the scope of this contribution to cover all mentioned aspects of cell culture monitoring. Additionally, some very recent publications give a very good overview over aspects in the field, e.g. Pohlscheidt et al. (2013), who particularly cover monitoring of physicochemical parameters as well as soft sensors, and Büntemeyer (2013) in the same volume who reviews off line monitoring. The present contribution focusses on key biologic and metabolic parameters whose monitoring is of highest importance for any cell cultivation process. Furthermore, it sheds light on emerging technologies and exploratory approaches towards monitoring those parameters as well as a few process implications of monitoring specifically these parameters. Additionally, for many technologies a short list of (partly subjective and not necessarily exhaustive) advantages and disadvantages are given.

7.4 Parameters and Technologies: Viable Cell Density, Total Cell Density and Cell Viability

Cell density is one of the most relevant process parameters in mammalian cell cultivation. This is due to the fact that all stages of cell cultivation, from establishing a simple sub-cultivation routine in basic cell culture to a defined process control-strategy in commercial production, are based on an accurate determination of cell density (e.g. Konstantinov 1996). One has to distinguish between viable and total cell density (VCD and TCD) as well as cell viability, the quotient of both.

Ideally, cell density and viability would be determined inside the culture vessel to avoid contamination from breaching the sterile barrier when sampling (Rehbock et al. 2008). Different strategies have been developed to meet the criterion of sampling-free analysis, e.g. measurement of optical density or capacitance, spectroscopy or indirect methods (e.g. off-gas analysis) via *in situ* probes. The drawback of most of those strategies is that they cannot distinguish reliably viable from dead biomass (Braasch et al. 2013), so viability determination is not possible or at least performed insufficiently. Potential alternatives are *in situ* microscopy and advanced NIR systems. Both have been reported to be able to discriminate between living and dead cells in an in-line setting (see Sects. 7.4.2.4 and 7.4.2.3), the former additionally providing morphological data of the cells. However, sampling and subsequent off-line or at-line analysis of cell density and viability with an appropriate method is predominantly performed as yet (Table 7.2).

Table 7.2 Methods for cell density and viability monitoring and their analysis method

Method/equipment	In-line	Online	At-/off-line
Trypan blue dye exclution		●	●
Flow cytometry		●	●
Impedance measurement			●
Capacitance measurement	●		
Spectroscopy	●		●
In situ microscopy	●		

In general, methods for cell density and viability monitoring can be differentiated into dye-based (e.g. trypan blue; fluorescence dyes) and dye-free (e.g. Coulter principle or capacitance) methods.

Many dye-based methods for viability monitoring, particularly those using trypan blue (see Sect. 7.4.1.1) and propidium iodide (see Sect. 7.4.1.2), rely on the condition of the cellular membrane (see below). A cell with a functional membrane is defined as viable, one with a compromised membrane dead (Altman et al. 1993; Browne and Al-Rubeai 2011). As much as this might be correct, especially in situations where necrosis is the cause of cell death, this definition neglects all cases where not passive, necrotic events but programmed, apoptotic processes or autophagy lead to cell death. Particularly in a bioreactor environment, apoptosis is accepted as the main cause of cell death (Ishaque and Al-Rubeai 2002; Singh et al. 1994; Zustiak et al. 2008). Apoptotic cells on their route to death and disintegration still possess an uncompromised membrane. An assay detecting cell viability accurately should therefore go beyond detecting cells with a compromised cellular membrane only. Fluorescence dye based assays, as described in Sect. 7.4.1.2, can be tailored towards those approaches. Moreover, Sect. 7.6 describes integrated methods for determining the cells physiological and stress related state.

7.4.1 Dye-Based Methods for Monitoring Cell Density and Viability

7.4.1.1 Trypan Blue Dye Exclusion

A very old, but still widely-used method for differentiation of viable and dead cells is the selective staining with trypan blue. This principle, investigated by Evans and Schulemann in 1914 (Evans and Schulemann 1914), relies on the cell membrane integrity dependent permeability for large molecules such as trypan blue. Viable cells (integrity of membrane) exclude the dye and appear unstained under the microscope, while the cytoplasm of non-viable cells with compromised cellular membranes is stained by the dye and appears (light-) purple-violet.

Hemoyctometer

For manual determination of viable and dead cell density, a cell suspension sample is mixed with a trypan blue stock solution and analyzed in a hemocytometer. Commonly used is a Neubauer chamber. The squares engraved on its surface are counted out with bright field microscopy. The viable/dead cell density as well as the viability of the sample can subsequently be determined.

Advantages

- Inexpensive

Disadvantages

- Time consuming
- User bias (user-to-user variation)
- Low number of counts (inaccurate measure)

Automated Trypan Blue Based Cell Counting

To overcome the disadvantages of manual hemocytometer-based cell counting, automated cell counters based on trypan blue staining have been developed.

Those machines, such as e.g. Vi-CELL (Beckman Coulter, Brea, USA) or CedexHiRes (Roche, Basel, Switzerland) are image-based cell analyzers using a fully automated liquid handling systems with an integrated flow through ("pseudo hemocytometer") chamber and a high-resolution image scanner. Image analysis algorithms (cell line dependent adaptation is required) enable the system to classify detected objects into debris, viable and dead cells, aggregates and other undefined objects. Besides the main parameters (viable-, total-cell density and viability), other parameters such as object-diameter can be examined. The cell diameter e.g. is a known indicator for deteriorating culture healthiness or ongoing apoptosis (Darzynkiewicz et al. 1997; Ishaque and Al-Rubeai 2002; Kroemer et al. 2009; Krysko et al. 2008).

Advantages

- Cost effective trypan blue assay
- Avoids variability inherent in manual sample preparation and counting
- No user-bias

Disadvantages

- High initial investment in equipment as compared to hemocytometer.
- Systematic errors can be caused by suboptimal settings of algorithm parameters.

Additional, general aspects of trypan blue based cell counting methods

Advantages

- Visual inspection of cell population
- Long term "gold standard"; acceptance by regulatory agencies

Disadvantages

- Serum and other FCS related substances can inhibit trypan blue (Bhuyan et al. 1976; Hu and el-Fakahany 1994).
- Staining artifacts can hamper interpretation of results.

7.4.1.2 Fluorescence Based Cell Density and Viability Determination via Flow Cytometry

Flow cytometry is a laser-based technique to simultaneously analyze various physicochemical and/or biological parameters of thousands of single cells in a cell population in a matter of seconds (see e.g. Al-Rubeai and Emery 1993 and Al-Rubeai et al. 1997 as an introduction). The technique has an enormous breath of applications in the life sciences (e.g. cell viability, cell number, analysis of the cell cycle, apoptosis, mitochondrial activity etc.). Analyzed cells can subsequently be sorted (fluorescence activated cell sorter; FACS) based on a specific characteristics.

Additional fluorescence-based solutions exist, e.g. the NucleoCounter, an automated image cytometer (Chemometecs; Allerød, Denmark) but not all can be described in this contribution. The following paragraph focuses on flow cytometry – not sorting – used for cell density and cell viability monitoring applications. Furthermore, flow cytometry will be revisited in a later section of this chapter as a means for monitoring cell stress/apoptosis.

In a flow cytometer, the cells are injected into a fast flowing sheath liquid stream. The cells are focused in the center of the sheath stream (hydrodynamic focusing) and pass cell by cell through the laser beam (flow cell). Scattered light (forwards and sidewards) as well as emitted fluorescence signals of the cell are detected and analyzed. Flow cell design can differ depending on the system used.

As mentioned above, flow cytometry has become a standard method for analyzing cell parameters like cell viability or/and cell density. It has to be noted that for absolute cell density determination (cell number per unit sample volume) an accurate determination of the sample volume is necessary and most flow cytometers nowadays, enable this. However, the applied principles can be different. Becton Dickinson (Franklin Lakes, USA) uses microprocessor-controlled peristaltic pump systems to monitor the sample volume pulled per run, whereas Partec (Bielefeld, Germany) measures the volume directly by mechanical means (volume defined by the distance between two platinum electrodes). Still, a different principle is applied by Guava (Merck, Darmstadt, Germany). In their systems, the classical flow cell has been substituted by a sheath fluid free microcapillary. The sample is "injected" due to direct aspiration, thus enabling accurate determination of sample volume.

For cell viability determination a broad set of suitable dyes are available (note that different cell lines might response differently to the same dye). It is important to distinguish between exclusion and inclusion dyes. The most prominent examples of exclusion dyes are propodium iodide (PI) or acridine orange (AO). Those dyes, similar to trypan blue, enter exclusively cells with compromised cellular membrane and bind, for example to nucleic acids in the case of PI/AO. The so-called inclusion dyes on the other hand, e.g. compounds like fluorescein diacetate (FDA), diffuse quickly into cells, after which they are cleaved by esterases to yield a non membrane-permeable, charged molecule, i.e. in this case, fluorescein. Undamaged cells retain fluorescein, whereas in damaged cells fluorescence weakens due to the dye leaking out of the cell.

In general, flow cytometry is used as an off-line or at-line technique. For further development of the method towards online monitoring and PAT, it would need to become automated in terms of sampling, staining and analysis. In cell culture technology, only few reports have been published on this topic (Kuystermans et al. 2012; Sitton and Srienc 2008). One of the major challenges besides establishing automated staining protocols is the sterile connection and sampling during the cell fermentation.

Advantages

- Rapid analysis, therefore high number of counts possible, resulting in low statistical error
- Apoptosis and more additional parameters can be monitored with the same equipment

Disadvantages

- Relatively high costs (initial investment and assay chemicals).
- Interferences are described in the literature (e.g. Altman et al. 1993). In addition to nucleic acids, PI may e.g. bind to glycosaminoglycans on the plasma membrane of viable cells.

7.4.2 Non-dye-based Methods for Monitoring Cell Density and Viability

7.4.2.1 Impedance (Electrical Resistance) Measurement

One of the oldest non-dye based measurement methods for cell density is the so-called “Coulter Particle Counter principle”. Here, the alteration of the electrical resistance caused by nonconductive particles such as cells (Coulter 1953; Gutmann 1966) is detected. The most commonly used systems based on impedance measurement are the Coulter Particle Counter (Beckman Coulter, Brea, USA) and the CASY cell counter (formerly Schärfe Systems/Innovatis, now Roche, Basel, Switzerland).

Both systems use more or less the same principle instrumental setup. In an isotonic liquid (PBS; CASYton) diluted cells are pumped through an orifice, separating two electrodes (chambers), the so-called sensing zone. As cells are less conductive than the liquid they are suspended in, each passing of a particle results in an increase of the electrical resistance across the sensing zone. This change in resistance causes a pulse (voltage or current, depending on the instrument) where the height of the generated signal is proportional to the volume, resistivity and shape of the particle (Matsushita et al. 1982; Freshney 2005). For cells of the same shape, a variation of cellular volume causes a change in size distribution; this can be exploited to discriminate between living cells and dead cells (dead cell = size of the nucleus; viable cell = size of the cell defined by its intact membrane).

Advantages

- Rapid analysis and therefore high number of counts possible resulting in low statistical error
- No user-bias
- Non-dye based measurement principle with no impact on viability by potentially cytotoxic dyes

Disadvantages

- Because of analyzing cells by examination of an electrical signal, no direct monitoring (visualization) of measured events is possible, this can lead to misinterpretation (aggregates/debris).

7.4.2.2 Capacitance Measurement

The principle of capacitance measurement, also termed radio-frequency impedance or dielectric spectroscopy, as a means of biomass determination, relies on the fact that cells with an intact cellular membrane in an aqueous ionic suspension can act as small capacitors when subjected to a periodically alternating electrical field (Carvell and Dowd 2006). Depending on the frequency and strength of the applied field, ions in the medium and the cytoplasm react. Positively charged ions are pushed in the direction of the field, whereas negatively charged ions are pushed in the opposite direction. The mobility of the ions is limited by the cellular membrane, which acts as a barrier for intra- as well as extracellular ions. Frequencies of 0.1–10 MHz applied to the cells result in a charge separation across the membrane at the poles of the cell (negatively charged ions positioned on the inside and positively charged ions on the outside at one pole and vice versa at the other pole), also termed polarization (Arnold 2001). The magnitude of that polarization is measured by the resulting capacitance in picoFarad (pF). Lower frequencies allow ions to travel until stopped by membranes, thereby creating a higher capacitance; higher frequencies reverse movement of ions before complete polarization of the membranes resulting in a lower capacitance. Directly proportional to an increasing cell number or

biomass, the number of polarized membranes and therefore the measured capacitance increases. Cell death is accompanied by a loss of integrity of the cellular membrane, which results in a free diffusive exchange of intra- and extracellular compounds and thereby does not contribute to the capacitance of the cell suspension. Therefore, the method does not allow the determination of the total cell density. Also, oil droplets, debris or gas bubbles do not contribute to the measured capacitance since they do not possess a cellular membrane. Nevertheless, at high concentrations, particles can reduce the net suspension capacity due to the reduction of the fraction of cellular volumes in the suspension. This is particularly important in microcarrier cultivations and might require calibrations in the presence of the normal concentration of carriers (Carvell and Dowd 2006).

The technology has found considerable acceptance in the industry (Carvell and Dowd 2006; Justice et al. 2011). Particularly for commercial bioprocesses, dielectric spectroscopy in-line probes are available from Aber Instruments (Aberystwyth, UK) and FOGALE Biotech (now Hamilton Bonaduz, Bonaduz, Switzerland). Both manufacturers provide stainless steel reusable probes (CIP/SIPable) as well as single-use probes embedded in single-use bag systems, e.g. for Hyclone STR (Thermo Scientific HyClone, South Logan, USA), Sartorius Cultibag and STR (Sartorius, Göttingen, Germany) Mobius STR (Millipore/Merck, Darmstadt, Germany).

The measurement principle of capacitance using dielectric sensors should not be mistaken for dielectrophoresis (DEP). DEP describes a principle where cells are separated based on their polarizability. The polarizability depends on conductivity and permittivity of the cell. Dead cells have a much higher conductivity (approx. 10^4 times) than viable cells. This is due to the fact that because of their interrupted cell membrane intra- and extracellular compounds can enter and leave by diffusion. Use of DEP for determining early apoptosis events in bioprocesses has recently been published by Braasch et al. (2013) and will be dealt with in Sect. 7.6.

Advantages

- Non-contact in-line measurement
- CIP-/SIPable; in situ analysis possible
- Non-optical/non-dye based method; no potential influence of dyes on viability

Disadvantages

- Non-image based analysis (correlation of spectroscopic data with off-line biomass measurements necessary).
- Opel et al. (2010) reported growth phase dependent variations in accuracy of mathematical models for capacitance and biomass correlation.

7.4.2.3 Electromagnetic Spectroscopic Measurements (NIR, MIR, Raman)

Electromagnetic spectroscopic techniques, such as near infrared (NIR), mid infrared (MIR), far infrared (FIR) or Raman spectroscopy are based on the principle that material matter can absorb energy after exposure to electromagnetic radiation. The energy of infrared radiation is in the range of the energy necessary to cause vibrations, rotations or stretching of molecular bonds. IR spectroscopy analyses the absorption of resonant frequencies characteristic for the molecular structure of a sample. Depending on the wavelength/wavenumber range in which this absorption occurs, different spectrometers using particular light sources and detectors have been developed.

An advantage of spectroscopic techniques in general is their capability for in-line (real time) monitoring using immersion probes capable of cleaning- and sterilization- in place (CIP/SIP). Spectroscopic methods deliver complex spectra, enabling the analysis of several parameters (physical product properties as well as chemical compounds) in parallel but do therefore require the application of advanced mathematical and statistical evaluation methods (chemometrics).

Two widely used chemometric methods are principal component analysis (PCA) and partial least squares (PLS). PCA can be described as a data-reduction method, used for decreasing the complexity of a data-set by breaking down the number of dimensions due to the generation of new variables (principal components; PCs). A PC consists of the linear combination of several original variables (dimensions). PCA is used for qualitative spectra evaluation. Regression methods as e.g. PLS are widely used for quantitative spectra evaluation by establishing a relationship between spectra (so-called latent variables; regions of the spectra) and the parameter to be quantified (e.g. cell density, metabolite concentration etc.) (Lourenco et al. 2012). Spectroscopy-based technologies e.g. such as NIR, MIR or Raman-spectroscopy have proven to be key PAT technologies.

Near Infrared Spectroscopy (NIRS)

Near infrared (NIR) spectroscopy determines absorption in the near infrared region (wave numbers 13,000–4,000 cm^{-1}) of the electromagnetic spectrum. NIR relevant functional groups of molecules in the sample are predominantly those containing a hydrogen atom (like O-H, C-H or N-H). It is actually the chemical bond whose "natural" vibration is moved from one energy level to another, causing so-called overtones and combination-bands in the NIR spectra (Simpson 2005).

NIR has become widely accepted in the pharmaceutical industry, up to now mostly for raw material testing (Rodionova et al. 2009; Hakemeyer et al. 2013). Moreover, there are several reports on using NIR for bioprocess control (Arnold et al. 2003; Cervera et al. 2009; Qui et al. 2014; Scarff et al. 2006), determining cell

density but most often metabolite concentration (e.g. glucose or lactate) during mammalian cell cultivation processes.

Sartorius Stedim (Göttingen, Germany) released in 2012 a NIR spectrometer which can be applied to standard stainless steel (as well as glass) bioreactors via a standard Ingold port for bioprocess monitoring and control (cell count, viability and glucose; Sandor et al. 2013).

Advantages

– No interference with water (as in MIR)

Disadvantages

– Resultant peaks are broad and overlapping, making interpretation of spectra difficult.

Mid Infrared Spectroscopy (MIRS)

Similar to NIRS, also MIRS is an absorption-based technique. Mid infrared spectroscopy covers absorption bands in the mid infrared range (wavenumber 4,000–400 cm^{-1}), caused by the excitation of fundamental vibrations of almost all chemical bonds. In MIRS, a very relevant spectral region is below 1,700 cm^{-1}, the so-called fingerprint region; those absorptions arise from the molecular skeleton (e.g. C-C or C = C) and are highly molecule-specific.

Despite of the fact that MIR signals provide more defined peaks than NIR in the absorption spectra (Landgrebe et al. 2010), reports on biotechnological applications of MIR are rare compared to NIR. This is partly due to the strong absorption of water at wavenumbers below 4,000 cm^{-1} as well as technical difficulties (Teixeira et al. 2009a). MIR is used for monitoring mammalian cell cultivations; e.g. Rhiel et al. (2002) monitored glucose and lactate concentration in a CHO fermentation by MIRS. However, reports on using MIRS for determining cell viabilities or cell densities are missing so far. Because of advantages of MIR (fingerprint regions), it is often reported in the context of metabolite/protein quantification (e.g. Sellick et al. 2010). These issues are elaborated further in the metabolite monitoring section below.

Advantages

– Absorbance is stronger and more distinct (narrower peak-shape) compared to NIR.
– Characteristic spectra (fingerprint regions).

Disadvantages

– Strong interference of water, causing masking of spectral information
– More expensive and fragile fiber optic compared to NIRS (Teixeira et al. 2009a)

Raman Spectroscopy

Compared to NIR and MIR which are based on detecting absorption of electromagnetic radiation, Raman spectroscopy is a scattering-based technique. Raman spectroscopy relies on the inelastic scattering of monochromatic light (usually from an adjustable laser source in the spectral range between near infrared and near ultraviolet light) due to the interaction of photons with the sample. Vibrational, rotational and other low frequency transitions in molecules caused by energy transmission from the light to the molecule (photon excites a molecule from ground- to a virtual higher energy-level) or vice versa (molecule relaxes and emits a photon) can be observed through frequency changes (Jestel 2005). Spectra that can be detected after irradiation and light scattering consist mostly of unaltered frequencies, those of the excitation light (Rayleigh scattering), as well as a fraction of shifted frequencies (Raman scattering). The frequency shift of excitation light and scattered light is molecule-specific. Therefore, Raman spectroscopy can provide information regarding the composition of the analyzed sample. However, Raman spectra are very complex and contain, unselectively, information on all molecules of the sample, it is therefore more or less impossible to simply "stare and compare" (Goodacre and Jarvis, 2005). For spectra evaluation, advanced mathematical and statistical chemometric methods (such as Principle Component Analysis, PCA; Hierarchical Cluster Analysis, HCA; or Partial Least Square, PLS) are required (Abu-Absi et al. 2011; Lourenco et al. 2012).

Raman Spectroscopy fulfills most of the PAT requirements for in-line process control methods, the technology therefore bears a high potential for application in cell culture and microbial fermentations as a means for biomass and metabolite determination. Examples from animal cell culture technology are metabolite monitoring (Xu et al. 1997) and recently published data for viability and VCD as well as glucose, glutamine, glutamate, lactate and ammonium monitoring in CHO cultures (Abu-Absi et al. 2011). Nevertheless, challenges of the method include the strong fluorescence of many biological molecules that may overlay Raman scattering signals and the generally weak Raman scattering of cell culture broths (Ulber et al. 2003). Commercial Raman probes are available e.g. from Kaiser Optical Systems Inc. (Ann Arbor, USA).

Advantages

- No interference with water (as in MIR)

Disadvantages

- Overlapping/overlaying of the Raman scattering bonds by fluorescence activity of several biological molecules in the sample.
- Bubbles from aeration can cause light scattering and attenuate signal intensity.
- Requires high power laser which increases costs and complexity of instruments (Lourenco et al. 2012).

General advantages/disadvantages of electromagnetic spectroscopy methods for cell density and viability determination

Advantages

- Can be applied to aqueous solutions without any sample preparation.
- Several parameters can be monitored with the same probe and simultaneously.
- Cleaning and sterilization in place (CIP/SIP) possible.
- In situ analysis possible.
- Non-dye based principle; does not interfere with cell culture.

Disadvantages

- Non-image based analysis (correlation of spectroscopic data with off line-/routine-biomass measurements).
- Calibration can be difficult; for interpretation of data chemometrical methods are necessary.
- Biomass measurement accuracy depending on growth phase (high accuracy during growth phase, low accuracy during stationary and decline phases).

7.4.2.4 In Situ Microscopy

In situ microscopy was first described by Suhr et al. (1995). It combines the advantages of non-contact microscopic inspection of cells with in-line monitoring of main process parameters as cell number, cell size distribution, morphological characteristics and – in the case of mammalian cells – viability. This information can be deduced directly from the monitored objects by using image evaluation. Therefore, no chemometrical methods are necessary for obtaining relative measurements of cell concentration and viability. For obtaining absolute measurement-values, suitable one point calibration procedures have to be carried out.

In general, an *in situ* microscope (ISM) consists of a video microscope which is directly immersed in the moving cell suspension. Microscopic images are subject to image analysis algorithms which select and count cells and measure morphological parameters. The original microscope as published by Suhr et al. (1995) employed fluorescence microscopy with incident light illumination to monitor *Saccharomyces cerevisiae*. For the monitoring of mammalian cell cultures, mainly two transmitted light *in situ* microscopic setups have been described, both of them employing transmitted light.

One of these ISMs employs a mechanically defined sampling zone that can be adjusted in volume by means of a sampling zone tube connected to a movable slide (Joeris et al. 2002; Höpfner et al. 2010). Inside this tube is an inner tube containing the objective, outside of which, a third tube – the probe tube – is placed. An LED emitting at a wavelength of 525 nm is placed at the top end of the latter inside the bioreactor. A linear stage, on which the CCD camera is mounted with cross-roller bearings, is fitted to a U-shape profile in combination with a second stage (see Höpfner et al. 2010 for details). Adjustment of the sampling zone as well as

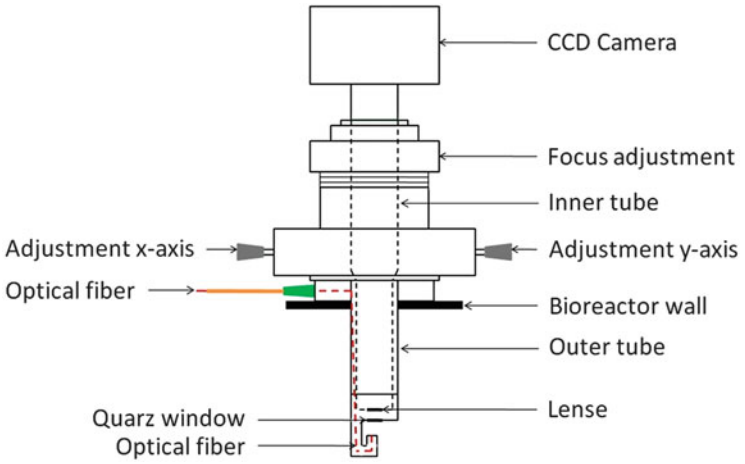

Fig. 7.3 In situ microscope as used by Camisard et al. (2002), Guez et al. (2004), Wiedemann et al. (2010 and 2011a, b)

focusing is carried out by two stepper motors. The complex set up can be removed from the probe tube to allow CIP and SIP operations of the bioreactor. This ISM has been used to monitor the total cell density of different types of animal cells including CHO and BHK as well as NIH-3 T3 cells cultivated on microcarriers (Rudolph et al. 2008, 2010; Höpfner et al. 2010). Also, an at-line setting of the device has been described (Akin et al. 2011). A proposed modification of this ISM is based on dark field illumination combined with image evaluation by support vector machine classifiers (Wei et al. 2007). Experimentally, this technique has been studied in off-line settings to distinguish viable from killed *Saccharomyces cerevisiae* cells in a mixture of both.

A different type of *in situ* microscope is based on non-contact-sampling by using optical depth of focus (Camisard et al. 2002; Guez et al. 2004). It consists of an objective connected to a CCD camera by an (inner) tube. This in turn is incorporated in an outer tube installed in a standard 25 mm Ingold port (see Fig. 7.3). The outer tube can be cleaned and sterilised when installed (CIP/SIP). No tubes have to be moved for operation, no moving mechanical parts or stepper motors are installed. No moveable or electric parts exist inside the bioreactor which is an advantage for cleaning validation and potential use in a GMP environment. An external luminescence diode generates flash illumination which is guided to the sampling zone by an optical fiber. Due to the short flash-duration of about one microsecond, the setup generates images of moving cells without blur. The sampling volume is defined optically by depth of field (Perrin 1909) and cell counting and characterization is performed by image analysis (Suhr et al. 1995). This ISM has been used for monitoring the cell density of Hybridoma (Guez et al. 2004) and Jurkat cells (Wiedemann et al. 2010).

This ISM as also been used to determine the viability of animal cells in an *in situ* and real time setting. Different image analysis principles have been used for this purpose. Guez et al. (2010) and Wiedemann et al. (2011a) describe analysis of the variance of greyness levels of cells for determination of viability whereas Wiedemann et al. (2011b) use the information entropy (E) of cell portraits for this purpose. Here, E is defined by $E = \sum p(i) \log_2 [p(i)]$ where i runs over all grey values present in the image and p is the probability of the grey values i occurring in the image (Gonzalez and Woods 2008).

Advantages

- Sampling free visual data in real time
- Dye-free determination of cell density and viability
- Additional morphological information of the monitored cells available

Disadvantages

- Image analysis algorithms need to be tailored to specific cell species, particularly in viability determination.

7.5 Parameters and Technologies: Metabolic Parameters and Recombinant Products

Since the 1990s, one of the major drivers in process development was to optimize the productivity of fermentation processes e.g. by prolonging the life span of the culture or by raising the specific productivity (e.g. Cotter and al-Rubeai 1995; De Jesus and Wurm 2011). This can, amongst other means, be achieved by optimizing substrate feeding to a culture, preventing a decline in specific productivity, preventing variation of product quality (e.g. glycosylation) and preventing cell stress or cell death due to a limiting factor.

To avoid such limiting factors, an accurate monitoring of key substrates like glucose and L-glutamine is necessary. Apart from monitoring substrates, metabolites such as potentially toxic by-products, e.g. lactate or ammonia, or the recombinant product are important factors to be monitored. In process development, this helps to determine needs and boundaries of processes, later in production it is an indicator of culture reproducibility.

It should be noted that analyzing product titer normally is accompanied by determining product quality attributes. However, a survey on the determination of such (biochemical) attributes is beyond the scope of this chapter. The same holds true for amino acid analysis beyond glutamine/glutamate. Particularly during process development, it might be feasible to extensively analyse the amino acid spectrum of cultures. This can be helpful when optimising culture media or determining optimal feeds. In industry and academia alike, amino acid profiling is often performed using ready to use kits, often GC-MS coupled, like EZfaast (Phenomenex, Torrance, USA). These methods will not be described in detail here.

Table 7.3 Methods for monitoring of metabolic parameters and recombinant products and their analysis method

Method/equipment	In-line	Online	At-/off-line
Metabolite analyzers		X	X
Spectroscopy	X		X
Automated systems for product monitoring			X

Substrate and metabolite concentrations can be measured using "classic" analytical chemistry methods employing photometry or titration. Product concentration classically is determined by enzyme-linked immunosorbent assays (ELISA) or chromatography (HPLC). Many of these assay formats are time-consuming, labor-intensive and difficult to carry out in a higher throughput setting as required by today's industrial biotechnology users. Therefore, the focus of the section below is on determining substrate and metabolite concentrations based on automated enzymatic (enzymatic-amperometric/enzymatic-photometric) or spectroscopic (IR) measurement principles, while concentrating on label-free, higher throughput capable automated systems for determining product concentration.

To this end, the majority of monitoring procedures are to date carried out off-line or at-line, due to a lack of reliable in-situ probes (Table 7.3). However, there are some applications of in-situ probes or commercial systems to connect an analytical device under aseptic conditions. Those configurations minimize risks (contamination, operator-depending variability) linked to sampling procedures. An example for such a device would be automated sampling devices (see below). Another prominent example of directly coupled systems is flow injection analysis (FIA) (Rehbock et al. 2008; Becker et al. 2007).

The monitoring of some parameters can have implications beyond the importance of the parameter as substrate or metabolite. A prominent example for this is Lactate Dehydrogenase (LDH). It is a strictly intracellular enzyme that in continuous cell lines mainly catalyses the conversion of pyruvate to lactate. Once LDH is released from the cells, its concentration can be monitored by NADH dependent spectrophotometric assays (Racher et al. 1990). Alternatively, automated LDH assays e.g. for the CedexBio (Roche, see below), exist.

The release of LDH has been described to be an indicator of cell death (Wagner et al. 1992). Therefore, it can be used as an indirect parameter for cell viability. More specifically, Chuan et al. (2006) describe a strong correlation of LDH release to caspase release and conclude that LDH can serve as an indirect indicator of apoptotic cell death in batch and fed batch cultures. It has also been used to measure necrotic cell death (Koh and Choi 1987) and is used in the industry e.g. to assess the influence of hardware characteristics (pipes, pumps, perfusion systems) on cell viability.

Furthermore, the release of LDH correlates with that of other intracellular enzymes (proteases, glycosidases) that can degrade the recombinant product. Indeed, it has been shown, that LDH release correlated with product quality decrease in cultures of BHK cells producing an IgG-IL two fusion protein

(e.g. Cruz et al. 2000). Chuan et al. (2006) showed that an LDH release linked sialidase release into the medium of EPO producing CHO cells leads to a reduced sialidation status of the recombinant product.

Lactate is another example of a monitoring parameter with large implications for process characteristics. Due to the Warburg effect, lactate is the main reason for acidification of continuous mammalian cell cultures. It has been known for a long time that the accumulation of lactate causes growth and productivity inhibition (e.g. Reuveny et al. 1986; Glacken et al. 1986; Glacken et al. 1988; Glacken 1988). Furthermore, the stoichiometric ratio of built lactate to consumed glucose (two, if all glucose is converted to lactate) is one indicator for the metabolic state of the culture.

Under some conditions, e.g. in the stationary phase of fed batch cultures or under low pH, lactate may be consumed rather than produced. This metabolic shift often has a positive effect on productivity (Hu 2012). Nevertheless, the mechanisms are not yet fully understood and the shift might not be easily reproducible even under controlled conditions. Lactate accumulation is still a challenge for industrial fed batch processes (Abu-Absi et al. 2014; Hu 2012; Le et al. 2012). There is an interrelation between lactate and osmolarity, the latter rising due to lactate accumulation and counteracting pH drop by base addition. Also, elevated CO_2 levels lead to base addition which in turn partially inhibits glucose oxidation, thereby raising lactate levels (Abu-Absi et al. 2014).

Lactate monitoring is also important for another reason: Small scale process development data may not translate easily to conditions in large scale bioreactors during scale-up. Lower degrees of CO_2 stripping and differences in cellular metabolism at large scale can lead to raised lactate concentrations (Abu-Absi et al. 2014). Smelko et al. (2011) and others report highest lactate levels in 15,000 L reactors when comparing a NS0 fed batch process across scales.

Taken together, the monitoring of such process parameters in combination with intelligent data mining can have a tremendous impact on process development and performance.

7.5.1 Automated Analyzers (Substrate, Metabolite and Product Monitoring)

Several commercial systems are available, e.g. BioProfile Flex (Nova Biomedical, Waltham, USA), CedexBio (Roche Diagnostics, Mannheim, Germany) or YSI 2700 (YSI, Yellow Springs Instruments; Yellow Springs, USA). All three analyzers are amongst others capable of determining the concentrations of glucose, L-glutamine, lactate as well as ammonia in aqueous solutions ($\leq$500 µL sample volume). The readout of the systems is different, with both the BioProfile Flex (except for ammonia) and the YSI systems using enzymatic-amperometric sensors with enzymes immobilized between membranes, as opposed to the CedexBio which

relies on enzymatic-photometric measurement principles (with free enzymes in solution).

Beyond such fundamental substrate and metabolite measurements, the CedexBio is capable of analyzing Lactate dehydrogenase (LDH; enzymatic-photometric), IgG (turbidity/photometric) and electrolytes (ion selective electrodes). The Nova BioProfile Flex is a multi-analyzer, which in addition to substrate and metabolite measurements enables the quantification of various electrolytes plus ammonia (ion selective electrodes), osmolality (freezing point depression), cell density and viability (trypan blue), off-gases (membrane-amperometric electrodes) as well as IgG quantification (photometric).

These bio-analyzers are most often used as standalone (off-line/at line) devices. Nevertheless, there are solutions available to connect them to one or even multiple bioreactors enabling aseptic sampling over a more or less unlimited period of time. Such auto sampling technologies are e.g. the Flex online auto sampler module (Nova Biomedical, Waltham, USA), whose configuration allows the connection of up to ten bioreactors to one BioProfile Flex. An alternative might be the SEG-FLOW sampling system (Flownamics, Madison, USA), which can draw samples out of up to eight bioreactor vessels and analyzes them by interfacing with e.g. YSI 2700 or Nova Biomedical analyzer systems.

7.5.2 Spectroscopic Methods (Substrate, Metabolite and Product Monitoring)

The principles of electromagnetic spectroscopic measurements such as NIR, MIR and Raman spectroscopy were already described in Sect. 7.4.2.3 (Cell density and viability monitoring). In brief, energy emitted by a light source can be absorbed by sample components (analytes) resulting in an analyte-specific attenuation fingerprint in the IR spectra. Mathematical and statistical evaluation tools like multivariate partial least square regression can be used to build models, correlating spectral information to analyte concentrations. Therefore, "training batches" are required, where the analyte concentration over time has been determined using conventional off-line methods.

7.5.2.1 Near Infrared Spectroscopy (NIRS)

There are several studies published evaluating the capacity of NIR spectroscopy in terms of substrate, metabolite and product-protein monitoring. Harthun et al. (1998) use spectral information to generate PLS-based calibration models enabling accurate prediction of glucose-, L-glutamine-, glutamate-, lactate-, ammonium- and recombinant human antithrombin III- concentration from uncleared cell samples in CHO cultures. Arnold et al. (2003) as well as Qui et al. (2014) use *in situ* and

on-line NIR probes for monitoring glucose, lactate and in the case of Arnold et al. additionally ammonina and glutamine. With Teixeira et al. (2009a) and Scarff et al. (2006), two exhaustive reviews summarizing recent applications of IR spectroscopy in cell culture technology are available.

Advantages

– "One probe fits many applications"

Disadvantages

– Low sensitivity (in the sub-gram region, as reported by Kornmann et al. 2004), make an application of this method for monitoring diluted molecules difficult

7.5.2.2 Mid Infrared Spectroscopy (MIRS)

The capacity of MIR spectroscopy for substrate and metabolite monitoring was demonstrated by Rhiel et al. (2002). They used *in situ* MIR spectroscopy to monitor lactate and glucose during 11–15 day CHO batch fermentations. Capito et al. (2013a, b) used off-line MIR spectroscopy to quantify antibody aggregates in therapeutic antibody purification processes as well as the quantification of host cell protein (HCP) from clarified cell culture supernatants. Sellick et al. (2010) demonstrate the use of off-line MIR to determine IgG titer from clarified CHO and NS0 cell culture samples via linear regression models (PLS).

Advantages

– Sensitivity as compared to NIR. Rhiel et al. (2002) reported detectability of glucose- and lactate-concentration changes of 0.5 mM.

Disadvantages

– Strong interference with water, masking important information in the spectra

7.5.2.3 Raman Spectroscopy

Abu-Absi et al. (2011) claimed reporting first results of in-line Raman spectroscopy for determining amongst others substrates (glucose, L-glutamine) and metabolites (lactate, ammonia) during CHO batch cultivations. Raman spectra were correlated to at-line measurements using PLS. Beyond that, there are some very recent studies available employing off-line Raman spectroscopy for e.g. predicting antibody titer (Ashton et al. 2013; Li et al. 2013).

As mentioned above, commercial Raman probes are available e.g. from Kaiser Optical Systems Inc. (Ann Arbor, USA).

Advantages

- Weaker absorption of polar substances such as water

Disadvantages

- Raman signals are in general weak (e.g. Ulber et al. 2003), therefore high concentrations of analyte compounds are required (Teixeira et al. 2009a).

7.5.3 Automated Systems for Product-Quantification

Screening capabilities, e.g. for product quantification, are required in some areas of animal cell culture technology. Especially in cell line- and process-development, large numbers of cell clones and cultivation conditions are assessed. Other than growth characteristics of the clones, one of the most important selection criteria is the specific productivity rate (Kim et al. 2012).

Beyond bioanalyzers as mentioned above (e.g. BioProfile Flex and CedexBio), which are capable of IgG protein-quantification, systems especially suited for high throughput protein quantification are available for such purposes. Biacore (General Electrics, Fairfield, USA), the Octet-Q (Pall FortéBio Corp., Menlo Park, USA) as well as the Caliper LapChip (Perkin Elmer, Waltham, USA) are examples and will be briefly discussed below.

7.5.3.1 Surface Plasmon Resonance (Biacore Systems)

These instruments measure molecular interaction in real time, while one molecule is immobilized on a chip surface and the second is free in solution. The sensor chip consists of a thin layer of gold on a glass surface. The gold layer is modified with a carbohydrate matrix on which proteins (the counterpart of the molecule to be quantified) are attached. The technique is based on a phenomenon called surface plasmon resonance (SPR): Polarized light is focused on the back of the sensor chip under conditions of total internal reflection, while a detector monitors the intensity of the reflected light. Under these conditions, a light component called *evanescent wave* leaks into the gold layer. At a certain angle of incident light the evanescent wave excites gold electrons. This results in surface plasmon resonance within the gold layer concomitant with a drop of the intensity of the reflected light at this angle. The angle of reflective light (refractive index) is amongst others, depending on the adsorption of molecules to the sensor surface (Schasfoort and McWhirter 2008).

Winheim et al. (2009) reported the development and qualification of a quantification assay for recombinant hIgG4 based on surface plasmon resonance measurement in a GxP environment. The authors report a higher precision and accuracy and less hands-on time with their Biacore-based assay compared to commercially available IgG-ELISAs. Beyond the application for quantification of immunoglobulins,

Zeder-Lutz et al. (1999) and Wendler et al. (2005) reported e.g. use of plasmon resonance to quantify biologically active recombinant biomolecules such as e.g. active hBMP-2 (human bone morphogenetic protein-2, Wendler et al. 2005)

Advantages

- Label-free method
- Ubiquitously applicable to proteins

Disadvantages

- Costly equipment (main device and sensor-chips)

7.5.3.2 Bio-Layer Interferometry (Octet Systems)

Protein quantification with the Octet system relies on bio-layer interferometry (BLI). It is based on the binding of macromolecules to a compatible ligand, depending on the molecule to be quantified, e.g. Protein A for IgG. Binding of an analyte molecule in the sample solution to a ligand immobilized on the biosensor surface increases the overall thickness at the biosensor tip. The interference pattern of white light, emitted by the device, lit down the sensor and reflected by two surfaces (an internal reference layer and a layer of immobilized ligand/protein on the sensor tip) is measured. A change in the number of molecules bound to the sensor tip causes a wavelength shift, detected in form of alteration of the interference pattern (Dayne 2012). The Octet systems allow fast and simple protein quantification, affinity or kinetic measurements, processing up to 96 samples in parallel. The specific quantification of proteins out of complex sample-matrices by BLI was reported e.g. by Li et al. (2011) The authors found that the Octet method used for antidrug antibody (ADA) quantification was ten times more sensitive than comparable ELISA assays. Further reports exist; e.g. Legmann et al. (2009) described the quantification of MAB concentrations in crude CHO cell culture samples using the Octet system.

Advantages

- Only molecules that directly bind to the biosensor surface are detected, providing high specificity.
- Label-free method.
- High throughput capable.
- Broad quantification range: 25 ng/m of – 2,000 µg/ml of hIgG using Protein A.

Disadvantages

- Costly equipment and consumables (sensor-tips)

7.5.3.3 Microfluidic Gel Electrophoresis (Caliper LapChip Systems)

The working principle of the LapChip system (Caliper Life Sciences, Hopkinton, USA, now Perkin Elmer, Waltham, USA) is based on classic gel electrophoresis, transferred to a microfluidic chip as described by Bousse et al. (2001). The LapChip system allows automated sample aspiration (from 96 or from 384 well plates), subsequent staining and terminal quantification. E.g. Legmann et al. (2009) reported the high throughput (HT) analysis of MAB integrity, purity and glycan-heterogeneity from mammalian cell culture samples on a LapChip system equipped with a HT Protein Express LapChip kit.

Advantages

– Combination of sensitivity of SDS-Page with speed of microchip technology

Disadvantages

– Costly consumables

7.6 Parameters and Technologies: Monitoring Cell Stress and Apoptosis

Many methods for apoptosis detection exist. In the opinion of the authors, particularly well-suited for standardized monitoring applications are methods based on flow cytometry and dye-based plate reader assays. Therefore, this section introduces both, at present widely used flow cytometry or microplate reader-based methods as well as exploratory (mass spectrometry or dielectrophoresis) instrumentation capable of monitoring cell stress/apoptosis in cell cultures. These methods aim at fulfilling general requirements for cell culture monitoring imposed by FDA and EMA, i.e. providing detailed information on a cellular level for an enhanced understanding of the cells physiological state (Read et al. 2009).

7.6.1 Flow Cytometry (FC)

The use of flow cytometry in the context of determining cell density and viability was already described in Sect. 7.4.1.2. Beyond such methods aimed at detection of plasma membrane integrity as an indicator for cell viability, FC became a powerful tool for assessing early indications of cellular stress or alterations in the cells physiological state. In this section, some of the – in the author's perspective – most interesting assay formats particularly in terms of process monitoring are highlighted.

The arguably most prevalent FC assay to observe cell stress and apoptosis is the detection of externalization of phosphatidylserine (PS) by FITC conjugated Annexin-V. The translocation/externalization of PS is an event found in cells

undergoing apoptosis and is considered an early marker of apoptosis (e.g. Krysko et al. 2008; Denecker et al. 2000). Nevertheless, externalization of PS is cell type specific and an event downstream of caspase (cysteine-dependent aspartate-directed proteases) activation (e.g. Fadeel et al. 1999); in other words it is an early, but not very early marker of apoptosis. Also, necrotic cells which have lost membrane integrity become positively stained by Annexin-V FITC. Additional staining with e.g. propidium iodide (PI) or 7-aminoactinomycin (7-AAD) allows for discrimination between viable cells which are FITC/Annexin-V negative and secondary stain negative, apoptotic cells (+/−) and late apoptotic or necrotic cells (+/+; e.g. Wlodkowic et al. 2012).

For early apoptosis monitoring, other markers can be employed. As both, intrinsic and extrinsic activation of apoptosis pathways, at early stages of programmed cell death very often lead to the activation of caspases −8 or −9, these enzymes are a feasible marker for detecting early apoptotic events. Fluorochrome-labeled inhibitors of caspases (FLICA) assays are broadly used for direct measurement of caspase activity (Abu-Absi et al. 2011). Membrane-penetrating FLICA molecules containing fluoromethyl ketones (FMK) bind to activated caspases since they also contain recognition peptide moieties specific for the caspase to be detected. Thereby, they are retained in the cell whereas unbound FLICA diffuses out of the cell and gets washed away. Necrotic and apoptotic cells can be identified in combination with an appropriate viability dye (PI, 7-AAD).

Another flow cytometry-based assay to monitor apoptosis assesses changes in the mitochondrial membrane potential (MMP; $\Delta\Psi m$). Intracellular compartments such as mitochondria require a function-related membrane potential. Disruption of the MMP, e.g. by physiological effectors like reactive oxygen species (Darzynkiewicz et al. 1997; Lee et al. 2008), has consequences for mitochondrial respiration, energy production and hence on cell survival. Declining of MMP is described as the point-of-no-return in apoptosis progression (Kroemer and Reed 2000). For monitoring MMP depolarization, JC1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide), a lipophilic cationic dye is widely used.

This dye shows so-called dual emission characteristics (Zamzami et al. 1995), meaning that at low concentrations JC-1 exists mainly as monomer (emission wavelength at 527 nm, green fluorescence) whereas at higher concentrations aggregates are formed, so-called “J-aggregates” (emission wavelength at 590 nm, red fluorescence). Healthy and apoptotic cells are differentiated by the fact that in case of healthy cells (high MMP) the majority of the lipophilic JC-1 is taken up by the mitochondria (Reers et al. 1995) where the red fluorescent aggregates are formed. Smaller amounts of JC-1 remain as green fluorescent monomers in the cytoplasm. The effect of accumulation in the electronegatively charged mitochondria rather than in the cytoplasm can be explained by the Nernst equation (Farkas et al. 1989). A collapsing MMP causes the diffusion and dissolving of the J-aggregates concomitant with a fluorescence ratio change from predominantly red (J-aggregates) to green (monomer JC-1).

Table 7.4 Methods for cell stress and apoptosis monitoring and their analysis method

Method/equipment	In-line	Online	At-/off-line
Flow cytometry		X	X
Microplate/multiwell plate-reader			X
Mass spectrometry			X
Dielectrophoresis		X	X

Advantages

– Multiplexing analysis

Disadvantages

– No online application (Table 7.4) for such early cell stress markers published so far (online applications only for viability, VCD and cell cycle analysis, e.g. Kuystermans et al. 2012; Sitton and Srienc 2008)

7.6.2 Microplate/Multiwell Plate-Reader

Besides the many available options to determine cell stress and apoptosis by flow cytometry, in some cases it might be more advantageous running those assays on a microplate/multi-well plate reader instead. Other than the higher sample throughput per time-unit, one advantage is the possibility to run not only fluorescence but also luminescence assays on these machines. The difference to fluorescence measurements is that in luminescence assays the emitted light is not the result of excitation by light of a different wavelength but of a chemical reaction. Compared to autofluorescence in fluorescence readings, there is actually no commonly used medium compound or endogenous cell constituent that exhibits auto-luminescence. This results in a lower background signal and concomitantly in a high sensitivity of the assay. It has to be taken into account though that these devices determine only average values of e.g. luminescence or fluorescence over the number of cells per well, whereas flow cytometry provides information on single cell level. In case the laboratory is equipped with both, flow cytometer and multi-well plate reader, it might be worthwhile comparing an assay in both to identify the optimal testing method for a specific application.

Multi-well plate readers are available from a range of suppliers (e.g. Promega GmbH, Mannheim, Germany; BMG Labtech, Ortenberg, Germany; Tecan Group Ltd., Männerdorf, Germany).

Advantages

– Higher throughput by less operation time

Disadvantages

– Only information on bulk/pool level, compared to flow cytometry analysis (Table 7.4)

7.6.3 *Mass Spectrometry*

An alternative method for monitoring cell stress/apoptosis in cell cultures is mass spectrometry (MS). Especially Matrix-Assisted Laser Desorption/Ionization (Reers et al. 1995) or Surface Enhanced Laser Desorption/Ionization (MALDI-; Karas and Hillenkamp 1988) Time of Flight Mass Spectrometry (TOF MS) could have the potential to become powerful methods for analysis of cell culture samples in terms of cell stress and ongoing apoptosis.

MS-biotyping applications, originally used in clinical and environmental microbiology environments to identify bacteria based on specific MS-fingerprints, are recently being used in the context of mammalian cell culture as well (SELDI-; Hutchens and Yip 1993).

In these MALDI TOF MS assays, the analyzed cells (sometimes referred to as "intact cells") get mixed with matrix and subjected to MS-analysis, a fast and label-free technique resulting in specific fingerprints/signatures.

Those signatures can be correlated with progressing apoptosis, as reported by Dong et al. (2011), Zhang et al. (2006) or Schwamb et al. (2013). Dong et al. identified two MS-peaks whose intensities correlated with the percentage of apoptotic cells in various adherent cell lines. Schwamb et al. presented the identification of a specific MS-signature consisting of 51 peaks, enabling the discrimination of unknown samples of suspension adapted CHO cell lines regarding their cell physiological state (viable, early apoptotic and late apoptotic/necrotic). The authors present a classification model built on the basis of this signature, allowing for prediction of viability changes 24 h earlier than with standard membrane integrity based monitoring and concomitant with the detection of an early apoptosis marker.

The application of SELDI TOF MS for mammalian cell culture monitoring has been introduced by Woolley and Al-Rubeai (2009a, b), reporting the identification of a biomarker for cell stress in CHO cell culture while profiling the supernatant of culture media. The identified peak at 7,7 kDa is reported to be correlating in signal intensity with the loss of viability.

Apart from monitoring cell stress and apoptosis, in the context of cell culture technology "intact cell" mass spectrometry has been used for clone characterization and stability assessment. Feng et al. (2010, 2011) report on screening recombinant CHO cell lines to determine high and low producers as well as fast stability assessment of clones by MALDI TOF MS.

Advantages

- Label free method
- Fast sample acquisition
- Detailed view into proteome/metabolome of the cell possible

Disadvantages

- At line; (Table 7.4) presently no online or in-line method available
- High investment for mass spectrometer; relatively high maintenance costs of equipment

7.6.4 Dielectrophoresis

Dielectrophoresis (DEP) describes the movement of a neutral particle in a non-uniform (divergent) electrical field (Pohl and Hawk 1966) not to be confused with the principle of electrophoresis, which describes the movement of charged particles in an uniform (parallel) electrical field.

Cells get polarized when exposed to an electrical field. This polarization causes intracellular "charge separation", due to the fact, that positively and negatively charged molecules inside the cells are pulled in opposite directions, causing a dipole. The intensity of those charge separations by a constant electrical field strength depends largely on the conductive and dielectric properties of the cells themselves (Diaz and Payen 2008; Nikolic-Jaric et al. 2013).

In a non-uniform field, due to the characteristics of divergent field lines, the local electrical field on both ends of the dipole (i.e. the cell) will be different. Thereby, depending on the strength of polarization, which again depends on cell physiological state, a force results that pulls the cell either towards the inner electrode (high-electrical field region; pDEP) or towards the outer electrode (weaker-electrical field region; nDEP) (Diaz and Payen 2008; Nikolic-Jaric et al. 2013).

DEP has been investigated mainly in cancer research e.g. by Becker et al. (1995), Diaz and Payen (2008), Gascoyne et al. (1997), or Labeed et al. (2003). Becker et al. and Gascoyne et al. demonstrated the separation of different cancerous cell types from blood using DEP, whereas Labeed et al. employed DEP to examine differences in the dielectric properties of non- and drug-treated cancerous cells. More related to cell culture monitoring are two recent studies by Nikolic-Jaric et al. (2013) and Braasch et al. (2013) utilizing DEP for the detection of apoptosis onset in CHO cells. The authors use a prototype DEP cytometer, to enable analysis of the dielectric property of single cells from a "homogenous" cell population. In their CHO cultures they observed at least two populations of cells distinguishable by their polarizability. Employing comparison analysis using standard apoptosis staining (Annexin-V) assays one of those populations could be associated with viable and the second with apoptotic cells.

Advantages

- A major advantage of DEP compared to capacitance probes is the fact, that DEP provides dielectric properties of individual cells not a mean value for a cell suspension.
- Low cost.

Disadvantages

- Only prototypic equipment available as yet

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Chapter 8
Serum and Protein Free Media

Michael Butler

Abstract For many decades bovine serum has been used as an essential component for the growth of animal cells in culture. However, the combined disadvantages of variability in composition, cost but particularly the potential for contamination with viruses or prions has been the driver for the substitution of serum with a more defined and animal-component free media. For some cell lines substitution with just a few simple ingredients can provide an effective liquid media for growth. However, for a number of cell lines finding a suitable serum-free formulation for growth has been very challenging. Because of the complexity of these formulations statistically designed methods have been adopted to ensure a rational approach to media design. This, as well as the increasing availability of microbially-produced recombinant forms of animal proteins has been significant in the development of animal-component free and chemically defined media. Sometimes chemically-defined media have poorer characteristics for growth promotion than the serum-based formulations that they replace. However, incremental steps of improvement are possible by the addition of key ingredients or by adaptation of the cells to newly formatted serum-free media.

Keywords Serum • Basal media • Chemically-defined media • Peptide hydrolysates

8.1 Introduction

Various biological fluids including serum, tissue extracts and homogenized chicken embryos have been used over the past 100 years to grow animal cells in vitro. It was in the 1950s that serious efforts were made to adopt systematic approaches to determine the nutrients required for mammalian cell growth. One approach was an attempt to analyze the contents of biological fluids. This was only partially successful because of the multiple components present at micromolar concentrations (Morgan et al. 1950).

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A second approach was to determine the minimal chemically-defined components that were essential for growth. This approach led to the development of Eagle's Minimal Essential Media (EMEM) (Eagle 1959). This consisted of 13 amino acids, 8 vitamins, 6 ionic species and supplemented with dialyzed serum to provide the undefined components required for growth. Although serum-supplemented EMEM was suitable for the growth of a number of cell lines, other cells required more complex formulations. By increasing the component concentrations of EMEM, basal media formulations such as Dulbecco's modification of Eagles medium (DMEM) were developed for the growth of other cell lines, to higher cell densities and for the propagation of viruses (Dulbecco and Freeman 1959). Enrichment of media with an enhanced range of nutritional components allowed clonal cell growth of selected cell lines, largely by the early work of Richard Ham who gave his name to the widely-used Ham's F-12 medium (Ham 1965). Sato had the ingenious idea of blending basal media formulations and developed the now commonly used 1:1 v/v DMEM/F-12 mix that has become widely used for the growth of multiple cell lines to high density (Jayme et al. 1997). However, despite the inclusion of up to 70 defined components, these chemically-defined media are still designated as *basal media* because they require supplementation with serum (typically 10 %) to sustain the growth of most cell lines,

This chapter focuses on the use of techniques to replace serum and the development of serum-free formulations.

8.2 The Advantages and Disadvantages of Serum

Serum, as the supernatant from clotted blood of bovine or equine sources has been found to provide high growth-promoting activity for a range of mammalian cell lines. This is a rich source of often unidentified components such as attachment factors, micronutrients, trace elements, water insoluble nutrients, growth factors, hormones, proteases, and protective elements (antitoxins, antioxidants, antiproteases), not provided by the basal medium and that promote rapid cell growth. Furthermore, the high albumin content of serum ensures that the cells are well-protected from potentially adverse conditions such as pH fluctuations or shear forces, which may occur particularly in large-scale cultures. However, supplementation of culture media with serum has many inherent disadvantages.

(a) *Batch-to-batch variation in composition*. The composition of serum is variable and undefined, which leads to inconsistent growth and productivity. Each batch of serum can vary in composition depending upon the diet and environmental conditions of the donor animals. This variation can cause significant differences in the growth-promoting characteristics of the serum, and ultimately causes significant differences in productivity of the cell-culture process.

(b) *A high protein content that hinders product purification*. The cells grown in a bioreactor secrete the product of interest (normally a protein) into the culture medium. If the culture medium contains serum, its protein concentration is already high. This causes difficulties in purification of the final product. Culture medium with a serum content of 10 % v/v has a high protein concentration that approaches 10 g/L. In comparison, the concentration of a recombinant protein secreted by the cells may only reach 0.1 g/L. This poses a problem in the purification process of the targeted protein within a large mix of serum protein. Furthermore, if the target is a specific monoclonal antibody, it may well be mixed with any other non-specific antibodies present in the serum and these are very difficult to separate.

(c) *The potential for product contamination*. The threat of contamination arises from unwanted viruses and mycoplasma that may be present in serum as well as the vectors of bovine spongiform encephalopathy (BSE, or "*Mad Cow Disease*"). Although there have been no proven cases of such contamination getting into the final product, no one wants to take the chance of manufacturing biopharmaceuticals that may have had contact with unknown agents of disease (Merten 2002). Prions as agents of BSE are of particular concern because of the difficulty of their removal. The incidences of new variant CJD (the human version of BSE) that have occurred in Europe are a great source of concern for the vaccine industry that has used serum and for which there is up to now no ideal substitute. Because of the concern over the potential human consequences of the presence of these contaminants in therapeutic products, most regulatory authorities have demanded the use of serum-free processes for biopharmaceutical manufacture.

(d) *Cost and availability*. Fetal bovine serum (FBS) is often regarding as the best available serum supplement for supporting cell growth. However, the cost is often prohibitive at $500–1,000 per litre and this can account for up to 95 % of the overall cost of the media. The most desirable source of FBS is from countries that have had minimal incidences of BSE such as New Zealand. However, there has from time to time been a global shortage of this serum and this can cause a problem in the continuity of a bioprocess.

(e) *Ethical concerns*. There is an increasing concern for the ethical treatment of animals that includes standard procedures needed to obtain serum from a bovine fetus.

8.3 Serum-Free Media

The challenge of formulating a serum-free medium is to identify and substitute all the components in serum that provide growth support for cells. Some cells are quite fastidious in their growth requirements and these requirements vary considerably from one cell line to another. Therefore, it has not been possible to design a single universal serum-free formulation to act as a serum substitute suitable for the growth

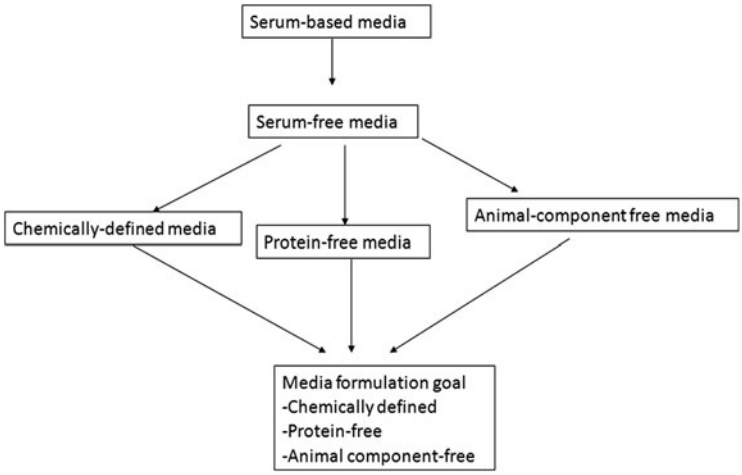

Fig. 8.1 A schematic showing the evolution of different media types

of all cell lines. In fact even different clones of the same cell line may require different formulations for optimal growth. As a result of this there are a multitude of different serum-free media formulations available, each normally directed for the growth of a specific cell line. Some formulations have been published. However, many are proprietary and are sold in liquid or powder form without an available list of components.

A serum-free formulation, by definition, means the absence of serum. However, within this definition there are various possibilities and classifications. Figure 8.1 shows the evolution 'of different types of media from those supplemented with serum to serum-free media with various characteristics. A chemically-defined medium is one in which all the components are known through well-defined molecular characteristics and may include proteins. A protein-free medium is one in which there is an absence of large proteins, although this may not be chemically-defined. An animal-component-free medium is one without components derived from animal sources but again this may not be chemically-defined. Each of these classifications is associated with potential advantages for growing mammalian cells in culture. Undoubtedly the most desirable medium is one which can be classified in each of these sub-types. That is: chemically-defined, protein-free and animal component-free. However, it is often the case that performance of the medium decreases with increased definition (Hodge 2005).

8.4 Basal Media

There are several rational approaches for obtaining a suitable serum-free formulation for the growth and productivity of a specific cell line. The general objective is to obtain a mixture of supplements that can substitute for the serum in a growth

media. However, one of the early considerations should be the basal media. Many of these media formulations were developed in the 1950s and 1960s and contain components of complex mixture of carbohydrate, amino acids, salts, vitamins, hormones, and growth factors. The most widely known are:-

- *Eagle's basal medium* (BME). This was originally designed for the growth of mouse-L and HeLa cells. There are several versions of BME which have been used for the growth of a wide range of cell lines. The medium needs to be changed at least every other day to support continued cell growth (Eagle 1955).
- *Eagle's minimum essential medium* (EMEM). EMEM has a higher concentration of amino acids than BME. It contains a balanced salt solution (Earle's), alternatively Hanks' salt solution can be used. This was developed as an improvement of BME for the optimal growth of a wide variety of clonal cultures (Eagle 1959).
- *Glasgow's modification of Eagle's medium* (GMEM). This is a modification of BME and contains 2X the concentrations of the amino acids and vitamins with extra glucose and bicarbonate. This media was originally developed for the growth of BHK21 cells (Stoker and Macpherson 1964).
- *Dulbecco's modification of Eagle's medium* (DMEM). This has four-times the BME concentration of amino acids and vitamins as well as addition non-essential amino acids and trace elements. A glucose concentration as high as 25 mM can be used in this medium for the growth of several cell types. The medium was first reported for the culture of embryonic mouse cells but has been used for a wide variety of applications for various cells, including primary mouse and chicken cells, and in virus production (Vogt and Dulbecco 1960).
- *RPMI 1640*. This was developed as a modification of McCoy 5A medium for the long-term culture of peripheral blood lymphocytes. It is now recognized as a general-purpose medium particularly for lymphocyte and hybridoma cultures [5].

These basal formulations contain published lists of up to 60 components and are available as sterilized liquids from media vendors. The basal media are normally supplemented with 10 % serum in order to promote maximum growth of specific cell lines. The serum may supplement the concentration of components already contained in the basal media. Therefore, one of the early steps in serum-free media development should be to optimize the basal medium. This is often done by blending existing formulations. For example, one choice which has proved popular is a 1:1 v/v mixture of DMEM and RPMI1640. DMEM provides high concentrations of amino acids and vitamins whereas RPMI1640 provides a long list of micronutrients to support cell growth.

8.5 Approaches for the Development of Serum-Free Media

8.5.1 Top-Down Approach for Serum Replacement

There are two basic approaches to developing serum-free media formulations. The first approach (top-down) might involve selecting an existing formulation for a similar cell line, supplemented with serum to obtain a reasonable level of growth. The content of serum could then be gradually reduced to cause a decrease in the cell growth to say 50 % of that originally obtained. At this point selected components could be added to the medium to restore the original level of cell growth.

The top-down approach is often easier to pursue since a working serum-free formulation can often be developed more quickly. Cell lines that belong to the same group, such as epithelial or transformed, often require the same growth factors for growth. Therefore a formulation that works for one epithelial cell line may work for another with minimal modifications to certain growth factors or hormones. For this reason serum-free formulations can be designed faster by this approach.

This method involves a systematic approach for the gradual replacement of serum by substitution with essential nutrients or growth factors. In this procedure the concentration of serum in gradually reduced to a level which around 50 % of maximal growth as measured by the cell yields after 3 or 4 days. This procedure may involve the adaptation of cells to gradually reduced levels of serum. Typically adaptation can be successful in reducing the serum concentration from 10 % to 2 %. At the lower serum level the growth rate will decrease until appropriate supplements are provided in the medium or until the cells adapt. Cellular adaptation can involve the synthesis of essential components by the cells.

The drawback to the top-down approach is that many components in the formulation may be unnecessary, and often inhibitory for growth. This can often result in the "capping" of the optimal performance of the medium (i.e.: the maximum growth may not be achieved) as improvements are hindered by the presence of unwanted compounds.

8.5.2 Bottom-Up Approach for Serum Replacement

The bottom-up approach involves the addition of potentially growth promoting components systematically to the media in an attempt to obtain incremental increases in cell growth. Although more labour-intensive and time-consuming, this approach can lead to higher quality media. Only the components that are required for growth are included in the formulation, allowing for greater control of optimizing the medium. Thus media developed in this way tend to have higher growth rates and are more easily improved since inhibitory compounds are less likely to be present.

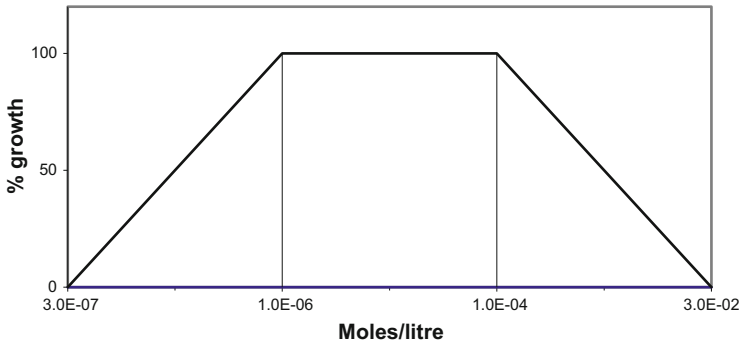

Fig. 8.2 The determination of the optimal concentration of a component

There is likely to be a wide concentration range over which any nutrient supplement is not limiting. Using Ham's approach (Ham and McKeehan 1979) the optimum concentration for the nutrient can be set at the mid-point of the broad optimum plateau of the growth-response curve (Fig. 8.2). This enables the concentration to be bracketed between a maximum and minimum value. This reduces the likelihood that the component will become growth limiting through the adjustments of other medium components. In the response curve shown in Fig. 8.2 the mid-point of the optimum concentration is 10^{-5} M. The concentration range of a supplement can be established initially by testing a broad range at tenfold increases e.g.: $\times 0.1$, $\times 1$, $\times 10$ and $\times 100$ of a nominal starting concentration. This can be followed by focus on a narrower range to obtain the optimum concentration for growth.

8.6 A Statistical Approach to Serum-Free Media Development

Because of the multiple potential components involved in the development of serum-free media it is almost essential to utilize an experimental design suitable for evaluating the relative importance of each factor. One of the most popular factorial "Design-of-experiments" (DOE) is the Plackett-Burman statistical approach, which was designed to be simpler than a full factorial experiment. This enables the evaluation of components to affect growth or any other measurable property in a small a manageable number of experiments. This screening method that can evaluate systematically a complex set of supplements for the promotion or inhibition of cell growth. A large number of components can be studied at once to determine which combination of factors is important for a serum-free formulation.

This statistical approach has been utilized in the design of various serum-free formulations. Castro et al. obtained a medium for CHO cells that resulted in 45 % higher productivity than previous formulations (Castro et al. 1992). From this study glycine, phenylalanine, tyrosine and BSA were shown to improve the specific

growth rate whereas other amino acids, such as methionine, proline and histidine enhanced the production of interferon-gamma. Other potential components, such as, insulin, arginine, aspartate, and serine produced an inhibitory effect on both cell growth and interferon-gamma production. Using a similar approach Lee et al. improved medium for CHO cell growth and erythropoietin production (Lee et al. 1999). This medium was based on IMDM. Supplements of glutamate, serine, methionine, phosphatidylcholine, hydrocortisone and Pluronic F68 were all identified as positive determinants for cell growth. The final optimized medium resulted in a 79 % higher product yield compared to the original serum-supplemented media.

A Plackett-Burman simple matrix is shown in Table 8.1 for the analysis of four potential media components. In this, 16 separate cultures would be established – normally in a multi-well plate. Each component is added at a high (+) or low (−) concentration. These values are chosen arbitrarily, although prior knowledge of the typical concentration effects of each component would be valuable. Culture 1 in the matrix contains all four components at their high concentrations whereas culture 16 has all four components at their low concentration. All other cultures are established with a mixture of high and low concentrations of the four components. Statistical analysis of the results would typically be performed of the cell growth following 3 or 4 days. However this approach is not limited to cell growth and can also be valuable in determining the effect of components on any culture parameter which might include productivity or glycosylation profiles. The primary goal of the method is to identify the primary components that influence the response – in this case the growth of cells. This is done by analysis of variance (ANOVA) and main

Table 8.1 A typical Plackett-Burman matrix

	Variables (components)			
Medium	A	B	C	D
1	+	+	+	+
2	+	+	+	−
3	+	+	−	−
4	+	−	−	−
5	−	+	+	+
6	−	+	+	−
7	−	+	−	−
8	−	−	+	+
9	−	−	+	−
10	−	−	−	+
11	+	+	−	+
12	+	−	+	−
13	−	+	−	+
14	+	−	+	+
15	+	−	−	+
16	−	−	−	−

+/− represents high/low concentration of component

effect plots. A main effect is identified when different concentration of a media affects cell growth. This type of analysis is best performed using statistical software such as that offered by SAS.

The steps involved in a typical Plackett-Burman experiment are outlined below:

(a) Select the standard basal medium or combinations analyzed for their ability to enhance growth.
(b) Establish two concentrations for each component to be tested : a high (+) and a low (−).
(c) Grow the cells in the serum-supplemented basal medium with serum (5–10 %) in a well-plate culture. Allow the cells to initiate growth (~24 h).
(d) Collect the cell pool from the serum-medium and inoculate into each of the 16 wells established according to the matrix in Table 8.1, each with the same basal medium plus combinations of media components (no serum).
(e) Include a positive control (serum-supplemented medium) and a negative control (medium with no supplements)
(f) Count the viable cells (by MTT assay or Trypan-Blue exclusion) in each well after a suitable time period (4 days).
(g) Calculate the variances from all of the effects from the single factors using suitable statistical software (e.g.: SAS). This should identify the components that were tested that have a significant effect on growth. The best combination of components can then be included in the serum-free formulation under development.
(h) Initiate an adaptation procedure for the cells to the new serum-free medium.

8.7 Mitogenic Components Needed to Replace Serum

There are specific groups of compounds that have been found to promote cell growth and serve well in serum-free formulations. In this section some examples of these are discussed but this is far from an exhaustive list.

8.7.1 Peptide Hydrolysates

These are obtained by the enzymatic hydrolysis of proteins derived from animal, plant or microbial sources and contain a variable composition of small peptides, which have proved valuable for many years as food supplements (Howard and Udenigwe 2013). Their use as cell culture media components has been widespread, although the batch-to-batch variability of their composition can be a problem. Meat hydrolysates such as Primatone RL have been shown to be particularly effective in promoting cell growth (Schlaeger 1996) but clearly this would be unacceptable in the current drive for the industry standard animal component-free media.

Yeast, soy, wheat, cotton and pea hydrolysates have been particularly widely used as individual components or as part of a defined cocktail to maximize the growth of a particular cell line.

Attempts to identify and isolate the active components of hydrolysates have proved particularly difficult because of their complexity and variability of composition (Michiels et al. 2011; Pasupuleti and Demain 2010; Hsueh and Moskowitz 1973). Analysis of a soy hydrolysate by HPLC and mass spectrometry identified 410 compounds of which 253 were assigned as peptides (Gupta et al. 2014). A statistical analysis of the correlation of these compounds and antibody production suggested that enhanced productivity occurred by the complex network of components rather than a single component. This type of analysis has proved difficult because of the variability of hydrolysate preparations from one batch to another. This has lead to the development of a predictive model as a screening tool for high performance hydrolysate lots (Luo and Pierce 2012). Without necessarily defining the composition of the hydrolysate, NMR fingerprinting can identify a pattern which could predict process consistency and product quality.

In order to meet the more stringent conditions of consistency of hydrolysates needed for culture media a novel hydrolysate production process can be employed to meet higher standards of control (Siemensma et al. 2010). The basis of the method developed by Sheffield is the use of a rationally designed animal component-free (ACF) enzyme cocktail that includes both proteases and non-proteolytic hydrolases. These enzymes can release peptides as well as primary components of the polymerized non-protein portion of the raw material. The enzyme cocktail ensures the release of not only the growth-promoting peptides and amino acids, but also key carbohydrates, lipids, minerals, and vitamins that may well play a part in the bioactivity of the final product. The use of ultrafiltration with typically a 10 kDa cut-off filter can add to the consistent quality of the final batch of the peptide hydrolysate.

8.7.2 Insulin and Insulin-Like Growth Factor

Insulin is a small polypeptide (5.7 kDa) that is known to have multiple effects on cell physiology, including membrane transport, glucose metabolism, and biosynthesis of nucleic acids and fatty acids (Komolov and Fedotov 1978). It is normally present in serum and so must be added as a supplement in serum-free media. Whereas many bioactive components are required specifically for certain cell lines, insulin appears to be a universal requirement for the growth of cells in culture. The stimulation of DNA synthesis by insulin is particularly important for cell growth and maintenance of the normal mitotic cycle (Komolov and Fedotov 1978; Simms et al. 1980). Although, insulin is an animal protein it is available commercially by manufacture from genetically-engineered microbial systems that enables it to be incorporated into an animal component-free medium formulation.

The concentration of insulin in culture media is usually around 5 ug/ml which is approximately $\times 10^3$ greater than serum. However this has been shown to be the minimal level required for significant growth of many cell lines (Chang et al. 1980; Florini and Roberts 1979; Simms et al. 1980). It is possible that the high insulin requirement is linked to cell metabolism in the presence of the high glucose concentrations added to most media. However, a more likely explanation is the instability of insulin under non-physiological conditions. It has been shown that 90 % of insulin can be reduced in 1 h at 37 °C (Hayashi et al. 1978), which may be due to the high redox potential related to the presence of such components as cysteine in the media. Replacement of cysteine by cystine decreases the reduction of insulin significantly.

Insulin-like growth factors (IGF) are naturally occurring peptides that have high sequence similarity to insulin and also have similar metabolic actions as insulin. However they are more potent mitogens and are required in media at lower concentrations to promote the same metabolic effects (Morris and Schmid 2000). Long R^3IGF-1 is a modified recombinant form of IGF which has been found valuable as a component in serum-free media. The modifications that include an amino acid substitution at position 3 as well as a 13 amino acids extension prevents inactivation of the molecule by specific IGF binding factors that may be secreted by mammalian cells. The modified form of IGF has been shown to $\times 200$ more potent and $\times 3$ more stable than insulin, both advantageous properties for media components.

8.7.3 Epithelial Growth Factor (EGF)

This is one of many naturally occurring growth factors with a potent mitogenic activity. It activates a signalling pathway by dimerization of a receptor (EGFR) embedded in the cell membrane and activation of a tyrosine kinase. During transformation cells tend to lose their sensitivity to EGF (Gopas et al. 1992). Therefore the factor is normally only included in the media of non-transformed and usually anchorage-dependent cells such as epithelial or fibroblast cells. Fortunately, recombinant forms of the factor are available from microbial sources including a recombinant Long EGF similar to the Long R^3IGF-1 described above and with which it may have synergistic activity (Simmons et al. 1995).

8.8 Transferrin: A Carrier Protein

This is a standard protein (~80 kDa) component of serum that is well-characterized for its importance in the transport of iron under physiological conditions. It is available as a single component from animal sources or as a microbially derived recombinant protein. Virtually every cell line has shown a response to the presence

of transferrin but the extent of the response is dependent on the availability of other forms of iron in the media. It was shown for mouse melanoma cells that the presence of transferrin enhanced cell growth $\times 15$ in the absence of iron but only by $\times 2$ in the presence of iron as $FeSO_4$ (Mather and Sato 1979). The value of transferrin has been shown to include the ability to detoxify potentially toxic trace elements in media (Barnes and Sato 1980), which is particularly important for low protein media. Some reports have shown that it is possible to replace the iron transport function of transferring by other forms of chelated iron such as ferric citrate or tropolone chelated iron (Metcalfe and Froud 1994.).

8.9 Attachment Factors

These factors are often termed extracellular matrix proteins and are essential for anchorage-dependent cells to allow rapid attachment to an available surface prior to growth. These protein factors such as fibronectin and laminin may be available in serum and may also be synthesised by many cell lines. However, they may be provided in the media or on a surface coated with one of the factors such as fibronectin. This can increase the speed of attachment of cells to the substratum, which may well preserve the viability of the cell population (Orly and Sato 1979).

Conclusion

The driver for the removal of serum as a component of a bioprocess for the production of biopharmaceuticals has been the recognized danger of viral and prion contamination in the final product. Regulatory approval would now be very difficult for a process that included animal-derived components particularly serum if there were an alternative. The difficulty in complying with this requirement is in the identification of media components that can provide the same growth promoting capacity as serum. Because of the extensive range of growth promoting factors in serum it has proved to be a universal supplement to basal media for growth promotion of almost any cell line. An equivalent non-animal sourced universal supplement has not been found. Rather, non-animal component media formulations have been designed for specific cell lines. The design of such formulations is not trivial given the long list of potential components and broad range of concentrations of each that could be tested. Plant and microbial sourced protein hydrolysates have proved useful components but their use is marred by variability of composition. The availability of recombinant forms of natural growth factors or major serum proteins has been particularly valuable as these can be isolated from microbial cultures and therefore designated non-animal-components. The difficulty of formulating serum-free media can be eased by statistical design-of-

(continued)

experiment procedures, although this may well require an extensive number of reiterative cycles before a working formulation can be found. At the present there are a number of serum-free media formulations available for suspension cell lines but relatively fewer for anchorage-dependent cells. This reflects the more exacting requirements of anchorage-dependent cells for attachment and growth factors. Although many serum-free media types are now available commercially their formulations are usually not in the public domain. Rather, the composition of the media is proprietary to the supplier. This is a relatively new phenomenon that did not occur in the early days of investigation of the nutritional requirements of cells and the development of basal media. However the secrecy surrounding the composition of serum-free formulations reflects the commercial reality which has developed with the range of high value products that can now be produced from mammalian cells in culture.

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Chapter 9
Glycosylation in Cell Culture

Maureen Spearman and Michael Butler

Abstract Glycosylation affects many general functional factors of a glycoprotein, such as stability, inhibition of proteolysis, solubility, aggregation, as well as other attributes critical to its use as a biotherapeutic. Therefore our understanding of the glycosylation pathway, factors that affect it, and our ability to manipulate glycosylation are essential in producing superior biotherapeutics. The complex synthetic pathway of N-linked glycosylation occurs in both the endoplasmic reticulum and Golgi and involves a large number of precursors and enzymes, leading to a large array of possible glycan structures. Thus, variability in glycosylation may be influenced by numerous factors that affect this pathway, such as host cell type, nutrient levels and supplements, dissolved oxygen, pH, temperature, and by-product accumulation, and thus must be closely monitored. Better analytical techniques have allowed linking specific glycan structures to functionality of glycoproteins, which have led to efforts to modify glycosylation through genetic engineering, sequence-interfering RNA (siRNA), glycosylation inhibitors or chemoenzymatic modification.

Keywords Glycosylation • Biotherapeutics • CHO cells • Genetic engineering • Culture conditions

9.1 Introduction

The glycosylation of biotherapeutics is now identified as a critical quality attribute that requires defining glycosylation for each drug to maintain consistent characteristics. Glycosylation profiles are unique to each glycoprotein but are also highly variable in the attachment of glycans at specific sites (macroheterogeneity) and also variation in structure of the glycans at each site (microheterogeneity), resulting in an array of glycans for each glycoprotein. Glycosylation can be affected by a large number of factors in cell culture production but are also defined by the host cell line. It is essential to maintain batch-to-batch consistency of glycosylation because glycosylation affects many attributes of glycoproteins such as solubility, thermal

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stability (Zheng et al. 2011), protease resistance (Sareneva et al. 1995), aggregation (Onitsuka et al. 2013; Rodriguez et al. 2005), and their characteristics as therapeutics such as serum half-life (Wright and Morrison 1997), immunogenicity (Chung et al. 2008; Noguchi et al. 1995), and efficacy (Sola and Griebenow 2010). Over the last few decades as analytical techniques have improved, specific glycosylation structures have become associated with functionality of individual biotherapeutics, one of the most significant being the antibody dependent cellular cytotoxicity (ADCC) of monoclonal antibodies (Mabs). This has led to the modification of glycosylation pathways through genetic engineering and other means to optimize the function of next generation biotherapeutics and close monitoring of glycosylation during production.

9.2 Glycosylation Structures

The most prominent form of glycosylation is asparagine-linked or N-linked glycosylation (Fig. 9.1). N-linked glycans are added to protein as a co-translational modification in the endoplasmic reticulum (ER) with oligosaccharide chains transferred en bloc to the nascent polypeptide chain from a lipid-oligosaccharide precursor, dolichol phosphate (Dol-P) (Kornfeld and Kornfeld 1985; Lennarz 1987; Stanley et al. 2009). Synthesis of the oligosaccharide begins on the cytosolic face of the ER membrane where the carrier molecule, Dol-P, is localized. A precursor molecule consisting of two N-acetylglucosamine (GlcNAc) molecules with a branched structure containing five mannose (Man) residues is produced through the sequential enzymatic addition of sugars via specific glycosyltransferases that use nucleotide sugars uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), and guanidine diphosphate-mannose (GDP-Man) as substrates. The Dol-P-glycan structure (Dol-P-P-$GlcNac_2$-Man_5) is enzymatically flipped from the cytosol into the endoplasmic reticulum where an additional four Man residues are added through a Dol-P-Man carrier and three Glc molecules are then added using Dol-P-Glc. The en bloc transfer of the glycan structure from the dolichol carrier to the protein occurs via the oligosaccharyltransferase (OST) complex.

N-linked oligosaccharides attach via the R-group nitrogen of asparagine at specific sites or “sequons” on the protein. Sequons are comprised of the sequence asparagine-X-serine/threonine (Asn-X-Ser/Thr), where X can be any amino acid with the exception of proline, and the third amino acid can be either Ser or Thr. Typically a glycoprotein will have a set of sequons that can be glycosylated, but variation in occupied glycan sequons leads to macroheterogeneity, or mixtures of glycosylated sites.

Glycosylation can also vary in microheterogeneity, which introduces alternative structures of individual N-linked glycans. Following the transfer of oligosaccharide to the protein the glycoprotein processing reactions, comprised of numerous exoglycosidases and glycosyltransferases, reconstruct the oligosaccharide as the glycoprotein passes through the ER and Golgi. Depending upon the specific

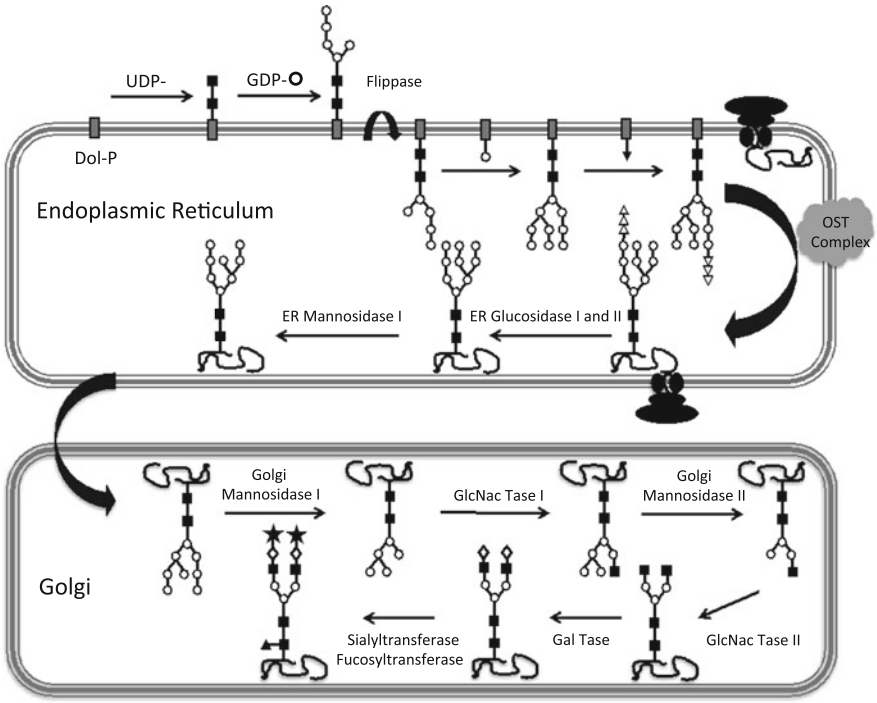


Fig. 9.1 Synthesis of N-linked glycans. A GlcNAc2Man5 is initially synthesized on a dolichol-phosphate (*Dol-P*) carrier from UDP-GlcNAc and GDP-Man on the cytoplasmic face of the endoplasmic reticulum (*ER*). The structure is flipped to the lumen of the ER where an additional four mannose residues and three glucose residues are added via Dol-P-Man and Dol-P-Glc. The glycan is transferred en bloc via the oligosaccharyl transferase complex to the nascent polypeptide and initial processing with the removal of the three glucose residues and one mannose residue occurs. Further processing reactions occur in the Golgi where several mannose residues are removed and addition of GlcNAc occurs, followed by removal of additional mannose residues and the addition of the complex oligosaccharides (GlcNAc, Gal, sialic acid and fucose). Further branching reactions may also occur to produce triantennary and tetraantennary glycans (not shown)

glycoprotein, some oligosaccharides may be maintained as high mannose glycans following the removal of the glucose residues in the ER. But most glycans are trimmed beginning with removal of one mannose residue in the ER and followed additional removal of mannose in the Golgi by an α1,2-mannosidase to reduce the $GlcNAc_2Man_8$ structure to the core molecule $GlcNAc_2Man_5$. An additional GlcNAc residue is then added, followed by the removal of two more mannose residues in the Golgi and the addition of other sugars to convert the structures into complex glycans that contain a terminal triplet of sugars consisting of N-acetylglucosamine, galactose (Gal) and sialic acid (N-acetylneuraminic acid, NeuAc or N-acetylglycolylneuraminic acid, Neu5Gc). Fucose (Fuc) can also be added in an α1,6 linkage to the first core GlcNAc residue or outer arm GlcNAc

residue. Further microheterogeneity may be introduced with a diverse array of glycosyltransferases and nucleotide-sugar donors which catalyze the addition of sugars residues at key enzymatic control points and lead to variable antennarity (branching) of the structure and extra sugar additions. Complex glycans are normally defined by their antennarity with two (biantennary), three (triantennary) or four (tetraantennary) branches. Sugar residues can also have different linkages, such as sialic acid that are generally attached via an α2,3 or α2,6 linkage and are in the terminal position. The three common types of oligosaccharides are high mannose, complex, and hybrid (Fig. 9.2), but within each of these groups, large amounts of variation can occur in antennarity, chain length or additional sugars (eg. fucose, lactosamine). It is estimated that up to 7,000 different structures are possible (Moremen et al. 2012). The control of the many glycosyltransferase enzymes are not well understood, but are affected by many factors such as pools of intracellular nucleotide sugars, Golgi transit time, pH, temperature and enzyme levels (Butler 2006; Moremen et al. 2012; Stanley 2011).

A secondary type of glycosylation is O-linked in which glycans are attached through an N-acetylgalactosamine (GalNAc) residue via a family of GalNAc transferase enzymes to the oxygen of Ser or Thr (Tian and Ten Hagen 2009). Other linkages have also been defined for specific proteins (Moremen et al. 2012). Some O-linked mucin glycans can be quite large but typically in recombinant proteins they are smaller than N-linked glycans and include many of the same monosaccharides and linkages. O-linked glycosylation is a

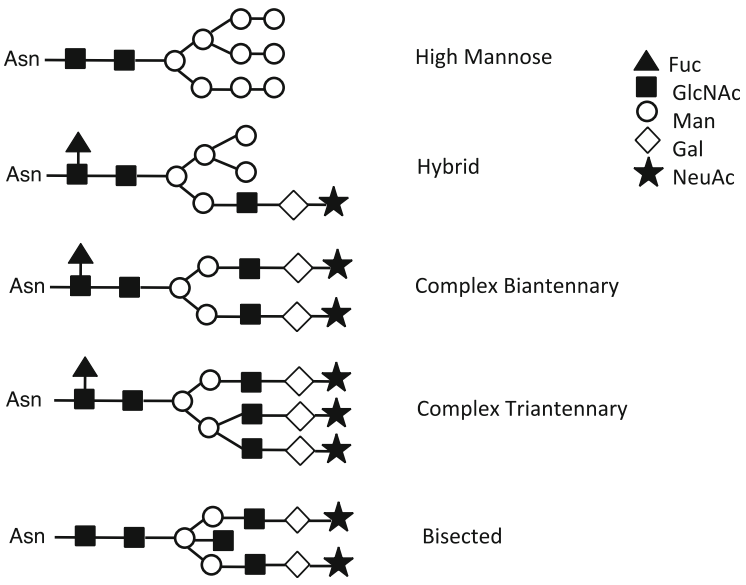


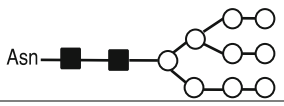
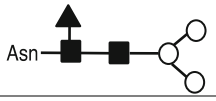
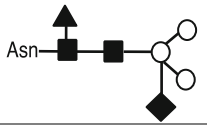
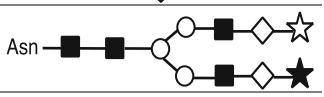
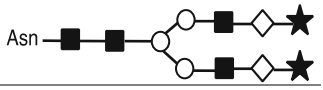
Fig. 9.2 Common N-linked glycans: high mannose; hybrid form with one arm high mannose and one arm with complex monosaccharides; biantennary complex (with terminal GlcNAc, Gal, NeuAc); triantennary complex and complex with bisecting GlcNAc

posttranslational event, involving only glycosyltransferases in the Golgi. Within the GalNAc-attached type there are eight groups of O-linked glycans based on their core structures. The consensus sequences of O-linked glycans are less defined, but regions of proline and alanine along with Ser and Thr may affect glycan addition. Synthesis involves sequential addition of individual sugars within the Golgi via glycosyltransferases using nucleotide sugar substrates. Core structures can be extended with additional sugars structures such as polylactosamine residues (GlcNAc-Gal) and fucose, and the terminal sugar residues are commonly sialic acids (eg. NeuAc). Control of the biosynthetic pathway is not well understood but may be controlled by localization of specific transferases through the Golgi compartments (Hang and Bertozzi 2005).

9.3 Cell Type Specific Glycosylation

Mammalian cell lines are chosen for biotherapeutic production due to their ability to glycosylate the protein closely to the native form (Table 9.1). Each host cell line has its own unique set of glycosylation machinery, which impacts the final glycan profile of the biotherapeutic. Chinese hamster ovary (CHO) cell lines have become the primary host cell system for production because of its rigorous growth in large-scale production, high product titer, ease of adaptation to chemically defined media, and similar glycosylation patterns to human cells. However, clonal variation in CHO glycosylation is a factor that must also be considered (Davies et al. 2013; van Berkel et al. 2009). One limitation of CHO cells is their inability to express the α2,6 sialyltransferase enzyme (Xu et al. 2011), and sialic acid is attached in only the α2,3 linkage. Natural human proteins have both the α2,6 and α2,3 sialic acid linkage. Another consideration is the incorporation of the sialic acid, N-glycolylneuraminic acid (Neu5Gc), in non-primate cell lines as a terminal sugar instead of N-acetylneuraminic acid (Neu5Ac). Humans have lost the ability to express enzymes to produce Neu5Gc, and it is therefore a potential antigen (Ghaderi et al. 2012). Other non-human cell lines may contain glycosylated structures that are antigenic in humans. In mouse cells the production of the antigenic structure, Galα1,3-Galβ1,4-GlcNAc, via an α1,3-galactosyltransferase enzyme make these less ideal as hosts for glycoprotein production (Jenkins et al. 1996). Plant, insect and yeast have the advantage of lower cost production, and higher titers, but have major differences in N-linked glycosylation. Plants have additional sugars (xylose and α1,3-linked fucose) (Ko et al. 2009; Webster and Thomas 2012); insects have short mannose glycans (paucimannose) (Ahn et al. 2008a); yeasts produce predominantly high mannose structures (Chiba and Akeboshi 2009). Genetic engineering can reduce the antigenicity attributed to non-human glycosylation by humanizing the glycosylation capacity in non-mammalian cell types (Castilho et al. 2011; Chiba and Akeboshi 2009; Durocher and Butler 2009; Loos and Steinkellner 2012; Wildt and Gerngross 2005). Alternatively, there is now some interest in using human cell lines.

Table 9.1 Comparison of N-linked glycosylation in different cell expression systems. (Fucose ▲, N-acetylglucosamine ■, Mannose ○, Galactose ◇, N-acetylneuraminic acid ★, N-glycolylneuraminic acid ☆, Xylose ◆)

Cell type	N-glycan	Structure
Yeast	High mannose	
Insect	Fucosylated core	
Plant	Xylosylated and fucosylated core	
Hamster mouse	Contain NeuGc/NeuAc	
Human	Contain only NeuAc	

The human cell line, HEK (human embryonic kidney), is commonly used for transient transfections in research and small-scale production. Recent work comparing transient transfections of HEK293 and CHO-S cells with 12 different proteins found significant differences in glycosylation patterns between the systems (Croset et al. 2012). In general, the size and number of glycoforms and the sialic acid content was found to vary in glycoproteins produced in the two cell types resulting in different isoforms. These differences emphasize the need for the development of human cell lines that are capable of large-scale production and have glycosylation patterns closer to human glycoforms found in vivo. PER.C6® is a human embryonic retinoblastoma cell lined developed for vaccine production and capable of high cell densities (www.crucel.com). It has been used to produce IgG with high amounts of fucosylated mono-galactosylated (FG1) glycan, similar to serum IgG (Jones et al. 2003). However, another monoclonal antibody produced in PER.C6® had much lower galactosylation with 60 % of the Mab agalactosylated (FG0) (Olovnikova et al. 2012), suggesting it may not produce similar glycosylation for all recombinant products, although differences in culture conditions could account for some variability. Human alpha-1-antitrypsin produced in PER.C6® had increased core fucosylation, increased tri- and tetraantennary glycans and changes in microheterogeneity at specific sequons compared to the plasma derived protein (Wang et al. 2013). A human fibrosacrcoma-derived cell line (HT-1080) can produce erythropoietin (EPO) with higher levels of tetra-antennary structures and none of the antigenic Neu5Gc is present (Shahrokh et al. 2011). Fusion of 293 cells with a B-cell line (Namalwa cells) yielded a cell line useful in transient and stable

clone production (Lee et al. 2013) which produces antibody with more terminal galactose, similar to human plasma (Seo et al. 2013).

9.4 Culture Parameters Affecting Glycosylation

Variability in the production process affects batch-to-batch consistency of glycosylation in both micro- and macroheterogeneity. Therefore it is essential to monitor and control parameters such as pH, oxygen, stirring rate and temperature, nutrient depletion and metabolic by-product accumulation, all of which can affect the intracellular glycosylation pathway that in turn control the glycan profile of a secreted glycoprotein.

The complex nature of N-linked glycosylation requires the cell to produce a large pool of precursor molecules necessary for the addition of individual sugar molecules by a huge array of glycosyltransferases. Many of these pathways are interconnected, but their regulation is not well understood. Therefore, much work has been devoted to determining the effect of media components and culture conditions on glycosylation of product.

Early work showed the depletion of nutrients during the course of batch cultures had time-dependent glycosylation effects. A reduction of glycan site occupancy of gamma-interferon during the batch culture of CHO cells (Curling et al. 1990) was shown in later studies to most likely be linked to glucose or glutamine levels (Hayter et al. 1992; Jenkins and Curling 1994; Nyberg et al. 1999). Glucose limitations in a chemostat culture produced an increase in non-glycosylated gamma-interferon, which could be rapidly restored with pulsed glucose additions (Hayter et al. 1992). Both reduced site-occupancy and truncation of N-glycans under low glucose conditions have been shown in several other recombinant glycoproteins and viral proteins (Davidson and Hunt 1985; Liu et al. 2014; Seo et al. 2014; Tachibana et al. 1996).

Intracellular nucleotide-sugars are the substrates for the addition of sugars to the lipid-oligosaccharide in the endoplasmic reticulum and also for the modification of the glycan in the Golgi. Early work suggested that glucose starvation caused intracellular depletion of glucose or a shortage of glucose-derived precursors of glycans resulting in a higher proportion of high mannose structures (Rearick et al. 1981). Low levels of glycosylation can be related to decreased concentrations of UDP-GlcNAc (the first sugar added to the dolichol-phosphate) in glucose-depleted or glutamine-depleted CHO cultures (Nyberg et al. 1999). Glutamine depletion reduced formation of glucosamine phosphate and glucose-depletion reduced synthesis of UTP, which are the precursors of UDP-GlcNAc. Recently, Mab produced in low glucose was also found to have reduced amounts of full length lipid-oligosaccharides and a concomitant increase in smaller lipid-oligosaccharides, resulting in an overall reduction is glycosylation (Liu et al. 2014). In the same study a strong correlation was found between galactosylation and sialylation indices and glucose uptake.

Yields in bioprocess production are now commonly greater than 10 g/L (Saraswat et al. 2013), and feeding strategies at high cell densities ($>10^7$/ml) are used to overcome nutrient limitations and maintain high productivity (Saraswat et al. 2013; Wurm 2004). Feed strategies may vary between stoichiometric feed, which is based on projected growth, or dynamic feeding which requires frequent measurement of the culture's metabolic state (Chee Furng Wong et al 2005). Maintaining critical nutrients such as glucose and glutamine at low concentrations results in the reduction in accumulated by-products such as ammonia and lactate (Khattak et al. 2010). However, nutrient balance for the maintenance of appropriate glycosylation may require different set-points of key critical nutrients and a tighter control to avoid short-term depletion of necessary nutrients that could cause variability of product glycosylation (Xie and Wang 1997) such as decreased sialylation and increased high mannose glycans (Chee Furng Wong et al. 2005). High throughput glycan analysis and real-time monitoring are an advantage for tighter control of glycosylation metabolites. Recent work with metabolomic analysis using NMR monitored the amounts of small molecules, which were correlated with glycosylation and adjustments made for optimization of glycan structures (Aranibar and Reily 2014). A combination of fluorescent lectin labelling and statistical analysis has found a correlation between glycosylation of cell surface proteins and monoclonal antibody glycosylation in CHO clones, which can be used to modify culture media for glycosylation optimization (Grainger and James 2013).

Glycan microheterogenity can be influenced by the addition of precursors and cofactors and used to enhance certain critical attributes of glycosylation for a specific glycoprotein. There are several examples of this in the literature. UDP-GlcNAc is an important intracellular nucleotide-sugar substrate for several GlcNAc transferases present in the Golgi. Supplementation of CHO cultures with glucosamine and uridine can lead to an elevated level of intracellular UDP-GlcNAc, (Baker et al. 2001), and enhance the antennarity of glycan structures produced in baby hamster kidney (BHK) cells (Gawlitzek et al. 1998; Grammatikos et al. 1998; Valley et al. 1999). However, the phenomenon is not universal for all cell lines, as antennarity was not enhanced in NS0 cells following glucosamine supplementation (Baker et al. 2001). Elevated UDP-GlcNAc also appears to cause a decrease in sialylation, which may be explained metabolically by the inhibition of CMP-sialic acid transport (Pels Rijcken et al. 1995). Also, replacing glucose with GlcNAc in the media of a human hybridoma, decreased the sialylation of the hypervariable region of the light chain of the antibody, with a $\times 10$ increase in affinity binding to its antigen (Tachibana et al. 1997). N-acetylmannosamine (ManNAc), a CMP-NeuAc precursor with high cell permeability has been used to enhance sialylation of gamma-interferon (Gu and Wang 1998). However, in other systems an increase of intracellular CMP-NeuAc was reported as a result of ManNAc feeding to either CHO or NS0 cells but without an increase in overall sialylation of a recombinant protein (TIMP1) (Baker et al. 2001). However, this strategy did change the ratio of N-glycolylneuraminic acid/ N-acetylneuraminic acid from 1:1 to 1:2 in the NS0 cells, a possible strategy for reducing antigenic potential. A more recent study has found that feeding CHO cells with specific sugar

with the appropriate nucleoside precursors, glucosamine (+/− uridine), galactose (+/−uridine) and N-acetylmannosamine (+/−cytidine), increased the sialylation of gamma-inteferon due to increases in respective nucleotide sugar levels (Wong et al. 2010). As well, terminal galactosylation of an antibody was increased by the addition of the precursors uridine and galactose and manganese chloride (Gramer et al. 2011). Higher levels of manganese, a cofactor for sialyltransferase and galactosyltransferase, have been found to increase overall galactosylation and also increase sialylation, as well as increase site occupancy of huEPO (Crowell et al. 2007). Macroheterogeneity can also be controlled by additions of manganese and iron, as site occupancy of t-PA (tissue plasminogen activator) increased with their addition (Gawlitzek et al. 2009). Thus it suggests that additions of precursors for glycosylation require that all precursors should be present in non-limiting quantities, with the appropriate cofactors for the glycosyltransferase enzymes, or imbalances in nucleotide sugar levels and glycosylation pathways may result in non-desirable glycosylation.

The trend in culture media is the movement towards serum-free and more recently chemically defined media. Adaptation of CHO cells to serum-free media results in changes to Mab glycosylation (Costa et al. 2013), and therefore must be a consideration when developing production platforms.

Several culture parameters are known to directly affect glycosylation of recombinant proteins and tight control of these factors is necessary to maintain consistently glycosylated product. Ammonia (NH_3) (or the ammonium ion, NH_4^+) can accumulate in a culture from cellular glutamine metabolism (glutaminolysis) and from the non-enzymatic decomposition of glutamine in the medium, and is inhibitory to cell growth, especially at high pH (Doyle and Butler 1990). Ammonia decreased terminal sialylation in a variety of recombinant proteins (Andersen and Goochee 1994; Yang and Butler 2000; Zanghi et al. 1998), and high levels of ammonia resulted in a shift of all glycoforms to higher pI values (Yang and Butler 2000). Low levels of ammonia (2 mM) also affected the sialylation of O-glycans (Andersen and Goochee 1995). There are two possible mechanisms suggested to explain the effect of ammonia: (1) enhanced incorporation of ammonia into glucosamine, a precursor for UDP-GlcNAc, increases the UDP-GlcNAc/UTP ratio, whereby UDP-GlcNAc competes with the transport of CMP-NeuAc into the Golgi and may decrease sialylation and (2) ammonia raises the pH of the Golgi, away from the optimal pH of the sialyltransferase enzymes (Valley et al. 1999). Glutamate can be substituted for glutamine, resulting in increased galactosylation and reduced ammonia (Hong et al. 2010)

Affects of ammonia on pH suggest that tight control of external pH conditions is necessary to maintain the internal pH of the Golgi for optimum activity of key glycosylating enzymes. Decreased galactosylation of Mab in CHO cells with increased ammonia was also thought to be due to decreased intracellular pH, thus affecting the galactosyltransferase activity (Hong et al. 2010). As well, galactosylation of Mab produced in the human cell line F2N78 was decreased with increasing pH (7.0–7.6). The pH of the medium also affected macroheterogenity in the distribution of glycoforms of IgG in a murine hybridoma

(Rothman et al. 1989b) and site occupancy of recombinant mouse placental lactogen-I from CHO cells (Borys et al. 1993) and a recombinant enzyme (Gawlitzek et al. 2009), but the pH ranges for optimum glycosylation varied for each production system.

Control of dissolved oxygen (DO) levels is an important parameter in maintaining optimal growth and metabolism of producer cells (Heidemann et al. 1998; Jan et al. 1997) but may also affect the glycan microheterogenity of a recombinant protein. In particular, an increase in sialyltransferase was observed at high oxygen levels that translated into increased sialylation of recombinant follicle stimulating hormone (FSH) (Chotigeat et al. 1994). However, others have found a decrease in sialylation of erythropoietin (EPO) at DO of 100 % (Trummer et al. 2006). Also, digalactosylated glycans (G2) of IgG gradually decreased from 30 % at 100 % oxygen, to around 12 % under low oxygen conditions (Kunkel et al. 1998). Galactosylation might be sterically impeded by the early formation of an inter-heavy chain disulfide or reduced DO may cause a decline in the availability of UDP-Gal. The redox potential of the culture is known to affect disulfide bond formation, which is critical for glycosylation at sites close to disulfide bridges (Allen et al. 1995; Gawlitzek et al. 2009; Rademacher et al. 1996).

Reduction of culture temperature below 37 °C increases productivity in many production systems (Fan et al. 2010; Rodriguez et al. 2010; Sunley et al. 2008; Trummer et al. 2006). However, lower culture temperatures can have variable effects on glycosylation. Small increases (4 %) were observed in glycan site occupancy of tissue plasminogen activator (t-Pa) at 31 °C (Gawlitzek et al. 2009). EPO glycosylation had reduced sialylation at temperatures of 31 °C and 33 °C (Trummer et al. 2006) and in another study a reduction of both sialylation and antennarity were observed below 32 °C (Ahn et al. 2008b).

As our understanding of specific affects of culture parameters improves, manipulation of culture variables by the modification of several factors and media additives can be used to tailor the glycosylation of the product. A recent example of this is the use of several culture parameters (sodium butyrate addition, reduced culture temperature, high pCO2 and use of sodium carbonate instead of sodium hydroxide for pH control) to reduce the amount of Neu5Gc incorporated into a recombinant protein produced in CHO cells (Borys et al. 2010). Cellular responses are often unique to the cell line and recombinant protein, and therefore culture conditions and parameters are not necessarily transferable for similar glycosylation patterns.

9.5 Glycosylation Engineering and Modification of Glycan Structure

Oligosaccharides are highly functional on most glycoproteins, with both microheterogeneity and macroheterogeneity of biotherapeutic glycoproteins playing a large part in efficacy through increased activity and half life, and reduced immunogenicity (Sinclair and Elliott 2005). Defining optimal glycosylation in relation to function has advanced with better analytical techniques. A goal now is to reduce heterogeneity in glycosylation to eliminate variability in product. As discussed, optimization of culture conditions can often be used to better glycosylation for a particular functional aspect of a biotherapeutic. However, the requirement for very specific homogeneous glycosylation structures or novel modifications to the glycan structure is unattainable by solely relying on specific cell lines and culture conditions. Genetic engineering allows directed modification of glycosylation in several ways (1) the addition of glycan sites (sequons) in the protein; (2) knocking out enzymes in the glycosylation pathway; (3) adding in additional enzymes to the cell system; (4) the use of mutant glycosylation cell lines and (5) combinations of these methods. Other ways of modifying glcycosylation are by using sequence-interfering RNA (siRNA), glycosylation inhibitors or chemoenzymatic modification, all tools that have been successfully used to alter glycan structure of recombinant proteins.

Erythropoietin (huEPO) is a biotherapeutic requiring a high degree of glycan branching and sialylation (with three N-linked and one O-linked glycans) to maintain its circulatory half-life (Fukuda et al. 1989; Misaizu et al. 1995). Glycoengineering was used to introduce two additional N-linked glycosylation sites (sequons) into the protein backbone, allowing hyperglycosylation of huEPO and increased the sialic acid content (Egrie et al. 2003; Elliott et al. 2004). The glycosylation changes were associated with a threefold increase in half-life and higher potency with a reduced frequency of administration in patients. The increase in sialic acid reduced the binding of huEPO to the receptor allowing increased half-life (Elliott et al. 2004). Genetic manipulation to increase sialic acid content of glycan chains by enhancement of precursors or sialyltransferase enzyme have increased sialylation in huEPO produced in stable clones, but have limited increase in efficacy (Bork et al. 2007; Jeong et al. 2008; Son et al. 2011). Interestingly, the introduction of sialyltransferase and CMP-sialic acid transporter genes by transient transfection did not increase the sialylation of huEPO in CHO and BHK cell lines (Zhang et al. 2010). The authors suggest that wild type populations (compared to selected clones) of these cells have sufficiently high expression of these glycogenes.

The generation of CHO glycosylation mutant cell lines (CHO-gmt) using lectin selection combined with zinc-finger nuclease technology is another method for glycan modification of biopharmaceuticals (Zhang et al. 2013). A highly sialylated form of huEPO was produced using glycoengineering of a N-acetylglucosaminyltransferase I (GnT 1) mutant cell line. Restoration of GnT 1 in a mutant cell line produced highly sialylated huEPO (Goh et al. 2014).

However, its efficacy has not been determined. Enhanced sialylation and fucosylation of huEPO was found with the introduction of a silkworm gene coding for the 30Kc6 protein into the recombinant CHO or addition of the protein into culture media (Park et al. 2012).

The function of monoclonal antibodies (IgG) is highly regulated by their N-linked glycans at Asn_{297} of the Fc region of the heavy chain, and modifications to their glycan have been intensely studied. The two glycans hold the Fc region in the required horseshoe shaped conformation (Arnold et al. 2007) (Deisenhofer 1981) (Padlan 1991) and if the glycan is removed a "closing" between the CH2 domains causing a reduction in FcγR binding capacity (Ferrara et al. 2006b; Krapp et al. 2003). The microheterogeneity of the glycans also affect function. The presence of α1,6 linked fucose on the core GlcNAc limits the interactions of the Fc region with the FcgammaIIIa receptor reducing the ability to elicit an antibody-dependent cell mediated cytotoxicity (ADCC) response with NK and peripheral blood monocytes (Shields et al. 2002). An increase in bisecting GlcNAc by over expression of GnTIII can increase ADCC (Ferrara et al. 2006a; Schuster et al. 2005; Shinkawa et al. 2003; Umana et al. 1999). This work has led to the production of glycoengineered Mab that has been approved for drug use (Ratner 2014). But it has also been shown that it is the loss of fucose and not the increase in galactose or bisecting GlcNac that is necessary for ADCC activity (Shinkawa et al. 2003). Galactosylation of the Mab is important in complement-mediated cytotoxicity (Hodoniczky et al. 2005). Several approaches have been used to reduce the fucosylation. Gene knock out of FUT8, coding for the α1,6 fucosyltransferase enzyme, has successfully created CHO host cell lines which produces Mabs with reduced fucose (Yamane-Ohnuki et al. 2004) and afucosylated Mab with enhanced ADCC activity has been approved for use in Japan (Beck and Reichert 2012). An siRNA (sequence interfering RNA) was used to prevent the expression of both the fucosyltransferase and GDP-mannose 4,6 dehydratase (GMD), an enzyme required for the production of GDP-fucose (Imai-Nishiya et al. 2007; Kanda et al. 2007a). Lectin selection with zinc-finger nuclease technology has produced a fucosylation mutant by knocking out the GDP-fucose transporter in a sialylation CHO mutant (CHO-gmt5) which already lacks the CMP-sialic acid transporter (Haryadi et al. 2013). The result is the production of Mabs that are asialylated and afucosylated. A highly unique glycoengineering method eliminated fucosylation of Mab by inserting bacterial GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD) into CHO cells that deflects the GDP-fucose precursors into GDP-rhamnose (von Horsten et al. 2010). Interestingly, "knob and hole" technology which produces heterodimers of Mab has shown that afucosylation of only one Fc region of the dimer is sufficient for ADCC activity (Shatz et al. 2013).

Sialic acid also reduces antibody efficacy by reducing the Fc receptor binding and ADCC activity (Kaneko et al. 2006; Scallon et al. 2007) and increases binding to dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) by changing the conformation of the Fc region (Sondermann et al. 2013). Glycoengineering of CHO and NS0 was used to express a secretable sialidase A to trim the sialic acid from Mabs (Naso et al. 2010). In some Mabs used

for anti-inflammatory therapeutics, the ADCC response is undesirable, and thus increased sialylation can be used to reduce it. Although CHO is the predominant cell line used in the production of biopharmaceuticals, its lack of expression of the 2,6 sialyltransferase enzyme (ST6), produces only 2,3-linked sialic acid glycans. Much work has been devoted to humanizing sialylation with the introduction of the ST6 gene but limited increase in 2,6-linked sialic acid, has been observed in the recombinant protein (El Mai et al. 2013). It is thought that increasing the 2,6 sialylation of Mabs has been difficult, due to accessibility of the ST6 to the glycan (Raymond et al. 2012). However, this can be overcome by the replacement of a phenylalanine residue with alanine in the Fc region of the Mab (Jassal et al. 2001; Raymond et al. 2012). In another report a mutation of Y407E (tyrosine to glutamate) residue in the CH3 region of IgG1 results in increased sialylation and galactosylation possibly through allosteric affects on the CH2 region and CH2-CH3 interface (Rose et al. 2013). Bispecific Mab with 70 % 2,6 sialylation was possible with overexpression of a gene for ST6 originally derived from a non-expressing CHO cell line transfected into the Mab producting CHO cell line (Onitsuka et al. 2012). This is thought to be due to a difference in the substrate-binding region of the CHO ST6 enzyme, allowing higher sialylation. These studies suggest that both Mab protein structure and enzyme specificity may play a large part in the addition of terminal sugar residues (Gal and NeuAc) on Mabs, and future glycoengineering will have to account for these parameters.

Other mutant CHO cell lines have been tested for their use in glyco-modification of biotherapeutics. CHO cell lines that produce predominantly Man_5 glycan structures have been produced using chemical mutagenesis and lectin selection (Zhong et al. 2012) or zinc-finger nuclease knockout with lectin-mediated selection (Sealover et al. 2013). These may be useful for the production of biotherapeutics, which require mannose-receptor uptake such as vaccines (Betting et al. 2009).

Additions of novel glycans to biotherapeutics can be used to alter their pharmacokinetics properties. Using protein engineering the polysialic acid domain of NCAM was added to a single-chain Fv antibody fragment to increase the half-life of the protein by 30-fold and increased tumor uptake by 12-fold (Chen et al. 2012). The addition of two glycan sites onto the hydrophobic CH2-CH3 region of Fc results in soluble monomers which result in a much longer half-life for Fab through neonatal Fc receptor (FcRn) binding and recycling (Ishino et al. 2013)

Glycoengineering in other host systems, such as yeast (Chiba and Akeboshi 2009; Wildt and Gerngross 2005), plants (Ko et al. 2009), insects (Ahn et al. 2008a) and also bacteria (Chiba and Jigami 2007) have been used to humanize the glycosylation of recombinant proteins, and often result in more homogenous glycosylation, which may be advantageous for specific biotherapeutic functions. This is beyond the scope of this chapter, but above reviews give in-depth information.

A secondary approach to optimize glycan structure for function is in vitro chemoenzymatic modifications of glycans, which can produce much more homogeneous glycosylation. Details of these methods have been described elsewhere (Mrazek et al. 2013; Wang and Lomino 2012). Trimming of N-glycans to the core

GlcNAc using endoglycosidases such as Endo H and EndoS provides a scaffold to re-build or re-attach another glycan. Early work rebuilt the glycan by sequentially adding sugars using appropriate nucleotide-sugars and glycosyltransferase enzymes but this can result in heterogeneity. Alternatively, highly homogeneous glycosylation can be accomplished by the en bloc transfer of large pre-formed oligosaccharides. Fully sialylated glycosylation of rituximab has been accomplished by a combination of chemical and enzymatic synthesis of the glycan with an en bloc transfer of activated glycan oxazolines to the protein (Huang et al. 2012). A mutated Endo S with glycosynthase activity allowed efficient addition of the glycan to the Fc site of the Mab. Introduction of a glucose residue to N-glycosylation site (NXS/T) using an N-glycosyltransferase allowed further addition of large glycan to the polypeptide by enzymatic transglycosylation (Lomino et al. 2013), and may be useful for creating unique glycosylation sites within a peptide to modify its characteristics.

A third method allows modification of glycan structure through the addition of glycoprotein processing inhibitors to the culture media during production. This can result in alternative glycan structures with each inhibitor specific for one of the processing enzymes: glucosidase inhibitors (castanospermine and methydeoxynojirimycin) result in $GlcNAc_2$-$Man_{8\text{-}9}Glc_{1\text{–}3}$ glycan; mannosidase I inhibitors (deoxymannojirimycin and kifunensine) result in high mannose structures ($GlcNAc_2$-$Man_{8\text{ or }9}$) and swainsonine, a mannosidase II inhibitor results in a hybrid glycans. Studies with altered glycan structures of IgG, found the glucosidase inhibitors and mannosidase I inhibitors could enhanced ADCC or FcγIIIa binding due to a decrease in the core fucosylation (Kanda et al. 2007b; van Berkel et al. 2010; Zhou et al. 2008). Also, a more open conformation of the Fc region was identified using X-ray crystallography of Mab produced in kifunensine with high mannose glycosylation and this is proposed to facilitate receptor binding (Crispin et al. 2009). Swainsonine, a mannosidase II inhibitor produces hybrid glycans but does not enhance ADCC which may be partially due to the presence of fucose on the hybrid glycan (Rothman et al. 1989a). Inhibition of fucosylation of antibodies and enhanced ADCC can also accomplished with addition of fucose analogues, 2-fluorofucose and 5-alkynylfucose derivative to cultures, which deplete cells of GDP-fucose (Okeley et al. 2013).

Conclusion

Enormous variability in the structure of glycans can exist from protein to protein and at glycosylation sites within a specific protein. Advances in the analytical techniques for glycoproteins and glycans over the past few decades have shown us how minor changes in glycosylation allows dramatic changes in the function of very important glycoproteins such as IgG. Biotherapeutics such as IgG and huEPO have given us good understanding of the importance of glycosylation at both microheterogeneity and macrohetergeneity levels,

(continued)

and have defined glycosylation as a critical quality attribute. The challenge now is to further our understanding of the general and specific affects of glycosylation on new biotherapeutics and improve current ones. The aim is to increase serum half-life and efficacy, lower immunogenicity and create greater homogeneity, and ultimately improve quality and reduce costs. This can be accomplished by further defining how culture conditions, nutrients and additives affect the glycosylation pathways and cellular metabolism, and by manipulation of glycosylation through genetic engineering and other means, leading to a tighter control over production and better overall glycosylation of product.

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Chapter 10
Modelling of Mammalian Cell Cultures

Nishikant P. Shirsat, Niall J. English, Brian Glennon, and Mohamed Al-Rubeai

Abstract Mathematical modelling of mammalian cells has always been for the purpose of control and optimisation of bioprocess. Therefore, most of the models have been unstructured, unsegregated and empirical. However, with the advancement in analytical techniques such as Raman spectroscopy and flow cytometry for monitoring the state variables, structured, segregated and mechanistic models have been developed to provide more understanding of the underlying mechanisms of growth and productivity in mammalian cells. This review describes models based on bioprocess classification, experimental and theoretical tools of modelling, modelling approaches – empirical, mechanistic and stochastic, and finally mathematical techniques used for modelling. The models are developed for a variety of reasons but the prime reason ought to be realism over generality and predictability. Therefore, models described here are reviewed with a biological perspective to assess their impact on our understanding of growth and productivity in mammalian cells.

Keywords Modelling • Mammalian cell culture • Non-linear model • Statistical model • Computational modelling • Segregated and nonsegregated model • Single cell model • Logistic model • Neural network

10.1 Scope of Bio-pharmaceutical Industry and Challenges

Bio-industry is expanding at exponential rate to meet the increasing demands for biopharmaceutical products. According to the market research conducted by BioPlan Association, the global biopharmaceutical industry is currently worth over $145 billion and should exceed $167 billion by 2015. The market for biopharmaceutical products is predicted to grow between 7 % and 15 % because by 2013 four drugs out of five top drugs will be protein-based (Whelan and Keogh 2012; ABB review 1/12). However, the process development costs have increased from $804 million in 2004 to 1,214 million in 2010 (Adams and Brantner 2010).

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Therefore, the biopharmaceutical industry is an expensive and formidable component of the overall 'bio-industry', and it is entirely dominated by mammalian cells because of their ability to carry out post-translation modification of biologicals for them to become biologically active (Butler 2005). However, there is a drive to improve efficiency and productivity, as well as reducing high experimental costs. Therefore, the ability to predict the precise behaviour of mammalian cell cultures under different growth conditions is highly desirable to improve both the efficiency and productivity of the underlying bioprocesses, not only for commercial reasons, but, perhaps more importantly, to counter the emerging competition from non-cell-culture technologies, such as in vitro ribosome display and transgenic animals (Duncan 2002). These improvements can be either be biologically-based, i.e., genetic manipulation of cells to increase intrinsic efficacy of a product, or culture-based, i.e., optimisation of culture processes to increase efficiency via purity, quality and productivity, all of which require the modelling expertise of engineers (Sidoli et al. 2004).

10.2 Critical Review of Mathematical Models of Biological System

In this broader context, it is worth reflecting on the potential and actual contributions of mathematical modelling to the biopharmaceutical industry. There is a "zoo" of mathematical models, in the biochemical engineering and mathematical biology literature, with a central objectivity being calculating numbers to 'fit' reasonably experimental data (Bailey 1998). The commercialisation of the bioprocesses modelling is dominated by bioprocess engineers, who perhaps have less knowledge in the underlying mechanisms of the process; many engage in developing Artificial Neural Network (ANN) techniques to develop empirical models to fit data (Montague and Morris 1994; Meltser et al. 1996; Chen et al. 2004; Ferentinos 2005). Therefore, it is not surprising that many biological scientists have a somewhat lesser-developed interest in mathematical modelling, because they are less aware of the potentially rich contributions of mathematical biology as a valuable research tool (Bailey 1998).

Biological or cellular improvements cannot be achieved without a deeper understanding of the underlying mechanisms of cellular growth and productivity, which is essential for operating efficient and cost-effective bioprocesses (Koutinas et al. 2012). Therefore, mathematical models are increasingly becoming central to understanding and improving cellular based processes. However, with the field of biotechnology shifting from method (knowledge-based) development to applications; this demands a systems-biology approach of detailed, mechanistic modelling; though the 'reductionist' approach has successfully identified most of the components and many of the interactions; unfortunately, it offers less convincing concepts or methods to understand how system properties emerge (Oberhardt et al. 2009). Arguably, the pluralism

of causes and effects in biological networks is better addressed by observing, through quantitative measures, multiple components simultaneously and by rigorous data integration with mathematical models (Sauer et al. 2007). Systems biology is an alternate to reductionist approach, in that this permits a systematic study of the complex interactions in biological systems. Systems biology has been used for parameter estimation, and is usually part of an iterative process to develop data-driven models for biological systems, which are expected to have predictive ability. Ashyraliyev et al. (2009) discuss how to obtain parameters for mathematical models by data-fitting. Systems biology provides an interdisciplinary approach, which combines experimentation based on explicit hypotheses with computational and mathematical modelling based on data-mining (e.g., genomics, proteomics, metabolomics etc.) (Makarow et al. 2008). Despite considerable developments in analytical and molecular biology over recent decades, Baily's (1998) observation "the development of mathematically and computationally oriented research has failed to catch up within biology" is as valid today as it was around 16 years ago, i.e., the understanding of experimental data has lagged behind the accumulation of data (Koutinas et al. 2012). This less-than-satisfactory situation will continue until there is radical change of attitude towards modelling. Below, we explore how such a change might take place.

The value of mathematics to life sciences is more than routine applications of statistics and calculus in solving problems; mathematical biology is about describing complex biological phenomenon in the simplest model without compromising reality and precision, in what Levins (1966) describes as the actions of a "simplest theorist". In this vein, it is not enough to create a mathematical model that "describes" biological behaviour but more importantly modelling is about how the "biological" mechanisms give rise to biological behaviour (Reed 2004). Therefore, mathematical models based on the end results alone, such as growth and productivity, do not further our understanding of the biological mechanism of mammalian cell cultures in different modes of operations (batch or fed-batch) or different culture media and different environments. Thus, it is obvious that mathematicians are not capable of researching any of these biological areas without the help of competent biologists in that area. In fact, for meaningful results, research in these areas should be led arguably by biologists who would define the problems, requiring mathematical solutions; mathematicians are expert at thinking through complex relationships and translating scientific questions into mathematical ones (Reed 2004). Some of the techniques are invaluable to the biologists and modellers working together to unravel the underlying mechanisms of the reactions so as to provide insight into factors affecting the process performance; these would include: screening of state variables for their biological significance in growth and productivity, in situ Raman spectrophotometry for simultaneous monitoring of multiple process parameters in real-time (Whelan et al. 2012), automated sampling and sample preparation steps for at-line flow cytometric analysis (Kuystermans et al. 2012), data processing (Motulsky and Chrstopoulos 2004), regression methods for estimating model constants (parameters) (Prajneshu 1999) and Design-of-Experiment (DoE) (ICH Q8R2 2009). These 'holistic' approaches require modellers to have some rudimentary knowledge of biology and the biologists to

understand the engineering requirements for monitoring and control of bioprocesses. Therefore, a brief introduction to mammalian cell cultures and the FDA PAT initiative (2004) for the pharmaceutical industry is included.

10.3 Classifications

Since there are innumerable models based on different modes of cultivation, requiring variety modelling approaches to suit a particular purpose, Tsuchiya et al. (1966) found the need to classify cultivation modes and Julien and Whitford (2007) developed this further.

10.3.1 Models Based on Classification of Bioprocesses

Tsuchiya et al. (1966) proposed a classification of bioprocesses for the benefit of engineers, who are interested in modelling the processes for monitoring and control. Their classification for microbes was adopted by Ramakrishna (1979) for mammalian cells and revised by Sodoli et al. (2004), who introduced the terms 'segregated' and 'unsegregated' bio-phases. Here, the classification of growth is extended to include types associated with each category of bioprocess (cf. Fig. 10.1).

The model can be structured or unstructured, segregated or unsegregated, deterministic or stochastic. Not all cells in a mammalian cell culture are alike; they are heterogeneous in composition. By classifying a model as being segregated or unsegregated, it is possible to account for the heterogeneity of the cell culture with regards to the cell age, size, growth rate and metabolic state. Referring to a cell culture as segregated implies that it is composed of cells in different stages of development and it is therefore heterogeneous. Conversely, an unsegregated model

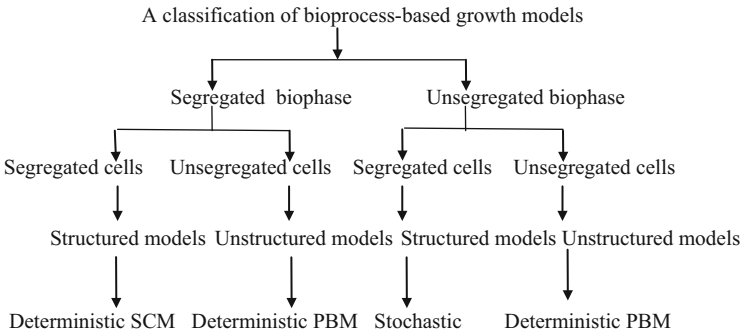

Fig. 10.1 Growth models based on bioprocess classification (Modified form of Sidoli et al. 2004)

views the population as consisting of identical "average cells" and uses a lumped variable such as total biomass per unit volume to describe the entire population. Segregated models build on the heterogeneous composition of a culture and offer the advantage of relating cell properties and biochemical activities within distinct parts of the population. Segregated models based on cell cycle relate the kinetics of growth, metabolic processes and product formation to the distribution of the population among the phases. Considering the differences between cells in the population, segregated model is more representative of the true physiological state of the cell. However, segregated models are also more computationally difficult to handle (Bailey and Ollis 1986). Variability within a cell culture can be accounted for by means of stochastic models that describe random processes. For systems in which the cellular processes are not subject to variability, a deterministic model is applied. Stochastic models use probability distribution functions to describe process dynamics at the cell and population levels. Since they account for randomness in the culture, they are more accurate than deterministic models but that is the case only as long as the systems contain a small numbers of cells (Tziampazis and Sambanis 1994). Thus, a segregated cell population is inherently heterogeneous but unsegregated is treated as homogeneous. However, according to Sidoli et al. (2006), the heterogeneous nature of structured Single Cell Model (SCM) approach is not strictly valid.

The models are broadly categorised as structured and unstructured and the choice of a particular model depends on the purpose for which it is employed. For example, structured models (Votruba et al. 1985; Dantigny 1995; Montesinos et al. 1997; Lei et al. 2001) are used to describe in more detail the intrinsic complexity of the system. Structured models attempt to explicitly describe segregated cell populations in terms of their intracellular contents, thus acknowledging that the cells are in different stages of development, i.e., cell properties and biochemical activities with distinct parts of the population (Tziampazis and Sambanis 1994). Structured models can provide a measure of the quality of the cell population and are based on more fundamental understanding of the processes (Sidoli et al. 2004). These models attempt to elucidate intracellular processes in that they possess structure either in the physical sense, namely the organelles, cell shape or size, or in the biochemical sense where biomass is subdivided into its intracellular biochemical components. Thus, biochemical data (i.e., metabolic flux) of the segregated cells in segregated bio-phase is used to develop structured deterministic Single Cell Model (SCM), while flow-cytometry data (cell-cycle phases and organelles) of segregated cells in unsegregated bio-phase is used to develop structured stochastic model (cf. Fig. 10.1). Sanderson (1997) developed a SCM of general applicability to mammalian cells by first modelling cellular function. Such models are useful for process understanding, but because of the complexity of measuring and monitoring a large number of intracellular state variables, they are of limited practical applications. Besides, the SCM approach is expensive (Sidoli et al. 2004). Shuler (1999), in his review of "single cell models: promise and limitations" states that SCM are inherently complex and hence computationally intensive. Structured models are more representative and informative than unstructured ones but they are also more difficult to handle mathematically (Bailey and Ollis 1986). They are useful

for process understanding but because of the complexity of measuring and monitoring a large number of state variables, they are of limited practical applications. Structured models can provide a measure of the quality of the cell population and are based on more fundamental understanding of the processes (Sidoli et al. 2004).

On the other hand, unstructured models do not account for the intracellular processes and acknowledge only implicitly the change of cellular physiological state with the environment (Fredrickson 1976). Unstructured deterministic population-based models (PBM) are derived from unsegregated cells in the segregated or unsegregated bio-phase on the assumption that all cells are at the same "average" stage of development (cf. Fig. 10.1). Thus, the biological basis of unstructured models is limited, and the mathematical equations involved are only phenomenological descriptions of the actual system. The unstructured models consider living cells as a single entity ignoring their intracellular metabolic flux (Provost and Bastin 2004) and hence provide a particular solution, and not a general one (Kontoravdi et al. 2010). They focus on specific outcomes that define the given bioprocess, e.g., substrate consumption, biomass formation and products of interest. Hence, in unstructured models, only a few state variables are considered, making it easier to apply them in practice. Therefore, the unstructured models are of limited applicability in situations where the cellular environment is highly dynamic in nature. Being highly empirical in nature, i.e., lacking physical knowledge of the system, they are primarily used in cases where only parameter estimation is required for developing the full model. However, these oversimplified models have proven effective in describing growth via interrelationship between growth and the state variables (Gaudy and Gaudy 1980; Bailey and Ollis 1986) and hence they are used widely for the design of on-line algorithms for process monitoring, control and optimisation (Provost and Bastin 2004). Besides, these unstructured PBMs are easy to build and they are useful in simulation studies because in subsequent *in silico* applications, the state variables are computationally tractable (Kontoravdi et al. 2010). It is far less expensive to run *in silico* experimentation than carry out actual experiments to identify the operating conditions, medium compositions and parameter spaces so as to obtain improved qualitative and quantitative information (Sidoli et al. 2004). However, there is a strong link between modelling principles of structured and unstructured models. Since, in structured models like SMC, the intracellular kinetics are in general not structurally identifiable without intracellular measurements (Provost and Bastin 2004), different studies have highlighted the possibility of deducing a simple model from a structured one by using model reduction techniques, thus, validating the macroscopic approach for modelling a bioprocess for monitoring and control (Provost and Bastin 2004; Haag et al. 2005).

With this in mind, a new trend in mathematical modelling has emerged by focussing on the so-called Metabolic Flux Analysis (Stephenopoulos et al. 1998), where intracellular fluxes are computed from the measurement of extracellular fluxes by using the stoichiometry of a metabolic network, governing the system. Provost and Bastin (2004) have described the application of metabolic flux analysis through the case study of CHO 320 cells cultivated in serum-free medium.

10.3.2 Classification of the Different Forms of Mathematical (Nonlinear) Models

Model forms used for monitoring, control and simulation are specified in Fig. 10.2. Non-linear models are broadly divided into categories: Qualitative, Mechanistic and Statistical (Julien and Whitford 2007). Qualitative models (cf. Fig. 10.2) can often be formulated even when the course of a culture process is not amenable for mathematical modelling, e.g., discontinuities occurring during medium exchange in repeated-batch culture (Julien and Whitford 2007). The simplest form is the "rule-based" model that makes use of "if-and-then-else" language to describe process behaviour. These rules are elicited from expert operators who know when and how to intervene when system behaviour is 'off-course'.

The most commonly used mechanistic models are derived from physical, chemical and biological parameters governing the processes (cf. Fig. 10.2). Model equations describing the parameters are developed from experimental data on the kinetics of metabolic reactions or metabolite and recombinant product levels or the more theoretical mass/energy conservation balances. A set of non-linear ordinary differential equations (ODEs) and/or partial differential equations (PDEs) with related algebraic equations are compiled to produce mathematical models to simulate real systems. ODEs refer to lumped parameters and are used to describe behaviour in one dimension, e.g., time, whereas PDEs refer to structured (distributed) parameters and hence account for spatial differences, e.g., substrate gradient in a large bioreactor. Segregated, structured models can be considered as unstructured, segregated because a model describes what an entire subpopulation does in that particular area. However, structured segregated models (Fig. 10.1) like SCM are more complex and hence more difficult to develop and solve, and their impact is very significant in large bioreactors where, for example, mixing kinetics and times may become critical to the success of bioprocesses.

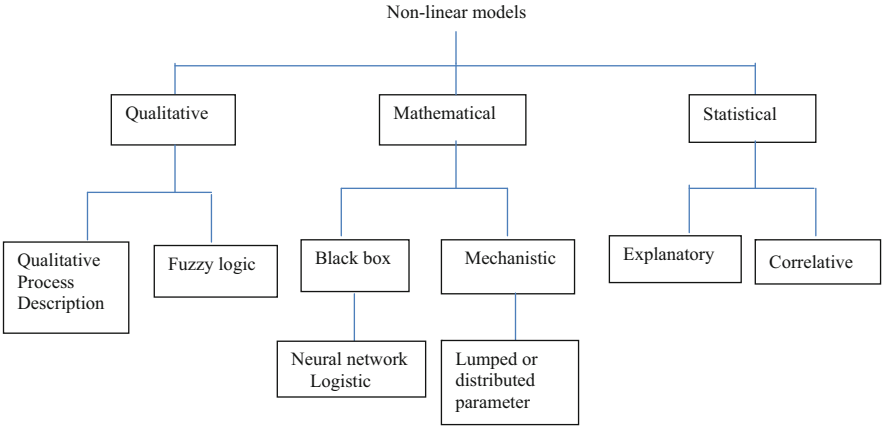

Fig. 10.2 Classification of nonlinear model forms (Adapted from Julien and Whitford 2007)

The term 'black box' or empirical model simply describe numerical relationships between system inputs and outputs (cf. Fig. 10.2); in this respect, these models are similar to correlative stochastic models. The parameters have no physical meaning or biological significance. However, as in many other areas of engineering, these relatively simple equations express accurately process trajectories (Bailey and Ollis 1986) and hence they are widely employed in the monitoring and control of bioprocesses. Neural networks (NN) are particularly suited for modelling complex non-linear processes (cf. Fig. 10.2). Neural networks approach is quite different to conventional cognitive computational approach in solving control problem. Neural networks are composed of a large number of highly interconnected processing units working in parallel, a concept originally inspired by the way human brain processes information (Julien and Whitford 2007).

It has been a long-standing practice to describe system dynamics by using continuous deterministic mathematical models, although they fall well short of this requirement of the reductionist approach to the modelling. It has been conceded that biochemical kinetics at the single-cell level are intrinsically stochastic (Ramkrishna 1979; McAdams and Aikin 1999) and hence, it is now generally accepted that stochastic models are vital for capturing properly the multiple sources of heterogeneity needed for realistic modelling of biosystems. Therefore, in recent years, the mathematical approach to computational system biology has been replaced by statistical methods (Wilkinson 2009). Stochastic models use probability functions to describe process dynamics at the cell and population. Since they account for random variation, they are more accurate than deterministic models (Tziampazis and Sanbanis 1994).

Statistics is the science concerned with linking model data and therefore, the success of systems biology vision depends on the accuracy of this data. For inferring the parameters of deterministic and stochastic biosystems models, statistical methods provide the best way to extract maximal information from the biological data (Wilkinson 2009). Furthermore, using statistical methods for estimating constants in the stochastic models by time course data makes it possible to describe biological dynamics in a quantitative framework.

The relationships between variables in the biological field are quite complex compared to the physical sciences, which obey natural laws, e.g., the law of gravity. Simple or multiple regression analysis is ideally suited, particularly for biological sciences where underlying mechanisms are not clear. Regression analysis enables to develop correlative models which indicate the 'functional' relationships, if any, between y, the response variable and one or more variables x_j, categorical variables (cf. Fig. 10.2). Statistical techniques such as stepwise regression (Draper and Smith 1998), multi-collinearity diagnostic tests (Myers 1990), Durbin-Watson statistics (Chatterjee and Price 1991) and Houston tests (Springate 2011) are used to develop explanatory models for testing research hypothesis of possible functional (causative) relationships between the categorical (independent) variables and a response

(dependent) variable. Furthermore, from previous theoretical or experimental work, it may be hypothesised that the relationship among several variables is of a given form, say a linear or a second-degree polynomial without specifying the numerical values of all of the constants in the equation. Regression analysis provides a systematic technique for estimating, with confidence limits, the unspecified constants from a new set of data or testing whether the new data are consistent with the hypothesis.

In this vein, Shirsat et al. (2013) have developed very recently segregated structured stochastic (correlative and explanatory) models, using cytometric data (cell-cycle phases and organelles). Thus, stochastic modelling of biological data is ideally suited for unravelling underlying mechanisms of biological phenomena via hypotheses testing (Shirsat et al. 2013). However, the findings have to be verified by further experimentation and their biological significance has to be consistent with prevailing theory or understanding (cf. Fig. 10.3).

10.3.3 Black-Box, Grey-Box and White-Box Models

Biologically-orientated mathematical modelling has to be knowledge-based i.e. a priori reasoning rooted in sound theoretical and evidence based understanding so that the resulting model is meaningful, uncovering partially or fully the underlying mechanisms of the biological phenomenon: it is less than satisfactory to have a priori knowledge, which enables mere fitting the experimental data (Reed 2004).

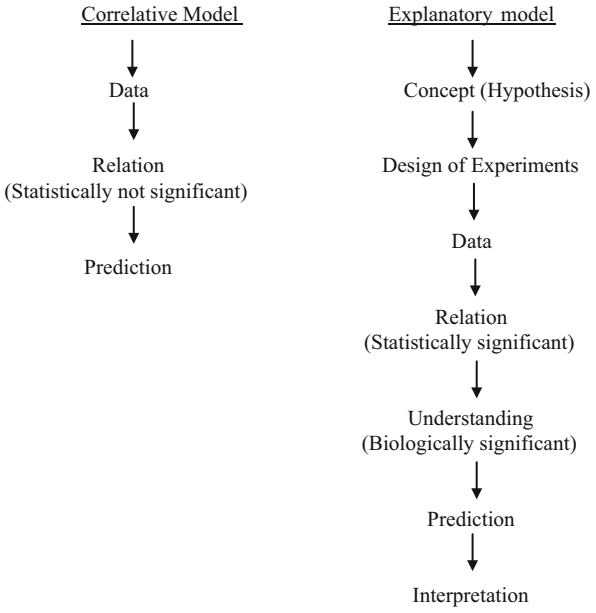

Fig. 10.3 Schematic representation of process of developing statistical models: correlative and explanatory

Therefore, models should be evaluated for their biological significance more so than their predictability. Thus, the significance, or otherwise, of model parameters may be used as a basis for classifying models into three categories: mechanistic, empirical and hybrid. The first class, mechanistic or 'white-box' models consist of mass balances of the state variables (biomass, substrates, inhibitors and products of interest) involved in a reaction scheme. White-box models are based on a priori knowledge of the interrelationship between the state variables. Thus, a mechanistic (white-box) model is based on certain knowledge-based assumptions about the type of growth, writing down differential or difference equations that represent these assumptions based on the first principles, and then solving these equations to obtain growth model. Here parameters have physical meaning and they are of biological significance; therefore, such models are useful in gaining insight into underlying mechanisms of the system and they are of immense help in efficient monitoring and control of processes. An empirical (black box) model, on the other hand, is where some input is given and some output is taken out; the parameters within the empirical model have no physical meaning nor are they of biological significance (Prajneshu 1999). In practice, grey box or hybrid model, form a compromise between black and white boxes, which combines the available a priori knowledge of white box with the reaction scheme and/or kinetics of black box, e.g., artificial neural network (Montague and Morris 1994; Meltser et al. 1996; Chen et al. 2004; Ferentinos 2005).

10.4 Cells, Cell Characteristics and Cell Lines

The use of animal cells for the production of recombinant proteins for medicinal purpose has boosted both development and application level in industrial production processes. The central object of a bioprocess is the cell. A living cell is a highly complex system which is often defined as the smallest autonomous biological unit. The main function of a cell is to survive, to reproduce and to manage itself. Living cell performs all these functions by building its own constituents and to provide its own energy through physical and chemical processes which constitute the cell metabolism. This latter consists of a network of thousands interconnecting reactions, the metabolic pathways, which are catalysed by enzymes and accurately controlled by regulation processes. Although it is known that many external factors and reagents influence the productivity of cells, little is known about the underlying mechanisms and therefore, optimisation of such factors is essentially empirical (Dutton 1998). Therefore, for the most part, the cell remains a "black box" with few inputs being monitored and their effects on outputs being poorly understood. Bacteria, yeast or animal cell cultures allow the synthesis of numerous products of interest for food or pharmaceutical sectors: vaccines, antibiotics, antibodies, wine, beer, industrial alcohols, yeast or enzymes for food technology. Therefore, a further research is required in cellular physiology because it is closely related to

cell behaviour, knowledge of which is essential in modelling and optimisation of productivity, as some intervention in waste treatment and pollution control (Moo-Young, 1995; Bailey and Ollis 1986).

Suitable cell lines for bioproduction display certain common characteristics: an ability to grow, unclumped, in suspension culture, stable and productive integration of heterologous DNA; capacity for desired posttranslational modifications; robust growth and high levels of productivity in variety of media; adaptability to a variety selection and production environments (Chu and Robinson 2001). Mammalian cells display many similar characteristics including protein expression. However, in some cell lines, specific differences can significantly affect in productivity. For example, glycosylation of a given protein can vary as expressed in various mammalian systems (Jenkins 2003). This is also true for clones sharing the same parents.

Biologically-based improvement in the performance of cell line comes through the genetic manipulation of mammalian cell involving recombinant technologies. More recently, enhanced interest in mammalian cell cultures is associated with recombinant protein technology developed in the 1970s and 1980s (Butler 2005). The first human therapeutic protein to be licensed from this technology in 1982 was recombinant insulin (Humulin from Genentech). However, *Escherichia coli* was used to produce insulin on large scale because it is fast growing and robust than mammalian cells. However, it was soon realised that recombinant therapeutics were more complex, requiring the post-translational treatment for their biological effectiveness. Only eukaryotic cells have necessary metabolic machinery for post-translation (Butler 2005). Biopharmaceuticals produced from mammalian cell bioprocesses are defined as recombinant proteins, monoclonal antibodies (MAb) and nucleic acid based products (Butler 2005). From 2006 to 2011, on an average 15 novel recombinant protein therapeutics have been approved by US, Food and Drug Administration (FDA) annually (Lai et al. 2013). Presently, most pharmaceutical companies use the methotrexate (MTX) amplification technology or the glutamine synthetase (GS) system for developing new mammalian cell lines (Lai et al. 2013). Although many mammalian cell lines have been used in the production, CHO (Chinese Hamster Ovary) cell lines are the most commonly used mammalian hosts in biological and for protein expression, especially MAb (Monoclonal antibody) are the most preferred choice (Whitford 2006). CHO cells are the preferred for large scale production of recombinant proteins for therapeutic use as they are fast growing; they can be readily transfected and can perform complex post-translational modifications required for biological activity (Kumar et al. 2007). They have also been used safely for the production of recombinant therapeutics for many years and as such they constitute a trustworthy system (Kumar et al. 2007). However, these applications require the use of a bioreactor. Indeed, such a system favours cell growth by creating a good environment. It monitors and controls the cell environmental conditions like gas flow rates, temperature, pH, dissolved oxygen (DO) level and agitation rate.

10.5 FDA PAT Initiative

Stringent rules have been laid down for the monitoring and control of bioprocesses for production of pharmaceutical products. Following the FDA Process Analytical Technology (PAT) initiative in 2004, the pharmaceutical industry changed from Quality-by-Inspection (QbI) to Quality-by-Design (QbD), which involves monitoring in real-time critical-process-parameters (CPPs), which have direct bearing on the quality of the product. Thus, it is step up from Quality by Analysis (QbA) to Quality by Design (QbD). QbD is a systematic approach involving predetermined objectives with emphasis on product and process understanding, which in turn are based on sound science and quality risk management (http://www.ich.org/LOB/media/MEDIA4986.pdf, 2009). Whereas QbD is needed for controlling the process, PAT is needed for monitoring the effectiveness of QbD control. The concept actually aims at understanding the processes by defining their Critical Process Parameters (CPPs), and accordingly monitoring them in timely manner (preferably at-line or on-line), thus being more efficient in testing while at the same time reducing over-processing, enhancing consistency and minimising rejects. It involves defining the CPPs of the equipment used to make the product, which affect the Critical Quality Attributes (CQAs) of the product and then controlling these CPPs within defined limits. This allows manufacturers to produce products with consistent quality and also helps to reduce waste and overall costs.

The major goal of PAT is to improve the understanding and control of the manufacturing process. Quality cannot be tested in products on-line; it should be built-in or should be achieved by design. PAT is seen as a system for designing, analysing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality. Process Analytical Technology (PAT), which is widely used in the biopharmaceutical industry, is an important tool for the implementation of QbD. PAT is used for monitoring and controlling the manufacturing process with appropriate feed-back or feed-forward controls as well as to facilitate the tracking and trending of the process operation to support post-approval, continual improvements efforts (Riley and Li 2011). Ganguly and Vogel (2006) published a case study of the application of PAT and scalable automation for bioprocess control and monitoring, where they conclude that mathematical modelling is an essential part of QbD because it provides scientific understanding of product and process, essential for monitoring and controlling bioprocess. A success of mathematical modelling depends on identifying key parameters in bioprocess.

10.6 Tools of Modelling

10.6.1 Screening of State Variables for Explanatory Correlation with Growth and Productivity

Screening of the state variables ought to be the starting point of all meaningful model building. The biological processes are inherently complex, involving numerous intracellular reactions culminating in growth and productivity. It is not possible to determine intracellular reaction kinetics without intracellular measurements (Provost and Bastin 2004). Therefore, the "metabolic flux analysis" approach is adopted to compute, via stoichiometry, intracellular fluxes from the measured extracellular fluxes. Knowledge of biology and biochemistry is required to decide which of the extracellular state variables are directly or indirectly linked to the growth and productivity. On the basis of a priori and/or theoretical knowledge, and experimental evidence, the biologists would be able to advice as to "which of the state variables are most likely to be significantly correlated to growth and productivity", i.e., research hypothesis. Therefore, experiments must be designed to collect the data on the selected state variables for multi-regression statistical analysis to confirm, or not, the hypothesis. These statistically significant state variables of biological significance must then be used for developing mechanistic or pseudo-mechanistic (i.e. hybrid) models. Shirsat et al. (2013) used this strategy structured, unsegregated stochastic models for growth and productivity of CHO cells.

10.6.2 Raman Spectrophotometry: Determination of State Variables

Routine monitoring and control of CPPs within the biopharmaceutical industry is currently limited to in situ sensors such as temperature, pH, dissolved oxygen, optical density and capacitance probes to monitor culture parameters for feedback control of cell culture bioreactors (Hanson et al. 2007). Most chemical, biochemical and biological properties are measured off-line. However, because of the delay between sampling and measurement, off-line measurements are unsuitable for control. Therefore, there have been intensive efforts over the last decade to develop methods for monitoring bioprocesses in real time. The method must preserve sterility and/or withstand sterilisation temperatures, be sensitive to low concentrations of analytes in complex growth medium, consume little or no volume and be stable over long period of cultivation (Whelan et al. 2012).

Reviews of various chemical, biological and optical sensors employed in process monitoring are published (Becker et al. 2006; Uber et al. 2003). Briefly the methods include inline and at-line techniques: biosensors and chemo-sensors, spectroscopy technologies, in situ microscopy, capacitance probes, software sensors, flow-injection analysis and high-performance liquid chromatography (HPLC). Of all

the available systems, optical sensors found to be most suitable for in-line measurements (Abu-Absi et al. 2011). Optical sensors are non-invasive, take measurements continuously without having to remove samples from reactors and can be steam sterilised in bioreactors. Infrared and Raman spectroscopic measurements appear to be the most promising for process monitoring due to their ease of sampling and potential to measure multiple analysis simultaneously (Moretto et al. 2011).

Raman spectroscopy has been successfully utilized to measure glucose, lactate, glutamine and ammonium and complex macromolecules in off-line samples (Cannizzaro et al. 2003; Enejder et al. 2002; Raman and Krishnan 1928). The technology relies on a light scattering phenomenon observed when a photon impinges upon an electron cloud of a chemical bond and is subsequently ejected. A Raman shift is observed only if the photon leaving the cloud has more (Anti-Stokes) or less (Stokes) energy than when it entered. Each net change in energy is indicative of specific chemical arrangements and can therefore be measured and correlated to the presence of specific constituents (Shimoyama et al. 1997; Adar et al. 1997). Raman spectroscopy is uniquely suited for monitoring aqueous bioprocesses because it provides well-resolved vibrational fingerprints of various chemical constituents and is relatively insensitive to water. With aqueous solutions, water peaks dominate the spectra of similar technologies, such as absorbance infrared spectroscopy, and is exceptionally problematic in NIR because the interference is concentration dependent (Mungikar and Kamat 2010). Raman spectroscopy does not face these difficulties with aqueous solutions primarily because water does not absorb laser irradiation nor attenuate the Raman scattered light (Adar et al. 1997; Li et al. 2010).

In order to comply with FDA-PAT initiative, Whelan et al. (2012) developed in situ Raman spectrophotometer for simultaneous monitoring of multiple process parameters in bolus and continuous fed-batch cultures. They used the method successfully to monitor in real-time glucose, glutamine, lactate, ammonia, glutamate and TCD (Total Cell Density) in a CHO 320 fed-batch cultures as required by PAT initiative.

10.6.3 Flow Cytometry for Determination of Cell Cycle Phases and Organelles

Flow cytometry is a general method for rapidly analysing a large number of cells individually by light scattering fluorescence and absorbance measurement. The power of this method lies in the wide range of cellular parameters that can be determined and in the ability to obtain time-course information on how these parameters are distributed in the cell population (Carroll et al. 2007; Mohd 2013). Thus, segregated cytometric data, representing different subpopulations, are ideal for developing unsegregated, structured model, which can provide more

information and understanding of the bioprocess (Riesberg et al. 2001). Such data is of immense importance for investigating via regression analysis the underlying mechanism of growth and productivity of CHO cultures, which could be used for optimisation of bioprocesses. Flow-cytometry analysis is based on the principle that apoptotic cells, among other typical features, exhibit DNA fragmentation and loss of nuclear DNA content. Use of a fluorochrome, such as propidium iodide, that is capable of binding and labelling DNA, makes it possible to obtain a rapid (within about 2 h) and precise quantitative evaluation of cellular DNA content by flow cytometric analysis (Riccardi and Nicoletti 2006).

Flow cytometry is a very powerful tool for measuring the distribution of cellular properties across the heterogeneous cell populations. Flow cytometry uses special techniques for staining cells and by carrying out analysis of light scattering and fluorescence signals, individual cells are differentiated with respect to their size, protein content, DNA content and other intracellular properties (Harding et al. 2000; Riesberg et al. 2001; Lloyd et al. 1999; Gueret et al. 2002; Naciri and Al-Rubeai 2006). Further advances in flow cytometry involve flow injection techniques to produce automated systems that provide on-line measurements of cell distribution properties (Abu-Absi et al. 2003; Kuystermans et al. 2012).

In the past, cytometric parameters: VCD (Viable Cell Count), cell cycle phases, mitochondria, golgi apparatus, endoplasmic reticulum and MAb (monoclonal antibody) were determined using off-line manual methods (Carroll et al. 2007; Mohd 2013). However, since the PAT initiative, it is imperative that biopharmaceutical industry implements process monitoring and improvements. Over the last decade several online and at-line monitoring technologies have been implemented in cell culture monitoring (Hams and Kostov 2002). Some of these technologies still face significant challenges of calibration and data analysis before they could be used in bio-manufacturing environment. Furthermore, it is vital that at-line automated sampling system does not contaminate the bioprocess (Kuystermans et al. 2012). Kuystermans et al. (2012) developed protocol requiring use of an automation platform consisting of both software and hardware elements that when combined implements a series of automated sampling and sample preparation steps for at-line flow cytometric analysis. Their method, in compliance with PAT, enables to investigate and monitor in real-time cell growth, viability, cell size and cell cycle of CHO cell culture.

10.6.4 Systems Biology in Computational Modelling

The aim of systems biology is to describe quantitatively the dynamic systems behaviour of complex biological systems involving the interaction of many components, similar to the reductionist approach in principle but different in practice (Wilkinson 2009). Systems biology is about ‘putting together’ rather than ‘taking apart’; it is integrative rather than reductionist. At the level of cellular modelling (SMC), time-course data on the abundance of many different biomolecules at the

single-cell level is required (Wilkinson 2009). Therefore, data is collected from diverse sources such as genomic, proteomic, metabolomics, bioinformatics, etc. Studying biological processes under this premise, systems biology combines experimental techniques and computational methods in order to construct predictive models. Wilkinson (2009) has published a review of stochastic modelling in computational modelling for quantitative description of heterogeneous biological systems. Two related developments are currently changing traditional approaches to computational systems-biology modelling. Firstly, stochastic models are being used increasingly in preference to deterministic models to describe biochemical network dynamics at the single-cell level. Secondly, sophisticated statistical methods and algorithms are being used to fit both deterministic and stochastic models to time-course and other experimental data. Both frameworks are needed to adequately describe observed noise, variability and heterogeneity of biological systems over a range of scales of biological organisation.

Mathematical modelling in the context of system biology refers to computational modelling and simulation of subcellular, cellular and macroscale phenomena, using primarily the methods from dynamical system theory (Makarow et al. 2008). One of the main purposes of the computational modelling approach is to test the explicit hypotheses about underlying mechanisms of cellular behaviour (Marakow et al. 2008). Hypothesis is an essential part of modelling because its validity or otherwise confirms the present day understanding but also sometimes it improves the present day knowledge by uncovering critical points. Dynamical systems theory is a mathematical tool to investigate nonlinear behaviour of complex biological systems. However, this investigatory approach, involving parameterisation, calibration and validation, is too expensive or even impossible to execute. Therefore, simulation is vital in computational model. Simulation can reveal hidden patterns and/or counter intuitive mechanisms in complex system. However, in both building and utilising models of biological systems, inverse problems arise on several occasions, for example, (i) when experimental time series and steady state data are used to construct biochemical reaction networks, (ii) when model parameters are identified that capture underlying mechanisms, or (iii) when desired qualitative behaviour such as bistability or limit-cycle oscillations is engineered by proper choices of parameter combinations (Engl et al. 2009). Unfortunately these inverse problems cannot be solved fully because of difficulties in modelling of complex biological systems (Brenner et al. 2010).

10.6.5 Regression Analysis and Estimation of Model Constants (Parameters)

Bioprocesses are inherently stochastic and, therefore, the deterministic models must include an error term (Prajneshu 1999). Least-squares may be used to fit a parameterised function to a set of measured data points by minimising the sum of

squares of the errors between the data points and function. The function is nonlinear when the parameters within are non-linear. Non-linear methods of least-squares fitting for parameter estimation apply to both linear and non-linear egression. However, minimisation of the residual sum of squares results in normal equations, which are non-linear in parameters. Since it is not possible to find an exact solution to non-linear equations, approximate analytic solutions can be found by employing iterative procedures to minimise the error between the function and the measured data points.

The main least-squares methods are: (i) Linearisation (or Taylor series) method, (ii) Steepest Descent method, (iii) Levenberg-Marquardt method. The details of these methods along with their merits and demerits are given in Draper and Smith (1998). Taylor series will converge very rapidly as it reaches near the true parameter values. Conversely, steepest-descent converges slowly even when it is nearing the true parameter values. The Levenberg-Marquardt method is the most widely used method for computing nonlinear least squares estimation of the parameters. This method is a compromise between the other two methods, combining successfully the best features of both and avoiding their serious disadvantages. The Levenberg-Marquardt method almost always converges and does not slow down (Prajneshu 1999). There are standard statistical packages, containing computer programmes to fit nonlinear statistical models based on the Levenberg-Marquardt algorithm. For example, SPPS has NLR option, SAS has NLIN option and IMSL has RNSSQ option to carry out least square estimate by Levenberg-Marquardt (Prajneshu 1999).

Procedures for solving non-linear equations require inputting initial values of the parameters. The choice of initial value is crucial. However, there is no set procedure for choosing initial estimates. The most obvious method for making initial guesses is a priori knowledge; alternatively, estimates calculated from previous experiments, known values for the similar systems or values computed from theoretical considerations. Prajneshu (1999) suggested four more methods: linearisation, solving a system of equations, using properties of the model and Graphical method. Of these four methods solving a system of equation is found to be the most effective. The method involves: if there are p parameters, substitute for p sets of observations into this model, ignoring error term (i.e. $\varepsilon = 0$). The function (lsqcurve) in MATLAB was used for solving these equations to determine the model parameters (constants). The function (MATLAB-lsqcurve), given the initial guesses, finds coefficients of x to best suit the nonlinear function fun (x, xdata) to the ydata (in the least-squares sense). The accuracy of the constants increases with an increasing number of observations.

10.6.6 Data Processing of State Variables

Raw experimental data are of little value because of non-normality of the error terms, together with unequal variances (Bernaerts and Van Impe 2004). Therefore, appropriate data transformation is required to stabilise the measurement error

variance is essential so that transformed data can be used for statistical analysis and model building. The main goal of non-linear regression analysis of the experimental data is to determine the time-course (i.e., 'trend' line/curve) of the bioprocess. The experimental values of dynamic bioprocess are prone to random variation and some experimental errors. In order to eliminate these errors and to represent the data as a standard curve, smoothing of the data points is done by polynomial regression methods, which minimises the differences between the observed and estimated values of the variable (Horiuchi et al. 1993; Motulsky and Chrstopoulos 2004). Thus, the model development is about modelling the trend line (standard curve) of the bioprocess because it represents the most probable behaviour of the cells. For instance, the data on the viable cell density (VCD) and MAb titre are non-linear and therefore, for linearisation, the data needs to be expressed as the logarithm of concentration rather than the concentration itself (Motulsky 2001) prior to transforming for subsequent modelling.

10.6.7 Design of Experiments

Mathematical modelling involves iterative cycle of: collection of available mechanistic process knowledge, experimental data collection, data processing, system identification and model validation (Ljing 1999). System identification involves detecting qualitative relationship between state variables from the mechanistic knowledge and experimental data. The quantitative description of such relationships is obtained through the structure characterisation and the parameter estimation (Bernaerts and Van Impe 2004). Experimental data is a main ingredient in modelling because it plays a vital role particularly in process structure characterisation and parameter estimation (Bernaerts and Van Impe 2004). However, mining of experimental data, describing process dynamics, is challenging because of the inherent heterogeneities in time scale and data type. Nevertheless, many have applied an array of classification and prediction techniques to investigate hidden process patterns. There are several techniques such as principal component analysis (PCA), partial least square regression (PLSR) and other automated techniques to capture the interactions between the process parameters (Le et al. 2012). These techniques are used for detecting state transition related to product and lactate formation, online monitoring, fault detection and diagnosis, scale-up assessment, process characterisation and root-cause analysis (Bachinger et al. 2000; Ündey 2004; Gunther et al. 2007; Kirdar et al. 2008). Other studies have used powerful supervised approaches such as decision tree (DT), artificial neural network (ANN) and support vector regression (SVR) to optimise a control scheme incorporating time-course data, predict the final process and reveal key parameters (Buck et al. 2002; Coleman and Block 2006; Charaniya et al. 2010) Among these multivariate analysis approaches, PLSR and SVR appear to be well suited to handle the various challenges associate with bioprocess data, i.e., high-dimensionality and co-linearity between various parameters (Le et al. 2012).

Data collection is a time-consuming, laborious and expensive exercise and therefore, Design-of-Experiment (DoE) approach is advisable to get sufficient, relevant information for modelling the bioprocess (Bernaerts and Van Impe 2004). DoE is a structured, organized method for determining the relationships among factors affecting a process and its output (ICH $Q8R_2$ 2009). DoE techniques help to identify qualitative relationship between the key variables in the bioprocess. DoE techniques enable modellers to determine simultaneously the individual and interactive effects of many factors that could affect the output results in any model The classical method, studying one variable at a time whilst holding all others constant (so-called 'One-Factor-at-a-Time'), is extremely inefficient and expensive (Stowe and Mayer 1966). It has been suggested that DoE can offer returns that are *at least* four to eight times greater than the cost of the experiments in a fraction of the time that it would take to run one-factor-at-a-time (Torbeck and Branning 2009). Shivhare and McCreath (2010) described practical consideration for DoE implementation in QbD. Mandenius and Brundin (2008) surveyed recent application of DoE methodology in the developments of biotechnological processes. Sercinoglu et al. (2011) demonstrated successfully the use of DoE tools in combination with an appropriate growth model to develop and test fed-batch strategies *in silico* before experiments are carried out in the laboratory.

Key to the success of DoE procedure is the selection of variables that are most likely to influence the outcome and the possible level at which they could be effective. Sanderson et al. (1999) and Sodoli et al. (2004) found that in order to harness the leaps in genomics/proteomics/metabolomics biochemical engineers must embrace the science of experimental design and its role in the model-building process. Similarly, Kontoravdi et al. (2010) stressed that an application of optimal experimental design is essential for successfully estimating the values of "sensitive" parameters and to extend the range of model' validity to the fed-batch conditions. However, the selection of variables and their levels is entirely based on experimenter's judgement and pre-knowledge of the process to be studied (Mandenius and Brundin 2008). Therefore, a collaborative approach between the modellers and biologists is imperative. The reduced set of experiments can be described mathematically as 2^{n-k}, where n is the number of factors to be investigated at the low and high levels, and k is the number of steps to reduce experimental design (Montgomery 2000). Mandenius and Brundin (2008) described several examples of bioprocess optimisation using DoE methodology to comply with the FDA-PAT initiative. Indeed, hypothesis testing could be made more rigorous by statistical techniques for decoding biological phenomena in conjunction with DoE methods for a given hypothesis, e.g., using fractional factorial designs on multiple levels (e.g., 2- or 3-) for screening purposes (Montegomery 2000). Amongst other DoE methods, the Plunkett-Burman designs (Stowe and Mayer 1966; Castro et al. 1992) and Bob-Wilson (Ergun and Mutlu 2000; Cebeci and Sönmez 2006) are the preferred choice, despite some of their (potential) shortcomings.

As an illustrative example of the power of DoE methods in mammalian-cell work, Castro et al. (1992) used a Plunkett-Burman design to test 23 variables with 24 experiments to determine which of the amino acids is most effective in

promoting the growth of CHO (IFNγ) cells and production of recombinant interferon (IFN)-γ. Castro et al. (1992) identified glycine is an important determinant of specific growth rate whereas for cell production bovine serum albumin (BSA), phenylalanine and tyrosine were also found to be important. The components important in the IFNγ production were BSA, sodium pyruvate, glutamate, methionine, proline, histidine, hydroxyproline, tyrosine and phenylalanine. However, insulin, arginine, arginine, aspartate and serine were found to inhibitory for growth and productivity.

10.7 Modelling of Bioprocesses

Levins (1966) suggests that population biologists have arrived at three strategies (at least) for building models to cope with the biological complexities (Levins 1966):

Type I models:	Generality is sacrificed for precision and realism
Type II models:	Realism is sacrificed for generality and precision
Type III models:	Precision is sacrificed for generality and realism

Thus, modelling is a trade-off between generality, realism and precision because a model addressing all three together requires a massive data and it is very difficult to construct and almost impossible to interpret (Levin 1966). The usefulness of any particular model depends on the modeller's goals. For example, to describe general ecological principles, it is usually necessary to sacrifice realism and precision; to describe a particular population, it is usually necessary to sacrifice generality. Thus, the choice of type depends on the modeller. However, the modelling maxim should be "simplest theory" (Levins 1966).

10.7.1 Growth and Productivity Models

The performance of cells, in large, cell cultures can be controlled and enhanced, provided that the system properties can be maintained at the required state. Mathematical models are especially useful for simulation, optimisation and control purposes. A realistic cell model will not only aid in optimising productivity but once such a model is formulated, prediction results can be obtained for metabolite concentrations that may otherwise be difficult to measure.

10.7.2 Principles Behind Model Formulation

In general, the quantitative description of a bioprocess in terms of a mathematical model involves formulation of three fundamental types of equations (Tziampazis and Sambanis 1994):

- Mass-Balance equations
- Yield equations
- Rate equations

The mass balance equations that are developed based on the reactor configuration and are essentially same for all cell systems if intrinsic kinetics of the culture is not taken into account. The yield and rate equations that describe cellular metabolism are independent of the reactor configuration. The yield equations are based on material and energy balances and relate the amounts/rates of the metabolite consumption and production. Although they may involve several underlying assumptions about metabolism, they have a theoretical basis and hence are reliable. The rate equations describe the kinetics of various processes, generally as functions of intracellular parameters and the composition of the extracellular medium. Being empirical in nature, rate equations are the most unreliable segment constituting a model (Tziampazis and Sambanis 1994). The process mass balances and the yield equations are not sufficient to fully describe the system. To remove the remaining degrees of freedom, formulation of rate equations or measurements of the rates on-line is required (Andrews 1993; Hu and Himes 1989). However, it is not always feasible to make on-line rate measurements but for a few compounds. Consequently, certain rate equations need to be specified. In the absence of sufficient knowledge, experimental results are utilized to obtain information about cellular processes and hypothesize kinetic expressions (Glacken et al. 1988).

10.7.3 Literature Review of Growth Models

For simplicity and clarity, the literature review of growth models follows the classification of bioprocesses (Fig. 10.1) and mathematical forms (Fig. 10.2). Previously-published reviews (Tziampazis and Sambanis 1994; Sodoli et al. 2004) cover the developments of Monod-type of unstructured, unsegregated and SCM-type structured and segregated/unsegregated growth models respectively. However, they do not mention variations of Verhulst logistic growth models, which are coming to prominence since Jolicoeur and Pontier (1989) developed a generalised a four-parameter logistic growth models. Therefore, these empirical unsegregated unstructured models for growth and productivity are reviewed under separate heading.

10.7.4 Unstructured Unsegregated/Segregated Models

The simplest way to model cell culture systems will be to consider an unstructured, unsegregated model. As these models do not require system details for their generation, they are essentially based on experimental data to derive information on cellular processes and postulate kinetic expressions. Generally, in the starting phase of model development for animal-cell-culture systems researchers and bio-engineers focused on empirical models.

For such a model, the growth rate expression can be written as (Shuler and Kargi 1992):

$$r_x = dN/dt = \mu N \tag{10.1}$$

where, r_x is the rate of cell generation, X is the cell concentration and μ is the specific growth rate.

The rate of cell growth is influenced by many factors such as temperature, pH, composition of medium, rate of air supply, etc. If all other conditions are kept constant then the specific growth rate may be affected by the concentration of a certain specific substrate (the limiting substrate or nutrient). Many cell culture processes exhibit saturation type kinetics, i.e., the rate of the process μ is limited by a certain factor when its concentration C is low, but the limiting effect disappears and the process rate reaches a maximum value μmax, as the concentration increases. The following empirical expression, i.e., Monod equation, (Similar to Michaelis-Menten equation for enzyme reactions) is used to relate the specific growth rate to substrate concentration (Shuler and Kargi 1992):

$$\mu = \frac{\mu_{\max} C_S}{K_S + C_S} \tag{10.2}$$

where C_s is the concentration of the limiting substrate (kmol m^{-1}) and the constant K_s is equal to the substrate concentration at which the specific growth is half of μ_{max} (h_{-1}). It is assumed that cells grow with constant cell composition and a constant cell yield.

Monod's Eq. 10.2 indicates the dependence of μ on C_s. One should note that Monod's equation is empirical and does not have any mechanistic basis. This equation is only valid for an exponentially growing or steady state culture under condition of balanced growth. The equation does not perform well during transient conditions. Despite its simplicity and no fundamental basis, it works surprisingly well in a large number of steady state and dynamic situations (Bailey and Ollis 1986). This characteristic has important implications in control of bioprocesses. Therefore, following the success of Monod models, several similar Monod-type models were developed to monitor and control the bioprocesses (Nielsen et al. 2003). It is obvious that the empirical Monod model for a single substrate will not fit all kinds of fermentation data. Therefore, the following models were developed by modifying the Monod model (Nielson et al. 2003):

$$\text{Teissier} \qquad \mu = \mu_{\max}\left(1 - e^{-C_s/K_s}\right)$$

$$\text{Moser} \qquad \mu = \mu_{\max}\frac{s^n}{s^n + K_s}$$

$$\text{Contois} \qquad \mu = \mu_{\max}\frac{s}{s + K_s x}$$

$$\text{Blackman} \qquad \mu = \left\{\mu_{\max}\frac{s}{2K_s}; s \le 2K\right\}; \left\{\mu_{\max}; s \ge 2K_s\right.$$

$$\text{Logistic law} \qquad \mu = \mu_{\max}\left(1 - \frac{x}{K_s}\right)$$

The above equations may serve useful purpose as data fitters and as control models in industrial fermentation, but it is arguable that their value is limited because they do not necessarily explain observed phenomena very well.

The situation is much more complicated where more than one substrate. i.e., multiple substrates influence the specific growth rate. Tsao and Hansen (1975) proposed a general multi-parameter, unstructured model for growth on multiple substrates:

$$\mu = \left(1 + \Sigma_i \frac{s_{e,i}}{s_{e,i} + K_{e,i}}\right)\prod_j \frac{\mu_{\max,j} s_j}{s_j + K_{s,j}}$$

where $s_{e,i}$ are growth-enhancing substrates and s_j are the concentration of substrates essential for growth. The presence of growth-enhancing substrates results in an increased specific growth rate, whereas the essential substrates must be present for growth to take place. The success of Tsao and Hansen (1975) for a large number of parameters to fit the data could be more coincidental than its successful application.

The following model was successfully applied for the growth of methanotropic bacteria on two substrates, i.e., oxygen and methane:

$$\mu = \frac{\mu_{\max 1}\mu_{\max 2} s_1 s_2}{(s_1 + K_{s1})(s_2 + K_{s2})}$$

Suppose if the concentration for both substrates reaches at a level (say $s_1 = 9$) where the rate is 90 % of its maximum, then the combined growth is limited to 81 %, of its maximum value, not a reasonable proposition. Therefore, Roels (1983) has proposed two alternatives, both of which can be generalised for application two limiting substrates:

$$\frac{\mu}{\mu_{\max}} = \min\left(\frac{s_1}{s_1 + K_1}, \frac{s_2}{s_2 + K_2}\right)$$

$$\frac{\mu_{\max}}{\mu} = 1 + \frac{1}{2}\left(\frac{K_{s1}}{s_1} + \frac{K_{s2}}{s_2}\right)$$

The above models will grow 90 % of the maximum value in the previously cited situation. The following model is developed where the growth is inhibited because of high concentration of the substrate or by the presence of metabolic product:

For high substrate concentration; $\mu = \mu_{\max}\frac{s}{s/K_i + s + K_s}$

For an inhibition by a metabolic product: $\mu = \mu_{\max}\frac{s}{s+K_s}\left(1 - \frac{P}{P_{\max}}\right)$

The two equations above may be useful models for including substrate or product inhibition in a simple way for both unstructured and structured growth models. In the extension of the Monod model, if the number of variables is not limited, then the validity of the model is lost outside the range of experiments on which the model was based.

Sometimes the metabolic end products, e.g., lactic acid, alcohol, ammonia, etc. inhibit cell growth, in the same way as enzyme inhibitors in enzyme reactions. The effect of an inhibitor on the growth rate becomes pronounced when its concentration reaches a minimum threshold value and growth rate is reduced as the concentration of inhibitor increases. Such effects can be modelled by analogy to the various types of inhibition of enzymatic reactions (Bailey and Ollis 1986). Of those inhibition models, the simpler and easier to use is that of purely non-competitive inhibition (Tziampazis and Sambanis 1994):

$$\mu = \mu_{\max}\frac{K_I}{K_I + C_I} \tag{10.3}$$

In the above equations, μ and μ_{max} have the same implication as mentioned before. i is the inhibitor concentration while K_i is the inhibition constant. Therefore, the analogous equation based on Michaelis-Menten is as follows (Nieilson et al. 2003):

$$\mu = \mu_{\max}\left(\frac{C_S}{K_S + C_S}\right)\left(\frac{K_I}{K_I + C_I}\right) \tag{10.4}$$

where C_I is concentration of inhibitor produced by the cells.

Most of the unstructured models are based on the determination of extracellular state variables (cf. Fig. 10.1). It is plausible that instead of one metabolite being the controlling factor, there may be a group of metabolites that control the metabolic processes within the culture. In that case, the expressions given above can be modified to incorporate additional terms (thus increasing the number of model parameters).

A typical cultivating medium for CHO cells consists of serum-free chemically defined CD-CHO medium (Bioscience, Ireland), supplemented with 25 μM methyl-amine sulphoxamine (MSX) (Sigma Aldrich, UK) and also glucose and glutamine,

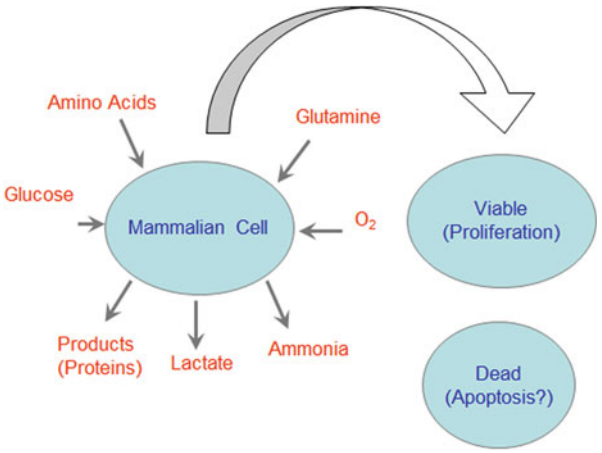

Fig. 10.4 Growth, death and metabolism of mammalian cell

the end metabolic products of which are lactate and ammonia, respectively (Fig. 10.4).

Therefore, the Monod logistic Eq. 10.18, used for modelling, becomes:

$$\frac{\mu_i}{\mu_{\max}} = \left(\frac{C_{G_i}}{K_G + C_{G_i}}\right)\left(\frac{C_{GTi}}{K_{GT} + C_{GTi}}\right)\left(\frac{K_L}{K_L + C_{Li}}\right)\left(\frac{K_A}{K_A + C_{Ai}}\right) \tag{10.5}$$

where μ, is growth rate, μ_{max} is maximum growth rate, C_G, C_{GT}, C_L and C_A are the concentration of glucose, glutamine, lactate and ammonia respectively, and K_G, K_{GT}, K_L and K_A are the corresponding Monod model constants and i = 1, 2, 3. . .. . ..n.

Multiplicative models, like Eq. 10.5, with concentration terms raised to various powers, introducing dependency of the parameters to other factors through empirical functions have been illustrated (Barford et al. 1992; Glacken et al. 1989). Factorial experiments (DoE) may be considered to study and model the individual and interactive effect of many variables, individually and interactively, on the process considered (Sidoli et al. 2004). These models are rudimentary but offer insight into which of the variables are important and need to be studied further. It has been argued that substrate uptake is often involved in substrate metabolism (a series of enzymatic reactions within the cell), and the value of K_s is also within the range of K_m, Michaelis-Menten constant (gL^{-1}). However, it is conceded that K_s in the Monod model is no more than an empirical parameter used to fit the average substrate influence on the cellular reactions. Thus, K_s represents the overall saturation constant for the whole growth process, i.e., all of the cellular reactions are pooled into a single reaction, which converts substrate into biomass. This is why the Monod model works so well in fitting the experimental growth kinetic data. Given the relative success of the Monod approach, several other unstructured kinetic models have been developed and proposed (Nielson et al. 2003).

Most Monod-type mathematical models (Gacken et al. 1989; Nielson et al. 1991; De Tremblay et al. 1992, 1993; Xie and Wang 1995; Dhir et al. 2000) were developed out of commercial necessity to improve the efficiency and productivity of the bioprocesses. Portner and Schafner (1996) carried out a comparison between selected unstructured hybridoma cell models rather than review them qualitatively. Quantitative assessment of the models involved error analysis. A careful comparison of the available data and models indicated that hybridoma cultures inheres not only a considerable error, either analytical or caused by metabolic changes, but also due to lack of understanding of the real metabolism (Portner and Schafner 1996). Therefore, they concluded that these unstructured models are empirical. However, these macroscopic (unstructured) models are of great importance in bioengineering for designing of on-line algorithms for process monitoring, controlling and optimisation (Portner and Schafner 1996). These unstructured, unsegregated, deterministic models disregard the inner workings of a cell (Provost and Bastin 2004) and hence provide a particular solution and not a general one (Kontoravdi et al. 2010).

When glucose is the limiting substrate (as in most fermentation processes), the value of $K_{s,}$ saturation constant is in the micro-molecular range (milligrams per litre). Therefore, its value is difficult to determine experimentally (Nielson et al. 2003). Hence, the $K_s s$ are selected from the literature (Nielson et al. 2003) and adjusted to fit the experimental data. However, a large variation has been observed in the published values of saturation constants because they (constants) do not reflect the effects of media and growth conditions (Portner and Schafner 1996; Kovarova-Kovatr 1998; Alexander 1999; Ferenci 1999). Portner and Schafner (1996) observed that in addition to analytical error, the most parameters showed a considerable range of scatter.

Therefore, Monod's Eq. 10.2 is considered to be purely empirical, with no biological basis (Roels 1983; Grady et al. 1999). However, this view is opposed by some microbiologists, who claimed that the empirical equation has some inherent deep meaning (Flickinger and Drew 1999). Nevertheless, there is a general consensus that the Monod equation can provide the most satisfactory curve "fitting" of the growth data (Kontoravdi et al. 2010). Bailey and Ollis (1986) have observed that the Monod equation is a great over-simplification, but for engineering purposes it expresses the interrelationship reasonably well, although the physical meaning of the model parameters is unknown, or perhaps does not exist. Therefore in defence of the Monod equation, Liu (2007) describes four representative theoretical approaches for deriving the Monod equation with a view towards offering deeper insight into the physical meaning of the equation. However, he concluded that Monod constants have no physical meaning or biological significance, other than regression based numerical values.

Despite this, using the published data of Chee et al. (2005), Shirsat et al. (2014) developed a Monod-hybrid growth model for CHO-IFN-γ cell line, in search of biological significance of Monod-type models. In the work of Chee et al. (2005), the cells were cultured in a 5 L bioreactor (B. Braun, Melsungen, Germany) with a working volume of 4 L culture medium (HyQ CHO MPS media from Hyclone). The cells were grown in glutamine-limiting substrate medium. Fed-batch operation

was performed using a modified online dynamic feeding strategy as described by Lee et al. (2003). Details of fed-batch and set-point control operations can be found in the published work of Chee et al. (2005). The Monod model described in the Eq. 10.5 was used by Shirsat et al. 2014 to model the batch data as a function of extracellular state variables, which were viable cell density (VCD), glucose, glutamine, lactate and ammonia. The model parameters were determined by regression and the resulting Monod-hybrid model was used to predict the growth rate μ in the fed-batch.

Shirsat et al. (2014) found that whilst the Monod-hybrid model gave a very good "goodness of fit" ($R^2 \approx 0.95$) for the specific growth rate (μ) of the batch, the fit for the fed-batch was poor ($R^2 \approx 0.57$). The culture medium for continuous fed-batch (CFB) with glutamine as a limiting substrate (0.3 mM) is specifically designed for maintenance and promotion of cell functions such as a substrate for protein synthesis, an oxidative fuel protein synthesis, inter-organ nitrogen transfer, etc. (Newholme et al. 2003). The culture grown with glutamine as the limiting substrate is prone to metabolic shift from growth to protein production (Korke et al. 2004; Kumar et al. 2007), as evident from the increase in the production of IFN-γ antibody from 3 mg/L in the batch culture to 27 mg/L in the fed-batch culture. Moreover, and more importantly, it was noticed that as the lactate concentration starts dropping (from 28 to 21 mM/L) from 152 h onward (i.e., the start of decline phase), the Monod-hybrid model for the growth rate (μ) starts deviating from the observed growth profile. However, the reduced form of the Monod-hybrid model, i.e. $(C_L/(K_L + C_L))$, for this region (152–249 h) found to be effective in bringing the estimated values of the growth rate (μ), in line with the observed ones ($R^2 \approx 0.91$) (Shirsat et al. 2014). Thus, the effectiveness of the reduced form of the model (152–249 h) suggests that the role of the lactate changes from being an inhibitor to a growth-supporting substrate (Nielson et al. 2003) in the decline phase, i.e., effectively describing the decline phase (Shirsat et al. 2014).

Mulkutla et al. (2012) carried out transcriptome (i.e., the set of all RNA molecules including mRNA, rRNA, tRNA and other non-coding RNA, similar to genome) and metabolic flux analysis on a fed-culture of mouse myeloma cell line (NSO) to unravel the factors which trigger metabolic shift. Their experimental results indicate that the shift to lactate consumption occurs upon the cessation of rapid growth and under conditions of low glycolysis flux and high extracellular concentrations. Mulkutla et al. (2012) concluded that lactate consumption in a fed-batch culture is an outcome of reduced glycolysis flux, which is a product of lactate inhibition and regulatory action of signalling pathway caused by reduced growth rate. Thus, the reduced form of the Monod-hybrid model is a mathematical description of "metabolic flux". This is a significant finding because the reduced form of the Monod-hybrid model is identical to the Michaelis-Menten enzyme kinetic equation. Furthermore, the Verhulst mechanistic growth model found to have the same goodness of fit for the fed-batch as the Monod-hybrid model, i.e., the combined effects of the Monod-hybrid saturation constants is the same as K, saturation carrying capacity in the Verhulst growth model and Monod-hybrid model pseudo-mechanistic. Therefore, the constants in the Monod-hybrid model

may not have physical meaning but they do behave as if they are of biological significance.

10.7.5 Structured Non-segregated Models

Although unstructured models may provide information on how the system properties affect the growth kinetics, they lack the level of detail necessary to understand the change in cell kinetics and/or physiology in response to change(s) in culture conditions because they (unsegregated models) do not consider intracellular processes. The accurate modelling of mammalian cell cultures is best approached by first modelling cellular function, i.e., Single-Cell model (Sidoli et al. 2004). Thus, the principle objective in SCM modelling is cellular function, which realised by using the techniques of Metabolic Flux Analysis (MFA) (Wang and Hatzimanikates 2006). The MFA involves balancing metabolites, hypothesising that the intracellular fluxes can be evaluated by measuring the extracellular fluxes. The metabolite balancing is based on stoichiometric model for the intracellular reactions and applying a mass balance around each intracellular metabolite, without any enzyme kinetic information (Caramihai and Severin 2013). The general defining relationship is the matrix form:

$$Sv = r$$

where S = stoichiometric matrix of the metabolic network, v = vector of unknown fluxes and r = vector of measured metabolic extracellular concentrations, whereas the metabolite intracellular concentrations is zero (Caramihai and Severin 2013).

Structured unsegregated models, collectively known as SCMs, offer the advantage of explicitly accounting for intracellular phenomenon such as cell cycle changes, alterations in cell size and shape; this feature is particularly important during transient or unbalanced growth conditions (Dorka 2007). In addition, cases where the rate of transport between organelles is potentially rate-limiting, SCMs explicitly consider intracellular spatial organisation within the cell. Under the SCM approach, a cell is divided into compartments on the basis of difference in composition/concentration. However, it is assumed that each compartment is a lumped phase, i.e., concentrations are constant throughout the compartment (Sidoli et al. 2004).

In order to describe these biochemical processes, the same basic engineering formulations are utilized, as discussed previously under unstructured non-segregated models, namely mass balance, yield and rate equations (Dorka 2007). Cellular metabolism can be incorporated into an SCM model by defining terms like (i) transport, (ii) primary metabolism, (iii) product synthesis, and (iv) cell growth and death with respect to the system (Sidoli et al. 2004). Transport processes include transport kinetics of biochemical species across membranes, including the compartmentalisation of the cell into distinct regions through which the transported

species flow (Sidoli et al. 2004). In addition to transport, primary metabolism and product synthesis relates conceptually to two or more linked compartments within which either particular metabolic pathways and/or entire metabolic cycles operate. Conservation equations written for each compartment and for each biochemical species, result in a set of ordinary differential equations (ODEs). Mass balance of each of the species (reactant) in each compartment is expressed as a set of ordinary differential equation (ODE). These ODEs are determined by reaction and transport kinetics. Therefore, the SCM approach is considered to be deterministic. Equation 10.7 illustrates the generic form of these conservation equations (Sidoli et al. 2004). The accumulation of any given component i depends on its flow into or out of the compartment j as a result of transport across the compartment barrier (first and second terms on the RHS of Eq. 10.6), generating intra-compartmental reactions and consuming the component (third and fourth terms on the RHS) and its dilution due to growth of the cell and thus increasing compartment size (last term on the RHS) (Dorka 2007):

$$\underset{\text{Accumulation}}{\frac{dC_{ij}}{dt}} = \underbrace{\left[\sum_{k=1}^{N_{in}} R_{ijk} - \sum_{k=1}^{N_{out}} R_{ijk}\right]}_{\text{Reaction}} + \underbrace{\left[\sum_{I=1}^{Ngen} r_{ijk} - \sum_{I=1}^{N_{con}}\right]}_{\text{Transport}} - \underbrace{\mu C_{ij}, \forall i, j}_{\text{Dilution}} \quad (10.6)$$

where C_{ij} is the concentration of the ith component in the jth compartment, R_{ijk} represents the transport rate of species i into or out of compartment j from the k_{th} source or sink compartment; N_{in} is number of source compartments; N_{out} is number of sink compartments; r_{ijl} – reaction rate of component i in compartment j in the l_{th} i-generating or i-consuming reaction; N_{gen} – number of reactions generating component i; N_{con} – number of reactions, consuming component i; (adapted from Sidoli et al. 2004).

Figure 10.5 represents compartmentalisation of the cell in the physiological context with cellular structures, such as organelles, pooled biochemical components or the cell environment being treated as compartments. However a problem with multi-compartment models is that the ODEs in Eq. 10.6 that represent distinct pooled concentrations do not give any idea of spatial distribution of concentration

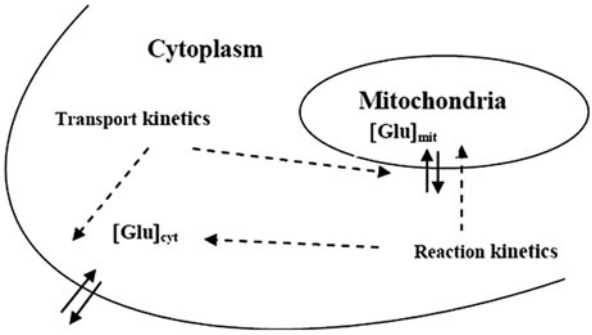

Fig. 10.5 Single cell compartmentalisation (Adapted from Sidoli et al. 2004)

gradients along the surface of the compartment but rather at a single point and do not represent physical dimensions or cell geometry. To overcome this drawback, instead of assuming constant concentration throughout a compartment as per the lumped phase assumption, a concession is made allowing concentration gradients to exist in some compartments. Hence, the resulting conservation equations will then include partial derivatives and additional diffusion equations that are required to compute the diffusive fluxes within the spatial geometry of each compartment. However, the addition of these terms and equations makes the model mathematically and computationally more complex (Sidoli et al. 2004). There are two ways in which an SCM can be applied, via a kinetic approach or via a stoichiometric approach.

10.7.5.1 Kinetic Approach

There are numerous reactions occurring within the cell at any time and further within the compartments themselves. Kinetic models follow a deterministic approach where one can account for intracellular dynamics by rate and transport mechanisms that give rise to a set of differential algebraic equations (DAEs), which are then integrated over the domain of interest, generally the culture time and results in well-defined time trajectories for all model variables (Gombert and Nielson 2000). The most commonly used form of expression to describe the reaction rates is the Michaelis–Menten equation. However, one must keep in mind that incorporating a greater level of detail into the system means that the more complex the reaction kinetics will be and the greater the number of parameters required as in the SCM. It is important not to over-parameterise the model as this will result in parameter-estimation problematic due to measurement noise and make the model very time consuming to solve. An additional drawback, in incorporating greater details in terms of complex dynamic expressions, is that it normally results in non-linearity in both parameters and variables which makes the models essentially intractable. In addition, kinetic models are unable to incorporate regulation and control of cellular activity, which requires dynamic simulation (Sidoli et al. 2004).

10.7.5.2 Stoichiometric Approach

Stoichiometric modelling represents an alternative to the kinetic approach. Such a model yields a static instance of metabolic activity and is represented by a system of flux balance equations based on reaction stoichiometry of a metabolic network with accompanying constraints on flux values. The resulting equations are solved as a constrained optimisation problem using some assumed objective; there exists a mathematical and therefore physiological feasible region in which a range of possible mathematical solutions or phenotypes are acceptable though not optimal (Gombert and Nielson 2000). The key advantage of stoichiometric models is that

they can account for competing reactions, which enables us to study the relative activity of certain pathways under various culture conditions. For example, the existence of more than one physiological steady state, known as steady-state multiplicity, has been observed by a number of investigators using stoichiometric models and metabolic flux analysis (Follstad et al. 1999; Europa et al. 2000; Cruz et al. 1999; Zhou et al. 1997; Linz et al. 1997; Paredes et al. 1999). Generally, most existing SCMs are only partially mechanistic and contain empirical mathematical functions that best seem to describe the observed phenomenon. This is due to the fact that many cellular processes that these models seek to describe have not yet been accurately described. The development of SCMs requires the incorporation and integration of either parts or the entirety of other smaller models each of which describe specific sub-processes. Utilising the most accurate sub-models that correspond to the components of cellular metabolism is, therefore, of key importance to the construction of a good SCM. On the whole, the SCM approach is a very effective tool to relate hypotheses about molecular level mechanisms to the whole cell and population response to changes in the local environment (Sidoli et al. 2004).

10.7.5.3 Single Cell Models (SCMs)

Sanderson et al. (1995) developed an SMC growth model for hybridoma culture. The model considers some 50 components in three distinct regions (the medium, the cytoplasm and mitochondria). It also includes consideration of metabolic features of glycolysis, glutaminolysis, the TCA cycle, the formation and utilisation of fatty and amino acids, competitive transmembrane transport, product formation and, cell growth and death. Sidoli et al. (2006) developed a coupled single cell (SCM) with population-balance (PBM) model for mammalian cell cultures. Their model captures increasing heterogeneity of the cells as in PBM and combine with intracellular metabolic flux. The model is highly parameterised with $>$700 parameters. However, the application of a systematic estimation procedure reduced the set of 700 to a manageable set, i.e., 14 parameters were found to be very estimable under batch and fed-batch modes. Model does not lend itself to traditional optimisation because of its complexity and therefore, an alternate practical solution was adopted for parameter estimation (Sidoli et al. 2006).

The kinetic form and parameter values were identified by an extensive series of factorial experiments and overall model was sufficiently simple for an analytical solution to be determined (Sanderson et al. 1999). Potentially the SMC models could be useful for analysis and prediction of experimental results (Chotteau and Bastin 1992), the optimisation of culture conditions (Glacken et al. 1988; Sanderson 1997; Bradford et al. 1992), and the investigation of fundamental metabolic processes (Bibila and Flickinger 1991a, b; 1992a, b). Sandrson et al. (1999) growth model consists of 90 ordinary differential equations (ODEs), 109 algebraic equations (AEs) and some 390 model parameters. However, overall model of Sanderson et al. (1999) includes 50 parameters, 90 ODEs and

130 AEs. On the other hand SMC model of Sidoli et al. (2006) comprises of 747 parameters of which only 263 are estimable.

10.7.5.4 Combination of Single Cell Models (SCMs) and Population Balance Models (PBMs)

All segregated (or corpuscular) models can be collectively grouped under the category of population balance models (PBMs). PBMs have been available since the 1960s and are the most mathematically concise way of elucidating the property variation of cells within a population and recognize the individuality of cells within a population. PBMs are further classified into (Mantzaris et al. 2001):

(i) Single- or Multi-variable,
(ii) Single- or Multi-staged, and
(iii) Mass or Age-structured

A single-variable model differentiates on the basis of one cell property which is generally cell mass. Whereas a multi-variable model uses more than one cellular characteristic to distinguish between cells and can be used to account for any number of biochemical constituents within the cell. Using a multi-stage model, it is possible to describe multiple developmental phases in the population with each stage of growth added representing each additional phase considered. Finally, if population differentiation is done on the basis of mass conservation laws, then the model is mass structured, whereas if cell age/maturity is used, the model is said to be age structured (Sidoli et al. 2004).

Three parameters are required in a generalized PBM, each having physiological significance. They are: (i) the single cell growth rates, (ii) the transition rates between each of the cell cycle phases, and (iii) the partition function. Together, these three process parameters define the collective state of the cell population (Sidoli et al. 2004; Tziampazis and Sambanis 1994). In essence, PBMs are a number balance on a cell population. Unfortunately, they are also particularly intractable because they are partial integral-differential equations and the accurate determination of model parameters imposes a more limiting restriction. Hence, in general, numerical methods must be used for their solution (Villadsen 1999). The SMC models examine in details an individual cell processes and reactions, in order to obtain more realistic behaviour of the cell, particularly physiology as reflected in cell metabolism. The SMC approach of the full metabolic network, involving a separate state variable for each intracellular species and a separate kinetic model for each intracellular reaction is commendable. However, it is unworkable in practice because the intracellular kinetics are, in general, not structurally identifiable without intracellular measurements in a single cell of dimension less than 1 μm. Therefore, Provost and Bastin (2004) a “reduced modelling” approach where the model is based on a set of macro-reactions which are compatible with the underlying metabolic network and supported by a preliminary metabolic flux. They develop a “reduce” SMC-type dynamic growth model, compatible with the

underlying metabolic network for CHO cells cultivated in stirred flask in a serum-free medium.

Kontoravdi et al. (2007) developed an unstructured mathematical model to describe cell growth and cell metabolism based on the underlying mechanism of amino acid metabolism, very similar to PBM-SCM approach. The network of amino-acid metabolism is based on existing biological knowledge as well as observed cellular behaviour. They found their model in good agreement with experimental data. Kontravdi et al. (2007) are of the opinion that their model can accommodate the inclusion of expressions for other cellular activities, such as production of recombinant viral vectors or proteins and therefore, it can be used as the basis for developing a model library for mammalian cell cultures.

10.7.6 Stochastic Models

Stochastic models use probability functions to describe process dynamics at the cell and population levels. A statistical approach is often required because of uncertainties surrounding some process variables. According to some, this is the only true measure of process uncertainty (compared with fuzzy reasoning). Probabilistic models are characterised by probability density functions of the process variables involved, with normal distribution being the most commonly used. Correlation models quantify the degree of similarity between two variables by monitoring their variations. System dynamics are not captured by statistical methods per se, but they play an important role in data mining and analysis, data compression, principal component analysis (PCA), and statistical process control (SPC). Since they account for randomness, they are more accurate than deterministic models (Tziampazis and Sambanis 1994). Thus, linear and non-linear regression methods are widely used in biological systems, particularly because of their complex nature involving several independent, dependent and interdependent variables. For example, the statistical methods have been used for monitoring of bioprocesses (Albert and Kinley 2001), the optimisation of the bioprocesses (Prajneshu 1998; Ergun and Mutlu 2000; de Alwis et al. 2007; Gao et al. 2007; Teng and Samyudia 2011), for the modelling productivity (Prajneshu 1998; Ergun and Mutlu 2000) and even for estimation of parameters in Monod model (Knights and Peters 1999). Horiuchi et al. (1993) developed the Data Base Simulation (DBS) method for simulating various time courses of batch and fed-batch cultures. The DBS method involves developing multiple regression models based on the culture data for simulation purpose. Horiuchi et al. (1995) also developed a hybrid simulation system for fermentation processes, combining a statistical procedure and fuzzy interface.

Structured and unstructured models based on biochemical data are non-corpuscular and therefore, they are unable to describe basic properties of eukaryotic cell cultures (Srienc 1999). For example, these models fail to predict the observed correlation between the quality of batches of baker's yeast and ratio of budded and unbudded cells (Dairaku 1985; Takamtsu et al. 1985), differences in

metabolism observed for cells in the budded and unbudded phases of growth (Kuenzi and Fiechter 1969), and the spontaneous synchronisation in yeast cultures grown at low dilution rates in a chemostat (Cazzador 1991). The corpuscular models are needed to understand these changes (Fredrickson et al. 1967; Fredrickson 1991; Nielsen and Viladsen 1992) because they take into account the effects of cell cycle and the environment on the behaviour of cell population (Srienc 1999). Therefore, these corpuscular models, based on mammalian cell cycle phases, are better indicators of cellular activities, physiology, morphology and even mechanical properties (Mitchison 1971; Needham et al. 1990; Ramirez and Mutharasan 1990; Henderson et al. 1992). Thus, the behaviour eukaryotic cells can only be understood through the physiological changes as reflected in cell cycle phases and organelles because the differences in the cytometric data of the batch and fed-batch can be attributed to the chemical and physical environment.

Flow-cytometric measurements provide information on the concentration of certain markers in cells with a high resolution in time and, if measured, also in space. This is ideal for the development of differential equation models which depend on high-quality time-series data. Therefore, Shirsat et al. (2013) used the cytometric data to develop unsegregated, structured, stochastic models for the batch and fed-batch cultures of CHOSVK1, describing, qualitatively and quantitatively, the growth and productivity in relation to the cell cycle phases and organelles (Fig. 10.6). The cytometric data consisted of cell-cycle phases, MMP/MM ratio (mitochondria), endoplasmic reticulum (ER) and golgi apparatus (GA). Fredrickson et al. (1967) developed a statistical framework (i.e., statistical model) for characterising the dynamic behaviour of the cell population based on a vector

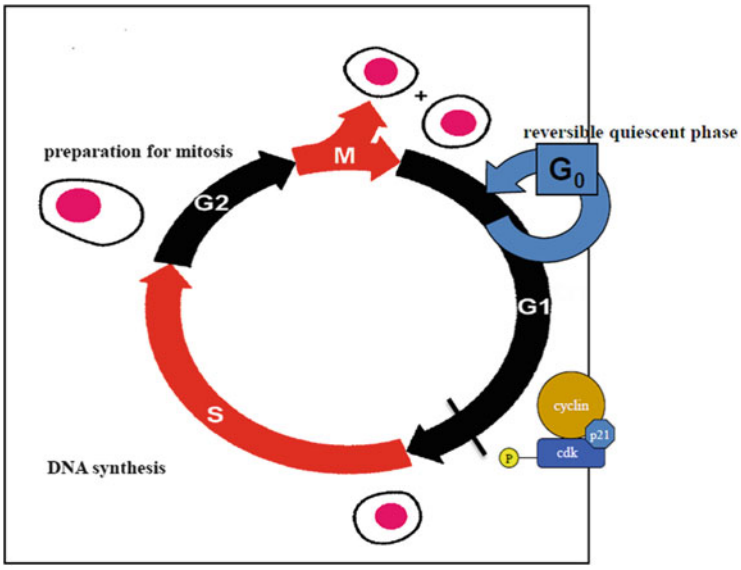

Fig. 10.6 Mammalian cell cycle

description of the physiological state of the cell. Statistical modelling is about relating stochastic or deterministic models to experimental data (Abu-Absi et al. 2003), and, as such, it is of key importance in system biology for uncovering relationships between the categorical variables and a given response variable.

Most biological systems are too complex to allow the relationship between the independent and dependent variables to be ascertained by experimental methods alone. Furthermore, the task is compounded where there is interdependency between the so called independent variables themselves. To address this, Shirsat et al. (2013) developed statistical methodology (cf. Fig. 10.3) to identify qualitative and quantitative aspects of the regulatory mechanisms underlying proliferation and recombinant protein production in cell culture. The regression analysis is best suited for uncovering the true functional relationship between the categorical variables and a response variable (Gold 1977).

Statistical modelling, like mathematical modelling, starts with a priori knowledge or experimental or theoretically based hypothesis. The literature survey indicated that cell cycle phases and organelles (mitochondria, ER and GA) are physiologically involved in the growth and productivity (Shisat et al. 2013). Therefore, Shirsat et al. (2013) constructed a general research hypothesis: "Cellular parameters measured by flow cytometry provide early indicators of the cell proliferation and productivity, thereby allowing sufficient time to manipulate the environmental conditions in order to enhance productivity" and they tested the hypothesis using the statistical methods. They extended statistical methods, used (Prajneshu 1998; Ergun and Mutlu 2000; de Alwis et al. 2007; Teng and Samyudia 2011) and for the modelling aphid production (Prajneshu 1998), are extended to the cytometric data to investigate the proposed hypotheses regards the underlying mechanisms for growth and productivity.

In all of this, Shirsat et al. (2013) developed correlative models for the growth and productivity using cytometric data. They used multiple linear regression method to develop multivariate correlative models of the type Eq. 10.7 because there are several categorical variables with the probability of being correlated to the response variables of cell number and MAb titre:

$$y_i = b_0 + b_1 x_{1i} + b x_{2i} + b_3 x_{3i} + \ldots\ldots\ldots\ldots\ldots b_p x_{pi} + u_i \qquad (10.7)$$

where b_0, b_1, b_2, $b_3 \ldots b_p$ are constants referred to as the model partial or simply as regression coefficients and u_i is a random disturbance. It is assumed that for any set of fixed values of x_1, x_2, x_3, $\ldots\ldots\ldots$ x_p that fall within the range of the data, the linear Eq. 10.1 provides an acceptable approximation of the true linear relationship between y and x, and u_i is the discrepancy in that approximation.

The regression coefficients b_i may be interpreted as increments in y corresponding to a unit increase in x_i when all other variables are held constant and, therefore, this interpretation holds independently of the actual units of x_i. The coefficients b_i are estimated by minimising the sum of squared residual, which involves minimising

$$S\left(b_0+b_1+b_2+b_3+.....+b_p\right)=\sum_{i=1}^{n}u_i$$

$$=\sum_{i=1}^{n}\left(y_i-b_0-b_1x_{1i}-b_2x_{2i}-b_{3i}-.....-b_px_{pi}\right) \tag{10.8}$$

$$b_0=\overline{y}-b_1\overline{x}_1-b_2\overline{x}_2-b_3\overline{x}_3-.....-b_p\overline{x}_p \tag{10.9}$$

where b_0 is referred to as intercept and bi as the estimate of the partial regression coefficient x_i. The coefficients of xi represent the contribution of an individual categorical variable to the response variable y. The above system of equations has a unique solution. When there are several competing independent variables involved in relation to a given dependent variable, stepwise multivariate regression method (Draper and Smith 1998) is used with a view to developing the simplest correlative model, i.e., finding the least number of categorical variables that can give the fullest description of the response variable (Chatterjee and Price 1991). The choice of state variables is based on the maximum R^2 value (Eqs. 10.10 and 10.11) for the least of number of categorical variables correlated to response variables.

A measure of "fit" of a model to the experimental data is defined as the coefficient of determination:

$$R^2=1-\frac{\sum_{i=1}^{n}\varepsilon^2}{\sum\left(y_i-\overline{y}\right)^2} \tag{10.10}$$

$$R^2=1-\frac{\sum_{i=1}^{n}\left(y_i-\hat{y}\right)^2}{\sum\left(y_i-\overline{y}\right)^2} \tag{10.11}$$

where R^2 = Measure of fit, y_i = An observation, $\hat{y}$ = Estimate of an observation, $\hat{y}$ = Average of all observations, ε = Error between observed and estimated.

Correlative models are of limited importance unless they are "statistically significant" (i.e., 'explanatory' models), explaining a possible functional relationship of the categorical variables with a given response variable (cf. Fig. 10.1). Therefore, Shirsat et al. (2013) developed explanatory models by subjecting the correlative models to three tests: the multi-collinearity diagnostic tests (Myers 1990), Durbin-Watson test (Chatterjee and Price 1991) and Houston test (Springate 2011). If in the correlative model, the categorical variables are collinear (>0.8–0.9 for correlation coefficient), then one of the fundamental basis of the regression analysis, i.e., "b_i represents increment in y per unit increase in x_i", is put at risk (Farrar and Glauber 1967). Therefore, multi-collinearity tests are *sine qua non*, i.e., essential for elimination of one of the two mutually collinear variables from the correlative model. Durbin-Watson (D-W) statistics for autocorrelation of residuals,

specifically lag-1 autocorrelation, can also used for hypothesis testing (Chatterjee and Price 1991). D-W statistics test for null hypothesis H_0 is that there is no first-order autocorrelation against the alternative hypothesis, H_1, that there is positive or negative first-order autocorrelation.

In the case of correlative models when the sample size is small, its effect on bias (systematic error) could be significant and hence sample size adjustment is essential. It has been found that with a sample size less than 25–30, the measure of 'true' error is unreliable. The two methods, for determining true errors, are: Dahlberg (1940) and Houston's "methods of moment" (1983). The Houston test has been found to be more effective because the sample size in the present work is less than 10 (Springate 2011). The results of explanatory model indicated that the underlying mechanisms of the growth and productivity of batch and fed-batch cultures (CHOSV1) can be explained by the (G1 + ER) and (G2 + ER) models respectively.

Shirsat et al. (2013) found that the increased number of cells in the G2 phase of the fed-batch culture is an indicator of the growth rate and the rate of cell division, whilst the increased number of cells in the G1 phase of the batch is associated with growth arrest and the decline of proliferative activity as a consequence of the decreasing amount of nutrients and increasing level of toxic by-products, which result in harmful conditions for the cells. Since the G2 phase is a period of rapid cell growth and protein synthesis which leads to cell division, all organelles including ER and Golgi have to be duplicated in mass and number to ensure their correct segregation between the two daughter cells.

The arrest of the cells in the G1 phase has a significant link with productivity. Protein production is dependent on the phase of the cell cycle and several genes such as those involved in ribosome genesis and protein translation are expressed highly in the G1 phase (Al-Rubeai and Emery 1990; Al-Rubeai et al. 1992; Moore et al. 1997; Yoon et al. 2003a, b; Fussengger et al. 1998, 2000; Fussengger 2001; Kaufman et al. 1999, 2001; Carvalhal et al. 2003; Ibarra et al. 2003; Fogolin et al. 2004; Bi et al. 2004; Trummer et al. 2006). Cells arrested in at the end of G1 phase cell cycle are metabolically more active and bigger in size than non-arrested cells (Catvalhal et al. 2003; Bi et al. 2004). Therefore, the arrest of cells in G1 phase is used in order to increase the productivity of recombinant proteins in number of commercial cell lines such as hybridomas and CHO (Al-Rubeai and Emery 1990; Al-Rubeai et al. 1992; Moore et al. 1997; Yoon et al. 2003a, b; Fussengger et al. 1998, 2000; Ibarra et al. 2003; Fogolin et al. 2004; Trummer et al. 2006). Thus, these statistical models capture the behaviour of the cells in response to their chemical and physical environment. Therefore, to improve the productivity, several techniques are employed to arrest the cells in G1 phase, e.g., (i) temperature shift to condition of mild hypothermia; (ii) cell-engineering-based approaches and (iii) chemical approaches all of which are discussed further by Kumar et al. 2007.

Shirsat et al. (2013) found that the number of cells in the G2 phase is an indicator of the growth rate and the rate of cell division, while the number of cells in the G1

phase is associated with growth arrest and the decline of proliferative activity as a consequence of the decreasing amount of nutrients and increasing level of toxic by-products, which result in harmful conditions for the cells. Since the G2 phase is a period of rapid cell growth and protein synthesis which leads to cell division, all organelles including ER and Golgi have to be duplicated in mass and number to ensure their correct segregation between the two daughter cells. A positive cellular response to the feeding strategy was manifested in terms of the increase in the mitochondrial activity and mass, and ER and GA contents. The increase in the mitochondrial activity in the fed-batch culture is consistent with the fact that protein production is an extremely energy-intensive process. Also the increases in the ER and GA contents of the fed-batch are consistent with a higher MAb productivity which requires a large amount of rough ER to fold the protein and many GA to prepare and package the protein secretion. Thus, the results of the study of Shirsat et al. (2013) indicate that the increase in the MAb concentration is not only due to the increased biomass but also due to increased cellular activity. Also, Shirsat et al. (2013) validated the statistical models against new data. Thus, linear multivariate regression analyses of cytometric data enable the development of predictive methodology of growth and productivity in the cell culture. G1 + ER and G2 + ER for the batch and fed-batch respectively, are of a biological significance because they help in explaining on the basis of experimental evidence the underlying mechanisms of the growth and productivity.

In general, feeding cells at periodic intervals can enhance the ER content and thus the expression of protein. Also, it enhances the ER protein folding capacity, which helps overcome the stress and foster survival, thus extending the exponential phase. Higher protein synthesis would lead to higher growth and MAb productivity. This cellular aspect is substantiated by the fact that the ER fluorescent intensity of cells grown in the fed-batch mode increased (24–176 h), whereas in the batch culture, it decreased from during the same period. Also, the higher productivity of the MAb is attributed to a longer (33 %) exponential growth phase in the fed-batch than the batch because most of the expression of protein takes place in the exponential phase (Al-Rubeai and Emery 1996; Mochida et al. 2000).

MAb productivity is at its peak during exponential phase (Lioyd 1999, 2002) but Agrawal et al. (1989) found that MAb productivity depends on the longevity of the cell life whereas Hayter found that the specific productivity (MAb/cell) is high in the decline phase. Kumar et al. (2007), Korke et al. (2004) found that arresting the cells in G1 phase (low temperature and addition of nutrients; low glucose and glutamine) can increase the cell life whereas (Coco-Martin and Harmsen 2008) suggested that cells should be grown in a large quantity and then use of cycline dependent kinase to slow down the growth. It is most likely that the higher productivity in the fed-batch cultures compare to the batch could be because of the arrest of the cells in G1 phase, resulting into metabolic shift from the cell proliferation to the productivity as well as the longevity of the cells.

10.7.7 Logistic Models

Most predictive models are shown to be based on variations of the Verhulst logistic growth models. Tsoularis and Wallace (2002) reviewed and compared several logistic models and analysed their properties of interest. They defined a generalised logistic function:

$$\frac{dN}{dt} = rN^{\alpha}\left[1 - \left(\frac{N}{K}\right)^{\beta}\right]^{\gamma} \tag{10.12}$$

where α, β, and γ are positive real numbers,

The first type of model, a mathematical one, is based on the Verhulst and Gompertz functions (Verhulst 1847; Gompertz 1825), which are extensions of the Malthusian (Malthus 1798) population model. The logistic function (Verhulst 1838; Robertson 1908) is one of the most classic growth models.

The simplest realistic model of population growth is the exponential growth model of Malthusian:

$$\frac{dN}{dt} = rN \tag{10.13a}$$

Solution of Eq. 10.13a gives:

$$N(t) = N_0 e^{rt} \tag{10.13b}$$

where N_0 and N_t population density at time $t = 0$ and $t = t$ respectively and r intrinsic growth rate. Verhulst (1847) developed a stable population model with a saturation level characteristic by introducing a multiplicative factor, $1 - (N/K)$, which represents the fractional deficiency of the current from the saturation level, K:

$$\frac{dN}{dt} = rN\left[1 - \frac{N}{K}\right] \tag{10.14}$$

The solution of Eq. 10.14 is referred to Verhulst-Pearl equation because Verhulst was the first introduced the logistic curve but Pearl (1920) was the first to use it to describe the United States population.

The solution of Eq. 10.14 is:

$$N(t) = \frac{KN_0}{(K - N_0)e^{-rt} + N_0} \tag{10.15}$$

The major advantages of this logistic function are its mathematical simplicity and biological interpretability (Wan et al. 2000). Therefore, the Verhulst logistic model

Table 10.1 The expressions of some commonly applied growth models. Parameters s and f denote the initial and upper asymptotic size respectively, while k is the growth coefficient. Other parameters have different biological interpretation

Model	Expression	References
Spillman	$W = f - (f - s) \exp(-kt)$	Spillman and Lang (1924)
Gompertz	$W = f \exp(\ln(s/f) \exp(-kt))$	Gompertz (1825)
Bertalaniffy	$W = (f^{1/3} - (f^{1/3} - s^{1/3}) \cdot \exp(-kt))^3$	Bertalaniffy (1957), Gille and Salomon (1995)
Logistic	$W = s \cdot f/\{s + (f - s) \exp(-kt)\}$	Verhulst (1838), Robertson (1908)
Richards	$W = s \cdot f/\{s^n + (f^n - s^n)\exp(kt)^{1/n}$	Richards (1959)
Janoscek	$W = f - (f - s) \cdot \exp(kt^p)$	Gille and Salomon (1995)

has played a central role in many aspects of theoretical and applied physiology (Krebs 1996).

Many other growth equations have been used in biological fields to describe a mathematical summary of time-course data on growth of organisms, organs or tissues (cf. Table 10.1).

However, the Verhulst model is asymptotic, describing only the exponential phase of growth (Jolicoeur and Pontier 1989; Goudar et al. 2005). In the 1930s and 1940s, Monod performed experiments on bacteria, fed on a single limiting-nutrient in order to see if the logistic equation could describe growth. He found it cannot describe the microbial growth (Parolini 2010). Incidentally, it is worth noting that the extrapolation of the logistic equation fails to fit somatic data satisfactorily in some cases (Ricklefs 1968; Cui and Lawson 1982a; Jolicoeur and Pontier 1992; Jolicoeur et al. 1992; Wan et al. 1998b). Extension of the models developed for microbial cell growth to animal cells should be treated with caution because the mammalian cells are a lot more advanced and complex (Tziampazis and Sambanis 1994). In reality, the specific growth rate $(1/N)\ dN/dt$ and N (population) is not necessarily linear (Cui and Lawson 1982a, b) and, therefore, in some cases, the logistic equation fails to fit somatic data satisfactorily (Ricklefs 1968; Cui and Lawson 1982a; Jolicoeur and Pontier 1992; Jolicoeur et al. 1992; Wan et al. 1998b). Therefore, to fit a growth-data set, many attempts have been made to construct a generalised form of the ordinary three- and four-parameter logistic equation (Richards 1959; Cui and Lawson 1982a; Jolicoeur and Pontier 1989; Jolicoeur et al. 1992; Jolicoeur 1999; Wan et al. 1998a). Notably amongst these is a four-parameter generalised logistic equation (GLE) developed by Jolicoeur and Pontier (1989) to describe exponential and decline phases of population of *Paramecium caudatum*:

$$X = X(t) = 1/[C_1 \exp(t/D_1) + C_2 \exp(-t/D_2)] \tag{10.16}$$

where t = time, X = expected population size at time t, D_1 = a time-scale parameter related to population decrease, D_2 = a time-scale parameter related to population increase, and C_1 and C_2 are two parameters which simultaneously control the relative rates of increase and decrease as well as curve height (population size). The four parameters C_1, D_1, C_2 and D_2 are all non-negative. Model Eq. 10.17 is

Table 10.2 Limiting forms of the model (16)

Condition	Equation	Model
$C_1 = 0$	$X = (1/C_2)\exp(t/D_2)$	Exponential growth
$D_1 \to +\infty$	$X = 1/[C_1 + C_2\exp(-t/D_2)]$	Logistic growth
$C_2 = 0$	$X = /(1/C_1)\exp(-t/D_1)$	Exponential decrease
$D_2 \to +\infty$	$X = 1/[C_1\exp(t/D_1) + C_2]$	Logistic decrease

closely related both to exponential growth and decrease and to logistic growth and decrease (cf. Table 10.1) for particular values (cf. Table 10.2) of its parameters ($C_1 = 0$, $D_1 \to +\infty$, $C_2 = 0$, $D_2 \to +\infty$).

Jolicoeur and Pontier (1989) conceded that their model (Eq. 10.16) in relation to the logistic curve model (Eq. 10.14) might be considered as more descriptive and less explicative (theoretical), i.e., the model (Eq. 10.16) is empirical. Since logarithmically-transformed data frequently do not have a constant variance, the model was fitted through a multiplicative-reweighted least-squares method. The parameters were estimated using the Jolicoeur and Heusner (1986) method.

Prajneshu (1998) developed the dynamical model for aphid population growth:

$$N(t) = ae^{-bt}\left(1 - de^{-bt}\right)^{-2} \tag{10.17}$$

where a, b and d are given by

$$\begin{aligned} a &= 2(r^2C + 2N_0)(b + r)(b - r)^{-1} \\ b &= (r^2 + 2N_0C - 1)^{1/2} \\ d &= (b + r)(b - r)^{-1} \end{aligned}$$

In the equation,

$$\frac{dN}{dt} = rN - \frac{N}{C}\int_0^t N(s)ds \tag{10.18}$$

the values of the original parameters r, C and initial population size population are given by

$$\begin{aligned} C &= a\left(2b^2d\right)^{-1} \\ N_0 &= a(1 = d)^{-2} \\ r &= \left(b^2 - 2N_0C - 1\right)^{-1/2} \end{aligned}$$

The statistical form of the model Eq. 10.17 with an error term added to the R.H.S was used for fitting the data:

$$N(t) = ae^{-bt}\left(1 - de^{-bt}\right)^{-1} + \varepsilon \tag{10.19}$$

The model parameters were determined by using Levenberg-Marquardt method for non-linear least-squares estimation.

The theoretical assumptions of the logistic equations, which are widely used in ecological studies, are too simple and open to much criticism (Smith 1974; Cui and Lawson 1982a) because the relationship between the relative growth rate (dW/dt) (1/W) and size W is not necessarily linear (Cui and Lawson 1982a, b). Therefore, Wan et al. (2000) developed a new generalised logistic model for mammalian somatic growth:

$$W = f - \frac{1}{\frac{b}{f} + \left(\frac{1}{f-s} - \frac{b}{f}\right)e^{kt}} \tag{10.20}$$

Substituting $t = 0$ in Eq. 10.20 yields $W = s$ and setting $t \to \infty$ gives $W = f$. Thus, parameter s and f are defined as the birth size and mature size respectively. All of the parameters were determined using the least-squares method (Jolicoeur 1999). The RSS (residual sum of the squares) values were used to evaluate fit (R^2, percent variation in estimated and observed values) of the models; the smaller a RSS value is, the better a model fit (R^2) to a data set.

The performance of the new model was compared with logistic, Gompertz, Bertalanffy, Spillman, Richards and Jonschek using 12 sets of somatic growth data. The results indicated that the Richards, Janoschek and the new model usually yielded lower values of RSS and higher values of R^2 than the logistic, Spillman, Bertalanffy and Gompertz models. Goudar et al. (2005) developed a four-parameter logistic equation to fit the batch and fed-batch time-course viable cell density profile of CHO cultures to estimate net growth rates from inoculation to the cell death phase. They also developed a reduced three-parameter form for nutrient uptake and metabolite/product formation. These logistic models constrained the fits to expected general trends, either increasing followed by decreasing (four-parameter) or monotonic (three-parameter). Goudar et al. (2005) developed a four-parameter logistic model because the standard logistic model (Eqs. 10.13a and 10.13b) cannot describe exponential and decline phases of mammalian cell population in the batch and fed-batch cultures.

Goudar et al. (2005) proposed a four-parameter generalised logistic growth equation for CHO cells:

$$X = \frac{A}{e^{Bt} + Ce^{-Dt}} \tag{10.21}$$

where X = viable cell density and A, B, C and D are non-negative model parameters (Jolicoeur and Pontier 1989). The contribution of exp(Bt) is minimal in the growth phase and when set to zero, Eq. 10.21 reduces to an exponential growth equation, A/Cexp(−Dt), with D as the specific growth rate. Similarly, neglecting

the contribution of Cexp(−Dt) during the decline phase of the cell, Eq. 10.21 reduces to A/exp(Bt) with B as the death rate. Thus, the parameters D and B represent the maximum growth rate μ_{max} and the maximum death rate k_{dmax}. Therefore, Eq. 10.21 can be rewritten as:

$$X = \frac{A}{e^{(k_{d\max} t)} + Ce^{(\mu_{\max} t)}} \tag{10.22}$$

The initial cell density, X_0, can be expressed as:

$$X_0 = A/(1 + C)$$

Equation 10.21 can also describe the successive ascending and descending of lactate concentration often observed in the fed-batch cultures (Goudar et al. 2005).

Goudar et al. (2005) estimated the parameter in Eq. 10.21 by minimising the sum-of-squares error (SSE) between experimental observations and model-predicted values. Equation 10.21, involving a sum of exponentials, can be inherently unstable. Therefore, Goudar et al. (2005) used three different algorithms for non-linear parameter estimation: Levenberg-Marquardt method (1965), the simplex approach (Nelder and Mead 1965) and the generalised reduced gradient method (Lasdon and Smith 1992). GLE models for the batch and fed-batch case gave very good correlation between experimental viable cell densities and model predictions. Goudar et al. (2005) claimed that the logistic equations for the time profiles of growth, nutrient and metabolites provide valuable information on cellular physiology. However, Hu and Peshwa (1991) are of the view that the kinetics rate of state variables cannot determine the physiological state of the culture because of the intrinsic complexity of animal cells.

The generalised logistic growth models (Eqs. 10.16, 10.20 and 10.21) are empirical, providing little insight into the mechanisms of cell growth. Therefore, Shirsat et al. (2014) revisited the Verhulst function with a completely different perspective. The Verhulst-modelling approach is simplistic, yet effective and mechanistic in nature (Wan et al. 2000). Shirsat et al. (2014) have described the derivation of the Verhulst growth model (Eqs. 10.23 and 10.24) from first principles:

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) \tag{10.23}$$

where N = cell densities/ml, K, carrying capacity or equilibrium (max) growth = cell density/ml and r (h^{-1}).

The Verhulst model constants, *K*, carrying capacity (or overall saturation constant), and *r,* intrinsic growth rate, are determined by plotting the experimental data, ln *N(t)* versus *(1/N) dN/dt*; the intercept on the ordinate axis gives the value of *r* (h^{-1}) (specific growth rate, μ) and the one on the abscissa represents the value of

K (ln VCD/unit volume) (Shirsat et al. 2014). Solving by separating variables yields:

$$N(t) = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e^{-rt}} \tag{10.24}$$

Equation 10.24 is called the Verhulst logistic equation, describing the exponential growth rate with defined limitations. Equation 10.24 is asymptotic to the carrying capacity, i.e., as $t \to \infty$, $N(t) \approx K$ with specific growth rate $(1/N)\ dN/dt$ decreasing linearly as the population increases, with slope $-r/K$, reaching zero at $N \approx K$. However, Eq. 10.24 describes the exponential growth phase only, and hence another equation is required to describe the decline phase. Experimental data considered by Shirsat et al. (2014) indicated that the cell growth and decline phases are symmetric at the point of maximum growth, N_{max} at time, t_{max} and, therefore, the rate of decline in the vicinity of the peak of the cell density can be approximated to the rate of growth. Thus, the decline phase can be described by replacing the intrinsic growth rate constant in Eq. 10.24 from $+r$ to $-r$, and $N_0 = N_{max}$, i.e.,

$$N(t) = \frac{K}{1 + \left(\frac{K - N_{max}}{N_{max}}\right) e^{r(t - t_{max})}} \tag{10.25}$$

Equations 10.24 and 10.25 can be expressed with a single equation using the Heaviside step function:

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) - \alpha N(t - t_m) H(t - t_m) \tag{10.26}$$

where H = Heaviside, $H(x = t - t_m) = \begin{cases} 0, x \prec 0 \\ 1, x \succ 0 \end{cases}$

$$N(t) = \frac{K}{1 + \left(1 - \frac{K - N_0}{N_0}\right) e^{-rt}} - \alpha(t - t_m) \frac{K}{\left(1 + \frac{K - N_{max}}{N_{max}}\right) e^{-rt}} \tag{10.27}$$

The constant, α, is based on the best 'fit' to the available data. Shirsat et al. (2014) obtained $\alpha = 0.001$ for batch culture and $\alpha = 0.00075$ for fed-batch culture in the case of their particular data.

The Verhulst growth models Eqs. 10.24 and 10.25 are population balanced (V-PB), where the limited resources along with the growth are implicit in K, the carrying capacity. In order to determine explicitly the effects of the nutrients, particularly limiting substrate, the Verhulst logistic equation was developed further to quantify the relationship between growth and the substrate (Edelstein-Keshet 2005). Thus, the reproductive rate k is simply proportional to the concentration C of the substrate,

$$\frac{dN}{dt} = K(C)N = kCN \tag{10.28}$$

where k, is constant of proportionality with dimensions, t^{-1},

$$\frac{dC}{dt} = -\alpha\frac{dN}{dt} = -\alpha kCN \tag{10.29}$$

and where α is units of nutrient consumed to produce one unit of population increment (i.e., yield $Y = 1/\alpha$). Integrating Eq. 10.29 gives:

$$C(t) - C_0 = -\alpha N(t) \tag{10.30}$$

At $t = 0$, $N(t) = 0$ and hence $Co = C(0) + \alpha N(0)$ is a constant. If the initial population is very small then Co is equal to the initial amount of nutrient, C. Thus, substituting for C in Eq. 10.30 yields:

$$K(N) = k(C_0 - \alpha N)N \tag{10.31}$$

The substrate-based logistic (V-SB), Eq. 10.31, appears in population dynamics in the following form (Edelstein-Keshet 2005):

$$\frac{dN}{dt} = r'\left(1 - \frac{N}{B}\right)N \tag{10.32}$$

where $N_0 = N(0) =$ initial population, $r' = (kC_0) =$ intrinsic growth parameter, rate of consumption and $B = (C_0/\alpha) =$ carrying capacity, cell density. The solution of Eq. 10.32 is:

$$N(t) = \frac{N_0 B}{N_0 + (B - N_0)e^{-r't}} \tag{10.33}$$

Thus, according to Eq. 10.33, the carrying capacity and intrinsic growth parameter are influenced by substrate concentration and the rate at which it is converted into biomass (Y). The values of B, carrying capacity and r', intrinsic growth factor are determined by substituting values for dN/dt, the cell populations, $N(0)$, $N(t)$ and initial concentration of substrate C_0 into Eq. 10.32 (Shirsat et al. 2014). Thus, it is possible to determine the course or trend of the growth after 24 h, and, since glucose is the growth substrate in all bioprocesses, the model can be used for optimising initial concentration. Thus, K and B (cell density/ml) are the carrying capacities (a function of media composition and mode of bioprocess, i.e., batch or fed-batch) and r and r_x (h^{-1}), are intrinsic rate factors (reflecting the physiological state of the cells and mode of operation: batch or fed-batch). The carrying capacities (Ks) are inversely proportional to intrinsic growth factors (rs) (Shirsat et al. 2014). The model constants, K and r, are derived from the growth data and therefore, they have

physical meaning and they are of biological significance. Hence the models, V-PB and V-SB, are classed as mechanistic (Prajneshu 1999; Wan et al. 2000). Thus, the Verhulst model constants, K and r, can be employed in large-scale production as reliable indicators of the growth and productivity, and are useful for optimisation of the cell cultures.

Shirsat et al. (2014) found that the values, as predicted by Verhulst growth models, V-PB and V-SB, correlated with the observed data ($R^2 > 0.95$ %) across the three cell lines: CHOK1 SV (MAb), CHO 320 (IFN) and CHOIFN-γ. This was also the case for mode of operation, batch and fed-batch as well as different bioreactor capacities: 50 ml, 3 L, 4 L and 15 L (Shirsat et al. 2014). Thus, the Verhulsts growth models are robust, not even affected by the volume and composition changes caused by the periodic or continuous additions of feed to the fed-batch cultures (Shirsat et al. 2014). Further, unlike Monod-type models, Verhulst growth models do not require the maintenance or death-rate terms to correct the predicted viable density to agree with observed values (Goudar et al. 2005; Shirsat et al. 2014). Thus, Verhulst growth models are ideal for optimisation of culture media and feeding strategy for large-scale production.

10.8 Neural Network (NN)

The modelling and simulation of complex systems, such as biological ones, can be very challenging because of limited knowledge for the underlying process. Therefore, one resorts to a general modelling framework, such as neural network models (Van Breusegem et al. 1991). These models are found to be useful for microbial systems (Van Breusegem et al. 1991; Syu and Tsao 1993) but they can also be extended to mammalian cell cultures (Tziampazis and Sabanis 1994). Neural networks (NN) are classified as "Artificial Intelligence" (AI). Briefly, neural networks can describe systems of any complexity by increasing the structure of the model. The latter consists of an input and an output level containing information about the input and output variables of the system. Between these two layers, there are several hidden layers (cf. Fig. 10.7).

Each intermediate level may either serve as a black box, or it can be constructed so as to accommodate some structural knowledge. The sum of each layer of weighted inputs is processed via linear or non-linear transfer function to form this layer's output. Neural networks (NN), like black box or empirical models, simply describe the numerical interrelationship between the system's inputs and outputs, and they may or may not have physical meaning of the state variables (Julien and Whitford 2007). The conventional computerised approaches for solving control problems involve programming based on a priori knowledge, otherwise computers cannot solve it (Julien and Whitford 2007). The neural networks cannot be programmed; instead, they must be "taught" to perform each particular task by presenting the network with training examples using historical process data obtained from previous culture runs (Komives and Parker 2003). To train the

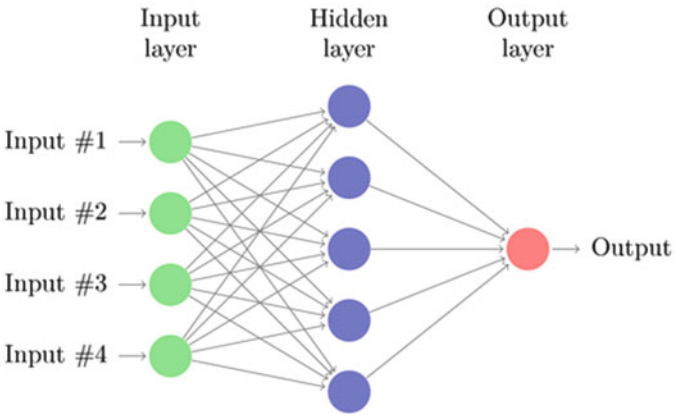

Fig. 10.7 Common architecture of feed forward neural network

ANN it is to get complete process information, corresponding to the NN inputs and outputs, from the data gathered in a set of fermentation runs. This set defines "an experimental space" and the ANN will predict outputs accurately only within this range and not beyond it (Caramihai and Severin 2013). Parameters are estimated by incorporating "learning" into the model so that repetitive feeding of the same data to the model results in parameter estimation. Once the model is formulated successfully, it can predict output for a new set of inputs. Syu and Esao (1993) demonstrated that these models, if properly built and trained, have good interpolating and promising extrapolating abilities for culture systems. Neural networks "learn" by example similar to the way biological systems learn by adjusting the inhibition and excitation effectiveness of synaptic connections between neurons. Because the network discovers how to solve a problem by itself, its operation can be unpredictable. However, an appropriately trained neural network can be thought of as an "expert" capable of analysing data and making estimations of process values.

The most commonly used NN architectures for process modelling and control are the feed-forward neural network (which allows signals to travel from input to output only) and the recurrent network (which can have signals traveling in both directions by introducing loops with an implied temporal dependence). One difficulty with recurrent networks is determination of the best network architecture with respect to the number of hidden units. Other proposed advanced network architectures include dynamic, fuzzy, and stacked neural networks. A dynamic neural network (DNN) adapts the static feed-forward network concept by using past process inputs and outputs to predict currently appropriate process outputs. Because the number of process variables and data are often limited, neuro-fuzzy networks combine fuzzy logic and neural network technology allowing "expert rules" to be added to data sets for improving overall model robustness. That can be very useful in bioreactor processes where controlled variables are often restricted to a limited range for design reasons (e.g., minimum or maximum achievable feed rates) or safety reasons (e.g. maximum allowable liquid volume height, vessel pressure, and

so on). Capturing real-life experiences from skilled operators (expert knowledge) for a problem domain augmented with a fair dose of common sense can compensate for sparse and noisy data, often resulting in a faster learning phase. Stacked neural networks have been proposed to further enhance model accuracy and robustness by aggregating several different networks, the output of which are determined by weighing each individual network output against the others for a final consensus (Julien and Whiford 2007).

Various applications have been studied in the literature. These include: (a) biomass and recombinant protein concentration estimation via feed-forward NN for a fed batch bioprocess with a recombinant *E. coli* (Warnes et al. 1998; Liu et al. 2008); (b) two types of NN (input/output and continuous externally recurrent) can control batch and fed-batch pyruvate production from glucose and acetate with a recombinant strain of *E. coli* (Zelic et al. 2006); (c) two NN also to control the submerged bioprocess of *Monascus anka* fungus cultivation, where the temperature and the dissolved oxygen are the inputs and the controlled outputs are the glucoamylase activity and the concentration of the red pigment (Chang et al. 2006); (d) NN-based soft sensor for online biomass estimation in fed bioprocess for polyhydroxibutirate production (James et al. 2002); (e) media formulation optimisation with genetic algorithm evaluated by ANN (Lara et al. 2006).

Cunha-Bakeev et al. (2005) used a Radial Basis Function (RBF) neural networks to estimate the process parameters in the CHO 320 culture, producing INF-γ. The selection of data was based on process knowledge. The data set matrix was separated into a training set (for modelling), a test set to determine the most appropriate network structure, and a validation set (to assess the efficacy of the model on independent data). Cunha-Bakeev et al. (2005) used the Root Mean Square Error (RMSE) as a criterion for identifying the best network structure and efficacy of the RBF model. They found that the estimation of cell count and product concentration was less accurate than that of the specific growth and production rates. However, the accuracy of cell count and product concentration can be improved if the estimates of specific growth and productivity were data-based models, thus combining a hybrid model scheme with mechanistic model.

10.9 Fuzzy Logic

Fuzzy logic is a qualitative approach to modelling for bioprocess control. Much valuable human knowledge is only available in a qualitative heuristic form (Caramihai and Severin 2013). It has been found that knowledge-based control structures using the human decisional factor (i.e., with a subjective element) offer sometimes better results than computer programme devoid of general knowledge. Consequently, 'intelligent' techniques (i.e., neural nets, fuzzy structures, genetic algorithms or expert systems) are capable of simulating human expert-like reasoning and decision making, dealing with uncertainties and imprecise information (Luebbert and Jorgensen 2001). However, control systems founded entirely on

the human subjective knowledge are less valuable than the control systems who utilise the objective information fitted by a conceptual model. Therefore, the literature recommends that intelligent control techniques are used only if the control structure based on quantitative models fails.

Frequently, different process parameters are controlled in order to follow predefined transitory trajectories. Such control strategies can be designed by a trial-and-error approach in combination with operator's experience and statistical analysis of historic data. Considerations include:

(a) One method for automatic bioprocesses control using AI is based on expert systems (ES) that reproduce the human operator' rules of action. The literature presents several examples how to transfer the knowledge from operators into knowledge-rule bases (Suzuki et al. 1997). The most used ES systems in bioprocess control are applied for supervisory control, or process monitoring and diagnosis. Moreover, ES logic is used to translate human language into a mathematical description. The parameters tuning is then regulated by phase detection based on if. . .then rules, conditional statements representing heuristic reasoning in which if expresses the condition to be applied and then-the action to be done. Of course, at the same time, it is not possible to calculate optimal parameter' values with this method. For example, (Hrncirik et al. 2002) an ES was developed in order to supervise a conventional control system applied to fed-batch baker's yeast cultivation and to surmount its limitation. Expert system BIOGENES can execute standard process control tasks (Hrncirik et al. 2002), but also advanced control tasks: process data classification; qualitative process state identification (metabolic state, process phase, substrate feeding); supervisory control through corrective actions.

(b) In another AI technique, the 'fuzzy' approach is based on fuzzy sets and fuzzy reasoning. In actual AI systems, fuzzy rules are often applied together with different types of models/parameter-/state- estimators. These fuzzy rules can be regarded as problem-specific basis function system (Mandenius 2004). Any variable can be a fuzzy variable, particularly recommended when it is not possible to define its value in a given situation. One define fuzzy sets in the form of a membership functions (between 0 and 1) in order to express what is likely to be considered as degree/level for a certain characteristic (high, medium, low). Relationships between fuzzy variables can be formulated with fuzzy logic operators (and, or, not) and processed by fuzzy logic. Fuzzy rules reflect the rules of thumb used in everyday practice and can be processed as 'if. . .then' expressions. With a set of fuzzy rules, considered as universal process approximates, the behaviour of a system can be described quite accurately. There are many applications, e.g. (i) hierarchical fuzzy models within the framework of orthonormal basis functions Laguerre and Kautz; (Campello et al. 2003); (ii) several important use of fuzzy control in the Japanese bioindustry by the companies Ajinomoto, Sankyo or Nippon Roche (Horiuchi 2002); (iii) the control of the α-amylase fed batch bioprocess with the recombinant *E. coli* to maintain glucose and ethanol at low concentrations

with two fuzzy controllers for feed rate control: feed forward and feedback (Honda and Kobayashi 2000).

(c) Because all types of information must be used in order to improve bioprocess control, e.g., mathematical/deterministic models, heuristic knowledge, rules-based reasoning, etc., a new control structure is developed in recent years – i.e., hybrid control systems (HCS). HCS acts on both parts of bioprocess control: conventional control systems (i.e., based on an a priori model) merge with AI techniques in a complementary way: if an a priori (mathematical) model exists, it will be preferred. If not, linguistic rules (i.e., expert systems/fuzzy techniques) will be used.

Generally it is necessary to design a control system, from which to choose (intelligent) control strategies, based on analytical methods for improving control performances. Zorzetto and Wilson (1996) developed Intelligent Control Structure (ICS) based on Hybrid Control Techniques (HCT). Most widely-used hybrid structures combine balance equations with ANN. For example, balance equations for substrate and cell concentrations couple with ANN for growth rate model for baker's yeast in fed-batch culture. Horiuchi et al. (1995) developed a hybrid simulation system for fermentation processes that combine a statistical procedure with fuzzy logic interface. They described the hybrid simulation ($t = t + \Delta t$) algorithm using a data-base simulation and fuzzy inference. Briefly, the Horiuchi et al. (1995) scheme is in two parts. Following the initial conditions, the first part – Fuzzy inference system entails: Calculate adaptability of simulated data to procedure rules → Identify culture phase → Determine state variables for regression models. The second part, Data base simulation system (Statistical procedure) is: Select data sets around state point in simulation → Determine coefficients of regression analysis → Calculate increments of state variables for time interval at Δt by Runga-Kutta method, and continue the data simulation until $t = t_f$.

10.10 Productivity Models

The main goal of the biopharmaceutical industry is to develop and produce protein-based drugs such as antibodies, which are in great demand. Although there is an abundance of growth models, models for productivity are scarce. It is not at all clear as to what extent the culture variables affect product formation because the study was carried out using different cell lines under divergent sets of growth conditions such temperature, DO and the composition of culture medium – nutrients, metabolites and other compounds (Dalili et al. 1990). However, simulation and optimisation of product formation require accurate quantitative descriptions of the process, which are important so as to develop predictive models relating to growth and death rate and the specific rate of substrate consumption (Seamans and Hu 1990). Antibody production appears to be non-growth associated kinetics, i.e., production continues in the decline phase (Hayter 1989; Agrawal et al. 1989) or

Table 10.3 Correlation for q_{MAb} with β denoting the growth dependent and α, the growth independent

Reference	Specific monoclonal antibody production rate, q_{MAb}
Frame and Hu (1991)	$q_{MAb} = \alpha + \beta_0\mu$
de Tremblay et al. (1992)	$q_{MAb} = \alpha + (\beta\mu/K_\mu + \mu)$
Lenardos et al. (1991)	$q_{MAb} = \alpha + \beta\mu_d$
Suzuki and Ollis (1989)	$q_{MAb} = {}_{(1 - fA)Kc + kAfA}$

negatively-growth-associated kinetics, i.e., an increase in productivity at a reduced growth rate (Korke et al. 2004; Kumar et al. 2007) or a combination of the two (Frame and Hu 1991; Linardos et al. 1991; Miller et al. 1986a,b). However, there are exceptions where productivity is hampered when growth rates are low (Gaertner and Dhurjati 1993).

Antibody production is affected by low nutrient levels (Gaertner and Dhurjati 1993) such as glutamine at low concentration is limiting in Monod-like manner (Dalili et al. 1990). This is contradicted by Korke et al. (2004) and Kumar et al. (2007), who found that in the glutamine-limited medium there is a definite metabolic shift from proliferation to protein synthesis. Lactate may inhibit Ab production following inverted Monod-type kinetics (Tziampazis and Sambanis 1994). However, the model simulation indicated that a high level of lactate can contribute to the reduction of glycolytic process (hence growth), as well as acting as a driving force for its conversion to pyruvate (Mulkutla et al. 2012). Thus, the metabolism in the cells shifts from proliferation to protein synthesis. The effect of ammonium may be modelled via non-competitive inhibition (Tziampazis and Sambanis 1994).

Table 10.3 shows a number of unstructured Ab productivity (Frame and Humodels); each one of them reflecting different behaviour of the cell lines investigated. Frame and Hu (1991) and de Tremblay et al. (1992) expressed cell-specific antibody production rate as a function of the specific growth rate. Dalili et al. (1990) based their model on a dependency on the glutamine concentration. Linardos et al. (1991) correlated linearly the antibody productivity to the death rate (Table 10.3) which is caused by the stress due to the exposure of the cells to low substrate concentration or high metabolite concentration. The range of validity for this has to be considered as very small because the data presented by Linardos et al. (1991) for batch culture do not validate the model (Portner and Schafer 1996).

Suzuki and Ollis (1989) developed a cell cycle model for antibody production on the basis of the knowledge that hybridoma cells produce MAb in the late G1-phase cycle, i.e., cell arrest in G1-phase leads to increase in the productivity with corresponding increase in death rate. Shirast et al. (2013) developed stochastic models for MAB productivity using cytometric data (i.e. cell cycle phases and organelles) of CHOK1SV cells. They found that productivity in the batch and fed-batch is statistically significantly correlated with (G1 + ER) and (G2 + ER) respectively. Since MAb productivity is non-growth associated (Agrawal et al.

1989), the most likely role of G1 phase arrest could be shifting the metabolic activities from proliferation to productivity.

An unsegregated, unstructured model for explaining growth and antibody production in hybridoma cultures was developed by Barford et al. (1992). Their work laid the foundation for more detailed and extensive models capturing cellular kinetics to a greater detail than had been done previously. The model was unique in the sense that: (i) a detailed stoichiometry (for catabolic, anabolic reactions and their interactive effects) was included and was capable of explaining both continuous and fed-batch behaviour, (ii) a large number of nutrients were simulated through the model and the nutrients were clubbed into six groups that were then solved for (Barford et al. 1992), (iii) the model was more extensive than previous models as it could simulate both fed-batch and continuous behaviour.

A number of unstructured models for cell-specific antibody production have been compared by Portner and Schafer (1996) for hybridomas in chemostat cultures. Specific growth rate, substrate concentration, serum and death rate have all been proposed as being the variables on which productivity depends, although which of these should be used and to what extent productivity is sensitive to them is very much cell line dependent. Non-segregated structured models have also been developed to describe product formation. 'Compartmental' models describe the system as a set of distinct pools which may be defined either by location or by kinetic behaviour. Interactions between compartments occur via unidirectional substance transfer, usually following first order kinetics with or without time delays (Noe and Delenick 1989). Other types of kinetic equations, such as Michaelis-Menten, can be incorporated, notwithstanding increasing mathematical complexity. Sambanis et al. 1991, developed a model incorporating chemical and physical structure to describe intracellular protein trafficking and secretion in a pituitary cell line.

Segregated models for mammalian cell cycle have been developed with population characterisation based on differences in cellular activities, morphology, and even mechanical properties (Mitchison 1971; Needham et al. 1990; Ramirez and Mutharasan 1990; Henderson et al. 1992). Segregated models can be enhanced by incorporating additional biological information to create models of higher structure. For example, cell-cycle theory can be combined with intracellular processes like the translation of messenger RNA (mRNA) to protein product (Suzuki and Ollis 1990). There also exist cell cycle models that take into account the reportedly different antibody synthesis rates in each of the cell cycle phases (Garatun et al. 1976; Liberti and Baglioni 1973; Mitchison 1971; Abraham et al. 1976; Ramirez and Mutharasan 1990). The advantage offered by these models is that no particular productivity function is needed. Instead, the productivity is described as a function of the population fractions in each cycle phase that are in turn dependent on the growth rate and can be described either with the aid of deterministic (Suzuki and Ollis 1990) or stochastic models (Linardos et al. 1991; Cazzador and Mariani 1993). An underlying assumption made here is that the synthesis rate is constant in each cycle phase; the culture conditions affect only the viable cell number and the distribution of cells among the phases. The validity of this assumption probably

depends on the particular system under consideration and the range of conditions employed (Dorka 2007). Shirsat et al. (2013) used cytometric data to develop unsegregated, structured models for growth (VCD) and productivity (MAb) of CHOSVK1 cultures in the batch and fed-batch modes.

Goudar et al. (2005) used a simplified form of Eq. 10.21 to describe the productivity and nutrient time-profile. Thus, setting $B \rightarrow 0$ in the Eq. 10.21 yields the logistic growth equation (LGE), which can be used to describe monotonically increasing product concentration, P.

$$P = \frac{A}{1 + C\exp(-Dt)} \tag{10.34}$$

The parameter D is a rate constant for concentration increase and definitions of A and C can be obtained by setting dx/dt = 0 and t = 0 in Eq. 10.34:

$$A = P_{\max} \bullet C = \frac{P_{\max} - P}{P_0} \tag{10.35}$$

where P_{max} is the maximum value of P and P_0 the initial value at t = 0. The equation can be rewritten in a more familiar form:

$$P = \frac{P_0 P_{\max}}{P_0 + (P_{\max} - P_0)^{-Dt}} \tag{10.36}$$

LGE models for the batch and fed-batch gave very good correlation between experimental productivity and model prediction.

Kontoravdi et al. (2007) developed a unifying approach to modelling of productivity in mammalian by combining all of the other approaches. Their model combines an unstructured model describing cell growth and death (Jang and Barford 2000) and cell metabolism (Jang and Barford 2000; Tatiraju et al. 1999) with a structured model of MAb synthesis and production (Bibila and Flickinger 1992; Tatiraju et al. 1999) and a compartmental model of protein glycosylation (Umana and Bailey 1997). Thus, there is no single model but a series of models together describing the MAb productivity.

Shirsat et al. (2014) extended the Verhulst population balanced (V-PB) growth concept productivity of monoclonal antibody (MAb) (V-Pr) by CHO-K1SV in the batch and fed-batch cultures. Thus, the Verhulst growth model becomes:

$$X(t) = \frac{K_X}{1 + \left(\frac{K_X - X_1}{X_1}\right) e^{-r_X t}} \tag{10.37}$$

where X_1 = Ln MAb at 24 h and $X(t)$ after t-hours, and, as before, K_X = productivity potential and r_x = productivity rate. The parameters, K_X and r_x, are determined in the same way as in the growth models, i.e., from the logarithm of MAb titre, Ln X,

to the specific rate of production, $(1/X)\ dX/dt$. The (x-) abscissa intercept of the resulting line is K_X, productivity potential and r_x, specific productivity rate is the (y-) ordinate intercept (Shirsat et al. 2014). Shirsat et al. (2014) found that though the batch and fed-batch cultures did not reach their full potential of growth as predicted by the Verhulst growth models, they did measure up to their full potential for antibody production, thus confirming that the antibody production is non-growth-associated and that the higher productivity in the fed-batch is attributed to the longevity of the cells in that culture rather than the viable cell numbers (Agrawal et al. 1989).

The Verhulst model rule for the growth "lower the intrinsic growth factor, higher the cell density" also applies to the Verhulst productivity model (Shirsat et al. 2014). Shirast et al. (2014) found that periodic additions of soy hydrolysate leads to a lower value of growth factor r for the fed-batch culture because of metabolic shift from proliferation to protein synthesis (Korke et al. 2004; Kumar et al. 2007). The metabolic shift is usually accompanied by a reduction in lactate production, a sign of slowing down of growth. The model simulation indicated that a high level of lactate can contribute to the reduction of glycolytic process (hence growth), as well as acting as a driving force for its conversion to pyruvate (Mulkutla et al. 2012). Also, high lactate level has been reported to exert an inhibitory effect on glycolysis enzyme phosphokinase (Mulkutla et al. 2012). Shirsat et al. (2014) have found that a lower intrinsic growth factor not only influences an increase in the carrying capacity K for growth, but, most importantly, it lengthens the exponential phase considerably. It has been shown that the expression of proteins like MAb reaches its peak in the exponential phase (Al-Rubeai and Emery 1996; Lloyd et al. 1999; Mochida et al. 2000), particularly when the feed contains soy peptides since it alleviates metabolic stress. Therefore, the longer the exponential phase lasts, the greater the MAb productivity, and, hence, several methods and techniques have been developed to slow down growth and extend the period of cell viability (Kumar et al. 2007). The Verhulst productivity model could be employed to evaluate the effectiveness of these methods and techniques. For instance, Shirsat et al. (2014) determined that the comparison of the Verhulst models for growth and productivity indicates that only a single exponential model is required to describe the productivity in the batch and fed-batch cultures. They found that the productivity profiles of the batch and fed-batch reached a plateau after reaching their productivity potential (K_x) exponentially whereas their growth profiles show a steady decline after reaching peak viable cell density exponentially. Thus, it appears that the cellular mechanism of the productivity may be different than the growth one.

Antibody production may be cell-specific as well as environmental (physical and chemical) conditions under which the cultures are cultivated (Dalili et al. 1990). Furthermore, antibody production seems to follow non-growth-associated kinetics, negatively-growth-associated kinetics (increased specific productivity at reduced growth rates), or a combination of the two (Frame and Hu 1991; Linardos et al. 1991). For example, Goudar et al. (2005) and Hayter (1989) found that the MAb production continues part of the way into the decline phase.

10.11 Critical Synopsis and Perspectives

It is evident from the range of models discussed above that modelling is arguably overly influenced by biochemical engineers, whose main motivation is to monitor and control bioprocesses. This approach has perhaps proven to be detrimental to the interest of biologists, who wish to elucidate underlying mechanisms of growth and productivity. With the introduction of the FDA initiative (2004), the monitoring of Quality-by-Inspection (QbI) has been replaced by Quality-by-Design (QbD), which involves monitoring in real-time critical-process-parameters (CPPs), which have direct bearing on the quality of the product. Therefore, Mandenius and Brundin (2008) have described several examples of application of DoE methodology to comply with FDA – PAT initiative by identifying the critical state variables for quality control and process optimisation. Thus, it is step up from Quality by Analysis (QbA) to Quality by Design (QbD). QbD is a systematic approach involving predetermined objectives with emphasis on product and process understanding, which in turn are based on sound science and quality risk management.

Structured and unstructured mathematical models are based metabolic flux as reflected in the kinetics of extracellular state variables. Unstructured models, derived from the kinetics of extracellular variables, are empirical and do not shed any light on the inner workings of the cell. Structured models, which are followed by unstructured ones, extrapolate the extracellular variables to intracellular metabolic flux (Provost and Bastin 2004). These structured models (Sanderson et al. 1995, 1997; Sodoli et al. 2004, 2006) involve a large number ordinary differential equations (ODEs) and algebraic equations (AEs) because of a large number of parameters (Sanderson et al. 1999) and, therefore, they are unwieldy, computationally intensive and almost obscure (Shuler 1999). Since no intracellular measurements can be carried out, the models are based on extracellular measurements and hence the SMC models are of a limited scientific value because they are inferential. Therefore, the "reduced" structured growth models of Provost and Bastin (2004) are preferable. They are quite informative and provide a firm basis for the design of on-line and optimisation of cell culture processes. However, the "reduced" models are only valid in the exponential phase.

The approaches to developing structured and unstructured models have been symptomatic, meaning based on effects rather than causes of the effects. The modelling has been about "describing" biological behaviour instead of what and how the "biological" mechanisms give rise to biological behaviour (Reed 2004). This requires the inputs of biologists, without whom no substantial progress can be made in advancing the bioprocess industry. For example, unstructured models have revealed that in the fed-batch case, the decrease in lactate concentration shifts the cellular metabolism from proliferation to productivity (Mulkutla et al. 2012). Therefore, this finding should be researched further with the help of biologists by investigating the corresponding changes in cell-cycle phases and organelles, particularly ER. Another example is that the CHO cells continue to produce MAb during decline or death phase (Hayter 1989), but this finding is connected with the

changes in cell cycle phases and ER. Kontoravdi et al. (2007) unifying approach of linking the cell dynamics with MAb synthesis for model-based and quality control is a way forward because it is attempting to relate observed changes in the extracellular phase with corresponding changes in the intracellular phase. Model simulation for macroscopic culture variables, such as nutrient and product concentrations, are in good agreement with experimental data and those for glycosylation are qualitatively correct. Despite the fact that the glycosylation results presented cannot be validated due to lack of experimental data, the proposed dynamic model paves the way for model-based product quality monitoring and control. Despite the fact that the glycosylation results presented cannot be validated due to lack of experimental data, the proposed dynamic model paves the way for model-based product quality monitoring and control.

An alternate to "metabolic flux" is "growth flux" (Shirsat et al. 2013), as reflected in the cell cycle phases. Cytometric data represents the whole dynamics of the cell-life cycle. Therefore, cytometric data of cell-cycle phases and organelles are the best indicators of cell physiology. Changes in cytometric parameters are in direct response to the physical and chemical changes in the growth environment. Thus, cytometric data is ideally suited for hypothesis-testing and developing stochastic structured models for growth and productivity. Cell phases provide natural compartmentalisation of the cells at different growth stages corresponding to changes in cell-cycle phases and organelles, and are hence connected anecdotally to growth and productivity (Al-Rubeai and Emery 1990; Al-Rubeai et al. 1992; Moore et al. 1997; Yoon et al. 2003a, b; Fussengger et al. 1998, 2000; Kaufman et al. 1999, 2001; Carvalhal et al. 2003; Ibarra et al. 2003; Fogolin et al. 2004; Bi et al. 2004; Trummer et al. 2006). Shirsat et al. were able to show that mechanisms of growth and productivity for CHO cells in the batch and fed-batch cultures are different, resulting in a high population and MAb titre for cells grown in fed-batch mode. The study also indicated how the cell cycle phases and ER can be manipulated to improve productivity.

The ultimate objective of modelling bioprocesses is to maximise productivity. Sidoli et al. (2004) stated that an increase in productivity could be via genetic manipulation of the cells or optimisation of cellular processes. The latter route requires a fuller understanding of underlying mechanisms of the bioprocess. However, biochemical engineers are interested primarily in using unstructured empirical models for monitoring and control of bioprocesses, requiring no knowledge of reaction mechanisms. Structured SMC models, based on extrapolation of extracellular state variables, do not shed any light on the inner workings of the cell. However, stochastic (structured) models based on cytometric data, Monod-hybrid (pseudo-mechanistic) growth models based extracellular data and Verhulst (mechanistic) growth and productivity models (V-PB and V-Pr), respectively, are quite promising and can provide insight into the mechanisms of growth and productivity (Shirsat et al. 2013, 2014).

The traditional Process System Engineering (PSE) approach is not readily applicable to the biopharmaceutical industry because of the complexity of biological systems and the limited understanding of the biological processes, both of

which lead to inadequate process models (Koutinas et al. 2012). Therefore, in the absence of proven model-based approaches, process optimisation in the biopharmaceuticals industry relies on extensive and, arguably less-than-necessary, experimentation. Arguably, the whole field of modelling of bioprocesses evolved from a single theory of Michaelis-Menten enzyme kinetics. It is too simplistic an approach and, therefore, it can be argued that little progress can be made until new non-Monod approaches are explored, such as cybernetic models presented by Ramakrishna (1983), the introduction of structure as defined by Fredrickson et al. (1970), and to the genetic level by Lee and Bailey (1984a, b). The concept of cybernetic modelling is an adaptation of a simple mathematical description of complex cell structure by assigning an optimal control motive (cause) to its response (effect) (Kompala et al. 1984). The model enables the observation of the behavioural response of the cells in a multi-substrate environment. Lee and Bailey (1984a, b) extended the concept to the level of nucleotide sequences. The key to their concept is an explicit connection between a particular nucleotide sequence and its affinity to a particular protein; the nucleotide sequence influences the corresponding transcription event, thus deriving a quantitative mapping from the nucleotide sequence to the overall phenotype. Bailey (1998) predicted that this new "genetically structured model" will be widely adapted with the support of advancement of techniques in genomics, proteomics, metabolomics, bioinformatics, etc. Therefore, this brings closer the tantalising possibility of optimising productivity based on biological knowledge. Koutinas et al. (2012) have described modelling based on this concept.

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Chapter 11
Mammalian Cell Line Selection Strategies for High-Producers

Darrin Kuystermans and Mohamed Al-Rubeai

Abstract With the increase in mammalian cell-expressed recombinant biotherapeutics, the process of accelerating the selection of generated stable mammalian cell lines is becoming a critical step in cell line development pipelines. The selection process is known to be a major bottleneck in obtaining a cell line from development into manufacturing, but with the current sales of biotherapeutics reaching close to US$125 billion with monoclonal antibodies being more than 50 % of the sales, there is no sign of a decrease in demand and the amount of cell line development projects in the pipeline will keep increasing. This means that more efficient cost effective cell line selection strategies are critical to meet demand for affordable biologics. The current advancements in the cell line selection process has helped in this regard, by reducing some of the labor and time required to reduce heterogeneity and determine clonality of a cell line expressing a quality biotherapeutic protein at the highest specific productivity possible. However, challenges remain in dealing with the sheer volume of cells that need to go through the screening process for the determination of a stable highly productive clone. This chapter will provide a summary of the methodology and strategies employed to select the desired cell lines that meet the demands of the biopharmaceutical manufacturing environment from manual selection to automated systems that aid in the mammalian cell line selection process.

Keywords Single cell cloning • Automation • Cell line selection • Bioprocess development • Biopharmaceuticals • CHO • NS0 • Flow cytometry • LEAP • Mammalian cells • Antibody

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11.1 Introduction

The biopharmaceutical industry has seen a rapid growth in therapeutic proteins, with the amount of blockbuster biologics on the market increasing by 65 % from 2006 to 2012 alone. Within those 6 years, for example, the total sales of recombinant biologics had doubled from US$63.8 billion to a staggering US$124.6 billion, with therapeutic antibodies being at the top of the class of biologic products manufactured, increasing from 34.5 % of total biologic sales in 2006 to 51.8 % in 2012 (La Merie Publishing 2013), with the predicted sales of monoclonal antibody (mAb) therapies reaching US$70 billion by 2015 (Chon and Zarbis-Papastoitsis 2011). With this increase in demand for clinical and commercial biologic drug substance manufacturing of mAbs and recombinant proteins through mammalian cell culture production platforms, the need for improved strategies to reduce development time whilst maintaining quality attributes and optimizing yields has been made a priority.

Biopharmaceuticals have employed new technologies to ensure that the mammalian cells selected for large scale manufacturing have the ability to produce recombinant glycoproteins with high efficacy and functionality at optimum expression levels. There are several mammalian cell lines that have been developed as expression hosts, with hybridoma cell lines being one of the earliest; however, for the purpose of biologic therapy production, the hosts have mainly been restricted to a small group of well-classified hosts. The Chinese Hamster Ovary (CHO) cell line (Cockett et al. 1990; Milbrandt et al. 1983) has been the primary host utilized for recombinant biologics production such as monoclonal antibodies with popular CHO cell lineages cultured being CHO-K1, DUXB11, and DG44. Apart from the CHO cell line, other cell lines that are commonly used for the production of recombinant proteins are mouse myeloma-derived (NS0) (Barnes et al. 2001; Bebbington et al. 1992), baby hamster kidney (BHK) (Carvalhal et al. 2001; Christie and Butler 1999; Geserick et al. 2000; Kirchhoff et al. 1996), and human embryonic kidney (HEK-293) (Baldi et al. 2005; Schlaeger and Christensen 1999). More recently, the human retina-derived (PerC6) (Jones et al. 2003) cell line, which has the ability to secrete high levels of recombinant product whilst having a low gene copy number (Butler 2005), has also joined this group of well-characterized hosts for biologics production. From these cell lines, the only non-anchorage-dependent cell is the NS0 cell line, while the others need to undergo an adaptation process to suspension culture. The media used for these cell lines can be either serum-free or chemically-defined protein free media for the purpose of process homogeneity, reproducibility, and safety. Chemically-defined protein free media formulations have become the preferred media (Lee et al. 1999; Zang et al. 1995; Sinacore et al. 1996) due to the high reproducibility and increased definition, which reduces process variability and makes it easier for a regulatory authority to approve the process. Early recombinant mammalian cell culture processes had titers below 1 g/L for antibody production (Birch and Onakunle 2005). With advances in culture media formulations and process technologies

(Kuystermans and Al-Rubeai 2011), productivities of 2–5 g/L in 'generic' fed-batch process are now routinely reported with extended culture such as perfusion processes reporting within 10–25 g/L (Kelley 2009; Chon and Zarbis-Papastoitsis 2011).

An important aspect of a biopharmaceutical glycoprotein product is the functional biological activity, which is usually directly affected by the glycosylation pattern, including trafficking and folding within the host production system (Scallon et al. 2006; Walsh and Jefferis 2006; Raamsdonk et al. 2001). Early studies first highlighted the importance of glycosylation in therapeutic proteins and stated that glycosylation profiles of proteins should be approached on a case by case basis during the development of a production process due to the profound influence of host cell type on posttranslational processing (Hooker et al. 1999; James et al. 1995, 1996; Jenkins et al. 1996). One of the main reasons that mammalian cells such as NS0 and CHO are utilized for therapeutic protein production is their unique ability to produce proteins with oligosaccharides attached to their serine/threonine (O-linked glycosylation) or asparagine (N-linked glycosylation) residues (Butler 2005; Seth et al. 2006). Glycosylation also goes beyond affecting the functional biological activity since, once secreted, the stability, aggregation, solubility, and immunogenicity of the protein may be affected, and insufficient glycosylation patterns can lead to rapid clearance in vivo (Kobata 1992; Sinclair and Elliott 2005; Willey 1999; Wyss and Wagner 1996; Sethuraman and Stadheim 2006). The use of mammalian cell lines other than human can cause immunogenic reactions in humans. Mouse cells generate Galα1,3-Galβ1,4-GlcNAc residues which are highly immunogenic in humans, due to the presence of α1,3-galactosyltransferase (Butler 2005; Jenkins et al. 1996). Glycosylation can also be affected by the culture environment (Chee Furng et al. 2005; Patel et al. 1992; Raju et al. 2000). Clone-dependent glycosylation is also seen due to processing inconsistencies where glycans can have frequent structural heterogeneities (Patel et al. 1992; Seth et al. 2006; Varki 1998). In addition, the enzymes present in a host cell line, which are required for glycosylation to take place, can be species-dependent. Terminal N-acetyl-neuraminic (NANA) sialylation is predominant in humans but varies in other species which tend to predominantly have N-glycolyl-neuraminic acid (NGNA) rather than NANA (Raju et al. 2000). CHO cells have an advantage in sialylation due to their high percentage of NANA sialylation rather than NGNA sialylation taking place on glycoproteins secreted from the cell line (Baker et al. 2001). To optimize all of these glycosylation patterns, glycosylation engineering may be used as a tool to modify the host production line in order to achieve near-human glycosylation. This may require only slight modifications in cells from mammalian origin other than human, but if the host is not mammalian in origin, it can be a complex task. Hamilton et al. (2006) achieved mammalian glycosylation in *Pichia pastoris* in order to produce functional recombinant erythropoietin. The yeast *Pichia pastoris* was able to secrete human glycoproteins with fully complex terminally sialylated N-glycans after removing all yeast glycosylation genes and introducing 14 heterologous human glycosylation genes; however, this technology still requires years of further fine-tuning, development and characterization before

being approved as a platform for therapeutic manufacturing. There have been similar attempts for insect-, and plant origin-based cell lines where the re-engineering involved can be complex (Hollister and Jarvis 2001; Cox et al. 2006), but the hurdles that are also required to get approval for such a cell line as a manufacturing platform can take many years.

Recently, the emergence of the ability for targeted genome engineering provided via programmable site-specific nucleases such as zinc finger nucleases (ZFNs) (Kim et al. 1996), transcription activator-like effector nucleases (TALENs) (Boch et al. 2009), and the clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonuclease Cas9 (CRISPR/Cas9) system (Mali et al. 2013a, b), an RNA-guided DNA endonuclease, has opened up further possibilities of cell line development for improving product yields. Given the ability to manipulate any gene or insert sequences of DNA at specific sites within the genome of the cell (Gaj et al. 2013), these programmable nucleases can be a powerful tool in the genetic engineering arsenal, as improvements in avoiding off-target effects are quickly being addressed to improve specificity and efficiency (Carlson et al. 2012; Ran et al. 2013; Mali et al. 2013a; Fu et al. 2014). The use of these genome editing systems is still in its infancy in regards to obtaining high-producer cell lines via genome editing; however, some work has already been done to demonstrate the applicability by engineering CHO cells via zinc finger nuclease-aided deletion of *Bax* and *Bak* genes to gain apoptotic-resistant cells (Cost et al. 2010).

The intent to culture mammalian cells as an industrial platform has always had its challenges, but the one seen as far back as the 1950s was the natural heterogeneity of mammalian cells, which was first observed when attempts were made to isolate them (Sato et al. 1957). What may seem to be the same mammalian cells in culture can still be cells with very different physiological characteristics and properties, including maximum viable cell numbers, integral viable cell times, specific growth rates and specific production rates of recombinant proteins (Barnes et al. 2001; Kim et al. 1998; Marder et al. 1990; Kim et al. 2001). This inherent heterogeneity seen in culture meant that biopharmaceutical companies have implemented strict procedures to determine ‘clonality’ in a cell line, in essence meaning that a cell line should be derived from a single cell (ICH Guideline 1995). Cloning procedures are carried out to reduce heterogeneity where a single cell, producing the recombinant protein of interest, is selected from a population of cells in order to ultimately obtain a homogeneous population of cells from a single clone; this population can be utilized for a specific finite amount of generations in the manufacturing environment without any change in expression level in terms of specific productivity, viable biomass and the recombinant product itself.

The selection of high-producing cell lines is known to be a costly process that can be a major bottleneck in getting a cell line from the developmental pipeline into the manufacturing environment. The methods employed to select these high-producers can vary, but the goal is to create a cell line that can be deemed stable due to its consistent product expression and suitable growth characteristics. The stability of a cell line is determined by several regulatory events within the cell, which can be separated into three phases (Barnes and Dickson 2006), where phase

one is the vector design, transgene-containing plasmid integration and the chromosomal environment. The second phase is mRNA stability and processing, which is crucial for regulating the expression of transgenes, as is seen with splicing events with XBP-1(S) that has been linked to the control cell line productivity (Shaffer et al. 2004; Sriburi et al. 2004; Yoshida et al. 2006), while the last phase is translational and secretory events where specific proteins within the secretory pathway can affect specific productivity (Smales et al. 2004; Alete et al. 2005). Although several regulatory events within the cell can determine stability, it is primarily assessed by the expressed protein being biochemically comparable, using available analytical techniques, from the initial cell selected to the final cell line produced, whilst observing consistent growth characteristics. Stability in this case can be classified as the limit of the in vitro cell age for production, which itself is a measure of time between vial thaws of selected cells to the harvest process from a bioreactor by measuring the elapsed chronological time in culture via the population doubling level of the cells. Thus, studies to determine cell line stability can be performed by evaluating the secreted quality and expression level of the therapeutic product for a cell line consistently attaining a high maximum viable cell number of over 50–60 generations from the establishment of the Master Cell Bank (MCB) to the end of the commercial manufacturing process in a bioreactor culture.

In order to obtain high-producing cell lines, certain host cell lines have adapted common expression systems that aid in the selection process. Some of these expression systems have become standard in industry, such as the DHFR (dihydrofolate reductase) and GS (Glutamine synthetase) systems. The DHFR system is used with CHO cells (Lucas et al. 1996), where a mutant CHO *dhfr*- cell line is transfected with a vector containing the target gene along with the *dhfr* marker gene. Selection occurs in hypoxanthine-, glycine-, and thymidine-free media. At the same time, methotrexate (MTX) concentrations are gradually increased to amplify the expression of the protein product due to MTX being able to inhibit the DHFR enzyme (Fig. 11.1). The GS system has been utilized in NS0 (Bebbington et al. 1992) and CHO (Cockett et al. 1990) cell lines, where cells are transfected with a vector containing the gene of interest in addition to the GS gene. Cells possessing the GS gene can synthesize their own intracellular glutamine; thus, these cells can be cultured in glutamine-free media with the addition of glutamate and the ammonia group provided by asparagine (Barnes et al. 2000). Unlike CHO cells, NS0 cells have no endogenous GS activity, making them the preferred cell line for use in this expression system. Selection can be carried out in the presence of methionine sulfoximine (MSX) which inhibits GS activity; as a result, the endogenous GS activity of CHO cells can be circumvented with the use of higher MSX concentrations and by inhibiting the GS activity, the gene of interest is amplified with a gradual increase in MSX concentration. In comparison to the DHFR system, one advantage of the GS selection system is that the cell cultures produce less ammonia, which can negatively affect the glycosylation of the recombinant protein (Yang and Butler 2000a, b); this is in addition to the GS system usually providing a shorter selection period due to the system requiring lower gene copy numbers for a similar expression level of the amplified transgene. Even though both MSX and

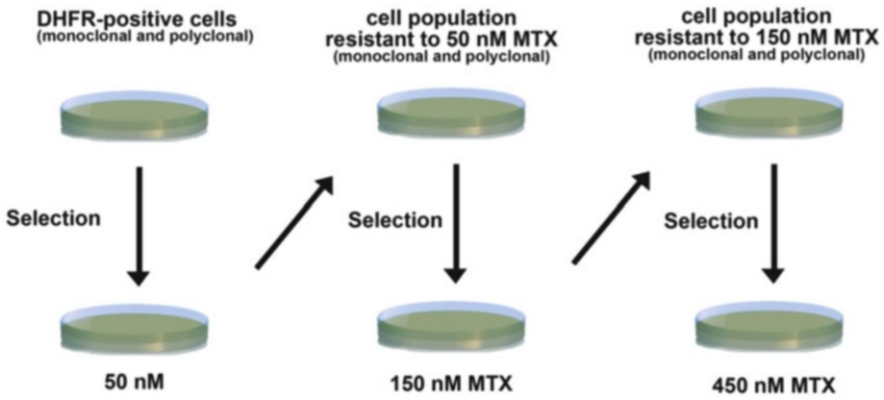

Fig. 11.1 MTX cell selection procedure. Illustrates the incremental step increases in MTX concentration and the selection of cell populations resistant to MTX in order to select the few cells with increased dhfr expression. After each round of MTX selection, the cells are exposed to even higher MTX concentrations

MTX selection aid in selection and gene amplification, both methods produce cell clones that are highly heterogeneous with only a few stable high-producer clones.

The major cost in the development of biopharmaceutical processes is the time required for cell line development, which traditionally involves the process of gene integration and amplification, with the use of selection agents such as MTX or MSX, followed by single cell selection using limited dilution single cell cloning (LDSCC) techniques. This selection process is laborious and time-consuming in nature (Borth et al. 2000; Carroll and Al-Rubeai 2004; Browne and Al-Rubeai 2007), using manual microscopy techniques to monitor the process and requiring several expansions of possibly hundreds to thousands of clones. In addition, the high-producing clones tend to be overgrown by the low or non-producers due to energy being diverted towards recombinant protein production instead of growth (Al-Rubeai 1999; Borth et al. 2000; Kim and Lee 1999; Kromenaker and Srienc 1994); these tend to be in the majority, since there can be a range of 40–90 % of non-producers, depending on transfection efficiency (Lee et al. 2006). Even after high-producer selection, another round of screening may be required to ensure stability of integration of the transgene before a limited scale-up of a number of selected clones can be carried out to evaluate batch and fed-batch growth profiles. This means that the longest steps in cell line development are the selection procedures, which can take anywhere from 6 to 18 months depending on cell line, expression system and selection method.

With the increase in blockbuster therapeutic glycoprotein approvals for commercial manufacturing, the industry is constantly looking to reduce the cost of marketing these biologics. Cell line development is one area that can greatly benefit from the faster selection of stable high-producers. Although expression technologies, improved media formulations and bioreactor culture systems have resulted in higher yields from smaller more intensified production runs, there is still much

development needed in the high-producer cell line selection process in order to reduce labor, development timelines and capital expenditure. This chapter will focus on those methods that are used in cell line selection from single cell screening approaches to recent advances in high-throughput cell screening technologies.

11.2 Manual and Semi-Automated Cloning Methods

11.2.1 Manual Single Cell Cloning

The first ever isolation of a single cell followed by clonal expansion (Sanford et al. 1961) was performed via capillary cloning and demonstrated that the environmental conditions proved to be a deciding factor for further clone growth. Low density cell growth of both primary and continuous cell lines can be near impossible without the proper conditions and Sanford et al. hypothesized that culture media required extensive modification by the living cell before the media was suitable for growth; therefore, a single cell would require far less volume than a population of mass culture for conditioning based on diffusional mass transfer mechanisms. Thus, at an initial high cell density, equilibrium is reached much earlier than at a low cell density.

The capillary cloning technique involved a single cell isolated from a Petri dish with a capillary tube (100 μm diameter; 5–8 mm long) that was heat-sealed on each end in order to reduce evaporation of the small volume of medium within and encourage cell growth. This technique created a micro-vessel environment, protecting the isolated cell from pH changes and media evaporation, while a conditioning environment was established for cell growth. Once growth was established, the capillary tube was opened and immersed in a culture dish allowing the cells to migrate into their new environment over time. The capillary cloning technique demonstrated that culture environment plays an important role and that generally low population cell densities in their standard culture environments could have low metabolite concentrations (growth-promoting substances i.e. hormones, growth factors, growth signals etc..) in order to condition the medium effectively for growth (Eagle and Piez 1962; Ham and McKeehan 1979; Sato et al. 1957).

There are several other techniques that can establish a conditioned medium environment for single cell cloning. One method to ensure that single cells have a proper environment for growth with the required conditioning factors has been to use feeder layers (Puck et al. 1956). Here, a feeder layer can be cells from the same cell line or from another cell line (e.g. 3T3 cells) that is irradiated or treated with mitomycin C when about 50 % confluency is reached, in order to induce growth arrest before seeding the cells as a layer for clonal growth maintenance. When single cells are grown over the feeder layer, the growth-arrested layer may secrete essential growth factors and nutrients to support clonogenic densities of isolated cells. Another method uses droplets of culture medium (Wildy and Stoker 1958;

Macpherson and Stoker 1962) to form a micro-environment for single cell cloning. This is done by forming approximately 10 μl droplets in a Petri dish and placing one cell in each drop via micro-pipetting, before covering the drops with liquid paraffin to create the environment for clonal growth to occur.

Although capillary, feeder cells and droplet techniques help in conditioning the medium for cloning, the development of custom media formulations specifically for the cell line being cloned allows for single cell cloning in larger volumes. In the 1960s, cloning without a feeder cell layer was accomplished when Chinese hamster cells were cloned in a Petri dish with 5 ml of defined synthetic medium (Ham 1965). However, this medium had poor reproducibility at low cell densities. It is now known that even within a specific cell line, single cell cloning medium may vary due to the heterogeneity of cells, which can affect reproducibility. Over time, better media formulations have been developed that have made it possible to isolate single cells at higher volumes with improved efficiencies (Lin et al. 2007; Kuchenbecker et al. 2007) and techniques such as Design of Experiments (DOE) have helped in the development of custom cloning media that are comprised of animal-free formulations. It is now possible to create an animal origin-free cloning media that is also developed for use in the entire biopharmaceutical engineering process from single cell cloning to commercial manufacturing.

One of the most common single cell cloning techniques is limited dilution single cell cloning (LDSCC), where a limiting dilution is prepared by culturing the cell line in a 96- or 384-well micro-titer plate at dilutions below one cell per well (Coller and Coller 1986). At the appropriate dilution, individual clones can grow in a single well. LDSCC requires the deposition of one to three individual cells in a well and a period of 1–2 weeks in culture allows determination of the small subset of cells that have expanded from a single cell. Supernatants of each well containing a single cell-based expansion are tested via enzyme-linked immunosorbent assays (ELISA) for a secreted glycoprotein such as an antibody; to guarantee monoclonality, multiple rounds of serial subcloning are performed. This method, although very popular, is laborious, since in addition to the three to four serial subcloning steps, a single round of selection can include anywhere from 10 to more than a 100 plates per screen. It is statistically possible to get a $p > 0.99$ after two rounds of LDSCC, but despite the repeated rounds of LDSCC, a monoclonal culture cannot be guaranteed, even when statistics dictate a high probability of clonality due to a phenomenon known as the persistence of mixed clones (Underwood and Bean 1988), where mixed clones can remain even after many cycles of re-cloning. In addition, low producers have a tendency for an increased growth rate due to less energy being diverted to production and more to growth, thus making up the majority of the population; also, if a well receives more than one cell, the additional cell(s) can have a conditioning effect and can also improve growth.

A technique with similarities to LDSCC is spotting (Clarke and Spier 1980). Here, a cell suspension containing about 500–1,000 cells/ml is used as a reservoir to obtain single 1 μl droplets using an open-ended sterile Pasteur pipette, so that these droplets can be deposited in a flat-bottom micro-well plate without touching the sides of the well to allow easy visualization during microscopic examination. An

alternative to the spotting technique, known as capillary-aided cell cloning (Onadipe et al. 2001), has been accomplished, where single 1 μl droplets, with the aid of a capillary pipette, are deposited in 48-well plates. Microscopic examination of each well is carried out independently by two scientists to determine that the wells contain single cells. Completing one round of capillary-aided cell cloning can replace two rounds of limiting dilution cloning to obtain a monoclonal cell line, giving a probability of monoclonality of $p > 0.99$. Capillary-aided cell cloning is one of the preferred methods of single cell cloning at Lonza Biologics, along with newer automated methods.

Adherent cells tend to be easier to differentiate from each other compared to suspension cells and have a tendency to form easily distinguishable macroscopic colonies from single cells, making some cloning techniques work much more efficiently. Two cloning techniques that work best with adherent cell colonies are based on colony lifts. One technique requires cloning rings, where ceramic or plastic cylinders are placed in order to isolate a selected colony. These rings have silicone grease on the bottom to create a water-tight seal in order to lift the colony selected from the plate. A variation on this technique uses agarose as a medium to seal the cloning rings around a selected colony from an adherent monolayer culture (Mathupala and Sloan 2009) to reduce the chances of lower cell viabilities, desiccation and cross contamination. The second technique requires the use of small filter discs of around 2–6 mm in diameter that are soaked in trypsin and place on the selected colony outgrowth for lifting. Once these colonies are lifted, by either technique, further propagation and evaluation of the clonogenic colonies can occur.

11.2.2 Semi-Solid Suspension Cell Cloning

Semi-solid substrates can be used for suspension cell cloning where monodispersed cell colonies tend to form due to the immobilization of cells in the media, allowing the isolation of selected cells using a pipette. The technique can be performed with various substrates which provide gelling properties and higher viscosities such as agar (Hamburger and Salmon 1977), agarose (Ayres 1982) and methylcellose (Davis et al. 1982). An advantage of semi-solid media comes from cells tending to secrete soluble protein products, since this can be detected and quantified (Gibson et al. 1993, 1997). Gibson et al. demonstrated that the standard preparation of cell culture media with 0.5 % agarose can be used to isolate clones that are high-producers from other clones in the culture by the use of a specific bivalent antibody to the secreted product. Adding an antibody that binds to the secreted product at a specific concentration produces a halo-like precipitate in the media due to the formation of capture-antibody/antigen complexes, which can be microscopically evaluated. When this method was first introduced (Gibson et al. 1993), it had poor mammalian cell growth and not all halos were easily viewed. The use of optimized capture-antibody concentrations, methylcellose instead of agarose and phase microscopy has resolved most of the difficulties

with this technique and it has been reported that the selection of clones can be done in half or a quarter of the time taken for LDSCC (Lee et al. 2006). Before the arrival of automated cloning techniques, semi-solid suspension cell cloning was the preferred method in biopharmaceuticals such as Janssen Biotech, Inc., which was formerly known as Centocor Biotech, Inc.

11.2.3 Semi-Automated Single-Cell Cloning

Micromanipulation devices have allowed further precision when working with microscopic samples and these devices have contributed to single cell cloning. The Quixell™ semi-automated system (Wewetzer and Seilheimer 1995) has taken the precision of the collection of cells through micromanipulation with fine pipettes and automated it to speeds that rival LDSCC. The Quixell™ system uses single cell manipulation via a glass micropipette controlled via a Z-axis drive to guarantee precise pipette positioning and a motorized microscopic stage, controlled manually through a keypad and joystick, to ensure single cell selection on an inverted microscopic setup. The system is a version of capillary-added single cell cloning, which is semi-automated, since it has the ability to use programmed destination locations within micro-wells on a plate. The micropipette can gently draw in a single cell or eject one via a Peltier mechanism, heating and cooling the volume of air in the micropipette. Two culture plates are placed on the stage, one a "source", usually a Petri dish type culture plate, and the other a micro-titer well "destination" plate, although variations of this setup can exist. The operator can use the joystick and keystroke movements to select a single cell from the "source" plate and transfer the cell to the "destination" plate. This system has a much better probability of achieving monoclonal cultures at a faster rate than LDSCC and early systems allowed a skilled operator to select and transfer a single cell in less than a minute. Wewetzer and Seilheimer determined the cloning efficiency (the percentage of viable seeded cells that formed colonies) of the Quixell™ system compared to LDSCC and gave similar results. To summarize, the Quixell™ system has been demonstrated to be reliable and minimally traumatic to cells (Wewetzer and Seilheimer 1995; Caron et al. 2000); however, when processing large numbers of single cells, it is still time-consuming, labor-intensive and overall a costly endeavor.

11.2.4 Fluorescence-Assisted Cloning and Selection

11.2.4.1 GFP and Other Fluorescent Proteins

After the discovery of the green fluorescent protein (GFP) from the bioluminescent Pacific Northwest Jellyfish, *Aequorea victoria* (Shimomura 1979), and the cloning

of the GFP gene in 1992 (Prasher et al. 1992), it became widely accepted as an excellent reporter protein given its fluorescence properties. This green fluorescing protein gains its fluorescence properties by absorbing blue wavelength light (peaked at 395 nm) and emitting green light (peaked at 509 nm) in the lower half of the visible spectrum. The main advantage of GFP is that the protein does not require any co-factors or substrates, except for oxygen, to fluoresce, thus making it ideal for examination in living systems and real-time imaging techniques. In fact, GFP can be used for quantitative analysis since it has been established that the fluorescence intensity of GFP and level of expression have a positive correlation (Subramanian and Srienc 1996; Meng et al. 2000). Although GFP is a commonly used reporter, there are many other fluorescent proteins that are either mutants of GFP or fluorescent proteins or mutants derived from other discovered life-forms apart from *Aequorea victoria* (Cormack et al. 1996; Kremers et al. 2011). The one major drawback of GFP is that it tends to decrease in fluorescence efficiency at temperatures higher than room temperature; thus, a point mutation variant commonly named enhanced green fluorescent protein (EGFP) has improved folding efficiency at 37 °C, allowing improved detectable fluorescence in mammalian cultures (Zhang et al. 1996; Cormack et al. 1996; Thastrup et al. 2001). The properties of GFP and its variants have thus given way to alternative selection methods that can improve cell selection efficiency; however, it must be kept in mind that the possibility of cytotoxic effects can exist with the use of fluorescent proteins like GFP (Liu et al. 1999; Baens et al. 2006), especially if these proteins are expressed at high levels. As a result, these proteins may be detrimental to the platform cells and produce an overall negative impact on cellular function.

When selecting recombinant protein-producing cell lines, GFP can be co-expressed with the recombinant protein of interest (POI) to act as a detection marker for expression of the POI and can also reflect expression levels. A common element that can be engineered into a plasmid to create a co-expression construct is an internal ribosome entry site (IRES), which is a ribonucleic acid (RNA) structural nucleotide sequence that enables translation initiation in the middle of a messenger RNA (mRNA) sequence in eukaryotes by recruiting ribosomes for the initiation of translation. Thus, two recombinant target proteins linked by an IRES element can be translated from a common mRNA and allow for the expression level to closely correlate with the relative expression level of the co-expressed protein. Internal initiation of translation was first observed for viral RNAs during picornavirus infection (Jang et al. 1988), in which 5' non-translated regions (5'NTRs) were shown to contain sequences that initiated translation of viral proteins.

With the use of IRES and fluorescence, the LDSCC technique can be modified, as shown in a 2010 study where researchers were able to identify clones with unstable transgene expression by the decline in EGFP fluorescence using a microplate reader for fluorescence detection (Freimark et al. 2010). Two bicistronic plasmids with IRES elements, one containing the recombinant target protein human vascular endothelial growth factor A (hVEGFA) and EGFP and the other containing human insulin-like growth factor 1A (hIGF1A) and EGFP, were used to correlate overall expression level of each recombinant protein with that of EGFP.

LDSCC was carried out and single cells were identified before incubation for 2–3 weeks, after which they were screened in a micro-plate reader. This method can reduce labor and time required to identify high-producers; however, although EGFP cytotoxicity was not detected for these clones, it still might have drawbacks, as specific productivity did not reach the high levels seen in commercial manufacturing cell lines. As a result, there may not have been enough EGFP to induce a cytotoxic effect in this study.

The Quixell™ method has also been modified to detect fluorescent proteins, and was setup to select cells transfected with bicistronic-based vectors co-expressing the transgene of interest and a fluorescent protein such as GFP (Caron et al. 2000). To avoid a cytotoxic effect, expression took place using a *tet*-regulated promoter (Gossen and Bujard 1992, 1995; Gossen et al. 1995) in order to allow doxycycline-induced expression for only 6–24 h, which was sufficient for fluorescence visualization. The combination of the Quixell and the fluorescent co-expressed proteins to assist in cell selection meant that target cells could be isolated at frequencies as low as 1/100,000 with a mean cloning efficiency of 40 % reached for both adherent and suspension cell lines (Caron et al. 2000). Although this technique has improved efficiencies with the use of fluorescent proteins, it remains a time-consuming process for commercial scale applications where thousands of clones need to be assessed and selected from a population of cells in order to isolate rare variants with the stable phenotype sought. Moreover, the use of co-expressing reporter proteins reduces the growth potential of cells as more energy needs to be diverted to protein production since two proteins are transcribed and translated at high levels.

11.2.4.2 Fluorescent Conjugates

Fluorescence does not need to be biological in origin. Compounds such as Fluorescein isothiocyanate (FITC), a derivative of the synthetic organic compound fluorescein, with an excitation peak wavelength of 495 nm and emission spectrum peak wavelength of approximately 519 nm, have been used in the cloning process when conjugated to MTX, forming F-MTX (Gapski et al. 1975). As MTX is a potent inhibitor of dihydrofolate reductase (DHFR), this property can be used in selection strategies where F-MTX can be used as a selection probe for MTX-resistant cells where increased fluorescence detection can correlate with increased transgene production (Kaufman et al. 1978). Kaufman et al. discovered that resistance to F-MTX was directly proportional to the *dhfr* gene copy number and in a later study (Kaufman and Sharp 1982) it was demonstrated that a *dhfr* cell line undergoing several rounds of selection pressure with increasing MTX concentrations could yield heterogeneous populations of low- and high-producers, with *dhfr* cells becoming resistant to MTX due to a decrease in membrane permeability or a change in the affinity for MTX (Assaraf and Schimke 1987). Ultimately, these heterogeneous populations of cells could contain up to 1,000 copies of the *dhfr* construct but, as some cells gain resistance to MTX, cloning needs to be done to select only the high-producers within the population.

Another method using FITC conjugates is with semi-solid media-based cloning, to look at cell secretion. Here, antibodies conjugated to FITC (or other fluorescent based molecules) can be mixed with a methylcellose media mixture to form a semi-solid media in order to detect secreted target recombinant proteins within the semi-solid media (Lee et al. 2006). Once the target protein is secreted into the semi-solid medium, precipitates can form with the corresponding fluorescently-conjugated antibody forming a halo that can be microscopically visualized around the cell or a colony of cells. This approach, which can be described as Fluorescent Labeling in Semi-Solid Medium (FLSSM), can be used to detect secreted proteins by fluorescently-labeled antigen or secondary antibody such as FITC-conjugated molecules in order to visualize high secretors (Caron et al. 2009). In a 2009 study of FLSSM in combination with the Quixell™ micromanipulator, it was found that a CHO and HEK293 cell population transfected to express either recombinant Insulin-like Growth Factor 1 (IGF-1) in both lines or Tissue-Plasminogen Activator (tPA) in CHO benefitted from FLSSM selection. After transfection of the cells, they were under hygromycin selection pressure for 2 weeks, after which they were either plated in a FITC- or Alexa 488-conjugated antibody-containing semi-solid media. Approximately 5 % of the population produced fluorescent halos around the cells. A Quixell™ system named the Quixell-FP™ was used to select fluorescent cells or colonies, so that the single cell clones could be chosen. By picking several clones, the secretion level compared to fluorescence intensity was assessed to give a positive correlation with the coefficient of 0.89. The FLSSM method in combination with Quixcell-FP™ can increase the efficiency of clone selection with high-productivity, by incorporating productivity measurements with single cell cloning; however, this still requires tedious selection work by skilled operators, making it a costly activity when it comes to screening thousands of clones on an industrial scale.

11.3 Selecting High-Producers with Flow Cytometry

The flow cytometer was invented in the late 1960s and advanced at a remarkable pace, evolving to the flow cytometer technology used today, which includes high speed cell sorting systems. Flow cytometer-based cell sorting systems first invented by Richard Sweet, Bill Bonner, Russ Hulett, Len Herzenberg, and others to carry out the sorting of viable cells, were an evolution of the first electrostatic cell sorter developed by Mack Fulwyler, based on the Coulter principle (Herzenberg et al. 2002; Fulwyler et al. 1969; Hulett et al. 1969). The first cell sorter was commercialized in the 1970s, starting with the Fluorescent Activated Cell Sorter (FACS) from Becton Dickinson, the company which trademarked the acronym FACS. The Becton Dickinson-based FACS instruments and other flow cytometric cell sorter (FCCS) instruments have become important research and analytical tools since their inception, with the term FACS becoming a generic term for any FCCS instrument. The history and development of cell sorters and flow cytometers is an

interesting read and several reviews have been written on the subject (Melamed and Mullaney 1979; Herzenberg et al. 2002; Givan 2004). The flow cytometer has the ability to perform high speed cell sorting of viable cells undergoing multiparametric analysis through the use of a polychromatic detection system. With this ability, flow cytometry has found many important uses from cell enumeration of rare cell phenotypes in heterogeneous populations to cell physiology and nanoparticle analysis. These capabilities mean that the flow cytometer is an excellent instrument for analyzing cell population molecular and biochemical events, which include selecting specific identified cells out of the population for further analysis.

The ability to use the flow cytometer as a research tool for mammalian cell culture in the context of biopharmaceutical cell lines has been of significant importance since the 1990s (Al-Rubeai et al. 1991; Al-Rubeai and Emery 1993), employed as a tool to study mammalian cell line physiology in bioreactor cultures and due to its role in improving cell line development approaches (Weaver et al. 1995; Al-Rubeai 1995; Holmes and Al-Rubeai 1999). The time taken for the development of biopharmaceutical protein production cell lines can be reduced via flow cytometry, where it is utilized as a high-speed cell sorter for selecting specific productivity phenotypes from a heterogeneous population, thereby allowing for speeds of cell selection that are not possible by manual visual detection. Although flow cytometers have mainly been used in the area of research and development, in recent years, it has been of particular interest for use in industrial processes with the introduction of process analytical technology (PAT) as a means of enhancing process monitoring and control. In this scenario, flow cytometry can offer the ability to further understand process kinetics that can lead to the improvement of the overall manufacturing process (Read et al. 2010; Kacmar and Srienc 2005; Sitton et al. 2006; Zhao et al. 1999; Kuystermans et al. 2012), with some of the physiological characteristics of culture such as cell cycle, cell size, pH-altered cell states and apoptosis data aiding in developing reproducible bioreactor processes at optimum controlled culture conditions.

11.3.1 Cell Line Selection Via Cell Sorting

The technology that forms the basis of a traditional flow cytometer instrument is a combination of fluidics, optics, lasers, and electronic signal processors that are linked by control and analysis software. One of the central parts of a flow cytometer that allows it to carry out its main function is the flow cell, where hydrodynamic focusing takes place, creating a single file of cells, allowing the interrogation of one cell at a time under ideal conditions. FCCS instruments typically use two methods of sorting cells. The first is the electrostatic method, which relies on vibration applied to the nozzle in order to produce droplets. By charging the stream of droplets containing a cell of interest, the droplets can be directed into the corresponding collection receptacle using high voltage deflector plates. The electrostatic method allows for multiple receptors such as tubes, multi-well plates and

slides to be used to deposit selected cells. The second method is known as the mechanical method and involves the cells of interest being captured by a catcher tube when the sort decision is made. The cells can be diverted towards the catcher by applying an acoustic energy pulse but may also be collected by moving the catcher tube into the stream on some systems. With the mechanical system, a concentration module may also be used after the catcher tube but before tube collection, or the cell can be directly sent to a collection tube. The electrostatic droplet sorter is the most common high-speed sorter design (Arnold and Lannigan 2010) and is used for most high-speed sorting applications.

With the development of FCCS technology, high-speed cell sorting provides the ability to enrich and purify cells from a heterogeneous population with great accuracy while depositing selected cells in micro-well plates up to 1,536-wells or on slides, in order to clone the cells. The selection of cells to aid in the cloning process via cell sorting can be accomplished in several ways that can be summed up as sorting based on intracellular reporter technology, cell surface capture technology, the gel microdrop technique, matrix-based secretion assay, or secretion and capture technology.

FCCS can involve the use of reagents (such as antibodies) that have the potential to contain transmissible spongiform encephalopathies (TSEs) or viral components; thus, it must be carefully utilized with platform cells that will potentially be used for commercial manufacturing. As a result, these reagents must be prudently sourced to avoid any difficult to remove contaminations further on in the development and manufacturing process.

11.3.1.1 Intracellular Reporters for Cell Sorting

One way to select high-producer clones via flow cytometry is by co-expression of a fluorescent reporter protein originally engineered into the construct developed for target recombinant protein expression. Fluorescent proteins are commonly expressed as reporters in FCCS experiments; a mutant of GFP (GFPS65T) was one of the first to be recombinantly expressed in mammalian cells for flow cytometry analysis (Lybarger et al. 1996). Compared to GFP, GFPS65T is better suited for flow cytometers equipped with 488 nm lasers since it has better fluorescence properties with 488 nm laser excitation and a faster rate of fluorophore formation (Heim and Tsien 1996). Hence, Meng et al. (2000) also used GFPS65T as a second selectable marker in a two promoter-based construct which involved the expression of DHFR and the desired protein via one promoter and GFPS65T via the other promoter in order to select high-producer CHO clones using a flow cytometer. A good positive correlation between cell productivity and GFPS65T fluorescence intensity was observed and the study also found that three rounds of sorting, each followed by 2 weeks of growth, gave higher-producing clones with sixfold differences in specific productivity compared to clones grown under selection pressure of increasing MTX concentrations grown over the same time period (Meng et al. 2000). Although the time period for both the manual and cell sorting-based

selection was the same, the amount of effort required was greatly reduced by not having to perform ELISA for the selection of high-producers; also, the possibility exists of applying further selection pressure in conjunction with cell sorting. This was demonstrated with CHO cells co-expressing a metallothionein (MT) GFP fusion and the target protein, where the isolation of high-producers could be achieved within 4 weeks (Bailey et al. 2002) due to the combination of MT-based gene amplification combined with FCCS.

The development of fluorescent reporters for FCCS-based clone selection has given good insight into the stability and metabolic regulation of protein expression. It has been shown that GFP concentration in recombinant NIH3T3 mouse fibroblast cells that reach confluency increases significantly compared to exponentially growing cells (Zeyda et al. 1999), which is most likely attributed to the reallocation of energy from biomass production to other cellular metabolic machinery such as protein production. This same pattern in metabolic energy reallocation can be seen when exposing CHO cell lines to lower culture temperatures, where growth is reduced and protein production may be elevated and a lengthened production phase can result in increased yields (Fox et al. 2004; Hunt et al. 2005). This also shows that the correct time-point should be taken when measuring GFP fluorescence from a culture as the metabolic and kinetic state of the culture will play an important role in determining protein productivity.

A large majority of biopharmaceuticals are Mab's and since they are heterotetrameric structures composed of equimolar light and heavy chain polypeptides, the efficiency of antibody assembly depends greatly on the ratio of expression of these chains. The ratio of heavy to light chains has been shown to affect final antibody production titers (Schlatter et al. 2005), thus it is important to select cell lines that also have optimal heavy to light chain ratios for Mab assembly. FCCS experiments which provided an insight into optimal antibody expression in CHO cells based on heavy and light chain assembly first used two-color sorting for GFP and yellow fluorescent (YFP) genes that were fused to a recombinant antibody heavy and light chain genes, respectively (Sleiman et al. 2008). The fluorescently-fused antibody chains were co-expressed via an IRES-based construct. With the aid of metal amplification, the dual fluorescing clones that were selected via FCCS showed a 38-fold increase in antibody production within 12 weeks when compared to the parental pool from which they were originally isolated.

A second study that used FCCS to isolate cells based on proper antibody assembly as well as high-production utilizing GFP fragments (Kim et al. 2012) had two rounds of FACS performed on CHO cells co-transfected with two co-expression plasmids, one containing the heavy chain and a fragment of the GFP sequence (GFPa) linked via IRES and the other containing the light chain and the other half of the GFP fragment sequence (GFPb). Proper antibody expression was indicated by the assembly and fluorescence of GFP from reconstitution of the fragmented GFP halves (GFPa + GFPb = GFP). The results indicated that the sorted CHO cell pools, sorted on the basis of reconstituted GFP fluorescence, had higher antibody productivity than the unsorted pools and antibody productivity was positively correlated with fluorescence intensity (Kim et al. 2012). A drawback of

using fluorescent proteins for flow cytometry selection strategies is that intracellular reporters tend to continue to be expressed by the cell and would be a hindrance in downstream removal validation when expressing recombinants targets bound for human-based treatments, as apart from the metabolic burden that an extra-recombinant protein can impose, it may cause the increased productivity of only the target recombinant protein being expressed.

The development of a recombinase-mediated cassette exchange (RCME) system in combination with FCCS has been considered a possible strategy to engineer and select a master cell clone (Qiao et al. 2009). Here, an interchangeable gene cassette can be removed or inserted into a specific genetic region within the stably selected master clone after several rounds of FCCS and RCME. After establishing the master clone, this clone can undergo RCME so that it does not retain the original fluorescent reporter protein expression used to isolate the high-producing clone during FCCS and instead has the gene of interest inserted in the genome for stable high-level expression of the target recombinant protein. A site-specific recombinase named FLP, isolated from *Saccharomyces cerevisiae* (Broach and Hicks 1980), mediates the double reciprocal crossover between two heterospecific recombination sites (RTs) that flank both the insertion cassette and target site on the genome (Schlake and Bode 1994). The use of a variant Flp enzyme mutant for improved performance and specific sets of functional target sites (FRTs) has been part of major efforts to improve the RCME system (Turan et al. 2011, 2013) making it possible to employ a tag and exchange strategy together with FCCS to select high-producer cells. A 2009 study by Qiao et al. showed that a master clone cell line could be produced from CHO-K1 cells in 2 months with the possibility of producing the production cell line in 1 month from the established master cell lines using FCCS to select and sort cells throughout the procedure. The basis of the procedure is that cell sorting is utilized to first isolate cells transfected with the *gfp* reporter cassette before the first RCME in order to preselect for cells with active transcription sites. In addition, the promoter is placed upstream of the FRT sites so that exchange cassettes are only expressed upon RCME integration. After the first RCME, where the *gfp* cassette is exchanged for a hygromycin thymidine kinase fusion gene cassette, FCCS counter-selection for cells that lost their fluorescence takes place, which is later restored with the introduction of a second GFP cassette with a second RCME and run through the cell sorter in order to obtain the master clones with a stable exchangeable loci. The overall results from RCME technology combined with FCCS are master clones that can achieve close to 100 % stable integration of the gene of interest into the genome with antibiotic selection and 10 % efficiency of integration without antibiotic selection (Qiao et al. 2009). The use of pre-engineered master cell lines can speed up the overall cell line selection procedure in combination with technologies such as FCCS and could be a potent tool for reducing development timelines.

Another approach for the application of flow cytometric sorting combined with RMCE technology is to select cell lines based on endoplasmic reticulum (ER) stress, as this was demonstrated to be a good indicator when a master cell line engineered via RMCE had an inducible GFP reporter construct under the

control of the GRP78 truncated promoter to indicate ER stress (Kober et al. 2012). When IgG Mab expression took place, the GFP ER stress reporter provided a correlation between the GFP fluorescence intensity and antibody expression level following the paradigm that overexpression of a recombinant protein would cause an ER stress response, hence GRP78 expression would increase correlating to the expression level of the target recombinant protein allowing for FCCS selection of high-producer pools from millions of cells. FCCS and RMCE can be a good combination strategy for enriching and selecting master clones, including the further development of stable high-producers with decreased genomic rearrangements of the gene of interest. However, further long-term stability studies might be needed on the master clones and derived high-producers to determine feasibility in commercial environments.

The use of F-MTX as a reporter in selecting high-producers can be adapted to a flow cytometry sorting strategy for DHFR cells, improving on LDSCC by reducing the labor and time required to carry out gene amplification of a cell population. By sorting cells with high fluorescence intensity of F-MTX, it is possible to obtain cells with high copy numbers of the *dhfr* gene (Yoshikawa et al. 2001), as demonstrated with CHO *dhfr*-cells transfected with a *dhfr* construct for producing human granulocyte macrophage colony stimulating factor. However, what must be taken into account is that in previous studies (Yoshikawa et al. 2000a), it has been observed that *dhfr* copy number is not always an indication of *dhfr* activity, as cells with one copy of the *dhfr* gene can have similar levels of DHFR expression as cells with many copies of the *dhfr* gene. Also with the addition of a gradual increase in MTX concentration, it is now known that highly-productive cell pools can be obtained with a high ratio of telomere-type cells (integration of the *dhfr* gene close to the telomeres), which have also been shown to have a high specific growth rate compared to other clones in the same population (Yoshikawa et al. 2000b). Thus, the positive correlation in regards to F-MTX fluorescence intensity to high-producer cell lines is after the gene amplification process and limited to cells with *dhfr* integration close to the telomeres tolerant for the cell sorting process to be able to identify the high-producers from the gene amplified heterogonous population without the use of LDSCC.

11.3.1.2 Cell Surface Expression

In the past, the specific productivity of some hybridoma cells had been observed to correlate with surface antigen expression (Sen et al. 1990; Charlet et al. 1995; Marder et al. 1990; McKinney et al. 1991). Upon further investigation of certain hybridoma lines that were separated based on the fluorescently-labeled antibody bound to their cell surface and compared to the specific antibody productivity (Marder et al. 1990), this correlation was further demonstrated with various isolated clones via flow cytometry sorting. The cell sorting demonstrated that clones with medium to high fluorescence intensity showed increases in the expression of antibodies based on intensity, but cells with low fluorescence had no detectable

antibody production. This correlation may depend on the phase of culture that the hybridomas are in (Leno et al. 1991; Charlet et al. 1995) and does not always stand true for all hybridoma cells (Meilhoc et al. 1989) or clones, as Marder et al. (1990) also observed that sorting bright surface fluorescing cells had outliers that did not always produce high-secreting clones.

Although, in some cases, cell surface expression can be used as a basis of the productivity of a hybridoma cell line, it was not until the early twenty-first century that other cell lines such as CHO were investigated for similar properties. To prove that the same correlation can be applied to secreted recombinant proteins from CHO cells, these recombinant proteins were targeted with fluorescent antibody stains in order to capture the recombinant protein on the surface of the cell during secretion (Brezinsky et al. 2003). After analyzing 23 CHO cell lines, the work demonstrated that a correlation also exists between recombinant protein productivity and the fluorescent intensity of cell surface-associated recombinant protein; further preliminary studies on NS0 cells showed similar results. For the main part of the study, in order to slow down the secretion kinetics and extend the time that the secreted protein was in contact with the cell surface membrane, the temperature was lowered to 4 °C (Fig. 11.2); however, the study also generated comparable signals at temperatures of 23 °C and 37 °C and it should be noted that previous stability studies using FCCS to sort hybridomas on the basis of surface-associated antibody content have found that fluorescence is stable for more than 1 h at room temperature (Kromenaker and Srienc 1994). The work by Brezinsky et al. (2003), reported 25-fold increases in the specific productivity without the use of prior gene amplification, and when MTX selection pressure was applied prior to FCCS, the resultant specific productivity was a 120-fold improvement. Further studies using this cell surface expression assay found that fluorescent signal was stable for 24 h at 4 °C but

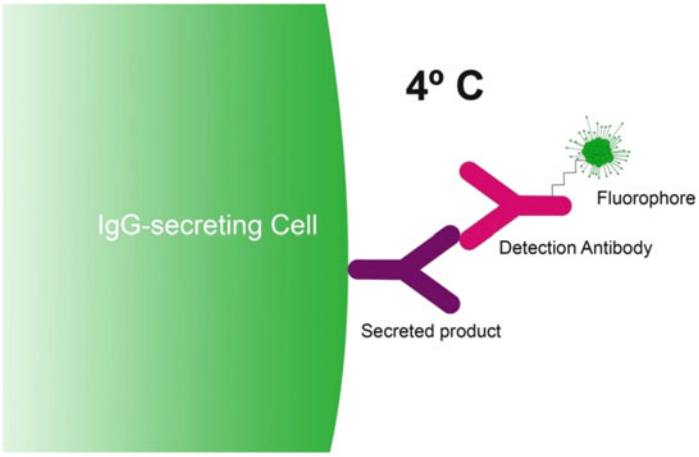

Fig. 11.2 When cells are transferred to a lower temperature of 4 °C, the conditions slow down the mechanism of product secretion from the secretion vesicle, which enables the surface capture and staining of secreted protein on the cell surface via a fluorescently-labeled antibody

decreased significantly at higher temperatures to approximately 2 h at 37 °C; in addition, it was observed that secreted product is trapped on the cell surface at all of the temperatures studied (Pichler et al. 2009), reacting similarly to an immunoprecipitation reaction. This FCCS technique of selecting high-producers via low temperature capture has also been applied to HEK293 cells (Song et al. 2011) and to generate high-producer CHO cells from a stable transfection pool within 2 months (Ye et al. 2010); however, the stability was compromised for the FCCS-enriched populations over time with the increased number of sorts that were performed. Overall, the low temperature capture method shows great promise for decreasing the time needed to select high-producers, but stability studies must be performed after FCCS enrichments to determine the optimal number of sorts before genetic instability becomes an issue as well as feasibility, as a commercial platform may be compromised.

CHO cells do not normally express CD20 as a cell surface protein; thus, by co-expressing CD20 with the POI, CD20 can act as a reporter protein for measuring the productivity of a cell via anti-cd20 antibody fluorescence during FCCS analysis. The genes encoding CD20 and the POI are linked by an IRES-containing bicistronic vector (Gurtu et al. 1996) to allow the simultaneous independent expression of both the POI and CD20 reporter originating from the same mRNA transcript. Since CD20 and the POI originate from the same mRNA transcript, the CD20 expression level can indicate a relative expression level for the POI (Liu et al. 2000), although most cellular energy resources will be targeted towards translation of the POI, as the CD20 protein is located downstream of the IRES site which is known to have a weaker translation efficacy (Mizuguchi et al. 2000) than mRNA transcript structures translated upstream of IRES. The FCCS screening using the CD20 reporter construct was performed in CHO DXB11 and DG44, providing a 96-well clone plating procedure that successfully eliminated unstable clones at an early stage of the cell line selection process whilst identifying high-producers (DeMaria et al. 2007). An advantage of this method is that, unlike the low temperature capture method, the CD20 antibody is widely available and does not require a specific antibody towards the POI. The drawback of this method, as with other methods employing separate reporter proteins that are co-expressed, is that this still ultimately requires cellular energy resources to place undue stress on the protein synthesis with the production of the CD20 reporter.

Further work done with a cell surface protein utilized as a reporter that is co-expressed with the POI, where the aim would be to lower the amount of translated reporter protein to still detectable levels in order to correlate with POI expression and reduce the overall impact of the reporter on POI production, has yielded positive results. By engineering an expression cassette containing a reporter with a non-ATG initiation codon in the open reading frame (ORF), and locating the POI gene directly upstream of the reporter ORF, it is possible to obtain both ORFs from a single mRNA transcript, although independently translated, with the aim of expressing the POI at a much greater frequency than the reporter protein. A study used this very concept to express CD52 variant as a cell surface reporter on CHO clones which do not normally express CD52, thus making it an ideal candidate for

cell selection via FCCS with FITC anti-CD52 antibody for detection (Cairns et al. 2011). Similar to the earlier study utilizing CD20 (DeMaria et al. 2007), the CD52 cell surface marker protein was a reporter but, unlike CD20, the CD52 cell surface protein has a much lower probability of translation compared to the target mRNA ORF coding the POI, in turn reducing diverted energy from POI production. Cairns et al. (2011) reported that the resultant FCCS selected CHO cell lines have a significant increase in productivity compared with those pools sorted under LDSCC conditions alone and that this FCCS method of high-producer cell selection has been attempted for ten different target molecules without any observed expression impairment. The non-AUG reporter system can be of great benefit in early cell development stages of selecting appropriate high-producers from a large pool of cells in only 1 or 2 h before further growth expansion in order to assess growth kinetics and long-term stability. As with most of the sorting methods discussed, multiple sorts can be run to improve upon cell line selection, but expression stability should always be monitored to assess suitability to the manufacturing environment.

11.3.1.3 Gel Microdrop Technique

The Gel Microdrop (GMD) assay for secretion requires that cells in a population are encapsulated in an agarose-based microsphere to form a porous gel matrix around the cell. Although not as common, other gel matrices such as calcium alginate could also work, in which gelation can occur with ion exchange instead of a temperature shift (Weaver et al. 1984). Since first reported by Gray et al. (1995) optically-clear gel microdrops (GMDs) with a high level of capture sites can be made up of a biotinylated agarose-like matrix incorporating streptavidin that can act as a bridge between the biotinylated matrix and biotinylated capture antibody. Formation of these GMDs creates a microenvironment where the recombinant POI is retained within close vicinity of the cell and can be detected via the addition of a fluorescently-labeled antibody that is also targeted towards the secreted recombinant POI. The generation of GMDs requires specialized equipment such as the One Cell Systems™ CellSys 100™ microdrop maker to carry out the process of emulsification of a two-phase system of aqueous agarose-based cell suspension, and a non-aqueous liquid such as dimethylpolysiloxane through vigorous vortexing allows for microdrop formation and solidification when the temperatures are cooled to 4 °C. By only introducing a low concentration of cells into the agarose mixture, a one cell per microdrop occupancy can occur based on Poisson statistics with a preparation usually resulting in 10–15 % of GMDs containing a single cell (Weaver et al. 1997). Since these drops range in size from 10 to 100 μm in diameter, with one cell occupancy mostly observed in a mean size drop of 25 μm, they are small enough for flow cytometric sorting. When these drops are sorted via FCCS, the fluorescent intensity that each microdrop emits can be correlated to the productivity of the encapsulated cell. Diffusion within the GMD can rapidly reach a steady state (Weaver et al. 1995) and take place at a rapid rate with molecules as large as 500

+kDa due to the microdrops' small size and permeability, while the capture antibody matrix that is formed acts like a selective net for the POI.

The technology encompassing GMD in combination with FCCS was first carried out with mammalian cell systems in the 1990s after being applied to microbial systems (Williams et al. 1987). Prior to using GMD for the selection of high-producers, the mammalian cell line studies with GMD involved separating secreting hybridomas from the non-secreting and low-producing cells (Powell and Weaver 1990; Hammill et al. 2000). Apart from the selection of secreting cells and high-producer populations, GMD technology in mammalian cells can be used to identify and select antigen-specific hybridomas from mixed populations (Gray et al. 1995; Kenney et al. 1995) and for cytotoxicity screening assays (Bogen et al. 2001). GMD technology has also been applied to the selection of high-producer mammalian cells in the production of cytokines (Atochina et al. 2004; Turcanu and Williams 2001).

With GMD combined with FCCS, selective sorting can isolate rare high-producer cells with reported increases in MAb protein production being in the range of twofold to fivefold (Weaver et al. 1997). However, the quantitative success of this assay depends on the GMDs capture sites not being saturated, which can be observed if the unoccupied GMD remain non-fluorescent to determine a baseline and can thus act as an internal control so that individual cell secretion can be monitored independent of microsphere size. After the FCCS procedure of sorting the selected encapsulated cells, the cells can be allowed to grow out of the gel or an enzymatic digestion of the agarose gel with agarose can take place to recover the viable cells. Although viable cells can be recovered, sometimes recovery can be stress-inducing for the cells and in the case of agarose outgrowth, may not always be successful.

11.3.1.4 Matrix Based Secretion Assays

The use of an avidin/biotin matrix system to enrich a population of high-producers has also been applied to another method apart from GMDs. Here the capture matrix is not within a droplet but rather on the surface of the cell, with the capture matrix generated for use in a method known as affinity capture surface display (ACSD). This technique was first described as a way to sort hybridomas and lymphocytes on the basis of IgM antibody (~600 kDa) and IFN-gamma cytokine (~34 kDa) secretion, respectively (Manz et al. 1995), but was soon modified to maximize the binding capacity of the matrix and ensure a greater capture surface area, reduce stearic hindrance, and use commercially-available reagents, thereby developing a refined method for selecting high-producer cell lines (Holmes and Al-Rubeai 1999).

The ACSD technique described by Holmes and Al-Rubeai (1999) demonstrated the effectiveness ACSD with NS0 cells secreting recombinant antibody, a cell line that is known to have viability issues when used with the GMD technique of isolating high-producers from a population. The capture matrix is composed of biotinylated molecules such as succinimidyl-6-(biotinamido)hexanoate NHS-LC-

biotin and a neutravidin bridge bound to a biotinylated antibody that is specific for the secreted POI. NHS-LC-biotin is a derivative of D-Biotin, containing a spacer arm off the valeric acid side chain, terminating in an NHS ester; the spacer arm provides a greater length compared to NHS-biotin, which contains no spacer arms (~13.5 Å length) and reduced steric hindrance of the whole modified biotin moiety (~24 Å length), resulting in better binding potential for secreted POI, since more binding sites are possible due to the increased surface area of the avidin linker. The capture process requires the use of a highly viscous medium of agar, methyl cellulose, or gelatin at 10 % w/v to minimize secreted recombinant POI diffusion, which can result in "cross-talk" between cells and ensure that secreted POI binds to the matrix of the cell that the POI was secreted from.

Once the capture process takes place at 37 °C, during a short incubation period of 10–30 min, the bound POI can be detected via the addition of an FITC-conjugated antibody and an FCCS procedure (Fig. 11.3a). One limitation that must be considered is the actual incubation time and that it should not exceed saturation of the secreted POI to the biotin moiety, as this would render the assay ineffective in correlating the fluorescent signal observed to specific productivity and assess high-producers from low-producers; thus, this must be carefully monitored for every cell line.

Further modifications to ACSD have been made by Lonza Biologics and the Al-Rubeai Lab. Lonza Biologics adopted ACSD and patented the modified technique for sorting of high-producers by replacing the capture antibody with either Protein A (Fig. 11.3b) and Protein G, which both bind specifically to the Fc regions of immunoglobulins and Protein L, binding through light chain interactions (Racher and Singh 2005). Here, the use of a binding polypeptide protein, which is a main component that is also used in chromatography resins for its affinity properties, is utilized instead of requiring the use of a specific antibody towards the POI. The binding polypeptide can be either Protein A (Langone 1982) derived from *Staphylococcus aureus*, Protein G derived from streptococcal (Sjobring et al. 1991) or Protein L derived from the Peptostreptococcus species, which has a binding affinity towards the kappa light chains of antibodies without interfering with the antibodies antigen binding ability (Kastern et al. 1992), gives further flexibility to target a variety of recombinant cost savings to the assay. Carroll and Al-Rubeai (2005) adapted the ACSD technique with the use of magnetic separation for selecting high-producers. Here, a non-FCCS technique was employed with ACSD, where a secreted antibody is captured by a biotinylated antibody that is specific for the secreted mAb, which, in turn, is detected by the FITC-conjugated antibody with affinity towards the secreted mAb followed by an anti-FITC microbead (Fig. 11.3c) that is added for magnetic separation (Carroll and Al-Rubeai 2005). The study found that ACSD combined with a magnetic separation method was also viable for the rapid isolation of high-producer cells when the results were assessed with flow cytometry analysis.

The advantage of the ACSD is that when compared to the GMD technique, the cells do not need to be encapsulated, and can have a higher survivability throughout

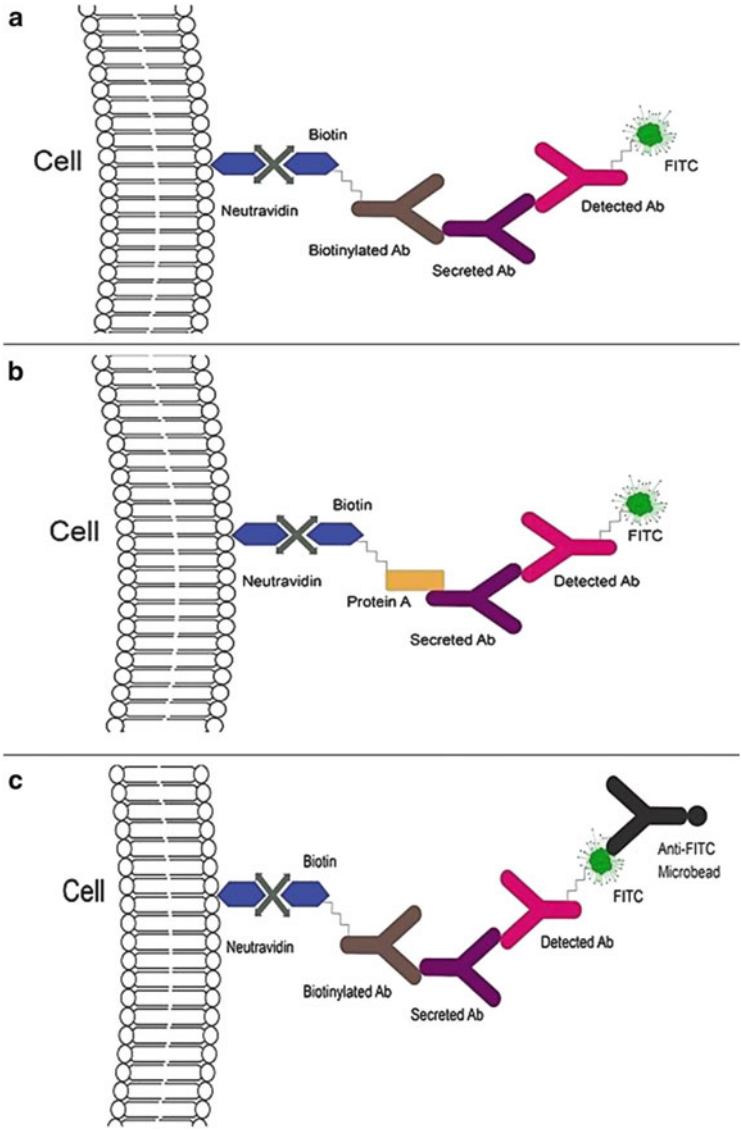

Fig. 11.3 The cell surface is biotinylated and the cells are allowed to secrete the product which is captured close to the surface of the cell in order to form a complex, thus aiding detection. With (**a**) affinity capture surface display (*ACSD*), the secreted product is captured by the biotinylated antibody that is specific for the secreted product. Since the capture antibody is anchored on the cell surface via an avidin or neutravidin bridge, the product is immobilized close to the cell surface and FITC-conjugated antibody is added to allow for product detection. With the modified ACSD approach (**b**), the biotinylated capture antibody can be replaced with protein A to enable the capture of the Fc portion of an antibody or Fc-fused protein that can be detected via an FITC-conjugated detection antibody that is specific for the secreted product. The use (**c**) of an anti-FITC microbead allows the ACSD strategy to be adapted to *magnetic column* cell selection, since cells that pass through a *magnetic column* can be captured if they are secreting the POI while non-secreting cells pass through the *column*

the process. ACSD allows for a larger percentage of cells to be analyzed for productivity as the majority of cells are labeled compared to only 5 % for GMD FCCS analysis; however, what must be considered is the surface area differences between matrix-based assays and GMDs, since with GMD, the saturation limit is theoretically an order of magnitude higher than that of matrix-based assays (Frykman and Srienc 1998). The timing of incubation is of critical importance; where 10–30 min can suffice in most cases before FCCS analysis to avoid saturation. When carrying out the FCCS analysis, it must be remembered to always account for cell size and the influence it has on the total fluorescence signal. Thus, forward scatter against fluorescent intensity plots can be included to help setup parameters and distinguish the influence of cell size on fluorescence signal obtained for selecting high-producers from the population.

11.3.1.5 Secretion Display Technology

One other flow cytometry sorting technique for high-producer selection is flow cytometry-based autologous secretion trap (FASTR). This technique is a patented technique (Fandl et al. 2008) trademarked under the VelociMAb™ name by the biotech company Regeneron. This method relies on the co-expression of the target antibody and a doxycycline-inducible membrane-bound cell surface Fc receptor (FcR) that binds to the Fc portion of the secreted target antibody when induced so that it can be displayed on the cell surface. Once cell surface display takes place, the high-producers are sorted via detection by an anti-Fc fluorescent molecule and an FcR blocking molecule is added to the sort to prevent any "cross-talk" of non-expressing cells. In order to address the problem of receptor saturation, the secreted antibody bound to the FcR complex is continually internalized by the cell (Browne and Al-Rubeai 2009). This technology can be used to select high-producers from a heterogeneous pool via FCCS and induction can be turned off once high-expressers are isolated, so that only the target antibody is secreted, reserving cellular energy resources for only the required tasks during commercial manufacturing and easing the load on downstream processing.

11.4 Advances in Productivity Screening and Clone Selection Technologies

11.4.1 Beyond ELISA

The use of ELISA as a step to determine the protein productivity of clones has been known to be part of the labor-intensive steps in the cloning and selection process; however, in recent years, advancements in the technologies used for POI detection have been able to reduce the time taken for this critical step to help to isolate rare

high-producing cell lines. Two of these are label-free technologies that have been commercially introduced, known as Homogeneous Time Resolved Fluorescence (HTRF) and Bio-Layer Interferometry (BLI). Both of these are much faster at giving protein titer results than conventional ELISA techniques, are label-free, and can have the analyses of POI, such as an Mab, integrated within automation platforms to further increase sample analysis and overall development throughput.

11.4.1.1 Homogeneous Time Resolved Fluorescence

The combination of Time Resolved (TR) measurements and Fluorescence Resonance Energy Transfer (FRET)-based assays has provided the means to carry out the technique that is now known as HTRF (Mathis 1995). With HTRF, two fluorophores exist, a donor and an acceptor, where the donor fluorophore is made up of rare-earth lanthanides with long emission half-lives (Selvin 2002) and the acceptor with a short emission half-life allowing for the transfer of energy between each other when in close proximity (Mathis 1999, 1993; Bazin et al. 2002), which is the basis of TR-FRET. HTRF eliminates a lot of noise and background that is inherently found in the typical fluorescent bioassay (Degorce et al. 2009), by introducing a time-delay of up to 150 μs between the initial light excitation and measured fluorescence, minimizing the introduction of short-lived fluorescence emissions from other materials such as other compounds, proteins and the medium itself, for example. When the donor fluorophore, in HTRF, fluoresces with a long emission due to the acceptor fluorophores short emission, it will be the only significant fluorescence detected due to the introduced time-delay for detection.

The central core of the energy donor in HTRF can be either a Europium cryptate (Eu3+ cryptate) or Terbium (Tb2+ cryptate), which is surrounded by a cage structure that acts like a receiver for both energy collection and emission at a specific fluorescent pattern (Degorce et al. 2009). Two of the main acceptors that have been developed for HTRF are near infrared acceptors known as XL665 (Mathis 1993) and d2 (Degorce et al. 2009). The acceptor XL665 is derived from the red algae and is a phycobiliprotein pigment of 105 kDa cross-linked after isolation for improved stability while d2 is similar in properties to XL665 but has an organic structure much smaller than XL665 reducing steric hindrance when needed. The emission wavelength of the donors are 620 nm while the acceptor is 665 nm, an advantageous property since the 620 nm wavelength can be used as an internal reference and emissions at 665 nm as an indicator of the biological reaction with the ratio being used in a ratiometric analysis to correct for well to well variability and any signal quenching (Dumont et al. 1996).

The use of HTRF in protein quantification such as for human antibodies and other therapeutic proteins has made this new technology a good replacement for ELISA assays in quantifying protein productivity of cell lines during the selection process. One specific assay can use an IgG antibody with the Fc portion conjugated to Eu3+ cryptate and an IgG antibody conjugated to XL665 (Degorce et al. 2009) in 96-, 384-, and 1,536-well plate formats, where the assay can be performed within

2.5 h, making it a time-saving and cost-effective alternative to ELISA. One example of a developed assay for MAbs has been demonstrated in a previous study (Idusogie et al. 2008) where the objective was to detect the MAbs or Fc fragments through *Staphyloccola* Protein A binding to Fc in culture supernatants. Here, the assay was performed seeking an inversely proportional response of fluorescence intensity to the concentration of detected and bound Fc in a sample which had an Eu3+ cryptate-labeled polyclonal rabbit IgG (Eu3 + IgG) with Protein A XL665 (PAXL665) added, so that when the FC protein is present in samples it will displace the binding of PAXL665 with Eu3 + IgG, reducing the TR-FRET response. The assay requires between 10 and 50 μl of cell supernatant and can be read on the appropriate plate reader assigned to read the two different wavelengths of 620 and 665 nm to provide a ratiometric reading (665/620 nm) above one. The study also observed that antibodies of slightly different conformations, which may affect the Protein A binding site, gave different results; thus, care must be taken when quantifying the productivity of clones if the Fc protein used as a standard is not the same. In the end, a standard curve would need to be generated with each experiment to determine sample titer and might hinder throughput in some automated systems unless it is built into the automation protocol.

HTRF assays can incur approximately the same cost as an ELISA assay, but the overall time saved compared to ELISA can make this a valuable tool when assessing clone productivity for cell line selection. As this assay can be integrated into an automation platform for high-throughput screening purposes, it will also save on the overall costs in comparison to ELISA at the same scale.

11.4.1.2 Bio-layer Interferometry

The use of alternative technology such as BLI has made it possible to carry out high-throughput screens of clone samples where the titer measurements may provide not only the specific productivity but can normalize the functional activity of the POI of each clone. BLI was first introduced in 2006 by the founders of ForteBio, an instrument manufacturer, as a technique based on phase-shift interferometry (Cooper 2006; Tan et al. 2008; Zuk et al. 2008). The principle workings of the method involve the detection of two light reflections from a single source, producing interference that allows for measurement of the aggregation of a target molecule on the surface of a sensor. The white light source generated is focused onto a proprietary optical coating of the sensor probe through an optical fiber, where the majority of light passes through the coating except for a small reflected amount. The coating would have biomolecules attached, such as Protein A, G, L, or an antibody or ligand targeted towards the POI and the reflected light is brought back through the optical fiber to a spectrometer with some of the wavelengths of light subjected to destructive or constructive interference. The interference produces an interference pattern across the visible spectrum which is correlated to the thickness of the biological layer on the optical coating and as the thickness increases, the interference pattern red shifts, indicating the change in the biological layer

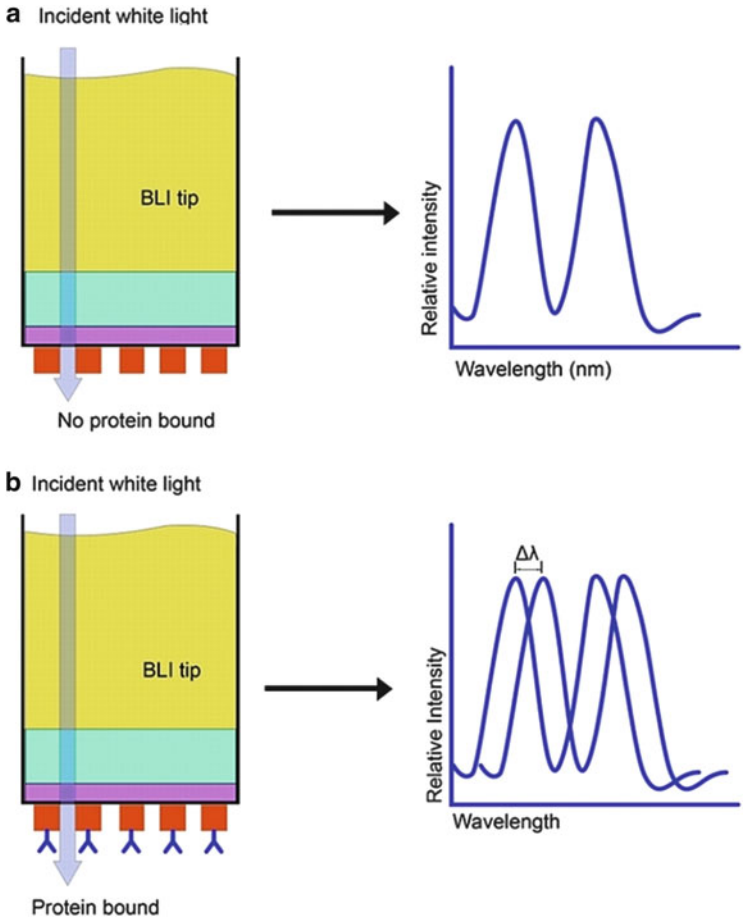

Fig. 11.4 Bio-Layer interferometry biosensor tip. (**a**) In this illustration, a protein A ligand (represented by *orange squares*) is immobilized on the biosensor tip surface where *white light* is shone down the biosensor. The light reflected back originates from the interface of the optical layer and from the surface of the biocompatible layer so that the two light reflections measure the optical thickness. (**b**) An antibody in solution produces an increase in optical thickness as it is bound to the protein A at the biosensor tip, which results in a wavelength shift, Δλ, which is a direct measure of the change in thickness of the biological layer in real-time, allowing for the measurement of binding kinetics, affinity and quantification

thickness in real-time (Fig. 11.4). As biomolecules associate or dissociate from the probe-coated surface, the change in interference patterns indicates the presence and concentration of a molecule in solution. Time course studies can also yield binding affinities giving binding specificity, with rates of association and dissociation.

Since BLI instruments eliminate the need for optical filters and binding measurements are not dependent on distance interactions found in FRET assays, this technique has become widely used in the biopharmaceutical industry for cell line

development and clone selection. Further advantages include the fact that crude samples can be analyzed, changes in refractive index in the medium do not hinder measurements, unbound molecules and flow rate do not affect the interference pattern with a number of studies done with BLI, demonstrating the real-time measurement of concentration and binding kinetics and affinities can be measured (Baselga et al. 2000; Che et al. 2009; Szolar et al. 2006; Abdiche et al. 2008a, b, 2009). To carry out these studies, ForteBio, currently a wholly-owned subsidiary of Pall Corp, has released a range of instruments geared towards BLI known as the Octet system, including a more portable single measurement instrument known as the Blitz. The Octet range of systems utilizes BLI to perform quantitative and kinetic analysis of the POI in culture media apart from other analytes of interest via the biosensor tip and can run 96, 384 and 1,536 setups depending on the system model and configuration.

A previous study has demonstrated that BLI shows a high degree of agreement with ELISA and that BLI gave a higher dynamic range of 0.4–50 μg/ml compared to ELISA (0.1–10 μg/ml) with the lower limit of BLI present due to BLI being biased towards over-recovery when lower sample concentrations were analyzed (Dysinger and King 2012). Overall, BLI assays shows good suitability for quantitative screening of cell culture media supernatants in cell line selection studies either as an ELISA replacement or complement.

11.4.2 Automation of High-Producer Cell Line Selection

11.4.2.1 Laser-Enabled Analysis and Processing

Scanning cytometry is a technology that encompasses some of the unique capabilities of flow cytometry with imaging technology, such as performing light scatter and fluorescence measurements that include spatial distribution of fluorescent probes and being able to run time course measurements by returning to a particular probed spatial target location for further evaluation or re-probing of the target location. The efforts of Kamentsky and Kamentsky (1991) and others helped to develop Scanning Cytometry technology and created a path to the development of instruments that are available today, which include instruments that have the capability of scanning cytometry combined with the ability to manipulate cells, such as laser capture micro-dissection (LCM) instruments (Bonner et al. 1997), laser pressure catapulting instruments (Buican et al. 1989) and, more recently, the Laser-Enabled Analysis and Processing (LEAP) platform from Cyntellect Incorporated, a company based in San Diego, California, USA. LEAP is a scanning cytometry instrument with roots in the semiconductor manufacturing industry (Koller et al. 2004) developed at Cyntellect Inc. The LEAP technology will be elaborated on as the platform can be utilized for cell line selection procedures with a high degree of automation, throughput and accuracy for the purpose of clone selection. LEAP also can be considered a combination of LCM and flow cytometry

cell sorting (Szaniszlo et al. 2006) allowing for a variety of high content analyses and manipulations that were not possible before its introduction.

The LEAP system is designed to carry out optical imaging of up to 100,000 cells per second and laser beam targeting at a rate of 1,000 cells per second, which enables high-speed imaging and cell manipulation. The optical imaging capabilities are greatly enhanced over conventional microscopy due to the flat field corrected F-Theta lens (Lin et al. 2008), providing a far larger area without any changes in focus or stage movements. The system has also incorporated a system of scanning galvanometric mirrors to allow for high-quality imaging at selected magnifications of a specified well (Hanania et al. 2005; Koller et al. 2004), with the large viewing field of the instrument providing direct viewing of one full well of a 96-well plate and four wells of a 384-well plate together with bright field and fluorescence imaging capabilities. The LEAP system is also designed with a pulsating laser with the ability to perform laser optoinjection assays and laser ablation to carry out live cell sorting. The optoinjection assays allow for high-speed microinjection of macromolecules via a pulsed laser at hundreds of cells per second at almost 100 % efficacy (Szaniszlo et al. 2006) and can be performed under sterile conditions, while the same laser can also selectively ablate cells, eliminating the selected cell from the population. The laser options on the instrument are an ultraviolet-355 nm and green-532 nm laser.

The Microsoft Windows-based LEAP software allows for gating of optically visualized phenotypes that can be automatically processed for both adherent and anchorage-independent cell lines. The system contains several software modules, with the main ones being Laser Targeting, Cell Counting, Cell Viability, Cell Xpress™, and the LEAP results Viewer; some of these modules contain further sub-modules (Lin et al. 2008). The multicolor images generated are used for real-time decisions that can be automated by setting the appropriate hardware and software parameters.

An LEAP cell line selection experiment can typically be setup with the use of Cyntellect™ 96- or 384-well plates. For suspension MAbs secreting mammalian cells, the plate containing the cells can be gently centrifuged at $51 \times g$ for 30 s, which will allow the cells to remain in the plate in a semi-attached state without damaging them (Szaniszlo et al. 2006), although laser power may require some adjustment to avoid cell movement while ablating cells. Optoinjection of gently centrifuged suspension cells has been shown not to remove the cells from a plate or slide surface (Szaniszlo et al. 2006; Lin et al. 2008) which makes this particular technique well suited for transfection experiments on suspension cells.

The LEAP system can be setup to identify high secretors after capture and staining of the secreted POI such as an antibody. The unwanted cells can simply be eliminated by laser ablation in each well that is analyzed. Currently, LEAP has been successfully demonstrated on several mammalian cell lines typically found in biopharmaceutical manufacturing environments such as CHO, NS0 and hybridoma cells, decreasing heterogeneity, and in some cases, being able to obtain clones with specific secretion rates of over 50 pg/cell/day, which is a 5- to 20-fold increase from the parental lines (Hanania et al. 2005). The instrumentation is gaining popularity in

both industry and academia as an alternative to flow cytometry for the purpose of selecting both adherent and suspension cell lines with a particular phenotype(s) that may be difficult to process via flow cytometry. Since LEAP can provide in situ measurements of individual cell protein secretion rates, the system is particularly well suited to the direct cloning of cells when selecting high-producers, reducing the amount of multiple serial growth and subcloning steps involved that are seen in other cloning procedures described in this chapter.

11.4.2.2 Cell Xpress Module

The standard Cell Xpress™ protocol for IgG-secreting cells is initiated by preparing a capture medium (Protein G in the appropriate culture medium) and adding the capture medium to the stock culture before plating the cells in 384- or 96-well plates designed for use in a LEAP system. After attaching the cell suspensions to the plate with gentle centrifugation, the plate is incubated overnight at the optimum culture conditions, where the IgG secreted by the cells is bound to Protein G, acting as a capture reagent in the vicinity of the cell, creating a halo around it; the highest secretors/producers generate the largest halos. The next day, the cells are stained with conjugated anti-IgG fluorescent detection reagent mixed with CellTracker™ Green (Thermo Fisher Scientific, Life Technologies, USA) as a halo detection reagent and live cell dye, respectively, and are incubated for 3–4 h before the removal of unbound reagents and are then washed by gentle aspiration so that cells do not move from their location in the wells (Fig. 11.5). Once the plates are washed to achieve the desired background fluorescence, analysis takes place via the Cell Xpress™ algorithm, which identifies viable cells and calculates the intensity of the surrounding halo before cell processing by laser ablation of unwanted cells. A good review of LEAP protocols is given in Lin et al. (2008) and includes further details on the Cell Xpress™ Module for cell line selection.

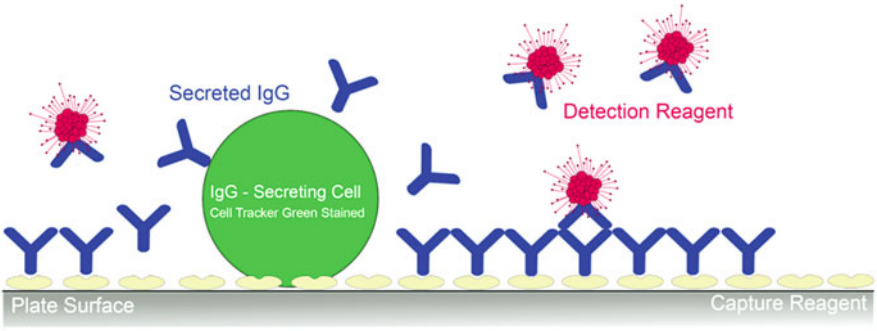

Fig. 11.5 Cell Xpress secretion assay. After an overnight incubation of cells with capture reagent, a halo is created around the cell because the secreted IgG is captured by the capture reagent surrounding the cell secreting the IgG. Afterwards, a detection reagent is added that binds to the secreted IgG

When single cell cloning is performed utilizing the Cell Xpress™ module, the high-producing cells are first enriched by eliminating all low-producers and the cells can be plated at one cell per well in a 384-well plate after the enrichment has been confirmed. The plated cells can then be further analyzed on LEAP using the Cell Xpress™ Module for determining and selecting viable high-producers. The advantage of Cell Xpress™ cloning is that it exposes the cells to less shear forces and stress compared to flow cytometry-based protocols due to lack of pressure and flow velocity (Shapiro 2003) and it can also perform a combination of analyses that can replace the manual ELISA and single cell cloning; however, parameters must be carefully set for laser ablation functions to ensure that the settings do not cause cell movement in the well whilst still performing the ablation function required.

LEAP cell selection represents a high throughput method that is gentle on the cells both for cell selection and quantitative measurements of POI secretion. The labor involved in LEAP experiments when using the Cell Xpress™ module for quantitative in situ detection of secreted IgG can be estimated to be between 1 and 3 h for a 2 day run, depending on the experience of the operator (Cresswell et al. 2009), making this less labor-intensive than the fully manual ELISA experiment for identifying highly-expressed recombinant protein producers from cloned cells.

11.4.2.3 Other Automated Screening and Selection Systems

In order to speed up development time and reduce labor costs, biopharmaceutical development operations have been implementing automated systems. As an example, the Merck & Co., Inc. have selected high-producer CHO cells based on a combination of a cell sorter, an automated imager known as the CloneSelect Imager™ (Molecular Devices, Sunnyvale, CA, USA) and the Freedom EVO™ liquid handling system (Tecan Group Ltd., Männedorf, Switzerland) for high-throughput cell line selection and development (Shi et al. 2011). Here, the cell sorter carries out the first selection screen of the high-producers after a transfection and amplification protocol has been carried out. The flow cytometry sorting operation requires staining with a fluorescent conjugated antibody towards the POI prior to sorting, as described earlier in this chapter. After the cells have been selected, they are grown in colonies in the CloneSelect Imager™, which allows the growth of cells whilst monitoring with time-lapse imaging to ensure the selection of clones derived from a single cell. The formation of the single clones over a period of 2 weeks is followed by an automated ELISA; this can be performed with the Freedom EVO™ liquid handling system. Other instrumentation such as the Octet 384-well system can also be incorporated into automated platforms such as the Freedom EVO™ for the automated selection of cells based solely on productivity or on both productivity and activity of the POI. Automation of cell line selection can be achieved through the use of several automated clone or colony selection systems. An automated workflow, as described here, can yield approximately

2,000–10,000 clones per operation cycle, as demonstrated by the Merck Lab (Shi et al. 2011); similar setups already exist in most commercial development labs.

Over the past several years, commercial entities have introduced several automated platforms that are focused on colony selection. These automated single colony isolation systems are marketed as the CellCelector™ (AVISO, Jena, Germany) and ClonePix FL™ (Molecular Devices, Sunnyvale, CA, USA), with both instruments being able to manage a large cell throughput. Both automated colony pickers use the FLSSM culture method to detect fluorescent halos around the high-producer cells in a colony and robotics to automatically pick and place the colony in a separate plate. The CellCelector™ culture plates are prepared using the FLSSM methodology and scanned on the instrument after 7–10 days of incubation using Cell D Software (Aviso/Olympus) for analysis. The software subtracts background fluorescence and determines the brightest clones before picking to transfer them to another plate (Caron et al. 2009). A similar procedure is employed by ClonePix FL™, an instrument that was originally developed by Genetix before acquisition by Molecular Devices. The ClonePix FL™ system first requires seeding of transfected cells at a low density in a semisolid media formulation combined with a fluorescent detection reagent known as CloneDetect™, an antibody probe that is specific for IgG conjugated to a fluorophore or any fluorescent conjugated antigen specific to the secreted POI in proprietary culture plates prior to instrument usage. After several days of incubated growth (7–10 days), the culture plates are placed in the ClonePix FL™ instrument and colonies can be selected based on "halo" intensity via the analyzed bright-field and fluorescent images that are digitally merged by the software for colony selection; this also enables the exclusion of fluorescent colonies that are too close to the non-fluorescent colonies, thereby decreasing the probability of selecting non-producers along with high-producers.

Recently, a single cell microarray chamber (SCMC) was developed (Yamamura et al. 2005) and adapted onto an automated platform for high throughput single cell analysis. The polymidethylsiloxane micro-chamber array chip has 31,360 wells on a total area of 1.39×2.23 cm^2, with a 30 μm micro-well diameter. Each cell occupies a single micro-well, which can be manipulated using a glass capillary and analyzed via the integrated fluorescent microscopic system all in one standalone unit (Yoshimoto et al. 2013; Yoshimoto and Kuroda 2013). The system workflow begins with the labeled cells in the micro-chamber being scanned with a CCD camera followed by fluorescent image analysis; if the micro-chamber well contains more than one cell, the image will be excluded, and selected cells recovered into an assigned well of a 96- or 384-well plate. For secreted antibodies, a cell-surface fluorescence-linked immunosorbent assay (CS-FIA) is applied for the labeling protocol. CS-FIA is a method that allows the formation of a trapping molecule around the cell surface made up of an antibody targeted towards the secreted POI conjugated to dioleoyl phospahtidylethanolamine-poly-ethylene glycol 2000 (DOPE-PEG2000). Once the DOPE-PEG2000-labeled cells are introduced into the micro-chamber, the secreted antibodies can be captured on the cell surface for detection with an FITC-conjugated antibody fragment via the CCD camera. The fluorescence detected correlates with the production rate and cells with

the highest amount of fluorescence can be automatically isolated via the software algorithm and robotics. The advantage of this system over automated colony pickers is since colonies are picked out, the issue of heterogeneity may still be present and thus 1 month or more may still be required to obtain candidate cells (Serpieri et al. 2010; Choi et al. 2010), while the SCMC system allows for individual cell evaluation and isolation of cells for further expansion, allowing a greater control of heterogeneity. The work of Yoshimoto and others showed that it was possible to select hybridomas that are high-producers in 1 day using the automated SCMC with daughter cells secreting even higher amounts of antibody after 2 weeks of culture.

While automation is becoming common practice, allowing for the delivery of high throughput bioassays in the research and development environment, there have only been a few automated platforms that can carry out the complete cell line workflow and require minimal operator intervention. The Cello™ system (TAP Biosystems, Royston, UK) is a prime example of such an instrument. The Cello™ robotic system can perform clone selection via automated clone screening and scale-up of static cultures. The Cello™ system software has a database to record all process steps and clone images along with any other associated data in order to document the culture and selection process. With 2–3 week-old transfectants loaded onto the Cello™ system, the instrument can screen single cell colonies and automatically select colonies for ELISA. This process can be fully automated and takes a minimum of 2 weeks with thousands of clones analyzed before ELISA is performed. In one study, the Cello™ system helped to generate high-producer CHO cell lines with fed batch culture IgG titers of 4.7–5.0 g/L (Lindgren et al. 2009), significantly reducing the manual workload and allowing several cell line development projects to be run in parallel via the automated cell selection system (Fig. 11.6). Apart from Cello™, TAP Biosystems has also been able to automate suspension shake flask cultures and a micro-scale bioreactor system, which are known as Sonata™ and AMBR™, respectively. These automated

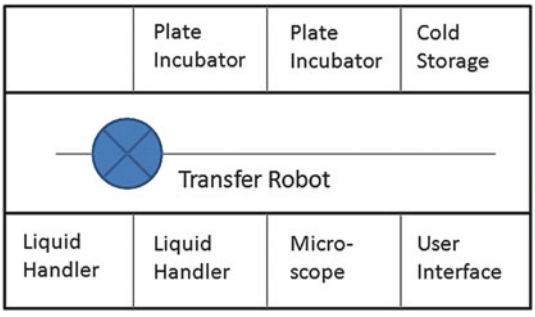

Fig. 11.6 The Cello™ Robotics system consists of a number of integrated modules that allow for the automated instrument cell culture system to handle mammalian cell culture in plates, enabling the efficient selection of optimal clones and cell lines. The modules are positioned on either side of a central area that houses the transfer robotic arm, as shown in the schematic

systems allow for further cell line selection at increased scales, whilst still being able to run a screen on several initial selected clones for the further evaluation of stability, growth and productivity at different culture conditions, such as in fed batch cultures. Since cell line selection can be one of the most labor-intensive steps in the development process, the assistance of the Cello Robotics and similar systems at varying scales allows for a reduction in labor expenses with the benefit of overall reduced long-term development costs.

Concluding Remarks

With the increase in the demand of mammalian cell-expressed biopharmaceuticals, a lot of work has gone into developing improved cell line selection systems to contribute to overall cell line development efforts and to reduce the costs and time required to select the appropriate cell lines. The cell line selection process can be carried out several times and in several steps of the cell line development process, such as from micro-well culture up to small scale parallel bioreactor runs performed to check productivity, where the specific production rate may still vary as productivity has many contributing factors including the culture environment. The traditional method of cell line selection is still considered to be LDSCC, but with the introduction of several new techniques over the last few years, biopharmaceutical manufacturers have adapted a combination of new technologies to reduce the time and labor required for the process, since most traditional methods do not provide the high-throughput screening capabilities to find clones with the proper growth, productivity and stability profiles in the fastest time possible.

As there are many cell physiological and metabolic factors that may contribute to a cell's overall profile, selecting a cell line with the desired characteristics for large scale bioreactor culture that produces a high quality POI at optimal quantities can be difficult to accomplish in a short time span without high-throughput selection techniques. It may be possible in the future, with a greater understanding of systems biology, to select such cells early on in the process; however, currently, a better approach may be to create engineered designer cell lines that are specifically bred for the stable high titer production of a POI after single-site integration, thus speeding up the initial selection process. The use of flow and scanning cytometry techniques has also greatly enhanced the cell line selection workflow, but there is still much room for optimization of these techniques. In addition, the use of automated selection systems has provided the possibility to screen thousands of clones providing a higher probability of selecting the best cell line for the production environment.

(continued)

The direction being taken by the cell line selection process is currently towards the increased use of automated systems which can be integrated to carry out the cell line selection process from transfection to small-scale bioreactor cultures after several clones have been selected and assessed in automated static and shake flask cultures. Although automation can be easily implemented, and can make the process more efficient, not every commercial entity is willing to dedicate the expense involved. Thus even with current technological advancements, the area of cell line selection still requires additional research to further advance the field in order to select the desired clone from a large population of cells in as short a time span as possible, thereby making it suitable for commercial scale production at a reasonable cost.

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Chapter 12
Building a Cell Culture Process with Stable Foundations: Searching for Certainty in an Uncertain World

Peter M. O'Callaghan and Andrew J. Racher

Abstract Considerable effort is expended during the mammalian cell line construction process to find a stably-transfected clone capable of supporting the large-scale manufacture of a recombinant therapeutic protein. Such a clone must synthesise a sufficient volumetric concentration of the product with the correct biochemical characteristics (glycosylation, structural integrity, etc.). Furthermore, this performance must be maintained over the extended time period required to support a manufacturing campaign in 20,000 L bioreactors. However, a significant proportion of recombinant clonal cell lines show production instability over long-term sub-culture, where volumetric product yield and/or product quality are not maintained. This instability can potentially extend product development timelines and can affect the ability of a manufacturing process to meet market demand. In the worse-case scenario it can also jeopardise patient safety if product quality is impaired. In order to prevent this, industrial cell line construction processes include long-term stability studies where several candidate lead clones are serially sub-cultured and monitored for signs of instability before selecting the final production cell line. The roots of production instability are varied, but the epigenetic silencing of transgenes at sites of host cell chromosome integration, and the direct mutation or loss of transgenes are prominent molecular causes. Considerable research has been conducted by both industry and academic groups into the molecular mechanisms underpinning instability, with an emphasis on uncovering early predictive markers of incipient instability as well as preventing its occurrence. In this article we present a detailed overview of the industrial experience of production instability and its impact on the manufacturing of recombinant therapeutics. We also discuss our current understanding of the molecular causes of cell line instability and how this has been used to mitigate the impact of this phenomenon through novel vector redesigns and cell line screening.

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Abbreviations

5-Aza	5-aza-2′-deoxycytidine
ACE	Artificial chromosome expression
ADCC	Antibody-dependent cellular cytotoxicity
CDC	Complement-dependent cytotoxicity
CHO	Current good manufacturing practice
cGMP	Chinese hamster ovary
CNV	Copy number variation
DHFR	Dihydrofolate reductase
DSB	Double strand DNA break
FACS	Fluorescence-activated cell sorter
GS	Glutamine synthetase
HAT	Histone acetyltransferase
HC	Antibody heavy chain
hCMV-MIE	Human cytomegalovirus major immediate early promoter/enhancer
IAA	Intracellular antibody
IR	Initiation region
IRES	Internal ribosome entry site
LC	Antibody light chain
LCR	Low copy repeat
Mab	Monoclonal antibody
MAR	Matrix attachment region
MCB	Master cell bank
MMBIR	Microhomology-mediated break-induced replication
MSX	Methionine sulphoximine
MTX	Methotrexate
NAHR	Non-allelic homologous recombination
POI	Protein of interest
qP	Cell-specific recombinant protein production rate
RIGS	Repeat-induced gene silencing
SDS	Sodium dodecylsulphate
SSA	Single-strand annealing
TF	Transcription factor
TSS	Transcription start site
UCOE	Ubiquitous chromatin opening elements
UTR	Untranslated region
WCB	Working cell bank

12.1 Introduction

The field of bioprocessing has seen major advances in recent years, mostly driven by a deeper understanding of the overall manufacturing process, and substantial optimisation of the media and feeds used during the bioreactor run (Fan et al. 2013). Now it is routine to generate clonal cell lines capable of producing harvest protein concentrations exceeding 5 g/L, with concentrations of 13 g/L being reported (Huang et al. 2010). However, one area of critical importance that has seen comparatively little progress is the control of cell line instability, which is broadly defined as a change in the critical quality attributes (i.e. product quality) of the recombinant protein over serial sub-culture, or a decline in the final product concentration. Although our understanding of the underlying molecular mechanisms causing instability of recombinant protein production in cells has improved, the primary method of eliminating unstable cell lines is still to perform long-term stability studies before selecting the manufacturing cell line. With the development of automated, high-throughput systems for clone screening it is now possible to survey large numbers of clones for signs of instability over long-term sub-culture with relatively little effort (Salmen et al. 2009; Browne and Al-Rubeai 2007; DeMaria et al. 2007). Nevertheless, the ability to develop and engineer production cell lines with predictable long-term stability would be a major advance in industrial biomanufacturing and would significantly reduce costs and variability during the cell line construction process, and accelerate the time to clinic (Porter et al. 2010). The last few years have seen a large number of published studies on this subject from both academia and industry, with some significant advances in both our fundamental understanding of this phenomenon, and also in our ability to predict unstable clones during the cell line construction process. No doubt over the next few years there will be further advances in this field as the Chinese hamster ovary cell (CHO) 'omics resources mature and give us new insights into the fundamental biology underpinning the performance of this system as a cell factory for biopharmaceutical manufacturing (Kildegaard et al. 2013). In this review we discuss recent developments in the understanding of stability in biomanufacturing cells lines, and review the current methods both for avoiding instability and for predicting its occurrence early in the cell line development timeline. As the majority of biologics on the market are currently manufactured using CHO-based systems (Li et al. 2010), this review will predominantly focus on the stability of recombinant CHO cell lines.

12.2 How Much Stability Is Required? Lessons from Industry

The consistency of recombinant protein production by mammalian cell expression systems is of interest from both academic and industrial viewpoints. The industrial viewpoint emphasises the consistency of product quality, which comprises identity, strength and purity. In contrast, in the majority of the academic literature the emphasis is upon understanding, and controlling, decreases in protein expression levels. Both viewpoints will be examined in this paper. The ICH Guideline Q11 (ICH 2012) states "the goal of manufacturing process development for the drug substance is to establish a commercial manufacturing process capable of consistently producing drug substance of the intended quality". Development of the manufacturing process "should identify any critical process parameters that should be monitored or controlled …… to ensure that the product is of the desired quality" (ICH 2009). The objective of both guidelines is to deliver consistent and reproducible clinical performance. It is generally believed that the inherent properties of the cell, events linked to the cell, and the expression vector contained in the cell, can all affect resultant product quality and safety (ICH 1997). Therefore, "effective quality control of these products requires appropriate controls on all aspects of handling the cell". Restrictions on the limit of in vitro cell age for production, i.e. the maximum age of cells used to manufacture a product, is an example of such a control. Testing of the cell bank for contamination with adventitious agents is another example.

Current analytical methods can detect even small changes in product quality, which enables highly sensitive monitoring of batch-to-batch consistency and variability in the manufacturing process. The analytical sensitivity does lead to the question about what is a significant change, as the linkage with change in clinical performance is generally less well developed and is difficult to test. Three factors have been identified as contributing to changes in product quality (Schiestl et al. 2011): inherent batch-to-batch variability; manufacturing process changes; and process drift. These factors can sometimes be the cause of cell line instability; other times the result of cell line instability, or both.

What are the consequences of a change in product quality? Antibody activation of effector function and the complement system by binding of the Fc with immune effector cell Fc receptors and C1q is influenced by Fc glycosylation. In an analysis of different commercial batches of Rituxan, Schiestl et al. (2011) reported that differences in the abundance of unfucosylated G0 glycans had a substantial impact upon ADCC potency, an essential part of the clinical mode of action. Studies have shown a linear correlation between the amount of afucosylated glycan with both FcγRIII receptor binding and ADCC. Therefore the ratio of afucosyl to fucosylated glycans is critical to achieve consistent efficacy, and changes as small as 5 % in afucosyl glycans can result in the order of twofold changes in ADCC. Changes in the degree of galacotosylation have been reported to reduce substantially the complement lysis activity of Campath-1H (Boyd et al. 1995) and Herceptin

(Hodoniczky et al. 2005). Terminal galactose residues have been shown to affect antibody binding to C1q and CDC with increasing cytotoxicity as the percentage of terminal galactosylation increases. Hence for antibodies where the mode of action is through effector function the levels of afucosyl glycans and the ratio of G0:G1: G2 should be controlled to minimise their influence on ADCC and CDC respectively. In summary, changes in product quality can result in altered biological activity and, potentially, clinical performance.

Host cell line and culture conditions used for expression of a therapeutic protein influence the glycosylation of the recombinant product (Lifely et al. 1995; Umaña et al. 1999). Although the manufacturing process is 'locked down' relatively early in the product life-cycle, theoretically giving a constant process and product, the process and product are sensitive to chemical entities present as contaminants in the raw materials used to manufacture the media and feeds used leading, again, to variability as different batches of media are used. This will not be discussed further in this manuscript. Similarly, the choice of host cell line is generally fixed early in the life-cycle of a product. However, although a cell line used to manufacture a therapeutic protein is derived from a single progenitor cell, the genotype and phenotype of the resulting cell line may change with increasing age of the cell line. This may, potentially, alter qualitatively and quantitatively the post-translational modifications made to the protein.

In summary, the biological activity and, potentially, clinical performance of a therapeutic protein may vary due to qualitative and quantitative changes in the post-translational modifications. One possible cause of this variability is the genotypic and phenotypic changes (reflecting changes at the genomic, transcriptomic and proteomic levels) that may occur as the cell ages. Thus cell age (measured as number of population doublings (Greenwood et al. 2004)) is a critical process parameter that needs need to be monitored and controlled to ensure consistent product quality. The ICH Guideline Q5D (ICH 1997) states explicitly that it "is important that a characterised cell bank provides a consistent product", with the consistency of product quality being the "primary subject of concern".

ICH Guideline Q5D (ICH 1997) describes the type of studies needed, as part of manufacturing process development, for the characterisation of a cell line for manufacturing a therapeutic protein. One dimension of this characterisation is an assessment of cell line stability, which encompasses "consistent production of the intended product". Figure 12.1 shows one possible workflow for a cell line stability study. Cells are recovered from the cryopreserved cell bank and sub-cultured, as independent lineages from individual vials using the commercial scale conditions (i.e. the same media, forward processing conditions, culture vessels, etc.), until the cell age, measured as number of population doublings, meets or exceeds the limit of in vitro cell age for production use. As a minimum, the in vitro cell age limit should encompass recovery of cells from the cell bank, establishment and maintenance of the cell culture that provides the inoculum for the seed bioreactors, and expansion of the cell mass through the seed bioreactor train to inoculate the production bioreactor. Alternatively, the cells may be 'rolled' in the seed bioreactor train to generate inocula for multiple production bioreactor cultures separated in time.

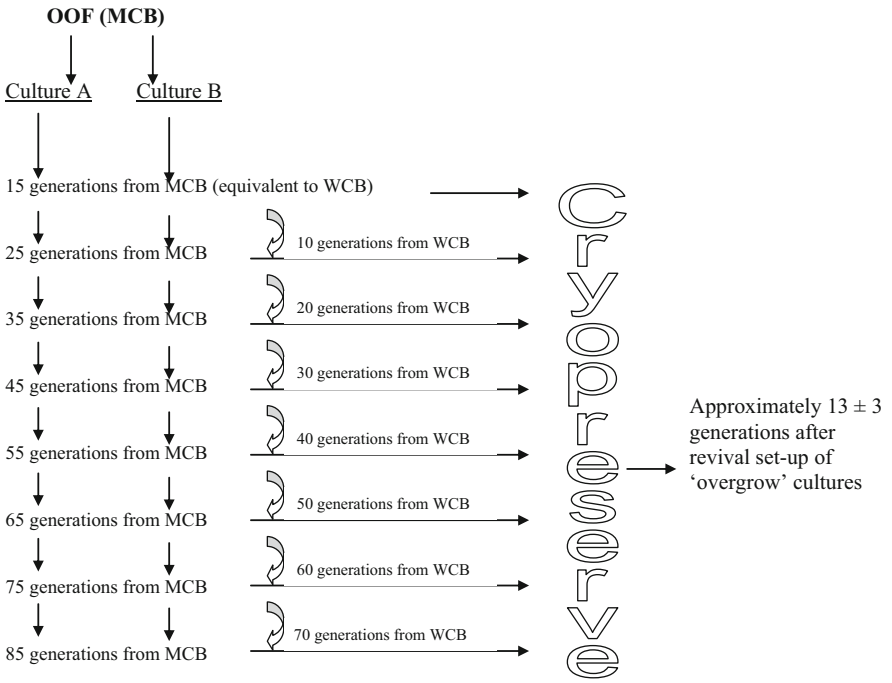

Fig. 12.1 Schematic of workflow for a cell line stability study encompassing about 100 population doublings. A vial of a characterised cell stock, in this instance a MCB, is used to create an independent lineage of cells. The cells are passaged in a model of the cGMP inoculum process, so that the cells are exposed to the same process environment as cells in the manufacturing process. Cell stocks are periodically cryopreserved. At the end of the study, cells of different ages are recovered from cryopreservation and evaluated in a model of the production bioreactor process. This approach eliminates a major source of experimental variability, i.e. differences in media batches. *OOF* out of freeze

Periodically, cell stocks are cryopreserved for further use. At least two time-points are examined: firstly, cells that have received a minimal number of sub-cultures from the characterised cell bank, typically a cGMP MCB; and secondly, cells "at or beyond the limit of in vitro cell age for production". The cells are evaluated in a model of the production bioreactor process.

The type of testing and test article(s) will depend upon the cell line and expression system, and product. Table 12.1 contains an example of the suite of tests used to assess stability of a recombinant cell line expressing a recombinant protein. The emphasis of these methods is on demonstrating consistency of product quality. Parameters describing cell growth and productivity will typically be assessed in parallel in order to assess process consistency, robustness and economy.

How is a stable cell line defined? One definition is that the product produced by a stable cell line must be biochemically comparable, using the analytical techniques available at the time, at the beginning and the end of the study. Routinely, this comparison is made using a combination of visual examination of the data,

Table 12.1 A representative, non-exhaustive suite of tests that could be used to demonstrate stability of a cell line used to manufacture a therapeutic protein. For all tests a comparison is made between the cells and product at a population doubling number close to the characterised cell bank, and the cells and product at the limit of in vitro cell age

Characteristic	Analytical method	Comment
Integrity of product		
Size variants	Reduced and non-reduced SDS electrophoresis	Masses of protein and subunit polypeptides; product fragmentation; disulphide shuffling
Charge variants	Isoelectric focussing	Native protein charge and variants e.g.: C-terminal lysine clipping; charged sugar profiles; amino acid deamidation
Charge variants	Weak cation exchange chromatography	As for isoelectric focussing plus isoaspartate deamidation variants
Size variants	Size exclusion chromatography	Proportions of protein monomers and aggregates
Post-translational modification	Maldi-ToF-MS analysis of PNGase F-treated product	Non-charged glycan species
Amino acid sequence	LC-MS of peptidase-treated product	Product sequence variants, post-translational modifications, and aglycosyl variants
Activity	Various	Biological function
Contaminants	Various	DNA and host cell proteins
Integrity of expression construct		
Gene copy number	qPCR	Gain or loss of gene copies
Structure of expression construct	Southern hybridisation analysis	Structural rearrangements within expression construct
mRNA lengths	Northern hybridisation analysis	Changes to transcription unit
DNA sequence	Reverse transcription of product mRNA	Mutations within exon(s) encoding product

understanding of the accuracy and precision of the assays, comparison with historical data, and experience. For example, product comparability by SDS electrophoresis analysis could be based upon demonstration of: no change in the number of bands; no gross changes, by eye, in mobility and relative mobility of the different bands; and the purity of the product being within a pre-defined range. Attempts have been made to develop summary statistics that reduce the dimensionality of these complex data sets. For example, the Z number (Hermentin et al. 1996) has been proposed as a parameter that describes protein glycosylation in terms of numbers of charged glycans. Such parameters have been suggested as being suitable for product consistency monitoring, although some workers have reported poor correlation with bioactivity (Yuen et al. 2011). Typically, even at the pre-biologics license application stage, there are insufficient data to undertake a robust statistical test of the hypothesis of equivalence. When a number of analytical methods are used to characterise the protein, the likelihood that differences in

product quality will be observed increases. In such cases, a risk analysis should be undertaken to understand the effect upon clinical performance. Changes in product quality during a cell line stability study occur at a low frequency. An analysis of data generated over more than 10 years from a large number (more than 100) of GS-NS0 and GS-CHO cell lines contained only three instances where the change in product quality (identity of product assessed by test such as those listed in Table 12.1) was considered significant (Lonza Biologics, unpublished data).

In terms of consistency of product expression, the authors would consider a change of less than 20 % in a measured parameter to indicate that the value of the parameter is consistent over the duration of the study. A value of 20 % is considered to balance the precision of the analytical methods with discarding too many cell lines due to a high false positive rate. The 20 % value is used to provide guidance: it is not an element of a formula in a system of logic designed to give a true-false answer. Analysis of more than 140 GS-CHO cell lines making different recombinant antibodies indicates that about 60 % show changes in antibody concentration of less than 20 % (Lonza Biologics, unpublished data). Not all changes are decreases, increases can be observed: also, the changes do not have to be linear with increasing number of population doublings. A large change in product expression does not automatically disqualify a cell line from use in cGMP manufacturing, provided that the product concentration observed at the end of the study is still capable of supplying the market demand for the product. The approximately 40 % of cell lines that exhibit large (>20 %) changes in product concentration does impact the selection and development of cell lines. It increases the number of cell lines that need to be screened to assure identification of at least one suitable cell line and extends the timeline for cell line development, as cell lines need to be screened in real-time. As cell line development is on the critical path to first-in-human studies, extending development timelines delays start of clinical trials. Timeline extension can be prevented by undertaking activities (e.g. preparation of cGMP cell banks) on a number of cell lines to mitigate the risk that the preferred choice is not stable. However, this will increase costs.

The design of cell line stability studies therefore provides information on the consistency of product quality, stability of product expression, and cell culture characteristics as a function of time of culture (expressed as number of population doublings). From a regulatory viewpoint, the stability study demonstrates that a manufacturing process will produce a consistent product when operated within the range of population doublings studied for a cell line. From a manufacturing and marketing viewpoint, stability studies provide the information required to decide if the projected market demands can be satisfied. They also provide information to ensure that the cell line productivity and growth characteristics make the process operable in a cGMP environment.

12.3 Impact of Long-Term Sub-Culture on Cell Populations

A stability study can characterise a cell line across a population doubling window of 100 doublings (Fig. 12.1). When a cell line is repeatedly sub-cultured over such an extended period of time (approximately 85 days for a GS-CHO cell line), this process effectively serves to positively select for fast growing variants within the original cell population. This can eventually lead to a gradual increase in average cell-specific growth rate of the culture over time (Beckmann et al. 2012; Heinrich et al. 2011; Kaneko et al. 2010). A good model for this process is the evolution of cancer, where a heterogenous pool of genetically-mutated cells competes for limited resources at the tumour site, with the fastest growing (or most robust) variants coming to predominate (Goymer 2008; Nowell 1976). Although a manufacturing cell line should have a fast rate of biomass accumulation with a high maximum viable cell concentration (among other characteristics), a general correlation between increasing cell-specific growth rate and a decrease in productivity has been widely observed, presumably due to the higher metabolic burden associated with high-level recombinant protein production (Beckmann et al. 2012; Kaneko et al. 2010; Jiang et al. 2006). Indeed, as a recombinant cell line or hybridoma loses productivity over extended culture due to *de novo* gene mutation, chromosome loss, or silencing, non-producer cells can rapidly overgrow the original population, particularly when non-producer status comes with a growth advantage (Kromenaker and Srienc 1994; Lee et al. 1991). Other metabolic changes with extended cultivation may also be observed such as increasing resistance to lactate, higher expression of glycolytic enzymes, increased rate of glucose uptake and an increase in the expression of anti-stress proteins (Beckmann et al. 2012; Hughes et al. 2007). In addition, there may be global changes to gene expression as the nature of the epigenome alters with serial sub-culture and increasing number of population doublings (Young and Smith 2001; Fairweather et al. 1987; Shmookler Reis and Goldstein 1982). All of these changes have the potential to impact product quality, and hence safety and efficacy. In essence, this is one cause of process drift.

Interestingly, some of the cellular changes over long-term sub-culture can only be observed under very specific conditions. A study by Bailey and colleagues (2012) uncovered one particular GS-CHO cell line that suffered a loss of batch culture product concentration after 40–50 generations without any significant change in recombinant Mab mRNA or gene copy number. This loss of productivity was only evident during the latter stages of batch culture in the older generation cells. Older generation cells had an altered metabolism together with an elevated level of the transcription factor GADD153 mRNA, indicative of increased cellular and specifically ER stress (Bailey et al. 2012). Related to these observations, Dorai et al. (2012) observed that unstable CHO clones were more prone to apoptosis, indicated by higher levels of the apoptotic markers annexin V and caspase 3.

Some of the consequences of long-term sub-culture derive from the fact that CHO host cell lines possess a significant degree of heterogeneity (Davies

et al. 2013), even when the cell line is purportedly clonal (Pilbrough et al. 2009). Heterogeneity is an inherent property of mammalian cells cultures, be it genetic or non-genetic in origin. This has been a highly active area of basic research in recent years, with some ground-breaking work revealing the underlying mechanisms and consequences of this property. Indeed, phenotypic heterogeneity even within clonal cell populations is a well-observed phenomenon in a wide range of cell types including bacteria (Avery 2005), yeast (Attfield et al. 2001), and mammalian cells, making the term "clonal heterogeneity" less an oxymoron and more a statement of an inescapable fact of biological systems. This clonal phenotypic heterogeneity can be explained by a combination of local micro-variations in the culture environment, random partitioning of organelles into daughter cells at cell division, and the stochastic "bursting" nature of gene transcription (Molina et al. 2013; Raj and van Oudenaarden 2008; Raj et al. 2006). Although a substantial proportion of this inherent cell-to-cell heterogeneity may have little or no impact at a functional (cell culture) level (Altschuler and Wu 2010), from a bioprocessing perspective the effect of variation in critical cellular attributes can be exacerbated by repeated passaging leading to substantial cell culture batch-to-batch variability, and problems with experimental reproducibility and robustness (Hughes et al. 2007). As such, efforts have been made to appropriately describe heterogeneity and to model the impact on industrial bioprocesses (Fernandes et al. 2011; Stockholm et al. 2007; Kromenaker and Srienc 1994; Lee et al. 1991). Perhaps the most critical aspect here is the potential for intra- and inter-batch heterogeneity of product quality attributes, such as N-glycosylation site occupancy, and qualitative or quantitative variation in the resulting glycoprofile (Damen et al. 2009). However, not all variation will have clinical significance, and it will be important to identify where heterogeneity truly impacts clinical performance and where it essentially constitutes background noise (Goetze et al. 2010).

The process of generating a stable cell line attempts to minimise or ideally eliminate heterogeneity by focusing on clonal isolates (for an example of a cell line construction process see Porter et al. 2010). However, an increasingly popular mode of protein production involves the use of heterogenous stable pools of transfectants, particularly for the rapid supply of material required in early stage testing (e.g. toxicology, immunogenicity, structural characterisation, etc.). In this method the bulk pool of stably transfected cells is propagated in selective media without any subsequent cloning. The transfectant pool is serially sub-cultured prior to scale-up for material supply. This method can be scaled up to 200 L scale using disposable bioreactors (Ye et al. 2010), or even to 400 L scale within a stainless steel stirred tank reactor (Lonza Biologics, unpublished data), indicating the considerable utility of this method in meeting the demands of early stage product development. Although this method has the significant advantages of higher product yields compared to the transient format, and faster speed over standard cell line construction, such pools are subject to declining productivity with increasing number of population doublings as non-productive cells in the pool overgrow the original population (Ye et al. 2010). A study by Du et al. (2013) analysed the heterogeneity present within MTX-amplified DHFR-CHO transfectant pools and

the effect of serial sub-culture by assessment of the Mab expression at the cell surface using flow cytometry. Most of the pools assessed during the study showed a normal (Gaussian) distribution in terms of cell surface Mab content (a proxy measurement for overall expression), characterised by a single peak encompassing approximately an order of magnitude difference in Mab expression. However, one of the pools analysed was characterised by two distinct populations of cells, one of which displayed very high levels of expression, and one which was clearly negative for Mab expression, the result of a genetic rearrangement of the Mab genes during culture. With further sub-culture the relative sizes of the two populations changed significantly, with the low/null expressing population increasing at the expense of the high expressing population, with a concomitant decrease in volumetric product concentration. This effect could be counteracted by using a FACS to separate the high from the low producing sub-populations and thereby rescue productive cells from within the original heterogenous pool. In a similar study Ye et al. (2010) used a FACS to enrich for high producers within stable pools, although such enriched pools are still highly heterogenous and subject to productivity decline over time. These studies illustrate in an exaggerated way the dangers inherent to processes based on heterogenous pools of cells, and suggest that "monoclonality" should be the starting point for methods that require the long-term growth of cell populations. The ICH guideline Q5D (ICH 1997) states explicitly that a cell line should be derived from a single progenitor cell.

12.4 Cell Line Instability and Its Impact on Product Quality

As mentioned above, it is absolutely essential for a manufacturing process that the quality attributes of the therapeutic product are maintained across the entire manufacturing process. During the expansion of the cells from the cGMP cell bank up to 20,000 L bioreactor scale, any (phenotypic or (epi)genetic) instability in the chosen host cell line could substantially impact on the final product, potentially in a way that could negatively impact patient safety. Perhaps reflecting the divergent interests of academia and industry, there have been relatively few studies investigating the impact of increasing number of population doublings on product quality by comparison with the number of published studies investigating the loss of productivity. Product quality characteristics such as N-glycan composition are determined by a very large number of genes which could theoretically be subject to epigenetic or genetic alteration over long-term sub-culture in a similar fashion to recombinant product genes (Kim et al. 2009). Despite this several studies have shown that recombinant protein glycosylation by mammalian cells is remarkably robust with increasing number of population doublings. For example, van Berkel and colleagues (2009) investigated human IgG N-glycoforms produced by the CHOK1SV cell line across 60 population doublings of cell culture and found no

major changes in product quality. Similar observations have been made with a larger number of antibodies and cell lines (Lonza Biologics, unpublished data). BHK-21 cells have also been shown to consistently produce a recombinant product (anti-epidermal growth factor receptor IgG1/interleukin-2 cytokine fusion protein) with the correct glycoprofile across 62 population doublings of cell culture (Cruz et al. 2002). Even when grown under different culture conditions for 80 days no significant change in glycoprofile was observed for this molecule, indicating that in the right cell line/clone this phenotype can be remarkably stable (Cruz et al. 2000). However, product quality is not restricted to just the consistency of the glycosylation profile, but also encompasses effects such as C-terminal variants (Luo et al. 2012), as well as changes to the actual amino acid sequence of the molecule. Indeed, direct mutation of the recombinant protein gene during the cell line construction process has been demonstrated to occur, with subsequent impact on the peptide sequence and overall product homogeneity (Dorai et al. 2007). Such issues serve to highlight the necessity not only for highly robust, reliable, platform processes, but also the need to closely monitor the process and product across the entire cell line development pipeline.

12.5 Genetic Instability and Its Impact on Biomanufacturing

Significant advances have been made in recent years in our understanding of the molecular mechanisms causing production instability of CHO cell lines. These mechanisms can be broadly described as being either genetic or epigenetic in origin, and these will be discussed in turn below.

The genetic stability of recombinant CHO cell lines has two essential components, namely the genetic stability of the integrated transgenes, and the overall "background" genetic stability of the host cell (O'Callaghan and James 2008). Genetic stability can be assessed using a number of techniques, most commonly by assessment of microsatellite mutation rates (Bhargava and Fuentes 2010; Eckert and Hile 2009; Hinz and Meuth 1999; Aquilina et al. 1994), determination of phenotypic changes conferred by mutation at specific loci (e.g. CHO hprt locus; Romney et al. 2001), or array comparative genome hybridisation (aCGH; Vauhkonen et al. 2006). More recent developments include the application of the newly available CHO genome resources for assessment of single nucleotide polymorphisms (SNP's) and other mutations (Lewis et al. 2013), and the development of an ultra-scale-down, microfluidic device for assessment of genetic stability at the single cell level by RT-PCR (Chen et al. 2012).

The background genetic stability of the host cell line is perhaps best understood in the context of the "mutator" phenotype characteristic of cancer cells (Loeb 2001). The genome of CHO host cell lines are well-known to be highly "plastic" with a substantial degree of instability and diversity, making this comparison highly

pertinent (Barnes et al. 2003; Lewis et al. 2013). The mutator phenotype of cancer cells is characterised by a large number of spontaneous random mutations arising from a fundamental inability to maintain genomic stability (Bielas et al. 2006). This impairment derives from an initial mutational event (or epigenetic change) that at least partially disables the cellular mechanisms of genome surveillance and repair (Schmitt et al. 2012; Negrini et al. 2010). Recent information from genome sequencing projects suggest that the genetic landscape of the various CHO cell lines used in biomanufacturing are in some ways broadly reminiscent of a population of cancer cells in the number and diversity of genetic mutations between them (Lewis et al. 2013; Schmitt et al. 2012; Xu et al. 2011). Indeed, the genetic landscape of biomanufacturing CHO cell lines encompasses changes at the nucleotide level as well as much larger-scale changes to the karyotype (Cao et al. 2012; Derouazi et al. 2006). The extent of these changes and the divergent histories of the various CHO cell lines has recently led to the suggestion that the cell lines in common use today are in some ways "quasispecies" (Wurm 2013). It is not unreasonable to assume that such a propensity for genetic mutation (induced or otherwise) would be highly likely to impact the production stability or product consistency of a cell line, particularly where de novo mutational events lead directly to the loss or mutation of transgenes.

Apart from the high level of mutations in CHO cell lineages, is there any direct evidence for a mutator phenotype in CHO cells? Mutator phenotypes have certainly been experimentally-induced in CHO cells, either through treatment of cells with mutagens (Chang and Little 1992; Romney et al. 2001), or via the overexpression of DNA polymerase β (Canitrot et al. 1998). It would certainly be interesting to quantify the spontaneous mutation rate of the various industrially-relevant CHO host cell lines. As pointed out by Wurm (2013), CHO cells "give rise readily to mutant phenotypes". Even if CHO host cell lines do not display the classic mutator phenotype, it may be that a similar phenotype is inadvertently induced as a result of growth in a synthetic culture environment. A study by Pohjanpelto and Holtta (1990) found that nutrient limitation during CHO cell culture was associated with an increase in c-myc mRNA and polypeptide, a protein well-known to be associated with genome instability and a mutator phenotype at elevated levels (Barlow et al. 2013; Prochownik and Li 2007).

As for the stability of the integrated transgenes, loss of recombinant genes over long-term sub-culture is a well-established mechanism underpinning production instability of mammalian biomanufacturing cell lines, including CHO and NS0 variants as well as hybridomas (Barnes et al. 2003). This phenomenon affects mammalian cell lines created using the commonly exploited DHFR and GS gene expression systems (for a schematic diagram of a GS vector see Fig. 12.2), and can occur even when cells are continuously cultured under chemical selection (MTX and MSX, respectively), a state that should theoretically serve to maintain the presence of the integrated genes (Beckmann et al. 2012; Kim et al. 2011; Heller-Harrison et al. 2009; Jun et al. 2006; Fann et al. 2000; Strutzenberger et al. 1999; Kim et al. 1998a, b). How can recombinant genes be lost from the genome? In order for recombinant genes to be incorporated into the genome a double-strand DNA

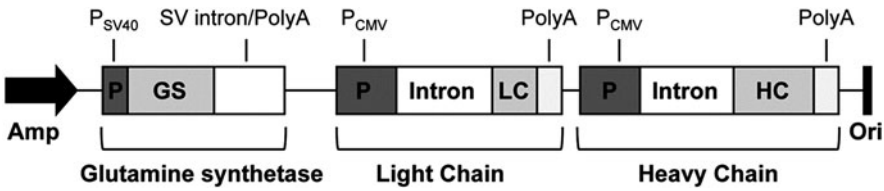

Fig. 12.2 Schematic representation of a typical GS (Glutamine synthetase) vector design used to generate recombinant IgG-producing GS-CHO cell lines. In this diagram the vector has been linearised prior to transfection by digestion with the restriction enzyme PvuI which cuts at a site located within the β-lactamase ampicillin resistance gene (*Amp*). The vector consisted of heavy chain (*HC*) and light chain (*LC*) cDNA cassettes each under the control of separate human cytomegalovirus (*CMV*) promoters (*PCMV*), with the GS selection marker driven by the SV40 promoter (*PSV40*). *Ori* indicates the bacterial origin of replication (Figure reproduced with permission from Kim et al. (2011))

break (DSB) must occur in the host cell chromosome(s) (Ohbayashi and Mitani 2006), and therefore by their very nature insertion sites may be inherently prone to DSBs and other genetic rearrangements. Indeed, it is likely that vector integration sites within NS0 or CHO host cell lines commonly localise to chromosome fragile sites (Barlow et al. 2013; Mondello et al. 2010; Glover et al. 2005). Certainly the preferential integration of exogenous vectors within chromosome fragile sites has been well demonstrated (Matzner et al. 2003; Mishmar et al. 1998; Rassool et al. 1991), and the specific growth conditions applied to CHO cells in the synthetic industrial environment may act to promote the formation of fragile sites if DNA repair or replication is impacted. Therefore, it may not be surprising that succeeding DSBs can lead to the deletion or mutation of incorporated transgenes.

When a DSB occurs there is an opportunity for copy number change arising through recombination repair mechanisms that utilise sequence homology to a greater or lesser extent namely, NAHR (ectopic homologous recombination), SSA, and MMBIR (Hastings et al. 2009b) (Fig. 12.3). All three mechanisms can operate where regions of LCRs, otherwise known as segmental duplications, exist, such as in recombinant CHO or NS0 cell lines. NAHR between LCRs within unstable genomic regions is thought to be responsible for a significant number of recurrent genetic disorders associated with the abnormal dosage of genes, such as Williams-Beuren syndrome and DiGeorge syndrome (Stankiewicz and Lupski 2002). In such human disorders unstable genomic regions contain LCRs that typically share >97 % similarity and are approximately 10–40 kb in length (Lupski and Stankiewicz 2005; Stankiewicz and Lupski 2002). Such characteristic features are likely to be highly similar to the genomic integration sites within recombinant host cell lines where multiple copies of expression vectors containing large numbers of repeated sequences (promoters, UTRs, HC/LC sequences etc.) are located (Kim et al. 2011; Barnes et al. 2007). Such LCR sites have a greatly increased frequency of CNVs caused by NAHR events. CNV, which in this context indicates loss of recombinant gene copies, can result from NAHR when a DSB is repaired using a homologous sequence from another (non-allelic) site in the genome, often

on the same chromosome, an event facilitated in recombinant cell lines when multiple vector copies are inserted at several sites in the genome (Fig. 12.3a). Where tandem duplications of a genetic element occur, a highly likely scenario especially in amplified cells lines, repair of a DSB can result in gene deletion when 5′-end resection during the repair process is not accompanied by 3′ stand invasion of a homologous sequence. This ultimately leads to loss of a repeat unit as well as the intervening sequence in a mechanism called single-strand annealing (Hastings et al. 2009b) (Fig. 12.3b). Several other mutational mechanisms exist that can cause genetic rearrangements and deletions (Hastings et al. 2009a, b; Gu et al. 2008). Replicative mechanisms such as replication slippage or MMBIR can also explain the partial or whole loss of transgene sequences (Liu et al. 2012; Hastings et al. 2009a, b). In some ways replicative mechanisms may be more powerful in explaining the non-recurrent, extremely complex nature of the genetic rearrangements seen in production cell lines (Cao et al. 2012; Liu et al. 2012; Hastings et al. 2009a, b; Gu et al. 2008; Barnes et al. 2007). These mechanisms would seem to suggest that a vector engineering strategy to reduce the number of repeat elements in vectors would give some benefit on stability by (theoretically) reducing the propensity towards recombination-based gene loss. For example, in the case of recombinant antibodies, different promoters and UTRs could be used for the HC and LC sequences. In this context it is perhaps interesting to note the observations of Kim et al. (2011), who speculate that the greater number of repeated elements (e.g. duplicated hCMV promoter and intron sequences) surrounding the Mab LC compared with the HC within certain GS vector designs following linearization (required for stable cell line construction), may contribute to the higher rate of loss of LC observed in some unstable cell lines (Fig. 12.2).

In some CHO cell lines very large changes in recombinant gene copy number have been shown to occur over long-term sub-culture, although it is not known whether such observations are the result of a gradual loss of copies or the result of a single large-scale genetic rearrangement (Kim et al. 2011). Where a loss of gene copies occurs frequently and independently among cells in an originally clonal cell line population (i.e. where copy number per cell consistently decreases across culture), such recurrent genetic rearrangements may perhaps be best explained by a NAHR mechanism. However, if this occurs more precipitously in a very low number of cells which subsequently overgrow the original culture, recent advances in our understanding of cancer genetics may again shed light on this bioprocessing phenomenon. Large-scale genetic changes called Complex Genomic Rearrangements are often observed in cancer cells (Zhang et al. 2009). However, in some cases there may be a sudden large-scale breakdown in genetic stability, a phenomenon called chromothripsis (Stephens et al. 2011). Chromothripsis occurs when multiple DNA breakpoints occur on a single chromosome leading to localised chromosome shattering and large-scale copy number changes (Liu et al. 2011; Stephens et al. 2011). Such phenomena may occur in CHO or NS0 cells in culture producing a substantial decrease in qP as surviving non-producing cells subject to chromothripsis overgrow the original pool of cells (Lee et al. 1991).

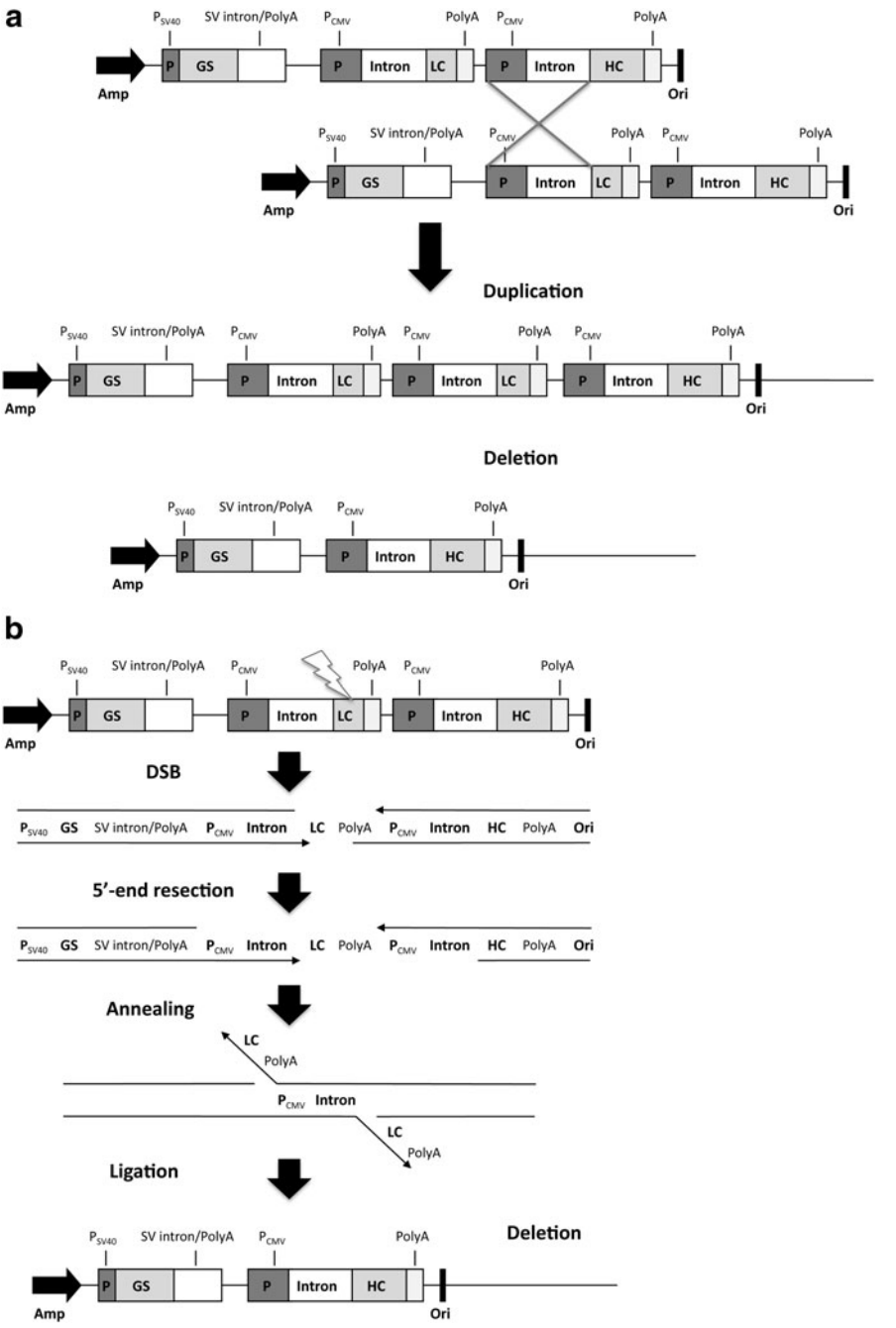

Fig. 12.3 Schematic representation of two possible mechanisms by which gene copy number changes can occur by homologous recombination. (**a**) Non-allelic homologous recombination (*NAHR*) can occur by unequal crossover if a recombination repair event uses a direct repeat as homology (e.g. repeated intron + hCMV-MIE sequences in a vector integration site within the genome). If such an event occurs, this can lead to duplication and deletion of the sequence located

The complex nature of the genetic rearrangements observed in production cell lines (Cao et al. 2012; Barnes et al. 2007), can mean that non-producer cells can overgrow the original population by virtue of genetic mutations that (for example) delete the recombinant protein product gene but leave behind the intact selective marker, thereby permitting continued growth even under selective pressure. Several efforts have been made to counteract this possibility by using a variety of technologies to directly link expression of the resistance gene to the product. For example, IRES elements (Kieft 2008) have been used for this purpose in CHO cells, which direct the production of recombinant multi-cistronic mRNAs containing the selectable maker gene and product gene ORFs on the same mRNA. This mRNA is then translated in 5′ cap-dependent and cap-independent (IRES) mechanisms to produce the recombinant products. Utilisation of such technology typically yields a reduced frequency of non-producer sub-clones and more efficient gene amplification (Ho et al. 2012; Li et al. 2007b; Pu et al. 1998). Although in the authors' experience, poor translation efficiency results in lower product concentration on average, especially for recombinant monoclonal antibodies (Lonza Biologics, unpublished data). Further development of a stringent selection system incorporating an IRES has been described by Van Blokland et al. (2011). In this work the encoded multicistronic mRNA consists of an upstream product gene and a downstream (mutant) zeocin selection marker which are physically linked by both a single IRES and an intervening sequence encoding for a short peptide. The inefficient rate of IRES-mediated translation initiation of the short peptide combined with the necessity for subsequent downstream re-initiation of translation (post-IRES) at the start codon of the selection marker theoretically ensures that only those clones with very high levels of transcription and translation of this entire construct can survive. This system allows the fine tuning of the selection strength since by increasing the length of the short peptide the rate of translation of the selection marker ORF is reduced (Van Blokland et al. 2011). As expected, this system produces fewer producer clones and a lower proportion of non-producers, but those that survive have high levels of production.

Fig. 12.3 (continued) between the repeated elements, which (in this example) could potentially include the LC sequence in a linearised GS vector. (**b**) Single-strand annealing (*SSA*). When 5′ end resection on either side of a DNA double strand break (*DSB*) does not lead to invasion of a homologous sequence, continuing resection can reveal complementary single-stranded sequences that can subsequently anneal. Deletion of the sequence between the repeats, plus one of the repeated elements, can then occur following completion of DSB repair by removal of flaps, gap filling and ligation. In the lower part of (**b**) each *single line* indicates a single DNA strand with polarity indicated by *arrows* on 3′ ends (Figure derived from vector map in Fig. 12.2, and based on information in Hastings et al. (2009b))

12.6 Epigenetics and Cell Line Instability

Epigenetic regulation of gene expression can be achieved via a wide range of chemical modifications to both the DNA and associated histones, including methylation, acetylation, phosphorylation and ubiquitination, and such changes are well known to have a role in cancer biology (Shen and Laird 2013; You and Jones 2012; Li et al. 2007a). As several recent studies have shown, in the absence of a significant change in the number or configuration of transgenes, cell line instability can still occur through the phenomenon of epigenetic gene silencing (Paredes et al. 2013; Yang et al. 2010; Chusainow et al. 2009; Barnes et al. 2004; Strutzenberger et al. 1999). However, the relative importance of gene loss verses transcriptional silencing in specific cell lines may depend on the host as well as the gene expression system used (Kim et al. 2011). Interestingly, in some cases both gene loss and silencing may occur (Osterlehner et al. 2011). Loss of productivity caused by epigenetic gene silencing is specifically recognised when the loss of qP is not associated with a loss of recombinant gene copies but with a decline in recombinant mRNA content. However, a decline in mRNA may not always be associated with a decrease in qP since there appears to be a threshold of mRNA content above which any decrease in mRNA copy number will not manifest as a qP decline (Barnes et al. 2004). This threshold level is thought to be the point at which the downstream biosynthetic reactions utilising recombinant mRNAs are saturated, and therefore only when the transcript copy number declines further will there be any phenotypic consequence. Cell line instability can be observed both in the presence and absence of selective pressure, although it may be both more likely and more precipitous once the selective pressure has been removed (Dorai et al. 2012; Kim et al. 2011; Osterlehner et al. 2011; Yang et al. 2010; Chusainow et al. 2009). Where a decline in recombinant Mab mRNA has been observed, it can affect both the HC and LC genes in cell lines created using both DHFR and GS expression systems, as well as in low and high productivity clones (Kim et al. 2011; Yang et al. 2010; Chusainow et al. 2009). Interestingly, where production instability is observed cell cultures never appear to completely lose the ability to manufacture the recombinant protein (at least over the duration of the analysis), with some cell lines stabilising at a much lower qP (Chusainow et al. 2009; Fann et al. 2000; Weidle et al. 1988).

The expression of stably integrated transgenes is strongly influenced by the site of genome integration (reviewed in Dickson 2009). The eukaryotic nucleoprotein chromosomal material is broadly divided into euchromatin and heterochromatin fractions. Euchromatin is characterised by an open chromosomal architecture and a high density of well-expressed genes. Condensed heterochromatin (including constitutive and facultative varieties) has a low gene density and is usually non-expressed or transcriptionally silenced. Whereas insertion of transgenes into euchromatin is associated with gene expression and productivity, insertion into heterochromatin is associated with gene silencing and low expression levels. Unfortunately it is likely that a subset of transgene insertion sites will end up located within heterochromatin, on the basis that it forms a substantial proportion

of the total nuclear chromatin. For example, the human nuclear genome contains approximately 17–20 % constitutive heterochromatin (Venter et al. 2001). Even if transgenes insert within euchromatin, they are not "safe and dry", since localised heterochromatin formation and chromatin condensation can occur, or even extend into euchromatic regions from neighbouring heterochromatin (Hawkins et al. 2010). Euchromatin and heterochromatin have a number of characteristic molecular hallmarks. In the case of transcriptionally-active euchromatin these include methylation of H3K4 (histone H3: lysine 4), H3K36 and H3K79, and hyperacetylation of histone lysines. For repressed heterochromatin these include methylation of H3K9, H3K27 and H4K20, and hypoacetylation of histone lysines (Kaelin and McKnight 2013; Richards and Elgin 2002). Histone acetylation influences the conformation of the chromatin; specifically, hyperacteylation increases the histone negative charge and thereby weakens the histone-DNA interaction leading to a more open chromatin state that is permissive for transcription (Kaelin and McKnight 2013; Krajewski and Becker 1998).

Direct methylation of cytosines within DNA CpG dinucleotides (CpG cytosine-5) is the most extensively studied DNA modification affecting gene expression in mammalian cells, and there are now several methods available for the high-throughput analysis of genome-wide CpG methylation, including in CHO cells (Wippermann et al. 2014). The vast majority of the eukaryotic genome is methylated at CpG dinucleotides. As a result CpG dinucleotides are actually underrepresented in the human genome based on the CG frequency due to the propensity of methylated cytosine to be converted to thymine by transition mutation following spontaneous deamination (Sved and Bird 1990). However, CpG islands, short regions particularly rich in CpG dinucleotides, are usually unmethylated (Bird et al. 1985). CpG islands are broadly defined by a higher than average GC content (>50 %) over a length of at least 500 bp, and a ratio of observed CpG's to expected CpG's above 0.6 (Siegfried and Simon 2010). However, there is some debate as to how exactly such regions are defined (Saxonov et al. 2006). In humans it is estimated there are around 25,000 CpG islands, whereas recent analysis of the CHO genome estimates the number of CpG islands to be 43,318, with 21,993 located within promoter and intragenic regions, although these numbers may change as bioinformatics analysis of the CHO genome matures (Wippermann et al. 2014; Illingworth and Bird 2009). Indeed, the sizes of the human and CHOK1 genomes are broadly similar (2.9Gbp vs. 2.6Gbp respectively), suggesting that there may be some scope for further adjustment to these figures (Xu et al. 2011; Venter et al. 2001). CpG islands have a very high frequency of association with gene promoters (Saxonov et al. 2006), and it is thought that the unmethylated state of CpG islands is essential for the expression of the associated genes. Indeed, the majority of promoter CpG islands are unmethylated (Illingworth and Bird 2009). How CpG islands maintain their unmethylated status is still largely unknown, although recent research suggests that the conformation of the DNA in the CpG island plays a role in protecting DNA from methylation (Ginno et al. 2012). Specifically, promoter CpG islands possess a characteristic R-loop structure downstream of the TSS caused by the distribution of Gs and Cs across the two DNA

strands (known as GC skew), and such a structure offers a degree of protection against the activity of the *de novo* DNA methyltransferase DNMT3B1 (Ginno et al. 2012). However, where CpG islands are methylated this is a key hallmark of heterochromatin and transcriptional silencing (Illingworth and Bird 2009). Methylation of promoter CpG islands is thought to inhibit transcription through occlusion of TF's; either as a result of methylation-induced changes to the chromatin conformation, or by directly impeding the ability of TFs to bind their cognate recognition sequences (Siegfried and Simon 2010). Methylation of DNA recruits methyl CpG binding proteins that subsequently have the effect of changing the local conformation of the DNA.

From a bioprocessing perspective the main upshot of this is that the presence of a CpG island in the most commonly used promoter, the hCMV-MIE, means that methylation-induced gene silencing is an existential threat to expression using this system. As observed by Kim et al. (2011), the hCMV-MIE contains a CpG island consisting of 29 CpG sites, the vast majority of which are known methylation targets. Several studies have observed an increase in hCMV promoter methylation across long-term sub-culture correlating with a decrease in productivity and a decline in recombinant mRNA copy number (Kim et al. 2011; Osterlehner et al. 2011; Yang et al. 2010; Chusainow et al. 2009). Kim et al. (2011) demonstrated that loss of qP with increasing number of population doublings in recombinant Mab-producing GS-CHO cells was associated with a decline in Mab HC and LC mRNA content and an increase in hCMV-MIE methylation of the promoter CpG island. This qP decline could be partially reversed by treatment of the cells with the DNA methylation inhibitor 5-Aza. Similarly, Yang et al. (2010) demonstrated that treatment of transcriptionally-repressed CHO cells with 5-Aza reduced the level of hCMV-MIE methylation, increased recombinant transcript copy number, and partially restored qP to early passage levels. However, although this phenomenon is well-characterised, it is still unclear which CpG sites in the hCMV promoter CpG island are most critical for reducing transcriptional output when methylated (discussed in Kim et al. 2011), although Osterlehner et al. (2011) have made some significant progress in identifying a specific CpG site whose methylation status is predictive of instability in a significant proportion of unstable clones (see below). A larger survey of clones is surely necessary to see if this is more widely predictive across different CHO cell backgrounds and gene expression platforms.

Where epigenetic gene silencing does occur, the likelihood of it happening may be influenced by the cell line background and the gene expression system used. Where many copies of a transgene lie in a tandem array within a single integration site the likelihood of gene silencing may increase since such arrays are subject to RIGS (Rosser and An 2010; Garrick et al. 1998). Where silencing of tandem repeats of recombinant genes does occur, McBurney and colleagues (2002) have demonstrated that large scale changes to chromatin architecture characteristic of facultative heterochromatin (hypoacetylation) are likely to be involved since treatment with histone deacetylase inhibitors such as trichostatin can prevent such silencing. Highly amplified cell lines, especially recombinant dhfr- cell lines subject to

stringent MTX-mediated gene amplification (Yoshikawa et al. 2000), are likely to have large tandem arrays of repeated transgenes which are perhaps likely targets of RIGS. GS-CHO cell lines tend to have lower gene copy numbers perhaps making them less likely to be subject to silencing by RIGS, although other mechanisms of silencing (CpG methylation) may be just as likely. In this way, the increasing use of cell lines with defined genomic integration sites (site-specific integration landing pads), that can reproducibly integrate single (or low) copy vectors using recombinase-mediated cassette exchange (Zhou et al. 2010; Gorman and Bullock 2000), may be the ideal system going forward as such landing pads can be targeted to euchromatin and are (theoretically at least) much less likely to be subject to RIGS and other forms of heterochromatin formation.

Direct DNA methylation is not the only epigenetic mark that is associated with heterochromatin and transcriptional silencing, as mentioned above. Recently, production instability in CHO cells caused by a decline of recombinant Mab mRNA content was correlated with a decrease in histone H3 acetylation (Paredes et al. 2013). Hypoacetylation of histone lysines, particularly histones H3 and H4, is a function of the relative activities of cellular histone acetyltransferase and histone deacetylase enzymes, and is a key mark of heterochromatin (Richards and Elgin 2002). In this context it is worth noting that several efforts to prevent or reduce the impact of epigenetic silencing of recombinant gene expression have been made. In terms of media development the addition of the short chain fatty acid sodium butyrate, an inhibitor of histone deactylases (Candido et al. 1978), is thought to increase productivity in some cell lines by inhibiting the silencing of transgene expression and/or by opening up the chromatin thereby facilitating transcription. Addition of sodium butyrate has been shown to boost CHO cell-specific protein production rate at least twofold, although it also has a substantial negative impact on growth and product glycosylation (Lee et al. 2013; Kim et al. 2012; Yee et al. 2008; De Leon Gatti et al. 2007).

To make further progress in our understanding of epigenetic gene silencing in CHO cells we need to develop a greater understanding of the underlying chromatin dynamics. Indeed, our knowledge of the CHO cell epigenome, nuclear dynamics, and the greater transcriptional network is extremely limited despite the recent advances in our understanding of the CHO genome and transcriptome. Since these aspects of the CHO cell are highly likely to impact the output from a biomanufacturing process, filling this knowledge gap is likely to affect our ability to meet the patient demand for protein-based therapeutics in the future. By confronting this knowledge gap we will enhance our ability to design next-generation gene expression technology for maximum utilisation of the inherent biosynthetic capacity of the CHO cell factory, and enhanced stability across culture.

12.7 Strategies to Combat Cell Line Instability

Although screening is a very effective tool for identifying unstable cell lines it is still a laborious and time-consuming process. So, with nearly 30 years of research on unstable production cell lines (Kaufman et al. 1985), are we any closer to identifying early predictive markers of instability? As described above, the industry standard hCMV-MIE promoter is often subject to methylation-induced silencing targeted to the promoter CpG island. Are there any early predictors of this process? An interesting study from Osterlehner and colleagues (2011) suggests that unstable cell lines have generally higher levels of hCMV-MIE CpG island methylation even before the instability has set in, making hCMV-MIE CpG island methylation status an early predictor of inherent instability. In particular, Osterlehner and colleagues identified a specific CpG site (−179 relative to the TSS) whose methylation status at early phase culture was predictive of the stability profile of 75 % of clones over long-term sub-culture, although recombinant mRNA content was not measured in this study. Unstable clones also tended to have higher transgene copy number, perhaps suggesting the involvement of tandem repeat silencing by methylation as an underlying mechanism in the cell lines studied (Osterlehner et al. 2011). Based on these findings the authors suggest that clones should be selected on the basis of low transgene copy number to minimise the chance of instability later in culture.

In certain clones the level of IAA also seems to serve as an early predictor of incipient instability. A study by Dorai and colleagues (2012) uncovered a correlation between the cell culture population IAA profiles (measured by flow cytometry) and incipient instability, where unstable cell lines displayed a heterogenous IAA profile with a minority secondary population of cells with much lower IAA even at early passage. With increasing number of population doublings the population of GS-CHO cells with low IAA increased as final culture titre declined (Dorai et al. 2012).

Methods have also been developed to assess clone production instability using cell surface markers. Feng et al. (2011) recently described the use of whole-cell mass spectrometry (MALDI-TOF) as a screening tool to identify unstable cell lines from their cell surface protein fingerprint. This method makes use of the fact that the recombinant protein secreted from the (CHO) cell will be present on the surface of the cell, roughly in proportion to its overall productivity. Similarly, DeMaria et al. (2007) developed a FACS-based screening method that identifies highly productive clones as well as unstable clones by virtue of the expression level of a cell surface-bound protein, CD20. This method is based on linking the expression of the recombinant POI to heterologous CD20 using an IRES, which coordinates synthesis of a bicistronic POI-CD20 mRNA and subsequently independent translation of the POI and CD20 (DeMaria et al. 2007). By this method unstable clones are rapidly identified by a decline in cell surface CD20 expression during the construction process, with the application of flow cytometry providing higher levels of throughput as well as accuracy in the identification of stable high producers.

Although predicting instability early in the cell line construction process is certainly advantageous in reducing risk and project resource requirements, the ultimate solution to this problem is surely the direct engineering of cell lines with significantly reduced propensity towards loss of productivity. Several strategies have been developed to this end with varying levels of success, reflecting the many and varied ways in which chromatin regulation influences both the expression and stability of transgenes. The large percentage of nuclear heterochromatin means that at least a subset of transgene integration sites are likely to be near such regions and thereby subject to silencing. Hence, several strategies have attempted to shield the transgene from the effects of nearby heterochromatin. One particularly ingenious tactic was developed by Chiu and colleagues (2003) where a fusion protein between a transcriptional activator HAT and a LexA DNA binding domain, was shown to generate a region of hyperacetylated chromatin 2.6 Kb in length, and maintain reporter gene expression despite the influence of a nearby silencing element (Chiu et al. 2003). In this (yeast-based) system the HAT activity could be targeted to the required genomic region by virtue of placing LexA binding sites next to the integrated transgene. This technology has been extended and applied to stable CHO-K1 cell lines by using a LexA-p300HAT fusion protein targeted to LexA binding sites located upstream of a CMV promoter prone to silencing (Kwaks et al. 2005). Using this system in combination with additional anti-repressor elements to enhance stability (described in Kwaks et al. 2003), it was possible to achieve high and stable levels of reporter gene expression in the absence of selection pressure, in contrast to control cell lines (LexA-p300HAT but no additional anti-repressor elements), which suffered a general loss of expression over extended sub-culture.

MARs are another element that has been extensively studied for the development of stable cells lines resistant to chromosome position integration effects and gene silencing (Harraghy et al. 2012). Such elements have been utilised for transgene expression for over 20 years (Phi-Van et al. 1990), although the exact mechanism by which MAR elements work has been largely unknown. However, recent work by Arope and colleagues (2013) has shed considerable light on the issue. The transcriptional enhancement and anti-silencing properties may spring from the synergistic effects of the characteristic MAR AT-rich microsatellite core sequence, which is associated with DNA bends and a high level of RNA polymerase II, together with the presence of several consensus binding sites for transcription activator proteins (such as NMP4, SATB1, Fast 1 and CEBP) (Arope et al. 2013). Very large numbers of MAR elements have been discovered in the human genome (Girod et al. 2007), and several elements from a range of genomes (human, chicken), have been tested to improve recombinant protein production (Girod et al. 2005; Kim et al. 2005; Kim et al. 2004; Zahn-Zabal et al. 2001). Most recently endogenous CHO MAR elements have been identified and utilised to augment gene expression in the CHO-K1 cell line (Chang et al. 2014). One recent development in the use of MAR elements involves the application of a MAR element in combination with both the dhfr-/MTX gene expression system and a mammalian replication IR (Noguchi et al. 2012). With this innovative system (termed IR/MAR-DHFR)

CHO cell lines (both DUXB11 and DG44 backgrounds) can be created that produce more recombinant protein and show a degree of enhanced stability over long term culture in comparison with the standard $DHFR^-$/MTX system on its own.

Finally, UCOEs have also been utilised within vector designs to enhance the stability of stable CHO clones and also to boost the productivity from stable transfectants pools (Dharshanan et al. 2013; Ye et al. 2010; Kwaks and Otte 2006). UCOEs are genetic elements isolated from the upstream regions of housekeeper genes and include unmethylated CpG islands and other promoter-associated elements. Such elements are proposed to act by creating a local chromatin environment that is conducive to high level transcription and resistant to both position effects and silencing, although some UCOEs are superior in this regard to others (Nair et al. 2011). When used to generate mammalian cell lines for manufacturing of a recombinant protein they have been shown to reduce the resource requirement during the cell line construction process by increasing the proportion of high expressing clones within the initial transfectant population (Benton et al. 2002). The stability of cell lines created using the UCOE system is also greatly enhanced relative to non-UCOE cell lines (Williams et al. 2005). Interestingly the efficacy of specific UCOE elements is strongly influenced by the associated promoter, presumably because of the specific interactions between the UCOE and the promoter in determining the local chromatin architecture (Nair et al. 2011).

Outside of epigenetic control elements, ACE technology has also been tested for production stability in CHO cells (Combs et al. 2011; Kennard et al. 2007, 2009a, b; Lindenbaum et al. 2004). In this modular system, utilising a satellite DNA-based artificial chromosome, multiple recombination acceptor sites, and a modified version of lambda integrase, it is possible to integrate transgenes into a predefined chromosomal location within the host cell line (Lindenbaum et al. 2004). This integration site, or "landing pad", is located within the ACE chromosome which is stably maintained throughout mitosis alongside the normal chromosome complement of the host cell line courtesy of its own centromeres and telomeres (Lindenbaum et al. 2004). By using this system it is theoretically possible to reduce the cell line construction timeline and the volume of clone screening by virtue of removing the inherent variability associated with random transgene integration into the native chromosomes of the host cell line (Kennard et al. 2007). A further advantage of this system is that in the absence of native host cell chromosome integration there is little potential to damage genes with critical roles in cell metabolism (Meisler 1992). The ACE landing pad should be demonstrably capable of supporting robust, high level transcriptional activity in the ACE host cell line with the added control over transgene copy number offered by changing the number of recombination sites and rounds of transfection. In terms of production stability across multiple population doublings, several publications have demonstrated in a limited cohort of cell lines that this system can support consistent product yields over timeframes necessary for industrial manufacturing, no doubt assisted by the presence of chromosome insulator elements (Sun and Elgin 1999) surrounding the transgene within the landing pad (Combs et al. 2011; Kennard et al. 2007). However, more extensive studies of recombinant CHO clones generated from a range of

CHO cell types (CHOK1SV, CHO-S and CHO-DG44) indicated that even with this system long-term production stability can still be an issue (Kennard et al. 2009a, b). Indeed, in some cases the entire ACE chromosome can be lost during serial sub-culture, particularly in the absence of selection pressure (Kennard et al. 2009a, b).

All of these technologies mentioned above tackle the problem by redesigning the gene expression technology. How about the cell line itself? With the further development and application of genome editing resources such as the CRISPR/Cas system (Cong et al. 2013), transcription activator-like effectors, and zinc finger nucleases (Wood et al. 2011), we may be able to reduce the underlying tendency of the cell towards instability by engineering genome methylation and recombination mechanisms. As the CHO genome resources mature, this may be the next step in the generation of cell lines with enhanced stability.

Conclusion

Cell line stability is an important issue for the industrial manufacturing of recombinant proteins using mammalian cells. The need to perform long-term stability studies on multiple cell lines during the cell line construction processes highly impacts time to clinic. In addition, the final chosen stable cell line may not always have the highest productivity, necessitating a greater number of batches to meet market demand. However, as has been described in this article, there has been a substantial volume of work published over the last 30 years from both academic and industrial scientists investigating the molecular causes of this phenomenon. This has led to the utilisation of new vector elements and designs, as well as the development of predictive tools to mitigate the impact of this problem. However, at the current time cell line instability is still a problem that is being actively managed rather than overcome within the bioprocessing industry. As industrialists involved in biomanufacturing, we expect that the on-going CHO 'omics revolution will give us new discoveries into the fundamental biology of the CHO cell, facilitating the intelligent redesign of expression vectors, and greater (metabolic, genetic) control over the cell lines themselves. As we simultaneously deepen our understanding of the manufacturing process design space, these two major on-going developments may converge to greatly enhance our ability to predict the performance of a host cell line from transfection to 20,000 L bioreactor.

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Chapter 13
Perfusion Processes

Véronique Chotteau

Abstract The interest for perfusion is increasing nowadays. This new focus has emerged from a synergy of a demand for disposable equipment and the availability of robust cell separation device, as well as the need for higher flexibility and lower investment cost. The cell separation devices mostly used today are based on filtration, i.e. alternating flow filtration, tangential flow filtration, spin-filter, or acceleration/gravity, i.e. inclined settler, centrifuge, acoustic settler. This paper gives an introduction to the basic concepts of perfusion and its practical implementation. It reviews the actual cell separation devices and describes the approaches used in the field to develop and optimize the perfusion processes.

Keywords Perfusion • Continuous process • Alternating flow filtration • Tangential flow filtration • Spin-filter • Inclined settler • Centrifuge centritech • Acoustic settler • Hydrocyclone • Floating filter

13.1 Introduction

A general trend in the biopharmaceutical industry is to favor fed-batch over perfusion mode (Chu and Robinson 2001). However recently the interest for continuous processes has increased. The main beneficial differences of perfusion compared to batch and fed-batch are a shorter residence time of the product in the bioreactor, higher cell densities associated with higher daily volumetric production, a longer cultivation length, healthier cultures and better control of the cell environment. A short residence time is more favourable for the product quality and is mandatory for the labile glycoprotein, e.g. factor VIII, enzymes, (Chuppa et al. 1997; Sandberg et al. 2006; Chotteau et al. 2001). Thanks to the higher volumetric production and longer cultivation length, bioreactors smaller than the ones used for fed-batch processes by at least one order of magnitude can be used to manufacture equivalent amounts of the product of interest (POI).

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Perfusion processes have also drawbacks. They are more challenging from technical and (consequently) sterility point of views. Large harvest volumes are continuously accumulating; necessitating further processing into down-stream and large medium volumes are needed. These drawbacks explain the historical predominance of fed-batch in the biopharmaceutical field. This is also clear from the more abundant literature available for the batch and fed-batch processes in comparison to perfusion.

The fact that perfusion processes use smaller bioreactors in production (mostly between 250 and 2,000 L) gives a perfect fit with the limited volume capacity of disposable equipment (see Chap. 15 by Eibl). The increasing use of disposable equipment associated with the emergence of more performing cell separation technologies (e.g. the ATF, see Sect. 13.3.2.2) enabling high cell densities have today changed the perception of perfusion in the biopharmaceutical field. The new interest in continuous processes is supported and reinforced by the large expertise, which has been accumulated from the commercial perfusion processes of labile proteins, e.g. factor VIII, (and stable biopharmaceuticals), see Kompala and Ozturk's report for a list of biopharmaceuticals (Kompala and Ozturk 2005).

A faster expansion of perfusion processes is unfortunately impeded today by the predominance of fed-batch, not to mention the predominance of antibody technology platforms based on fed-batch operation and sustained by existing large (and very large) -scale equipment. Accordingly the skill of the cell culture experts is predominantly in fed-batch processes, and the same goes for the literature/technical information and for Contract Manufacturing Organisations.

Applications of perfusion mode other than for the manufacturing of biopharmaceuticals have recently been reported and are increasingly used in the industry, such as cell bank manufacturing (Tao et al. 2011; Seth et al. 2013; Clincke et al. 2013a) or high cell density seeding of production bioreactors, i.e. '*N-1 step*', (Pohlscheidt et al. 2013; Padawer et al. 2013; Yang et al. 2014; Hecht et al. 2014). Furthermore perfusion processes can be advantageously used for the production of proteins, required as research tool. These latter processes obey hard time constraints but do not require to be optimized to reduce their cost of goods (COGs) and are most often based on low-producer (non optimized) cell lines. In that case, the product manufacturing obtained at high cell density can compensate for the low cell specific productivity while the constant environment of the perfusion is more favourable for the product quality and stability. These processes can use medium renewal in excess compared to the cell demand and therefore require limited process tuning saving labour of development.

In perfusion mode, the culture medium is continuously renewed while the cells are retained inside the bioreactor using a dedicated device, see Fig. 13.1 for a schematic representation. In many cases, the cells are grown until a selected density is reached, then this cell concentration is stably maintained at so called 'steady-state'. The cells are maintained in growing stage while their density is controlled by partially discarding the cell broth in 'cell bleeds' (or 'bleeds'). Alternatively, or in combination, the cell growth is arrested or slowed down for instance by decreasing the temperature. 'Chemostat' refers to a process where the cell broth is continuously

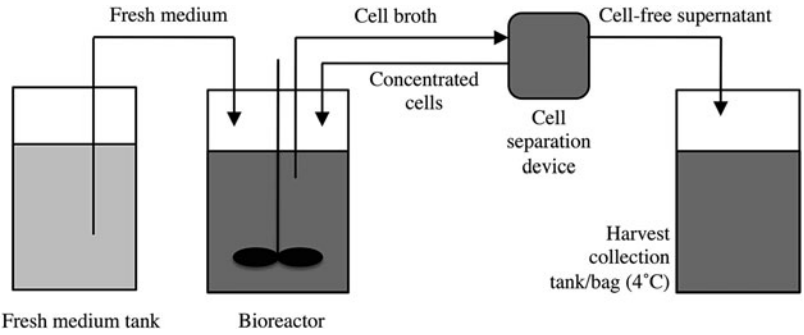

Fig. 13.1 Schematic representation of a perfusion process: Using the cell separation device, the cell-free medium is continuously removed from the bioreactor into the harvest line while the fresh medium is automatically re-fed into the bioreactor. The bioreactor level is maintained constant. Here an external separation device is represented. The cell broth is removed from the bioreactor by a pumping or suction effect. The separation device separates the cell-free supernatant from the cells, which are returned to the bioreactor at a more concentrated density, while the cell-free supernatant is processed to the harvest collector

removed without using a cell separation device at a rate equal to the growth rate. This mode is more common for microorganism fermentation but it is used for mammalian systems as well, mainly as a tool to understand and develop continuous processes.

Perfusion provides a continuous renewal of the culture medium, generating a constant environment to the cells. This is beneficial for several factors: the cell metabolism and growth, cell health and the product of interest (Ryll et al. 2000; Mercille et al. 2000; Tang et al. 2007; Voisard et al. 2003). As represented in Fig. 13.1, cell-free medium is continuously removed from the bioreactor into the harvest line while fresh medium is automatically re-fed into the bioreactor; the bioreactor level being maintained constant. The medium renewal continuously provides nutrients to the cells, so that their depletion can be avoided, as well as a continuous partial removal of unwanted components: the metabolic by-products, i.e. lactate and ammonia, the carbon dioxide produced by the cells and the HCO_3^- from the alkali added for pH control. Finally the product of interest (POI) is continuously removed from the bioreactor (except in the hybrid fed-batch and perfusion concept, see Sect. 13.3.2.2), and can be stored in a cooled harvest tank. This is beneficial for the product quality and stability, and indispensable for the labile products. The constant environment provided by the perfusion mode is also beneficial for other types of mammalian cell cultures like stem cells, primary cells or tissue culture (Serra et al. 2012; Rodrigues et al. 2011).

The work and resources required to develop a perfusion process varies depending on the purpose of the process. A sub-optimal process, requiring limited efforts, can be acceptable for a rapid POI production for Discovery/Research, in the case of a process developed for pre-clinical and early clinical phases under hard time constraints. The process can then be further optimized in preparation for the commercial phase (Meuwly et al. 2004). More work will be needed when

developing a manufacturing process where the production yield and the product quality are critical. Obviously even more efforts will be necessary if the POI is unstable or sensitive. Concerning the cell manufacturing for cryopreservation or inoculation of a production bioreactor, the accent is put on cell healthiness (i.e. high viability), high cell density and cell stability. High viability is critical for the subsequent operation steps since low viability could obviously jeopardize the next step but also cell death could be associated to cell selection, resulting in a cell population shift.

After this Introduction, the present chapter will present the practical aspects of implementing a perfusion process in Sect. 13.2. Then Sect. 13.3 will review the main cell separation devices used today. This will be followed by a description of the approaches to develop a perfusion process, see Sect. 13.4. Finally the future perspectives are discussed in Sect. 13.5.

13.2 Basics of the Practical Implementation of Perfusion Processes

In a perfusion process, the culture medium is continuously renewed by removal of conditioned medium compensated by feeding of fresh medium while a cell separation device retains the cells in the bioreactor. The practical implementation of the perfusion process requires that the volume of fresh fluid (i.e. medium, additives, alkali) added to the bioreactor is exactly equal to the volume removed from the bioreactor consisting of the spent medium, the cell bleeds and the sampling volume. A typical method is to pump out the supernatant at a given rate corresponding to the perfusion rate, which should take into account the cell bleeds, and to automatically feed the fresh medium in order to maintain the culture volume constant (using feedback control). It is recommended to add the fresh medium at a slow pace to minimize the impact of the fluctuations of the spent medium removal and to avoid affecting the temperature of the culture. Alternatively, the fresh medium can be fed at a given rate and the spent medium is automatically pumped out based on the culture level. Most operators prefer the first alternative since instrument failure might cause an empty bioreactor while the second alternative will provoke overflooding of the bioreactor. An alternative option is to feed and harvest using calibrated pumps without feedback control, however this requires thorough manual monitoring/checking to avoid varying bioreactor volume or even bioreactor wash out (so it is not an option for manufacturing processes). Recall that identical pumps do not always provide exactly the same flow rate, a fact enhanced by the tube fatigue after long operation periods.

The bioreactor volume can be measured using a level probe or a load cell, taking into account a conversion factor from weight to liquid (often approximated to 1 kg/L). The perfusion rate (D), expressed in number of reactor volumes per day or RV/day [unit $= \text{day}^{-1}$], is the daily rate at which the medium is renewed. During

operation, the actual perfusion rate should be checked at least daily. It can be measured based on the fresh medium consumption (volume or weight) or on the sum of exhausting fluids (volume or weight) consisting in harvest, cell bleed and samples. It is recommended to check its value based on at least a couple of hours of operation or 1 day, to average the small fluctuations in the system.

For cultures of cells in suspension, perfusion can be applied directly after the inoculation however the culture is often initiated in batch mode, e.g. at cell density $0.3–0.6 \times 10^6$ cells/mL. The perfusion should be started when the cells are still in exponential growth phase, ensuring that no limitation has occurred, typically at a cell density of 1 or 2×10^6 cells/mL. The perfusion is often initiated at 1 RV/day, which is a set point adopted in many perfusion processes and can support cell densities up to 20×10^6 cells/mL in many cases of CHO cell-based process using a well performing culture medium – this is of course process specific. It is a convenient starting value for the development of a new process. 1 RV/day perfusion rate is also suitable to adjust the operation parameters of the perfusion such as the medium and harvest pump rates. Notice however that, at low cell density, 0.5 RV/day or less can be enough in many cases. It is possible to start at higher cell density, shorting down the time required to achieve the target cell density, but the perfusion should be initiated immediately at inoculation in that case. Notice that the inoculation cell density has not the same major impact as in a fed-batch process due to the application of the perfusion.

13.3 Cell Separation Devices

The present text presents the existing devices for cell separation not integrated as a part of the bioreactor, which are reviewed in Chaps. 4 and 15. Several detailed reviews (Castilho and Medronho 2002; Voisard et al. 2003; Kompala and Ozturk 2005; Warnock and Al-Rubeai 2006; Griffiths 2001; Woodside et al. 1998) have been previously published giving a deeper view of the existing cell separation techniques. The present text aims at updating or complementing this knowledge more than repeating it and is focussing on used techniques more than those at an explorative stage.

In mammalian cell perfusion, the separation device can be based on different physical principles: (i) filtration, (ii) acceleration/gravity, (iii) electric field, see (Castilho and Medronho 2002) for review. The separation device can be mounted internally in- or externally to the bioreactor. For several technologies both alternatives exist, however in large scale they are usually mounted outside the bioreactor. The advantage of mounting the separation device internally is the compactness of the system for sterilization operation and foot-print, and in certain cases, e.g. filtration, to avoid pumping the cells using a peristaltic pump. The main advantage of mounting the separation device externally is to have a better access to the device in case of decreased operation performance, alleviating system cleaning or regeneration, or in case of failure, allowing replacement or

re-sterilization. This advantage leans in favor of external mounting instead of internal when possible.

13.3.1 Basics About Factors Characteristic of the Separation Devices

Several factors characterize a cell separation device: the perfusion rate capacity, the cell density capacity, the re-circulation rate, the mechanical stress, the residence time, the cell retention and the POI retention inside the bioreactor.

Several devices consist of a re-circulation loop integrating the separation device, e.g. tangential flow filtration, acoustic settler. The cell broth is circulated in this loop (using for instance a pump) at a rate called the re-circulation rate. Obviously, this rate has to be higher that the perfusion rate. It can be higher by one or two orders of magnitude.

The cell separation process often implies mechanical forces, and can therefore potentially damage the cells. This effect can be due to the separation device, to the re-circulation of the cell broth in a tubing system and, in some devices, to a peristaltic pump. In a tubing system, the cell damage can occur due to the wall shear stress, accentuated by the transition edges of the connectors. The detrimental effect of the fluid circulation and pumping is not the velocity it-self but what is occurring at the boundary of the fluid in movement with the still solid parts, i.e. the tube wall, where the fluid velocity is null. This effect is the wall shear stress. Some POI can also be sensitive to shear stress.

The residence time in the separation device can impact the process since the cells are outside the controlled environment of the bioreactor. For instance, oxygen shortage is rapidly occurring (within minutes or seconds depending of the cell density). Very long residence times can even result in substrate shortage and metabolic oscillations.

The cell retention is the yield of the number of cells retained in the bioreactor over the total amount of cells (i.e. sum of the cells released into the harvest line and the ones retained in the bioreactor).

The POI can be unintentionally retained inside the bioreactor by the cell separation device (instead of passing to the harvest line). This is typical for filter-based technologies. A gel layer is formed on/in the filter membrane, which can reduce the pore size or even block the pores. When the pore size is sufficiently small the POI can be partially retained. Devices other than filter-based can also some times suffer from POI retention due to cell accumulation. The POI retention is monitored by comparing the POI concentrations in the bioreactor and in the harvest line.

13.3.2 Cell Separation Devices Based on Filtration

Cell separation by filtration has been used since decades. 'Classical' filtration where the cell broth flow is orthogonal to the membrane or dead end filtration is not suitable for perfusion since filter clogging happens very quickly. Several alternative filtration technologies have been developed to overcome this by putting in motion the filter or the cell broth: spin-filter, tangential flow or cross-flow filter, floating filter, vortex-flow filter. The shear created by this movement helps to free the filter pores. Filter fouling is an inherent drawback of any filtration technology. Fouling results in degraded filtration performances in terms of filtered fluid flow rate but can also generate a selective retention of the POI.

13.3.2.1 Spin-Filter

The spin-filter was introduced by Himmelfarb et al. (1969). It consists of a cylindrical screen, which is continuously rotating together with the bioreactor impeller or independently, see Fig. 13.2. The cell-free supernatant enters in the spin-filter while the cells are retained outside. The permeate is pumped from the spin-filter cage into the harvest line. It was first introduced mounted inside the bioreactor. The alternative of external placing is preferred today in particular for large-scale bioreactors. Outside the bioreactor, it can be replaced or cleaned and re-sterilized in case of fouling. Fouling of spin-filter has been often described. It has been showed that it is caused by a deposition of nucleic acid and dead cells (Esclade et al. 1991; Mercille et al. 1994).

The operation parameters are critical for the fouling: material such as hydrophobic plastic should be preferred to stainless steel for the screen (Avgerinos et al. 1990; Esclade et al. 1991). The addition of silicone polymer antifoam has been reported to rapidly cause filter fouling (Emery et al. 1995). Higher rotation speed of the spin-filter helps to reduce the fouling but can be associated to increased cell damage (Deo et al. 1996; Vallez-Chetreanu et al. 2007). Higher perfusion rates result in increased fouling (Tolbert et al. 1981). This factor is unfavorable for high cell densities since these require higher perfusion rates. As Yabannavar (Yabannavar et al. 1992) predicted experimentally, Figueredo-Cardero et al. (2009) showed by CFD that the liquid flow direction through the spin-filter screen is not only inwards the spin-filter cage but also outwards; the perfusion rate being the difference of both flows. The study showed as well that higher rotation speeds increased the outwards flow while increasing the perfusion rate had only a limited effect of reducing this flow. Vallez-Chetreanu et al. (2007) showed that larger filter pore size can improved the filter longevity and that applying ultrasound-vibration to the spin-filter had a positive effect to reduce the fouling, resulting in a doubled culture period.

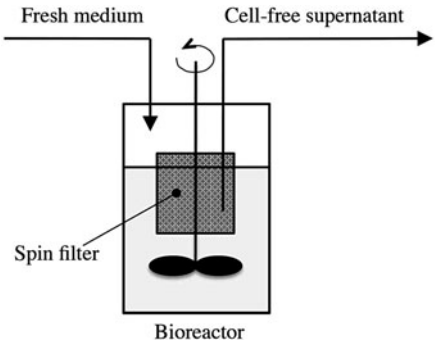

Fig. 13.2 Representation of bioreactor equipped with an internal spin-filter. The spin-filter consists of a cylindrical screen, which is continuously rotating. The cell-free supernatant enters in the spin-filter from where it is pumped into the harvest line

13.3.2.2 Tangential Flow Filtration (TFF) and Alternating Tangential Flow Filtration (ATF)

To minimize filter fouling, the flow of the cell broth can be tangential to the filter surface instead of orthogonal like in a spin-filter or a 'classical' filter, e.g. depth filter (de la Broise et al. 1991; Velez et al. 1989; Caron et al. 1994; Kawahara et al. 1994). The tangential movement reduces the filter fouling since the particles are not pressed into the filter membrane. The first tangential systems used in animal cell culture were based on membranes, i.e. cross-flow filtration. Later, scientists adopted hollow fibres (HF) instead since these are offering a much larger surface area. 'Tangential flow filtration' or TFF refers nowadays to perfusion based on HF used in tangential flow mode.

TFF

In the TTF system, the cell broth is pumped in the lumen of the fibres using a peristaltic pump. The cell-free supernatant passes through the fibre membrane in a direction orthogonal and radial to the fibres, see Fig. 13.3. All the fibres are caged together in a shell from which the supernatant is pumped into the harvest line. Several tens or hundreds of fibres are mounted in parallel in a cartridge. The scale-up is made in first hand by increasing the number of fibres in the cartridge. The fibre pore size is often 0.2 μm, the size for sterile filtration or microfiltration. Alternatively 0.45 μm is adopted. In the 1990s, other sizes of several μm were tested as well in cross-flow filters (de la Broise et al. 1991; Kawahara et al. 1994). Smaller pore size, i.e. ultrafiltration (UF), has been recently used, e.g. 50 kDa cut-off filters used for the production of MAb retained in the bioreactor (see below).

Several studies (Smith et al. 1991; Hiller et al. 1993; Zhang et al. 1993; Kyung et al. 1994) were published in the early 1990s using cellulose ester HF achieving varying densities of hybridoma or HEK293 cells from a few millions cells/mL to a maximum of 10^8 cells/mL by Kyung. Several years later, Cortin et al. (2004) and Galvez et al. (2012) successfully produced adenovirus in HEK 293 cell system. In 2011, Clincke et al. published a comparative study of perfusion in wave bioreactor

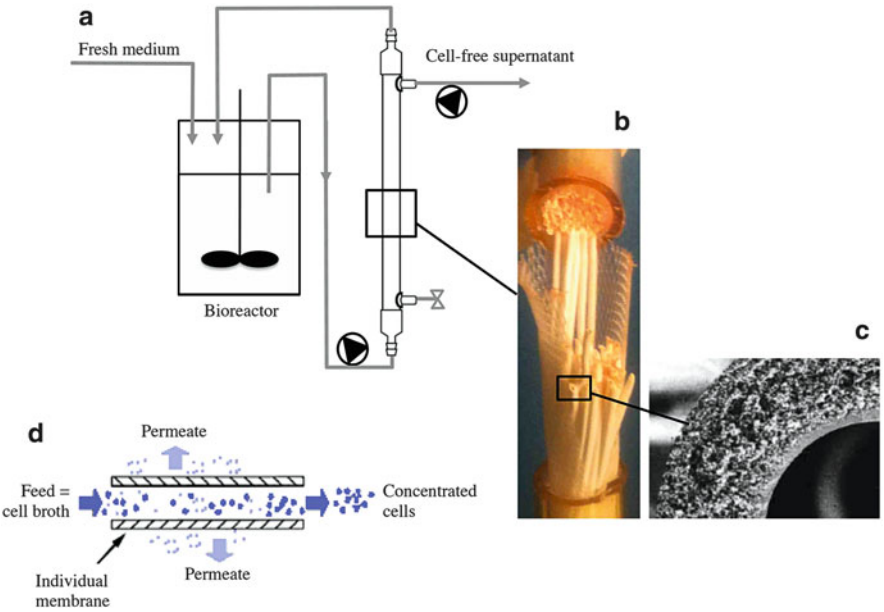

Fig. 13.3 Perfusion using a hollow fibre filter in TFF mode: (**a**) Schematic representation. The cell broth is pumped into the HF. The cell-free supernatant (permeate) is pumped into the harvest tank while the concentrated cells are returned into the bioreactor. (**b**) Photo of a HF (GE Healthcare) cut to show the detail of the polysulfone hollow fibres in the solid outer shell. (**c**) Detail of an ultrafiltration hollow fibre of GE Healthcare (*photo courtesy of GE Healthcare*). (**d**) Schematic representation of the principle of the tangential flow filtration occurring in a hollow fibre. A peristaltic pump pushes the cell broth in the fibre lumen. The liquid can pass through the fibre membrane while the larger particles, i.e. the cells, are retained inside the fibre. Exiting the fibre, the cell broth is more concentrated than at the inlet

using TFF or ATF using polysulfone HF (Clincke et al. 2011, 2013a, b). They established steady state cultures at $\approx 25 \times 10^6$ cells/mL or 1–1.2 $\times 10^8$ cells/mL during more than 2 weeks and reached a maximal density of 2.14×10^8 cells/mL.

Membrane Fouling

TFF systems have proved to reduce fouling compared to the spin-filter. However membrane fouling leading to a partial retention of the POI has often been observed. Hiller observed that up to 20 % of produced antibody was retained inside the bioreactor in a hybridoma perfusion process and installed a back flow flushing, which could reduce the membrane fouling (Hiller et al. 1993). Zhang et al. (1993) coated the HF with polyethylene glycol before autoclaving to reduce the protein binding since polyethylene glycol had been reported to be effective for low protein adsorption and low cell binding. They also installed a back flush of the HF. These

operations prolonged the HF usage but could not completely prevent the membrane fouling/blockage.

ATF

The alternating tangential flow filtration (ATF), which is using the hollow fibre filter in a different way, was introduced by Shevitz in 2000 (Shevitz 2000). In this system, a diaphragm pump pushes and pulls the cell suspension in the fibres in an alternated way with a cycle of around 1 min, see Fig. 13.4. No peristaltic pump is necessary so this is gentler for the cells compared with the TFF. Furthermore the alternating flow creates a back flush in the filter membrane, which is favourable to reduce the filter fouling. We compared the ATF and TFF performances and showed that the POI retention by the membrane was systematically less severe using the ATF (Clincke et al. 2013a). The product retention was however not totally absent. We have also observed higher or lower retention for different MAb's using the same process and medium hence the POI retention is product specific. Typically, during several days after a new HF has been installed, the POI is freely passing into the harvest line; hence the POI concentrations in the bioreactor and in the harvest line are similar. Then the bioreactor POI level starts to increase compared to the harvest line. This divergence is more pronounced using TFF than ATF.

Several studies have been published based on the ATF technology. In bioreactor size up to 70 L, Yuk et al. (2004) produced recombinant oncolytic adenoviral vector in HeLa cells adapted to suspension and infected at 10 or 20×10^6 cells/mL. Bleckwenn et al. (2005) obtained a cell density of 4.4×10^6 cells/mL of HeLa cells anchored on microcarriers Cytodex 3. Jardin et al. (2007) produced secreted alkaline phosphatase using Sf-9 cells maintained at steady-state cell density around 20×10^6 cells/mL during 43 days. Using the vaccinia virus expression system, they produced gp120, HIV envelop coat protein, and enhanced green fluorescence protein.

We performed a series of experiments in a wave bioreactor connected to an ATF2 using a MAb producing CHO-K1 DHFR$^-$ cell line (Clincke et al. 2011, 2013a, b; Chotteau et al. 2014a). We developed and studied this system in several runs maintained at 20×10^6 cells/mL by cell bleeding during up to 2 weeks. Cell densities of $1.23–1.32 \times 10^8$ cells/mL could finally be reached. Recently, we developed similar processes in stirred-tank bioreactor for two other MAb producing CHO-K1 cell lines, a CHO-K1 DHFR$^-$ line and another CHO-K1 line using an anti-silencing region expression system. We maintained the cultures at steady-state by cell bleeds around 20×10^6 and around 1.1×10^8 cells/mL during 1 week, then densities up to 1.9×10^8 cells/mL were finally obtained (Chotteau et al. 2013; Zhang et al. 2014b). Padawer et al. (2013) and Yang et al. (2014) showed the advantage of implementing perfusion-based high cell density inoculation (N-1 stage) of fed-batch production bioreactor for several CHO cell lines producing MAb's or fusion protein. Padawer grew cells around 20×10^6 cells/mL and inoculated a production bioreactor up to 5×10^6 cells/mL ($25 \times$ higher than control

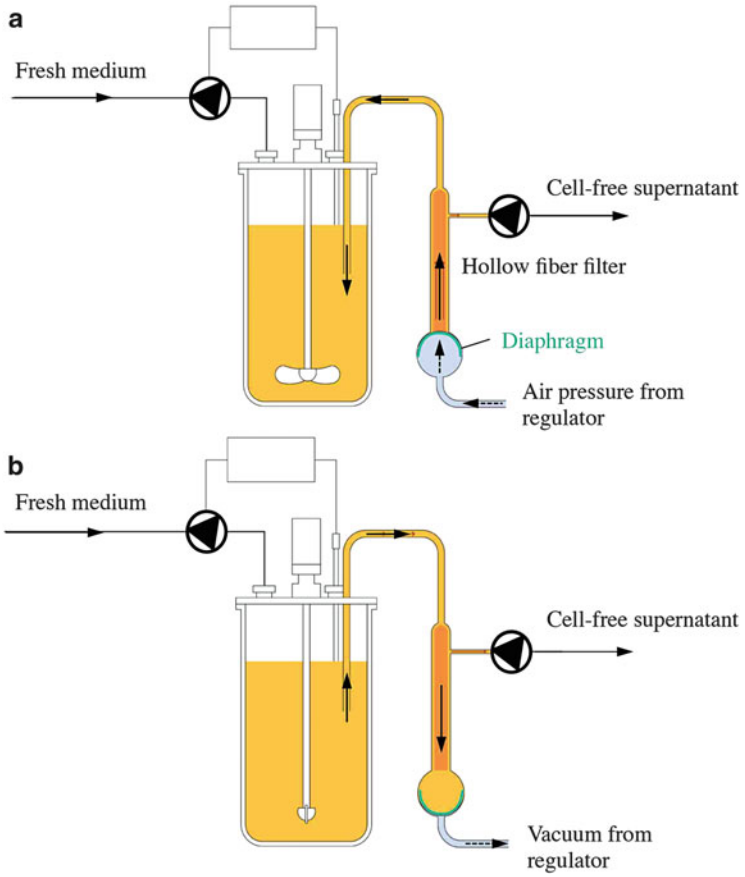

Fig. 13.4 Schematic representation of the alternating movement of the ATF technology: (**a**) The diaphragm is pushed towards the HF by pressurised air from the ATF regulator. The cell broth is pushed back into the bioreactor. (**b**) The diaphragm is pulled outwards the HF by the regulator vacuum effect. The cell broth is pulled outside the bioreactor into the HF

process) without affecting the process performances. In Yang's report, cell broths from 40×10^6 cells/mL density cultures were used to inoculate reactors at 10×10^6 cells/mL resulting in final product quality equal or superior to the control process. Genzel et al. (2014) compared the production of influenza A vaccine in an ATF-based perfusion process using several types of hollow fibre cartridges in two cell lines, avian AGE1. CR cells and human CAP cells, reaching up to $50 \times$ 1E6 cells/mL.

Ultrafiltration

The concept of continuous filtration to concentrate high molecular weight components, i.e. retaining the product of interest in the bioreactor and eliminating low molecular weight components (e.g. toxic by-products, lactate and ammonium) was introduced in continuous dialysis bioreactor (Kurosawa et al. 1991; Amos et al. 1994). The same principle is now used in industry using ultrafiltration HF perfusion, i.e. XD Process Technology at DSM, achieving very high cell densities (Zijlstra et al. 2008), however experimental details have not been disclosed. In a wave bioreactor study, Clincke et al. observed a similar cell growth until 10^8 cells/mL density using microfiltration and using 50 kDa UF membrane connected as TFF or as ATF (Clincke et al. 2013a). In this UF process, the permeate was pumped at a much lower negative pressure due to the smaller fiber pore size, eventually causing a complete fiber fouling around 10^8 cells/mL density.

The advantage of this process is to retain the product of interest inside the bioreactor so that harvesting of the bioreactor is performed once instead of weekly/bi-weekly harvests of the perfused permeate. This process cannot be as long as conventional perfusion due to the risk of degradation of the product of interest or even the risk of aggregation from high protein concentration. Noticeably as well, the cell clarification of these very dense cultures at the end of a run poses new challenges.

Shear Rate

In the ATF or the TFF, the cells are exposed to shear. The shear is usually characterised by the shear rate, γ, as follows

$$\gamma = \frac{4q}{\pi R^3} \tag{13.1}$$

where q = flow rate in the lumen of one fiber; R = lumen radius of a fiber.

Zhang et al. (1993) observed cell damage for a shear rate $\geq 1{,}266\ s^{-1}$ in 1 L hybridoma cell perfusion using a HF of mixed cellulose ester of 0.2 μm pore size. Maiorella et al. (1991) observed no cell damage up to a shear rate of $3{,}000\ s^{-1}$ in cell separation for harvest from Sf-9, hybridoma and myeloma cultures using cross-flow filtration. Clincke et al. applied shear rates of 1,000, 2,400 and $3{,}400\ s^{-1}$ in CHO cell perfusion without observing an adverse effect (Clincke et al. 2013b). During this study, blockage of the hollow fibers was never observed.

13.3.2.3 Floating Filter

Perfusion in wave bioreactors can be operated using a flat floating filter. The filter is placed inside bioreactor and the wave motion creates shear. The compactness of the

system alleviates the operations. Using this system, cell densities of 20×10^6 hybridoma cells/mL, 27×10^6 CHO cells/mL and 1.5×10^8 PerC6 cells/mL (this latter obtained in 1 week) have been reported (Tang et al. 2007; Tao et al. 2011; Adams et al. 2011). A drawback of this technology is a high tendency for filter clogging at high cell density.

13.3.3 Devices Based on Acceleration or Gravity

Several technologies are based on acceleration or on gravity with or without enhancement of the cell separation. Gravity is exploited in gravity settlers where the cell broth is vertically pumped upwards with a flow slower than the cell sedimentation velocity. Applied to single cells in suspension, the sedimentation is very slow causing a very long cell residence outside the controlled conditions, which is unfavourable for the cells. Hence these devices are only recommended when the cells are anchored on microcarriers so that the sedimentation velocity is sufficiently fast (since the microcarriers have a higher density compared to the single cells). The cell sedimentation velocity is increased in the inclined settler and in the acoustic settler. Separation by centrifugal acceleration is used in centrifuges and in the hydrocyclone.

13.3.3.1 Inclined Settler

Inclined settlers (Fig. 13.5) include several inclined plates or lamellae on which the cell sedimentation occurs more rapidly than in free cell broth due to a convection phenomenon, called Boycott effect (Boycott 1920; Kinosita 1949). The cell broth is pumped in the lower part of the settler; the sedimented cells are sent back to the bioreactor; and the cell-free supernatant is pumped from the top part of the settler into the harvest tank.

The maximal perfusion rate increases with the settling area. Inclined settlers have the advantage of minimizing the shear on the cells and a certain ability to eliminate the dead cells from the bioreactor. Disadvantages are limited perfusion rates, long residence time in the cell separation device (32 min or the cells in suspension in the settler and 1.46 h for those adhering on the lower plate (Searles et al. 1994)), and that the cells have a tendency to adhere to the plates. Applying a coating on the lamellae mitigates this last effect. Application of a vibration and chilled plate are also used to reduce the cell adhesion (Searles 1994; Hecht et al. 2014). A heat exchanger to decrease the cell broth temperature to room temperature is advantageously implemented to reduce heat convection problems in the settler and reduce the cell metabolism. Lipscomb et al. (2004) used an inclined settler where the bottom plate was chilled (4 °C) to reduce the cell adhesion as well as back-pulsing was daily applied to prevent cell build-up in the drain port. Batt reported cell density of 10×10^6 hybridoma cells/mL (Batt et al. 1990). Choo

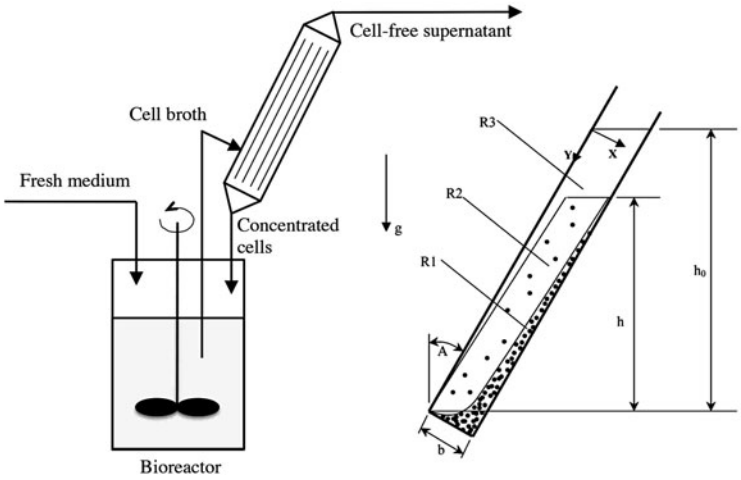

Fig. 13.5 *Left*: Schematic representation of an inclined settler connected to a bioreactor. The cell broth is pumped in the lower part of the settler; the sedimented cells are sent back to the bioreactor; and the cell-free supernatant is pumped from the *top* part of the settler into the harvest tank. *Right*: Illustration of the physical principle of the inclined settler reproduced from Xu and Michaelides (2005), permission of reproduction Taylor & Francis. Three parts are distinguished: *R1*, the sediment layer region; *R2*, the dilute suspension region; and *R3*, the clear fluid region

maintained a NS0 culture at 8×10^6 cells/mL during 53 days with a cell retention $\geq$98 % (Choo et al. 2007). Pohlscheidt reported cell density of 16×10^6 CHO cells / mL in 3,000 L bioreactor at 1 RV/day with a cell retention of 85 % (Pohlscheidt et al. 2013).

Shen and Yanagimachi (2011) studied the characteristics of inclined settler by CFD (computational fluid analysis) approach. They concluded that the settling behavior of the particles depended of the harvest flow, not of the inflow rate or the ratio of the plate length to the plate width. To improve the performances, they advised to decrease the spacing between the plates.

13.3.3.2 Acoustic Settler

In the acoustic settler, the cell sedimentation is enhanced by a temporary cell aggregation, see Fig. 13.6. An acoustic wave is applied to a chamber where the cell broth has been pumped. The cells are concentrated at the resonance nodes of the acoustic waves. When the wave is interrupted, the aggregated cells sediment rapidly, falling back into the bioreactor. The device is operated in cycles of filling the chamber, applying the wave and interrupting the wave to allow cell sedimentation. One issue of this technology is the power needed to create the waves and the associated heating effect, which can be damageable for the cells. Although this has been solved for small scale by air cooling and at pilot scale (200 L) by water circulation, this remains an issue for larger scale-up. A detailed review of the acoustic settler has been published by Shirgaonkar et al. (2004).

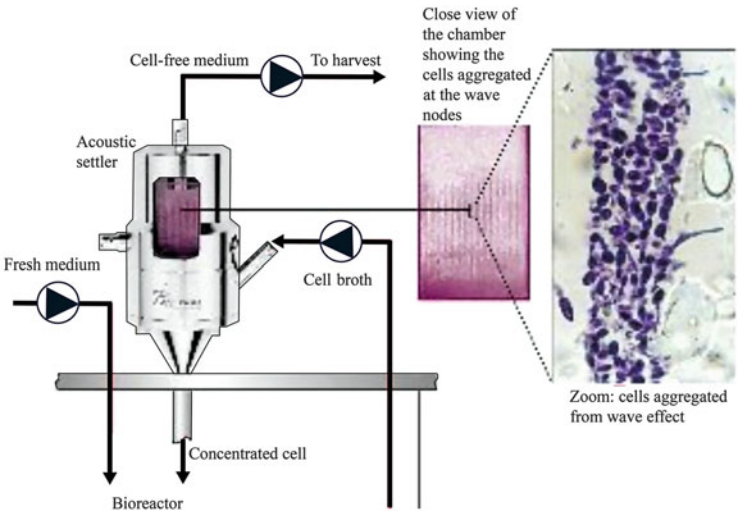

Fig. 13.6 Acoustic settler. Representation of the settler BioSep and the chamber where the cells are accumulating at the nodes of the wave (Courtesy of Applikon Biotechnology)

Sf9 cell densities of 30×10^6 cells/mL and 40×10^6 cells/mL have been reported using the acoustic settler (Zhang et al. 1998; Gorenflo et al. 2004) and Gorenflo obtained 10×10^6 CHO cells/mL in 100 L scale (Gorenflo et al. 2002). An improvement of the technology was brought by installing an air-driven system able to suck the cell broth into the settler chamber and to back flush the cells into the bioreactor, avoiding the use of a peristaltic pump and enabling to empty the chamber completely (Gorenflo et al. 2003). Using this system, they maintained a cell density of 10×10^6 cell/mL during 110 days at 95 % viability. The same group optimized the settler setting parameters to maximize the cell retention (Gorenflo et al. 2005b). Dalm studied the effect of the re-circulation using the pilot scale settler system (Dalm et al. 2005). She concluded that oxygen depletion could occur at re-circulation rate $\leq$6 RV/day and that this depletion caused a slight increase of the lactate production. In a study of the feed and cell bleed, the same group obtained a density of 42×10^6 hybridoma cells/mL (Dalm et al. 2004).

13.3.3.3 Centrifuge

Centrifugation is an efficient way to separate the cells from liquid. It can also separate dead from living cells efficiently. Operating centrifugation sterilely and continuously during weeks or months is however challenging. The Centritech centrifuge provides this function while the adaptation of stack centrifugation to perfusion requirements has not really succeeded. Originally developed in Sweden, the Centritech technology is today commercialized by CARR/Pneumatic Scale

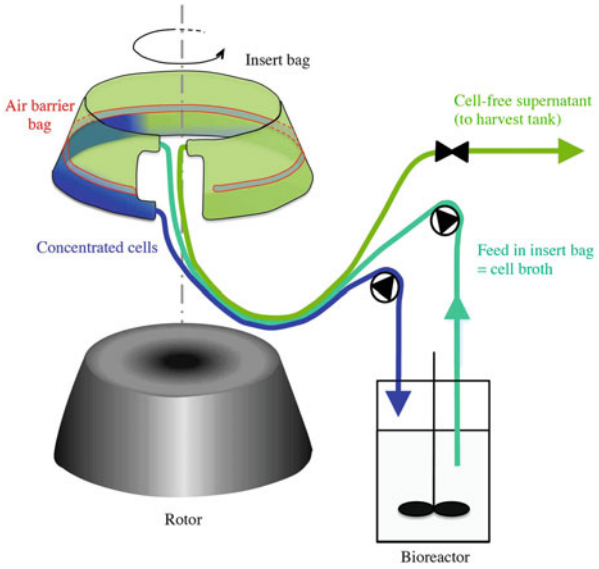

Fig. 13.7 Schematic representation of the principle of operation of the centrifuge Centritech. The cell separation occurs in the disposable insert bag fixed on a rotor continuously in rotation. A cycle of about 1 min allows sequentially the following moments: (1) the cell broth enters into the insert bag while the air barrier bag is deflated allowing free passage of the cell broth in the hole insert bag, (2) the supernatant is separated by centrifugal force and by the inflated air barrier bag (in *red*), which physically separates the supernatant from the concentrated cells, (3) the concentrated cells are sent back to the bioreactor. This cycle is repeated indefinitely beginning again by operation (1) and so on

Angelus, USA, after several rounds of acquisitions and purchases (e.g. Kendro, Heraeus, Sorvall).

In this device, the cell separation occurs in a disposable insert bag mechanically fixed on a conical rotor in continuous rotation, see Fig. 13.7 for an illustration. The conical shape provokes the cell concentration at the largest part of the rotor. An air barrier bag is inflated to physically separate the supernatant from the concentrated cells. The cell broth, the supernatant and the concentrated cells are automatically admitted into- or removed from the insert bag in repeated cycles. The insert bag together with the connecting tubes to the bioreactor and the harvest tank is disposable. This last feature makes this device quite attractive not only for the biopharmaceutical manufacturing but also for harvest of virus production. Another advantage is that, when correctly operated, it is gentle for the cells. Unfortunately, it suffers also from several drawbacks. The operation parameters require quite some optimization and the operators need to be well trained. The insert bag and in particular the connecting tubes are submitted to high mechanical fatigue from a constant rotation (made possible by using the same principle as cables connected to rotating radar). The quality of the insert bag is therefore critical. Unfortunately at each acquisition of the Centritech technology by a new actor, the major product

developments have been to change the colour of the instrument as well as to relocate and modify the insert bag manufacturing causing repeatedly failure in the bag quality and delivery.

Described in the early 1990s (Apelman 1992; Apelman and Bjorling 1991; Chatzisavido et al. 1993), it has been adopted in some commercial perfusion processes for the production of labile proteins, e.g. Shire (two CELL Centritech systems connected to a 2,000 L bioreactor), Swedish Orphan Biovitrum (D-domain deleted factor VIII Refacto® manufacturing for Pfizer at 500 L scale). Two models of the Centritech centrifuge are available, the LAB II for small and pilot scale (12–259 L/day perfusion rate and up to $253 \times g$) and the CELL Centritech (144–2,880 L/h, up to 320 g). The maximal perfusion rates are however lower in practice. They are decreasing when the cell density is increasing and are dictated by the volume of concentrated cells in the insert bag, the time for cell concentration per cycle and the number of cycles per hour. The small-scale centrifuge is a good tool to predict the large-scale centrifuge however it is not perfect due to the different dimensions of these devices. The high purchase price of the small-scale machine is an impediment to the purchase of numerous centrifuges, limiting the capacity for process development. The residence time in the Centritech centrifuge is about 2 to 9 min (Chotteau et al. 2002), of which the residence time in the separator it-self is less than 1 min. The total residence time is highly depending of the length of the connecting tubes, which should be minimized. Observe that the total residence time is not longer in the large centrifuge compared to the small one since larger diameter tubes are used at larger scale. Several studies have reported a damageable effect of the Centritech on the cells (Johnson et al. 1996; Chotteau et al. 2002; Kim et al. 2007, 2008). The cell damage increases with the rotor speed and the applied g force. From studies supervised by T. Björling at Pharmacia-Upjohn, it was concluded that this effect was not due to the pumps as claimed by Kim et al. (2008). It is quite probably not due to the acceleration it-self but rather associated to a wall shear stress in the insert bag. Hence higher acceleration provokes higher damage. The cell damage can be significantly reduced by using the centrifuge in intermittent mode, i.e. several minutes per hour or so (Johnson et al. 1996).

13.3.3.4 Hydrocyclone

A detailed description of the hydrocyclone for mammalian cell culture can be found in Castilho's review (Castilho and Medronho 2002). Hydrocyclones are often used to separate solids from liquids or liquids with different densities in chemical processes. The device allows continuous cell separation and has the advantage of not including any mechanical part in movement. It was introduced in cell culture processes by Jockwer et al. (2001). Briefly, the cell broth is introduced tangentially at high flow rate in a conical device where a double vortex is created, separating the heavier particles (i.e. the cells) at the bottom end and the supernatant at the upper end. A drawback is that the cells are submitted to a high pressure drop, which is damageable

Table 13.1 Comparison of perfusion devices from published information and from a user perspective

Properties	Spin filter	ATF	TFF	Incl. settler	Acoust. settler	Centritech
Separation efficiency of viable cells	100 %	100 %	100 %	85–99 %	85–99 %	99 %
Elimination of dead cells (+ + = elimination)	− −	− −	− −	+ +	+ +	+ +
Potential for high cell density at small scale (1E6 cells/mL)	10	200	200	16	42	20
Protein recovery after separator (+ + = high yield)	− −	–	− −	+ +	+ +	+ +
Residence time (min) of cells in separator and connection tubing	0[a]	1–2	1–2	≈30	3–14	2–9
Risk of cell accumulation jeopardizing operations (+ + = low risk)	− −	+ +	+ +	− −	+	+
Simplicity of operation (+ + = simpler)	–	+ +	+ +	–	+	− −
Easiness to optimize the operation parameters for new process (+ + = easier)	+ +	+ +	+ +	− −	–	− −
Scalability	+ +	+ +	+ +	+ +	–	+ +
Possibility for re-sterilization	Yes[b]	Yes	Yes	Yes[b]	No	Yes
Disposable	No	No[c]	Yes	No	Yes[d]	Yes
Purchase cost (− − = more expensive)	+	+	+ +	+	+ +	− −
Running cost (− − = more expensive)	+	− −	− −	+	+	–

[a]If device placed external to bioreactor
[b]If device placed external to bioreactor
[c]Has been announced by Refine, USA
[d]A disposable acoustic settler has been newly launched, CytoPerf, by Iprabio/Charter Medical, USA

to the cells. This has been studied for several cell lines using different hydrocyclone designs and operation settings (Pinto et al. 2008; Elsayed and Wagner 2011).

13.3.4 Conclusion About the Cell Separation Devices

Today quite a few cell separation devices are available, some are largely used, others are under development or have been abandoned. The large scale systems, which are used for suspension cells, are the ATF, the inclined settler, the Centritech, the TFF and the spin-filter. Table 13.1 shows a comparison of several devices from

published information and from a user perspective. One can see that the HF-based technologies are the only ones able to support very high cell densities. They have the drawbacks of POI retention by the filter and no capacity to eliminate dead cells. On another hand they do not require extensive tuning and training for operation. The comparison of the ATF and the TFF indicates that the POI retention is lower using the ATF while very high densities can be easier to achieve with the TFF. This latter is using only a pressure pump while in the ATF the diaphragm pump has a double effect of pressure and vacuum, which can be more challenging. Another factor for both ATF and TFF is that the wall shear stress associated with this technology is important and might not be compatible with all types of cells and POI. The Centritech causes also wall shear stress however in a different way and with a much smaller contact surface. The high price of the equipment (even at small scale) and the varying quality of the insert bag have been highly unfavorable for the spreading of this technology. This has been reinforced by the high requirement of training and optimization. The inclined settler requires also training and optimization, but mostly, the limitation of this device resides in the perfusion rate capacity, the cell density capacity, the high residence time and the cell adhesion on the lamellae. Many labs have purchased and are using the acoustic settler for small-scale developments but unfortunately the large-scale device is not available. The spin-filter is used in lab's where the competence is present but nowadays it is not an attractive option.

13.4 Development of a Perfusion Process

Different types of perfusion processes can be distinguished:

- *Stable cell density with growing cells:* The culture is initiated by a growth phase during which the cell density is rapidly increased until a given target. The cell density is then maintained stable at this level, 'steady-state', by cell bleeds performed at a rate compensating the cell growth. The cell viability is maintained as high as possible while the cells are in growing stage. This is one of the main strategies used in perfusion field. Industrial processes are operated on this principle for months (Heidemann et al. 2000).
- *Stable cell density with growth-arrested cells:* Similarly to the first strategy, the culture is initiated by a growth phase until the cell density target is reached. The cell growth is then slowed down or completely arrested in order to achieve a stable cell density (Chuppa et al. 1997; Ducommun et al. 2002b; Ahn et al. 2008). Cell growth arrest is known to be potentially associated with a higher cell specific productivity (this is cell line specific).
- *Increasing cell density:* This strategy consists in increasing the cell density until a pre-determined target or until a physical limitation is encountered (Clincke et al. 2013a, b; Kuczewski et al. 2011; Adams et al. 2011).

The process development can be divided in two main parts:

(i) Optimization of the perfusion parameters: selection or optimization of the type of perfusion, the medium and nutrient feeding strategy (e.g. perfusion rate, feeding of additives), the cell density target, the environmental parameters (such as pH, temperature, dissolved oxygen concentration);
(ii) Optimization of the cell separation process: selection of the cell separation device and tuning of its parameters.

The selection of the final process is based on the integrated/combined information of parts (i) and (ii) and takes into account the cell or process stability, the COGS of the whole manufacturing process (i.e. up-stream and down-stream) and the failure risk.

13.4.1 Systems for Process Development

13.4.1.1 Screening Systems

The optimal system for process development is of course a bioreactor connected to a cell separation device. However in order to generate information more rapidly, screening systems can be used. Small vessel cultivation systems aimed at screening a larger numbers of conditions can be used for a pre-determination of parameters, followed by confirmation or refining in bioreactor scale. Typically, medium selection and effect of medium components can be screened saving labor and time. Most often, the main trends observed in batch culture will remain true in perfusion culture, e.g. a favorable effect of a plant hydrolysate on the POI production observed in batch culture will most likely be confirmed when applied in perfusion process (Heidemann et al. 2000).

Perfusion process can be simulated using systems like shake flasks, spinners or 50 mL tubes with vented cap in so-called pseudo-perfusion process (also called semi- or quasi-perfusion). In pseudo-perfusion, the daily medium renewal is operated manually: the culture is centrifuged, the supernatant is discarded partially or totally and the cells are re-suspended in fresh medium. With this system a quasi steady-state of the cell density can be created if the cells are partially discarded on a daily basis. If the cells are totally retained, an expanding cell density is generated, which will rapidly be limited in terms of aeration for instance. An alternative screening system is semi-continuous cultures, in which the cell broth is partially replaced by fresh medium every day at a rate equal to the growth rate. These are simulated chemostat; the volume of renewed medium and the amount of discarded cells are not independent. Compared with the pseudo-perfusion their operation is less labor consuming but they offer less flexibility, do not simulated perfusion (but chemostat) and attention has to be paid to avoid cell wash out. To conclude this paragraph, it is important to recall that these screening systems are not controlled so that variations and limitations in environmental parameter (e.g. pH, DO) can occur. In particular, these systems are not adequate to simulate high cell density processes.

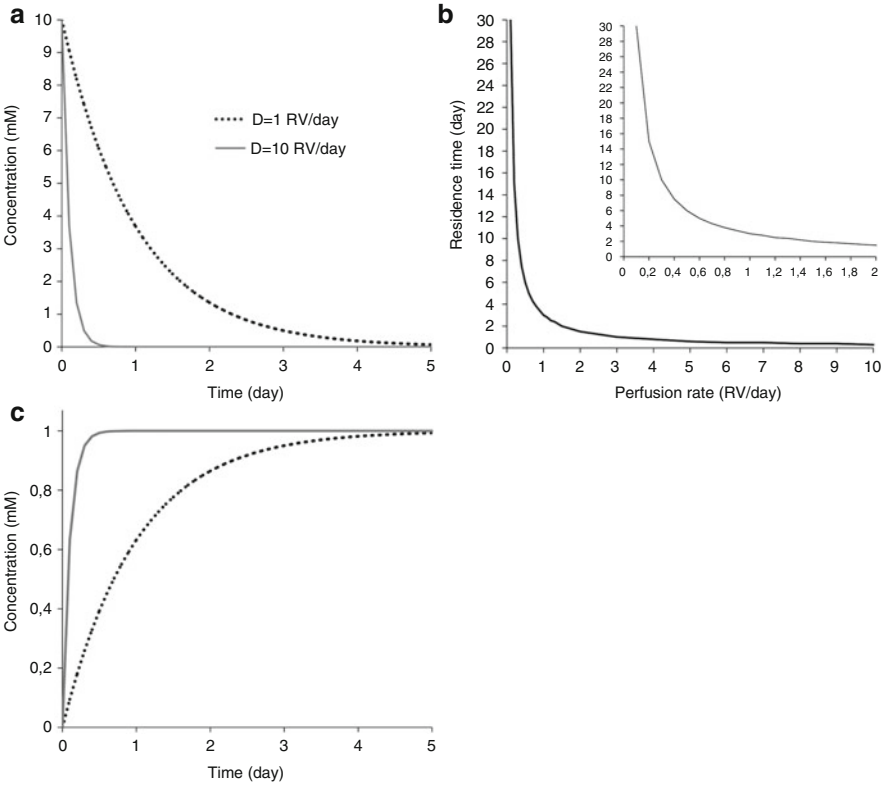

Fig. 13.8 (**a**) Simulation of the evolution with time of a component concentration (originally present at 10 mM) in a culture in perfusion mode (in absence of addition or production) for perfusion rates of 1 (*dotted*) or 10 RV/day (*continuous*). It takes 3 days for the component concentration to decrease to 5 % of its initial concentration at perfusion rate 1 RV/day or the residence time at perfusion rate 1 RV/day is 3 days. (**b**) Residence time of a component in perfused bioreactor as a function of the perfusion rate with zoom in the corner. (**c**) Evolution with time of the concentration of a new component (added at 1 mM concentration) in a culture in perfusion mode for perfusion rates of 1 (*dotted*) or 10 RV/day (*continuous*)

A main difference between the pseudo-perfusion system and real perfusion is the concentration kinetics of a new component present in the feed medium, i.e. its concentration evolution with time independently of the cell consumption or production, as well as the residence time of a component. Figure 13.8 illustrates this kinetics; starting from a null concentration, the concentration of a component reaches its input value asymptotically. It takes 3 days at perfusion rate 1 RV/day for a complete medium renewal since the fresh medium is constantly diluted in the culture. Contrary, in a pseudo-perfusion, the entire medium volume is renewed at once for the same apparent rate of 1 RV/day. Due to this difference, a partial medium renewal, e.g. of 50 %, can be adopted instead of a complete medium renewal. Another difference is that pseudo-perfusion is literally speaking a repeated

batch regime although with small variations. For this reason, daily medium change better mimics the perfusion than e.g. medium renewals every second day. However this latter option is sometimes adopted to reduce the labor burden.

In systems absent of limitations and in growth phase, batch, semi-perfusion, semi-continuous, chemostat and perfusion systems exhibited comparable cell specific production and consumption rates given that the abundance of the nutrients is similar (Schmid et al. 1992; Henry et al. 2008).

13.4.1.2 Bioreactor and Scale-Down Model

Bioreactor systems are used for the development of the perfusion processes and most of the parameters can easily be studied in scaled-down bioreactors. Exceptions are parameters such as the shear stress and the deleterious effect of bubble/gassing, for which scaled-down studies are more challenging. The study of the cell separation device it-self has to take into account the limitations of the targeted large scale for the parameters like the liquid flows or the power. The optimization of the perfusion process parameters (except the cell separation device) can be performed using small-scale bioreactors equipped with a cell separation device different from the one used at large scale (given that the separation device does not affect these parameters). For instance, the perfusion process (not the cell separation process) can be studied using an acoustic settler while the large-scale system is not an acoustic settler.

A perfusion process can be studied by sequentially changing one parameter at a time in a culture stabilized in steady-state (Griffiths and Pirt 1967; Miller et al. 1988; Hiller et al. 1993, 1994; Bollin et al. 2011). An example is given in Fig. 13.9 where Miller et al. (1988) studied the effect of pH on a hybridoma culture. Typically after a transitory period of some 3–5 days, the process will stabilize and give information of the effect of the studied parameter, which can be monitored during some 4–6 days. Then a new value of a parameter to be studied will be applied and so on. Notice that these numbers of days are given as a rule of thumb but the culture parameters such as cell density, viability, metabolites concentration, etc. should be monitored to confirm that the transient period is terminated and that a new stable state has been reached. In particular, longer transition periods can be necessary if a depletion has occurred or if a longer cell adaptation has to take place. Recall that depletion can lead to lag phase and cell apoptosis. In that latter case, it can be recommended to perform a large cell bleed and renewal of medium: typically reducing the cell density to half or to one third of its value and doubling momentarily the medium renewal (over a half- or 1 day for instance). Another obvious case where one should be cautious when applying a sequential parameter change is if growth arrest has occurred since the cells will need more time to switch to growth state. Finally Angepat et al. (2005) tried to shorten the intervals of parameter changes. They observed that the transient effects of parameter changes applied during 3 days, were in agreement with the steady-state behavior so that this could be exploited to reduce the time for process development. They also studied

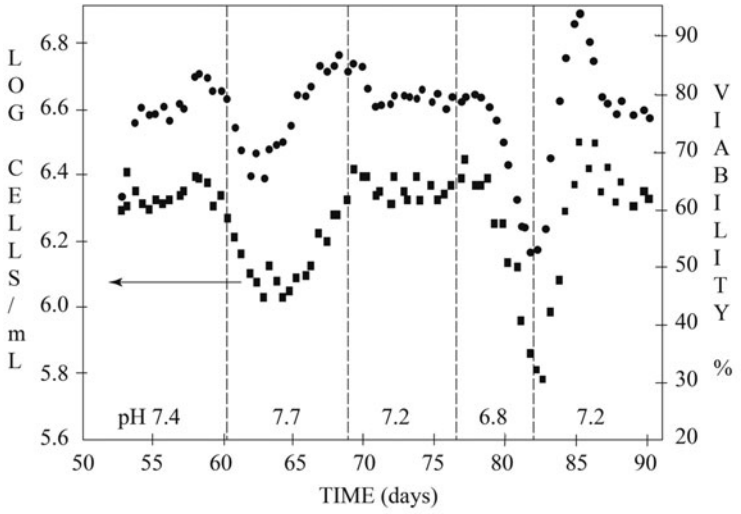

Fig. 13.9 Effect of pH in a continuous hybridoma culture: viable cell density (*squares*), viability (*circles*) (Reproduced from Miller et al. (1988), permission of reproduction from John Wiley and Sons)

the effect of 1 day-changes and concluded that this gave more variability and could be suggested only to see trends.

13.4.2 Optimization of the Process Parameters

Optimizing a process aims at maximizing the yield while maintaining the quality attributes. To maximize the yield, i.e. the volumetric production, high cell density is a key factor. The process space depends on several factors, which dictates the boundaries inside which this production optimization is taking place. These factors are (quoted in part from Konstantinov et al. (2006)):

- the maximum allowable residence time of the POI in the bioreactor. This factor is important for labile proteins.
- the maximum perfusion rate, which can be achieved by the cell separation device
- the minimum achievable cell specific perfusion rate (CSPR) with satisfying POI quality. Too low CSPR can cause improper quality, e.g. too low glucose availability can affect the glycosylation
- the maximal cell density achievable for the cell line in the selected bioreactor, a factor limited by the physical capability of the bioreactor. Typically, the maximum oxygen transfer rate or the CO_2 removal can be these limiting factors and are specific of the cell line, the POI and the process.

- the maximal cell density supported by the cell separation device with satisfying performances
- the cell diameter. At very high cell densities, the cells occupy a non-negligible fraction of the liquid, e.g. around 45 % at 2×10^8 CHO cells/mL (Clincke et al. 2013b). Hence larger cells occupy a larger fluid fraction for the same cell density.

13.4.3 Medium Selection

Comparably to batch or fed-batch processes, in perfusion the culture medium includes all the necessary components to sustain the cell growth and production of POI. Nowadays optimized serum-free and chemically defined media achieve very good performance and provide not only these necessary components but moreover can give enhanced cell growth, cell survival and POI production. The culture media developed for fed-batch cultures can be used for perfusion. Feed concentrates, aimed at fed-batch processes, can be advantageously supplemented to base media, for instance to improve the POI production. Co-factors (metal ions) or vitamins can be required for the production of some non-antibody glycoproteins. For instance factor VIII requires metal ions such as copper for its activity so it should be included in the medium in stoichiometric balance. This is in particular critical when serum-free media are used since serum brings metals in the albumin. Screening of base media, feeds and particular component addition can be performed in batch screening format. Confirmation of the effects and refinements can be performed in semi-perfusion or semi-continuous screening systems allowing larger screening possibilities before final confirmations in perfused bioreactor operation.

Reducing the medium usage without compromising the process performance can be desirable by optimizing the medium composition and using concentrated media (Runstadler 1992; Hiller et al. 1994; Ozturk 1996; Konstantinov et al. 2006; Yang et al. 2014).

13.4.4 Perfusion Rate Strategy

The perfusion can be ‘dissected’ into three important functions:

- To provide the nutrients.
- To eliminate the toxic by-products.
- To reduce the POI residence time in the bioreactor, important factor for labile proteins.

The optimized perfusion rate for each of these functions is not necessary the same. In the case of labile proteins the maximal allowable POI residence time will dictate D’s lowest limit.

Different concepts can be applied to determine the perfusion rate

- (i) based on the cell density or cell specific perfusion rate (CSPR)
- (ii) based on the availability of a main substrate in the culture
- (iii) based on the concentration of a by-product in the culture
- a combination of some or all items (i), (ii), (iii)

When the perfusion process has been optimized resulting in a well-balanced process with efficient substrate usage and at steady state, (i), (ii) and (iii) converge. Hence a process, which has been developed for instance based on (i) can be controlled using (ii) for manufacturing as Meuwly reported (Meuwly et al. 2006).

13.4.4.1 Perfusion Rate Strategy Based on CSPR

The cell specific perfusion rate is an established strategy where the perfusion rate is a linear function of the cell density (Ozturk 1996; Konstantinov et al. 2006):

$$\text{CSPR} = \frac{\text{perfusion rate}}{\text{cell density}}$$

Using a CSPR allows avoiding the depletion of components in the culture. A well-tuned CSPR can also help minimizing the production of toxic by-products. To identify the CSPR, varying conditions of perfusion rate and/or cell density can be applied. An example is given in Fig. 13.10 from a study of Dowd et al. (2003) where the CSPR was varied. The metabolic rates increased as a function of the CSPR due to a higher glucose and nutrient availability. At very high CSPR, above 0.2–0.3 nL/cell/day, this correlation saturated; the cell specific rates had an inflection and plateaued due to the fact that the nutrients were in excess. The volumetric t-PA production represented as a function of the CSPR had a similar profile with an inflection and a plateau above 0.25 nL/cell/day. Hence 0.25 nL/cell/day was the optimum CSPR for this process, i.e. the smallest CSPR with highest cell specific POI productivity. In batch cultures, higher abundance of nutrients is associated with higher rates of consumption of these nutrients (e.g. glucose, glutamine) and higher rates of production of their associated by-products (e.g. lactate, ammonia). When these nutrients are delivered in large excess, they might not cause an increase in their specific consumption rates anymore, showing a saturation of the metabolic rates as a function of the nutrient concentration. Translating this known behavior into perfusion system, higher nutrient availability by higher renewal rate causes higher consumption until saturation is reached. On another hand too low CSPR can cause apoptosis (Nivitchanyong et al. 2007).

Applying the CSPR method Clincke et al. obtained more than 2×10^8 cells/mL (Clincke et al. 2013b). In this process glucose and glutamine were fed separately from the medium at a stoichiometric rate such that their residual concentrations in the bioreactor were maintained at low levels. Using this method, when a process is

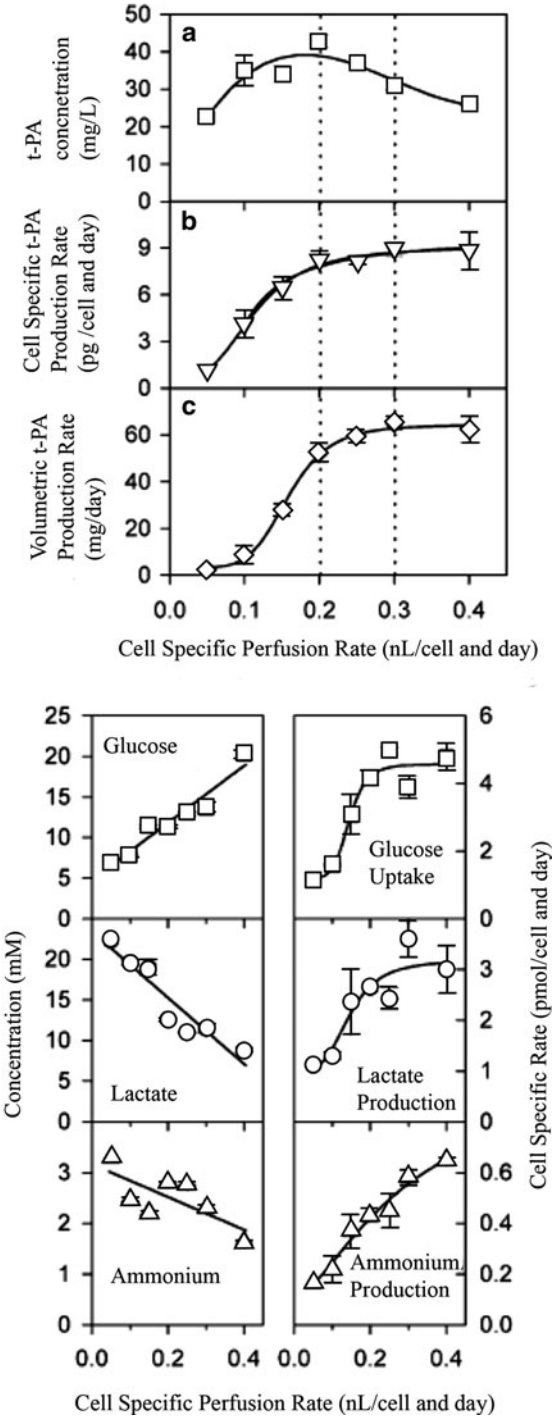

Fig. 13.10 Steady-state recombinant protein production as a function of cell specific perfusion rates (*Top*). In (*a*), the t-PA concentrations, in (*b*), the cell specific productivity and in (*c*), volumetric productivity. Steady-state glucose, lactate and ammonium concentrations and cell specific rates as a function of cell specific perfusion rates (*Below*) (Reproduced from Dowd's report (Dowd et al. 2003), permission of reproduction Springer)

established and the consumption rates known, these main substrates can then advantageously be delivered together in the fresh medium formulation.

In order to reduce the COGS of a sub-optimal process developed to produce the material for early clinical trial, Meuwly et al. (2004) studied the effect of decreasing the perfusion rate of a CHO cell-based process in packed-bed bioreactor. He observed that a 25 % reduction compared to the original level was satisfying for the yield and quality of POI while 50 % reduction caused a 30 % productivity loss however without alteration of the quality.

13.4.4.2 Perfusion Rate Strategy Based on Main Substrate Measurement

The control of the perfusion rate can be based on the consumption of a main substrate present in the medium such as the glucose (Dowd et al. 2001; Wang et al. 2002; Meuwly et al. 2006). From daily glucose concentration measurement, the perfusion rate is increased or decreased in order to maintain the residual glucose concentration constant in the culture. This approach requires that the cell specific glucose consumption rate is constant so it should be applied when steady-state culture is established.

The oxygen uptake rate (OUR) has also been used to control the perfusion rate, given that the OUR is a good indicator of the physiological state of the cells (Kyung et al. 1994; Galvez et al. 2012).

13.4.4.3 Perfusion Rate Tuning for Removal of Toxic By-Products

In case the lactate or ammonia concentrations are reaching unfavorable levels, the perfusion rate can be increased to remove these by-products (Handa-Corrigan et al. 1992). A graphical representation of the effect of lactate or ammonia concentrations on the growth rate or the cell specific production rate can provide guidelines for the selection of limits of these by-products in the process (Clincke et al. 2013b). Finally more sophisticated approaches such as metabolomics can provide a deeper understanding of the production processes (Vernardis et al. 2013).

13.4.5 Cell Density: Target, Monitoring and Control

13.4.5.1 Selection/Optimization of the Cell Density

Two decades ago, the cell density in perfusion processes was around a few millions cells/mL in many cases. A standard in industry today is to target around 20×10^6 cells/mL but there is a trend towards much higher cell densities where the benefit of perfusion can be fully exploited. It is probable that today many industrial processes

are targeting 50–80 $\times$ 10^6 cells/mL. We have developed processes in wave bioreactor and in stirred tank bioreactor where steady state cell densities around 1–1.3 $\times$ 10^8 were maintained by cell bleeds (Clincke et al. 2013b; Chotteau et al. 2014b). The metabolism and MAb productivity were similar at these concentrations compared to the control steady state cultures at 20 $\times$ 10^6 cells/mL. In packed bed processes, the densities which have been achieved are higher than in suspension as reviewed by Meuwly (Meuwly et al. 2007) where ranges of 1–5 $\times$ 10^8 cells/mL have been reported. From this perspective, it seems that the challenge resides now in scaling up these very high cell densities while using low perfusion rates to minimize the liquid handling.

13.4.5.2 On-line Cell Measurement or Estimation for Perfusion Monitoring

The on-line information of the cell density is a precious tool for perfusion processes. The methods described hereafter are generally used in the field and more detailed information can be found in other chapters.

Evaluation of the Biomass Using a Probe

The cell density can be measured on-line with commercial probes based on the dielectric properties of the cell, i.e. bioimpedance, or based on the optical density (OD). The OD probe measures the turbidity, which gives information about the interference of particles present in a fluid, in other words the total cell density given that these particles are only cells. Wu found that the prediction of the cell density by Aquasant probe was satisfying up to 20 $\times$ 10^6 cells/mL when the culture had a high viability and a low level of cell debris but deviated otherwise (Wu et al. 1995). Gorenflo mounted an OD detector in the acoustic settler and showed a linear correlation of the cell density with this signal up to 20 $\times$ 10^6 cells/mL density (Gorenflo et al. 2005a).

Dielectric spectroscopy (called also capacitance or bioimpedance) is often used to monitor the cell density in culture. When applying an electrical field in the culture, the cells with intact membrane become polarized (contrary to disrupted cells), increasing the capacitance. This information is exploited to evaluate the biovolume. This measurement is more reliable than the OD since it is less sensitive to the contamination of particles and dead cells. It is often used to monitor the biomass in perfusion (Noll and Biselli 1998; Ducommun et al. 2001). Carvell and Dowd (2006) found that a linear relationship of the bioimpedance information and the biomass was observed mostly under 20 $\times$ 10^6 cells/mL using an Aber probe. We found that that the cell density estimated by an EVO probe (Fogale) was linear with the biovolume up to 1.6 $\times$ 10^8 cells/mL CHO density measured by concentrating an exponentially growing culture from shake flask (Zhang et al. 2014a), see Fig. 13.11.

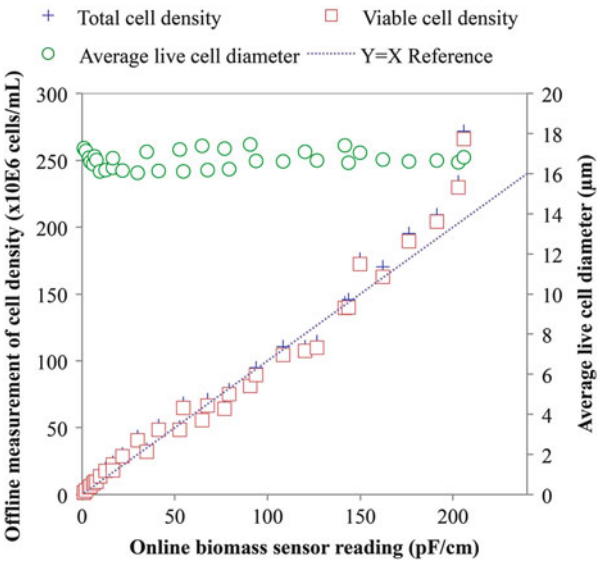

Fig. 13.11 Cell density measured using bioimpedance probe EVO (Fogale) measured in CHO suspension samples obtained by concentrating (centrifugation) an exponentially growing culture in shake flask. A linear correlation of the bioimpedance information and the biovolume is observed up to 1.6×10^8 cells/mL density (Zhang et al. 2014a)

Indirect Evaluation of the Biomass Based on the Cell Metabolism

Several groups have reported monitoring the cell density indirectly by monitoring the consumption of oxygen or glucose (Kyung et al. 1994; Meuwly et al. 2006).

13.4.6 Cell Arrest

An alternative way to maintain the cell density is to arrest the cell growth. Cell arrest obtained by hypothermia or addition of a chemical, e.g. butyrate, has been reported for perfusion; given that the cell specific productivity is comparable or increased and that the protein quality is correct or improved (Ducommun et al. 2002a; Chotteau et al. 2001; Chen et al. 2004; Kim et al. 2004; Angepat et al. 2005; Oh et al. 2005; Meuwly et al. 2006; Ahn et al. 2008; Zhang et al. 2014a). Another advantage of lowering the temperature under 35.5 °C is that the proteolytic activity is lower compared to 37 °C (Chuppa et al. 1997).

13.4.7 Protein Quality

During the culture, the harvest is stored in a cooled harvest tank allowing an enhanced preservation of the POI quality. The proteolytic activity is highly reduced at 4 °C. During the process development, the evolution with time of the POI quality in the cooled tank is studied according to the quality attributes important for the

POI, i.e. analyses/characterization. This study together with logistics and COGS factors will contribute to the decision of the harvest frequency for the process. The constant environment of the perfusion contributes to the stability and improvement of the quality attributes with time, e.g. glycosylation (Goldman et al. 1998), sialilation (Goh et al. 2014), aggregation (Rodriguez et al. 2010).

If small variations in the POI quality occur, like minor variations in the distribution of species, a common procedure in industry is to pool different POI batches issued from different harvests and from different culture runs in order to reconstitute the POI according to the specifications.

13.4.8 Effect of a Peristaltic Pump and Wall Shear Stress in the Re-circulation Loop

The effect of the wall shear stress (from the cell broth circulation in the tubing system) and the peristaltic pump in the re-circulation loop should be minimized to avoid cell damage. Using larger tube diameter reduces the wall shear stress since a smaller portion of the fluid is in contact with the wall but unfortunately it increases the loop volume and therefore the residence time in the loop. The tubing of the re-circulation loop should be shortened and the diameter selected to compromise between short residence time and low volume. Ideally, the residence time should not be longer than 1 or 2 min.

Similarly, using a larger pump reduces the detrimental effect of the pump since a smaller proportion of the fluid is in contact with the tube wall when a roller is pressing the tube. To minimize the effect of the passage in the pump head, it is recommended to favour larger pump suitable for the given process settings and to use a pump head with two rollers, i.e. not four. Similarly, variations in tube diameter and transition edges should be avoided.

The effect of pumping the cell broth in the re-circulation loop, where the cell separation device has been short cut, can be evaluated by monitoring the cell health and growth. Clincke et al. found no detrimental effect of pumping the cell broth in the re-circulation loop used in a TFF-based wave bioreactor culture system (4 L bioreactor volume, 1 L/min re-circulation rate, 0.25 L re-circulation loop volume) in a range of 1.5–2.75 L/min (Clincke et al. 2013a).

13.5 Future Prospects

Perfusion processes are today receiving a renewed of interest, a trend encouraged by Health Authorities. The increasing use of disposable equipment associated with the emergence of more performing perfusion technologies enabling high cell densities have today changed the perception of the continuous process in the

biopharmaceutical field. These processes are more flexible and require lower investment costs compared to the large bioreactor facilities. Processes integrating continuous up-stream and down-stream operations are explored and will probably be the processes of tomorrow (Grandics et al. 1991; Kumar et al. 2006; Warikoo et al. 2012; Vogel et al. 2012).

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Chapter 14
Single-Use Bioreactors for Animal and Human Cells

Stephan C. Kaiser, Matthias Kraume, Dieter Eibl, and Regine Eibl

Abstract Single-use (SU) bioreactors are being increasingly used in production processes based on animal (i.e. mammalian and insect) and human cells. They are particularly suitable for the production of high-value products on small and medium scales, and in cases where fast and safe production is a requirement. Thus, it is not surprising that SU bioreactors have established themselves for screening studies, cell expansions, and product expressions where they are used for the production of pre-clinical and clinical samples of therapeutic antibodies and preventive vaccines. Furthermore, recent publications have revealed the potential of SU bioreactors for the production of cell therapeutics using human mesenchymal stem cells (hMSCs).

This chapter provides a perspective on current developments in SU bioreactors and their main applications. After briefly introducing the reader to the basics of SU bioreactor technology (terminology, historical milestones and characteristics compared to their reusable counterparts) an overview of the categories of currently available SU bioreactor types is provided. SU bioreactor instrumentation is then examined, before discussing well-established and novel applications of SU bioreactors for animal and human cells. This includes descriptions of the engineering characteristics of often-used types of SU bioreactors, covering wave-mixed, stirred, orbitally shaken systems and fixed-bed systems. In this context, the scaling-up of geometrically and non-geometrically similar SU bioreactors is also addressed.

Keywords Single-use bioreactors • Cell cultures • Wave-mixed bioreactors • Stirred bioreactors • Orbitally shaken bioreactors • Fixed-bed bioreactors • Sensors • Scale-up • Human cells • Animal cells • Virus-like particles • Stem cells • Microcarriers • Optical sensors • Cultivation bags • Computational fluid dynamics • Engineering characterization • Leachables/extractables • Power input • Mixing • Mass transfer

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Abbreviations

1D	One-dimensional
2D	Two-dimensional
3D	Three-dimensional
bDtBPP	Bis(2,4-di-tert-butyl phenyl) phosphate
BEVS	Baculovirus expression vector system
CFD	Computational fluid dynamics
CHO	*Chinese Hamster Ovary* (cells)
CMO	Contract manufacturing organization
DoE	Design of experiments
DO	Dissolved oxygen
EDR	Energy dissipation rate
EVA	Ethylene vinyl acetate
hMSCs	Human mesenchymal stem cells
hADSCs	Human adipose-derived mesenchymal stem cells
hBM-hMSCs	Human bone(marrow)-derived mesenchymal stem cells
HPTS	Hydroxypyrene trisulfonate acid
LED	Light-emitting diode
mAbs	Monoclonal antibodies
MDCK	*Madin-Darby Canine Kidney* (cells)
MEMS	Micro-electro-mechanical systems
NK	Natural killer (cells)
PE	Polyethylene
PET	Polyethylene terephthalate
PIV	Particle image velocimetry
pO_2	Partial pressure of oxygen
pCO_2	Partial pressure of carbon dioxide
PTFE	Polytetrafluoroethylene
PVC	Polyvinylchloride
QbD	Quality by Design
RANS	Reynolds averaged Navier-Stokes (equations)
RFID	Radio-frequency identification
RT	Ruston turbine
SBI	Segment blade impeller
Sf-9	*Spodoptera frugiperda* (subclone 9)
SU	Single-use
TBPP	Tris(2,4-di-tert-butylphenyl) phosphite
VLP	Virus-like particle
WCB	Working cell bank
WVB	Working virus bank
WFI	Water for injections

Symbols

Latin symbols

Symbol	Unit	Description
a	–	Constant in Eq. 14.28
b	–	Constant in Eq. 14.28
B	m	Width of the culture bag
c	–	Constant in Eq. 14.28
C	–	Constant in Eq. 14.1
c_H	–	Mixing number
$c_{O_2,L}$	$kg \cdot m^{-3}$	Dissolved oxygen concentration
c_S	m	Impeller distance
D	m	Vessel diameter
d_0	m	Shaking diameter
$d_{B,32}$	m	Sauter diameter of gas bubbles
D_{O_2}	$m \cdot s^{-1}$	Diffusion coefficient of oxygen
d_R	m	Impeller diameter
F_G	$m^3 \cdot s^{-1}$	Gas flow rate
$Fl_{z,p}$	–	Primary axial flow number
$Fl_{z,s}$	–	Secondary axial flow number
g	$m \cdot s^{-2}$	Gravitational acceleration
H_C	$kg \cdot m^{-3} \cdot Pa^{-1}$	Henry coefficient
H_L	m	Liquid height
h_R	m	Off-bottom clearance of the impeller
k	s^{-1}	Rocking rate
k_La	s^{-1}	Specific (liquid-side) mass transfer coefficient
L_C	m	Characteristic length
N	s^{-1}	Shaking frequency
N_C	s^{-1}	Critical shaking frequency
Ne	–	Power number (Newton number)
N_R	s^{-1}	Impeller rotational speed
P	W	Power input
p_{O_2}	Pa	Partial pressure of oxygen
p_{CO_2}	Pa	Partial pressure of carbon dioxide
r	m	Radius, radial coordinate
R_r	m	Reverse point of axial flow
Re	–	Reynolds number (for stirrers)
Re_{crit}	–	Critical Reynolds number
Re_{SR}	–	Reynolds number for shaken reactors
Re_{WR}	–	Reynolds number for wave-mixed reactors (rockers)
Q_G	vvm	Aeration rate
t	s	Time
$t_{m,95\%}$	s	Mixing time (for 95 % homogeneity)
t_R	s	Hydraulic residence time

u_{tip}	$m \cdot s^{-1}$	Tip speed
V	m^3	Volume
v_G	$m \cdot s^{-1}$	Superficial gas velocity
V_G	m^3	Gas volume
V_L	m^3	Liquid (working) volume
v_z	$m \cdot s^{-1}$	Velocity in z-direction
Wo	–	Womersley number

Greek symbols

Symbol	Unit	Description
β		Parameter in Eq. 14.3
$\dot{\gamma}_{NT}$	s^{-1}	Shear gradient (local velocity gradient normal to flow direction)
Δx	m	Maximum fluid element displacement
ε_T	$m^2 \cdot s^{-3}$	Turbulent dissipation rate
η_L	$Pa \cdot s$	Liquid dynamic (molecular) viscosity
λ_e	m	Kolmogoroff microscale of turbulence
μ	h^{-1}	Specific growth rate
ν_L	$m^2 \cdot s^{-1}$	Kinematic viscosity
π	–	Circle number (≈ 3.14159265)
ρ_L	$kg \cdot m^{-3}$	Liquid density
ω	s^{-1}	Angular velocity

14.1 Introduction

In contrast to their conventional counterparts made of glass and/or stainless steel, SU bioreactors have a cultivation container fabricated from plastic materials. Generally, the cultivation vessel is pre-assembled, beta- or gamma-irradiated for sterilization and delivered by the vendor ready-to-use. After one use, it is decontaminated and discarded (Eibl et al. 2010a). While the cultivation containers in micro-, milliliter- and small liter scales are most often made of rigid polycarbonate plastics, larger systems consist of flexible two-dimensional (2D) or three-dimensional (3D) bags whose contact layers are made of polyethylene (PE) or ethylene vinyl acetate (EVA) films. In the case of cultivation bags, stainless steel trays or customized support containers, incorporating heating blankets or double jackets for temperature control, are required to shape and fix the bags.

The development of SU bioreactors was initiated by Fenwal Laboratories' (today Fresenius Kabi) invention of plastic blood bags made of polyvinylchloride (PVC) in 1953. Further milestones include the introduction of plastic flasks and dishes for routine work in cell culture laboratories during the 1960s, the invention of polystyrene multitray systems (Schwander and Rasmussen 2005) and hollow fiber bioreactors (Knazek et al. 1972) during the 1970s. Due to the availability of

different hollow fiber bioreactors (e.g. FiberCell, Amicon, Endotronics systems) (Whitford and Cadwell 2009) and two-compartment dialysis membrane bioreactors (e.g. CeLLine, MiniPerm) during the 1980s and 1990s, the production of diagnostic and therapeutic antibodies in the one and two-digit mg-range became possible (Hopkinson 1985; Falkenberg 1998; Marx 1998; Brecht 2010). The breakthrough for SU bioreactors was marked by the introduction of wave-mixed systems, the first model of which, the WAVE BIOREACTOR, was launched in the late 1990s. Its superiority for the cultivation of animal cells was demonstrated in several comparison studies (Eibl et al. 2010b), which led to the development of the WAVE BIOREACTOR 1000 and further wave-mixed bag bioreactors from various suppliers (see Sect. 14.3.1). Since the mid 2000s, different stirred bag bioreactors (see Sect. 14.3.2) have become commercially available and today, together with stirred rigid bioreactors (see Sect. 14.3.2), make the largest group of SU bioreactors. Various types of bioreactors, which differ from the wave-mixed and stirred mixing principle (see Sect. 14.2.1), are also currently available.

If SU bioreactors are selected properly and operated correctly, more flexible, safer, greener, cheaper and faster production processes can be achieved compared to reusable bioreactors (Aranha 2004; Ott 2011). In fact, steam sterilization prior to inoculation and costly, time and labor intensive cleaning procedures become obsolete, eliminating the need for aggressive, corrosive cleaning agents and water for injections (WFI) used in final rinse cycles. Furthermore, the need to validate the cleaning processes and the risks of cross contamination in multiproduct manufacturing facilities are also reduced, which enables more flexible and quicker product changes, as well as reducing costs.

However, cell growth and product quality/quantity can be affected by chemical, biological and physical properties of SU cultivation containers. This has already been demonstrated for bags made of PE films used for cultivations with serum-free, protein-free and chemically defined culture media (Altaras et al. 2007; Kadarusman et al. 2005). The strength of this effect depends on several factors, including the sensitivity of the production cell line, the components contained in the culture media, the initial cell density and storage time of the PE bags. As shown in Fig. 14.1, the film of the bag consists of multiple layers.

Due to the absence of serum albumins as carrier molecules in serum-free culture media, interactions between media components (e.g. cholesterol and fatty acids) and the contact layer may occur, which can limit cell growth or inhibit product secretion (Kadarusman et al. 2005). The most important concerns are related to cytotoxic leachables, which migrate from the film material into the culture broth and inhibit cell proliferation and product expression (Horvath et al. 2013; Wood et al. 2013). Using *Chinese Hamster Ovary* (CHO) cell lines, Hammond et al. (2013) identified bis(2,4-di-tert-butyl phenyl) phosphate (bDtBPP) as a potential leachable that decreases mitochondrial membrane potential and suppresses cell growth at concentrations as low as 0.1 $mg \cdot L^{-1}$. This compound is generated during gamma irradiation (25–45 kGy) by the degradation of tris(2,4-di-tertbutylphenyl) phosphite (TBPP, often referred to by its trade name Irgafos 168), an antioxidant stabilizer that is commonly added to PE resins. Consequently,

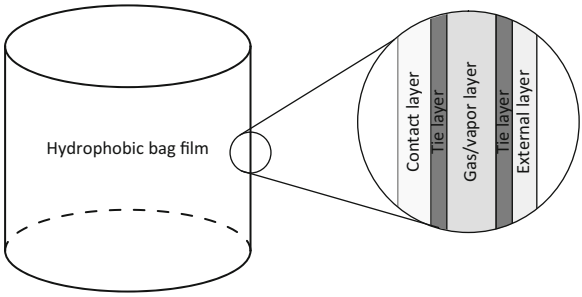

Fig. 14.1 Typical composition of a multilayer bag used as a bioreactor container. The functional layers in a multilayer film include (1) the contact layer, which provides an inert surface, (2) the gas/vapor barrier layer, which limits the diffusion of gasses and vapors and is typically made of Ethylene vinyl alcohol, (3) the external layer, which improves the mechanical performance of the film, and (4) the tie layers between the previously mentioned layers, which bond them together through physicochemical interactions

in-house performance studies, such as the recently published DECHEMA bag test study (Eibl et al. 2014b), with cultivation bags and applied production cell lines / cultivation systems are required to identify potential cytotoxic leaching if SU bioreactors are to be implemented successfully. Consistent, complete and traceable quality control of bag supplies helps to reduce the requirement for bag tests at customers sites and improves the acceptance of commercially available SU systems in production processes.

14.2 Overview of Current SU Bioreactors on the Market

Due to the rapid development of SU bioreactors in the last two decades, users can now choose between a multitude of types which differ in the design of the cultivation container, instrumentation and scale. Based on their design, SU bioreactors can be distinguished as follows: flexible bags, rigid dishes, tubes, cartridges, flasks and vessels. Small scale systems mostly use either very little or no instrumentation. But as size increases systems are equipped with sensors to monitor and control key parameters (see Sect. 14.2.2). In general, these systems are connected to a control unit and no external equipment, such as incubators, is required to maintain optimal process conditions for the production organisms.

Currently SU bioreactors with working volumes of up to 2,000 L are commercially available (Löffelholz et al. 2013a). Eibl and Eibl (2010) distinguish between small-volume systems (screening scale), medium-volume systems (benchtop and pilot scale) and large-volume systems (production scale). They presented an approach for categorization based on power input and classified SU bioreactors into static and dynamic systems. Static systems, such as t-flasks and multilayer flasks, are exclusively used on small and medium scales, because mass transfer is

limited, leading to lower cell densities and product titers compared to dynamic systems.

14.2.1 Categories/Classes of SU Bioreactors

In Fig. 14.2, an approach to the categorization of dynamic SU bioreactors according to their mixing and power input, which has been developed by the DECHEMA's temporary working group "single-use technology in biopharmaceutical manufacture", is presented below.

The largest group of SU bioreactors is mechanically driven which can be further subdivided in stirred, oscillating and orbitally shaken systems. Stirred SU bioreactors (see Sects. 14.3.2 and 14.3.3) consist of cylindrical or cube-shaped vessels and are mostly bubble aerated. Centrically mounted, rotating stirrers are predominantly used, hence baffling is desirable, in particular for larger scale implementations, in order to improve mixing. However, under the majority of operating conditions baffles are not required. The stirrers are usually magnetically driven and mechanically coupled to seal the shaft. Tumbling stirrers, as used in the Nucleo Bioreactor from ATMI (max. 1,000 L culture volume), are less common, although they provide thorough mixing at low specific power inputs (Zambeaux et al. 2007). In oscillating systems, mixing is driven by horizontal oscillation (wave-mixed bag bioreactors, max. 500 L culture volume, Sect. 14.3.1), by vertical oscillatory rotation (BayShake bioreactor, max. 1,000 L culture volume (Kauling et al. 2013)) or by vibrating perforated discs (Saltus Vibromix bioreactor, max. 1,000 L culture volume (Werner et al. 2010b)). Similar to the orbitally shaken SU bioreactors (max. 200 L culture volume, Sect. 14.3.4), oscillating systems using either bubble or surface aeration are available.

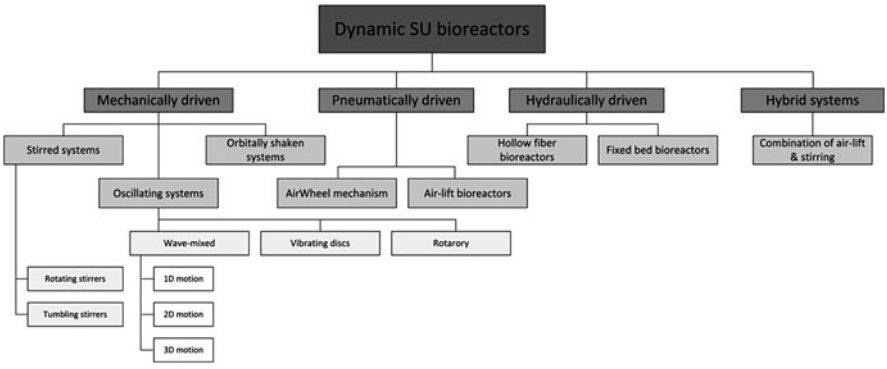

Fig. 14.2 Power input-based categorization of dynamic SU bioreactors for animal and human cells. The classification, produced by the DECHEMA's temporary working group "single-use technology in biopharmaceutical manufacture", distinguishes between mechanically driven, pneumatically driven, hydraulically driven and hybrid driven SU bioreactors

The mixing and aeration of pneumatically driven bioreactors is achieved by direct gassing of the liquid with air or gas by an aeration device integrated into the cultivation bag. An example is the PBS bioreactor series (max. 2,500 L culture volume (Schultz and Giroux 2011)), which is agitated by Air-Wheel® technology and aerated through a dual sparger (Kim et al. 2013; Lee et al. 2010). The hybrid CellMaker Plus system (max. 50 L culture volume) from Cellexus combines the principles of a bubble column and stirring (Shukla and Gottschalk 2013).

As shown in Sect. 14.4, microcarriers can be used in mechanically and pneumatically driven bioreactors if the applied cell lines require immobilization to achieve desired product quantities and qualities. Alternatively, hydraulically driven hollow fiber bioreactors (max. 2.1 m^2 growth surface) incorporating a pump or the recently introduced fixed SU bed bioreactors (max. 500 m^2 growth surface, Sect. 14.3.5) can be used (Brecht 2010; Kilian 2013).

14.2.2 Instrumentation of SU Bioreactors

Instrumented bioreactors either operate with in-situ sensors, which are in direct contact with the process fluid and therefore have to be sterilizable, or ex-situ sensors which allow non-invasive control. Sterilizability of ex-situ sensors, such as optical sensors that take measurements through a transparent window or classical sensors that are fitted outside of the sterile barrier (Lindner et al. 2010) is not an issue. Important parameters for process control and automation that can be monitored in traditional cell culture bioreactors include primarily physical properties, such as temperature, pH value, dissolved oxygen (DO) and carbon dioxide concentration, stirrer or rocking speed, gas and liquid flow rates, pressure, foam/level and vessel weight. Furthermore, advanced parameters such as power input, off-gas composition and biological parameters (including cell density, concentrations of substrates, products and metabolites, redox potential, conductivity etc.) are monitored in heavily instrumented systems, which are mostly found in R&D environments. However, currently only a few SU solutions are commercially available for determining advanced parameters (see Table 14.1).

The first challenge to overcome when implementing SU sensors in bags or rigid plastic vessels is related to the beta- or gamma-sterilization processes (Bernard et al. 2009). The sensor has to be inserted into the bag and must withstand the sterilization process without loss of functionality and sensitivity. Furthermore, the sensors must be inexpensive, if they are to be disposable. Alternatively, traditional probes can be used, but these have to be cleaned and sterilized externally and are subsequently connected to the SU bioreactor via aseptic couplings. An example of this concept can be seen in the stirred S.U.B. from Thermo Fisher, where autoclavable probe assemblies using Kleenpak connectors are used.

The most important parameters to be measured in SU bioreactors are DO and pH. Physically similar sensor designs and apparatus are used to measure both parameters, although the optics, chemistry of the sensor patch and methodologies

Table 14.1 Selected available SU sensors and their specifications for the measurement of biological and physical parameters

Parameter	Working principle	Manufacturer/ vendor	Specifications
Dissolved oxygen	Optical (fiber optical)	PreSens[a]	Measurement range 0–100 % O_2 (detection limit: 0.03 % O_2)
			Resolution ±0.1 % O_2 at 20.9 % O_2 (±0.01 % O_2 at 0.21 % O_2)
			Drift at 0 % O_2 <0.03 % O_2 within 30 days (sampling interval: 1 min)
		Ocean Optics, Inc.[d]	Measurement range 0–100 % O_2
			Detection limit: 0.05 % O_2 in gas and 0.02 ppm in liquid
			Resolution ±0.05 % at room temperature
	Optical (LED and large area photodiode)	Finesse Solutions, Inc.	Measurement range: ~ 0–52.5 % O_2 (250 % air-saturation)
			Precision: 0.55 % or <3 % (whichever is greater) of reading at O_2 levels <21 %
			Operating temperature: 4–45 °C
			Accuracy at 20 °C: <±1 % at 20.95 % O_2
pH value	Potentiometric	Sartorius Stedim Biotech SA and Metroglas	Measurement range: 0–11 pH
			±0.1 pH units precision (pH 2–9)
			Response time: <60 s (until drift <0.6 $mV \cdot min^{-1}$)
	Optical (fiber optical)	PreSens	Measurement range: 5.5–8.5 pH
			Response time (t_{90})[b]: <120 s
			Resolution at $pH = 7 \pm 0.01$ pH
			Accuracy at $pH = 7 \pm 0.05$ sensor spot calibration
			Drift at $pH = 7 < 0.005$ pH per day (sampling interval of 60 s)
		Ocean Optics, Inc.	Measurement range: 5.0–9.0 pH

(continued)

Table 14.1 (continued)

Parameter	Working principle	Manufacturer/ vendor	Specifications
	Optical (LED and large area photodiode)	Finesse Solutions, Inc.	Measuring range pH 5.5–8.5
			Rel. accuracy ±0.1 pH unit over ±0.5 pH range centered at 1-point stand. pH value
			Response time (t_{90}, agitated): <90 s
			Drift: <0.05 pH units over 21 days (sampling rate: once every 5 s)
Dissolved carbon dioxide	Optical (fiber optical)	PreSens	Measurement range: 1–25 % CO_2 at atmospheric pressure (1,013.15 hPa)
			Response time (t_{90}) at 20 °C: <3 min for changes from 2 % to 5 % pCO_2
			Resolution at 20 °C: ±0.06 % at 2 % CO_2 and ±0.15 % at 6 % CO_2
			Drift at 37 °C in a CO_2 incubator with 100 % rel. hum.: <5 % of reading per week
Pressure	Piezoresistive effect (electrical resistance of a metal or semiconductor caused by mechanical strain)	PendoTECH	−0.48 to 5.2 bar measurement range
			15–40 °C operating temperature
			<±5 % measurement accuracy
			Sizes: Luer, ¼″–1″ hose barb
		SciLog	−0.34 to 4.1 bar measurement range
			±20 mbar measurement accuracy
			Sizes: Luer, 3/8″–1/2″ hose barb, ¾″–1″ tri-clover
		Finesse Solutions, Inc.	0–0.48 bar measurement range
			±1.4 mbar measurement accuracy (at 25 °C, including drift 21 days)
			±1.4 mbar sensor drift over 21 days
	RFID-tag-based transducer with pressure-sensitive membrane[c]	GE Healthcare	−0.34 to 2.3 bar
			±17 mbar measurement accuracy

(continued)

Table 14.1 (continued)

Parameter	Working principle	Manufacturer/ vendor	Specifications
Flow rate	Ultrasonic	Levitronix	Measurement range[d]: 0–80 $L \cdot min^{-1}$
			Accuracy of $\pm 0.188\ L \cdot min^{-1}$ below 1 $m \cdot s^{-1}$ and ±1 % above 1 $m \cdot s^{-1}$
	Infra-red reflection	Equflow	Measurement range[d]: 0.1–20 $L \cdot min^{-1}$
			Accuracy: 1 % of reading
	Coriolis	PendoTech	Measurement range[d]: 5–24,000 $g \cdot min^{-1}$
			Accuracy of ±1 % of rate + zero offset stability (0.06–20 $g \cdot min^{-1}$)
			Max. operating pressure: 80–120 psig
Glucose, Lactate, Glutamine	Enzymatic oxidation and electron transfer from analyte to electrode (anode)	CITsens Bio	Measurement range: 1–60 $mmol \cdot L^{-1}$
			Resolution: 0.1 $mmol \cdot L^{-1}$
			Detection limit: 1 $mmol \cdot L^{-1}$
			Precision: $\pm 1\ mmol \cdot L^{-1}$
Conductivity	4-electrode conductivity cell	SciLog	Measurement range: 1–200 $mS \cdot cm^{-1}$
			Accuracy: $\pm 2.5\ mS \cdot cm^{-1}$ of full range
		PendoTECH	Measurement range: 0.1–50 $mS \cdot cm^{-1}$
			Temperature normalization to 25 °C
			Accuracy: $\pm 0.1\ mS \cdot cm^{-1}$ from 0.1 to 2 $mS \cdot cm^{-1}$ and ±5 % of reading at 2–50 $mS \cdot cm^{-1}$

[a]Data depend on the sensor material
[c]Equilibrated sensor kept in well stirred solution at 37 °C
[c]Under development
[d]Different sensor sizes available

differ. The majority of SU sensors for measuring DO are based on the principle that oxygen quenches the fluorescence of a fluorophore in a dynamic and well-defined manner (see Fig. 14.3). The sensing patch is typically illuminated by one or more properly filtered light sources (e.g. LEDs). The dye, commonly a ruthenium-based or platinum-based fluorophore with a lifetime of longer than 10 ns that is incorporated into a silicone matrix, emits light that differs from the incident light in wavelength, phase, and intensity. If oxygen is present near the fluorophore, the oxygen molecule receives the excess energy via non-radiative transfer, resulting in

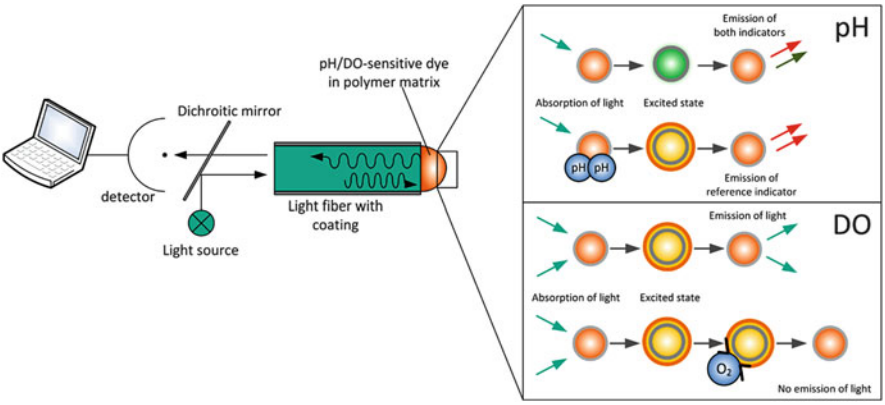

Fig. 14.3 Schematic of the general setup of a fiber-optic sensor with a dichroic mirror. The principle of fluorescence dependent pH and DO measurement are also provided (Adapted from Lindner et al. (2010) and PreSens' website www.presens.de)

a reduction or quenching of the fluorescent signal. The magnitude of the phase shift and intensity difference depends on the DO level in the surrounding liquid. A photomultiplier or photodiode is used to capture the emitted fluorescence after separating it from the excitation light using a dichroic mirror (Glindkamp et al. 2010). Because of possible errors caused by ambient light conditions, background noise and changes in incident intensity, fluorescent intensity is less desirable than phase shift as the primary measurement variable. Despite the fact that phase shift is preferred, it is more difficult to measure electronically (Qualitz 2009).

In contrast to oxygen-consuming Clark electrodes, optical sensors can be used in diffusion limited zones where electrochemical probes would decrease the oxygen concentration (Armstrong 1994). Furthermore, optical sensors can be miniaturized, which enables measurements to be taken in small volumes of less than 1 mL (Rao et al. 2009). Sensing electronics are extremely stable and only need to be calibrated once a year (Qualitz 2009). Due to the more laborious calibration procedures required for the proper use of conventional probes, the setup of optical sensors is substantially less time-consuming than for conventional probes, since they arrive pre-calibrated from the vendor. Commercially available optical oxygen sensors, e.g. from PreSens (Germany), Ocean Optics, Inc. (USA) and Sartorius Stedim (Germany), are irradiatable without loss of sensitivity. The measurement ranges of these sensors are between 0 % and 100 % pO_2 (see Table 14.1).

Long-term stability is a key requirement of optical sensors and should be at least guaranteed for the shelf-life of the cultivation bags. However, limited long-term process stability, which is mainly affected by photobleaching, is the most pronounced disadvantage of optical sensors. This can, however, be compensated for by modifying the dye. According to Lai et al. (2004), the photostability of highly electron-deficient, multiple fluorinated platinum porphyrins is significantly enhanced compared to non-substituted fluorophore. As an alternative approach,

optical filters are integrated into the large area photodiodes utilized in TruFluor™ sensors provided by Finesse Solutions, Inc. This reduces the light intensity required and, therefore, minimizes photo-degradation of the active sensing element. The sheaths of both TruFluor™ DO and TruFluor™ pH sensors can be pre-inserted into SU bioreactor bag ports prior to gamma-sterilization (Paldus and Selker 2010).

Fiber-optic pH sensors contain either fluorescence- or absorption-based pH indicators. The latter include phenol red and cresol red indicators (Mills et al. 1992). Frequently used fluorescing dyes include fluorescein derivatives and hydroxypyrene trisulfonate acid (HPTS) (Munkholm et al. 1988; Mills and Chang 1993; Fritzsche et al. 2007), which exhibit two excitation wavelengths corresponding to the acid and its conjugate base. The pH can be determined from the ratio of emission intensities using a pair of high intensity LEDs (one ultraviolet and one blue) that excite the patch. While fiber-optic pH sensors can – like other chemosensors – be miniaturized and offer short response times, disadvantages of fluorescence-based pH sensors include cross-sensitivity to ionic strength and limited measurement ranges (about 3 pH units).

A wider pH range was obtained by Li et al. (2006), who immobilized modified fluorescent aminophenylcorroles in a sol-gel matrix. A recently introduced SU pH sensor, which was jointly developed by Sartorius Stedim Biotech SA and Metroglas and utilizes a potentiometric pH measurement, offers a range of pH measurements from pH 0–11 with ±0.1 precision (Bernard et al. 2009). However, the pH sensor must be kept in a wet environment during storage, before and after gamma sterilization. For this purpose, a patented encapsulation device was developed that enables the insertion into Flexel® 3D media bags without affecting sterility. Specific gels were chosen for both the internal and external reference electrolytes, which enable measurement in any orientation, while the short probe body simplifies packaging and handling (Bernard et al. 2009).

Fiber-optic sensors can also be utilized for the measurement of dissolved carbon dioxide (pCO_2) (Uttamlal and Walt 1995). Based on the Severinghaus pCO_2 electrode principle, the sensor consists of a pH sensitive dye (e.g. HPTS) in an HCO_3^- buffer solution. This is encapsulated in an expanded polytetrafluoroethylene (PTFE) support, which is held at the distal end of an optical fiber by a gas permeable membrane. A pH change in the indicator solution, which is related to the pCO_2 by the Henderson-Hasselbalch equation, is produced by CO_2 crossing the membrane. PreSens pCO_2 sensors utilize the Dual Lifetime Referencing method as an internal reference, where the signals of an analyte sensitive indicator and an inert reference indicator with very different luminescence lifetimes are superimposed on one another (Klimant 2003).

Pressure is another crucial, safety-related process parameter, since an overpressure situation, e.g. resulting from a clogged vent filter, can easily rupture the cultivation bags, resulting in batch loss. However, SU bioreactors are often not compatible with traditional stainless steel pressure gauges. While the pressure in some systems is controlled by a re-usable pressure sensor outside of the sterile barrier, static and dynamic pressures of gases or liquids in SU bioreactors can be accurately measured by low-cost micro-electro-mechanical systems (MEMS) that

are based on piezoresistive pressure measurements. The sensor elements are typically integrated in a Wheatstone bridge circuit, through which an applied pressure gives a proportional output voltage (Bink and Furey 2010). Pressure sensors from PendoTech and SciLog® for measuring the pressure inside tubing are commercially available and have similar measurement ranges of up to 5.2 bar. For small-scale systems, sensors originally designed for medical applications can be integrated into flexible tubing in a flow-through mode using Luer or hose barb adapters. Larger diameters also have tri-clover connections. The repeatability, accuracy, and robustness of the MEMS in these sensors were found to be satisfactory (Clark and Furey 2007). Sensors are accurate to 20 mbar for pressures up to 1 bar and 2.5 % of the reading value for pressures between 1 and 1.51 bar. Since the signal from the sensors is not a traditional field output signal, such as 0–20 mA or 0–10 V, an intermediate device is required to display and transmit the sensor readings to a process control system (Furey 2007).

To measure the pressure in the headspace of flexible bags, the TruTorr™ provided by Finesse Solutions, Inc. can be used. Based on integrated, gamma radiation-resistant memory chip technology, the sensor compensates for temperature and is self-calibrated for immediate use.

In the future, an alternative to piezoresistive sensors may be provided by recently developed passive radio-frequency identification (RFID) sensors (Surman et al. 2011). They consist of a pressure sensitive flexible membrane, an RFID-tag-based transducer and a layer that modulates the electromagnetic field generated in the RFID sensor antenna. Multivariate analysis of the measured impedance of the sensor provides temperature-independent pressure response. However, no commercial solution for pressure measurements in SU equipment based on RFID is currently available.

Further process parameters detectable by SU sensors include conductivity (SciLog, PendoTECH), flow rates (LeviFlow), capacitance (Fogale Nanotech, Aber Instruments) and total protein concentration (Schneditz et al. 1989). The conductivity sensors provided by both SciLog and PendoTECH are pre-calibrated according to pre-determined cell constants, which are stored on each sensor's chip for out-of-the-box, plug and play use. Flow rates are most commonly measured by ultrasonic signals, where the ultrasonic wave is accelerated or decelerated depending on the flow direction, thus providing a direct measure of the liquid velocity, as in the LeviFlow® sensors provided by the Levitronix GmbH. In contrast, Equflow flow sensors are based on infra-red reflection signals that are monitored by an ultra-light-weight turbine rotor. PendoTech also offers a SU Coriolis flow meter.

Information on biomass concentration can be obtained using turbidity. The use of backscattering light can increase the range of linear correlation for higher particle concentrations. However, while turbidity sensors only give a measure of total biomass concentration, capacitance sensors can provide specific information on viable cell mass. The latter are based on the fact that the non-conducting (intact) cell membranes allow a build-up of charge in an alternating electrical field.

Furthermore, the capacitance signal is not sensitive to gas bubbles, cell debris and other particles in suspension (www.fogalebiotech.com).

To monitor metabolization of glucose, glutamate and/or lactate during cultivation, CITSens Bio sensors can be used, which are based on an enzymatic oxidation process and electron transfer from the analytes to the anode (Spichiger and Spichiger-Keller 2010). In contrast to a number of alternative sensors on the market, where hydrogen peroxide production is measured and, therefore, sufficient oxygen is required, the function of CITSens Bio sensors is not affected by oxygen concentration. Furthermore, the by-products that are produced are of exceptionally low concentrations. Depending on the bioreactor, the sensors are built into the original cap of T-flasks, roller flasks, shake flasks or bag-reactors and are gamma-sterilized before being delivered to the customer. However, storability is limited to about 21 days (15 days for lactate) as a result of sensor instability (the specified storage time at 5 °C is 6 month) (www.c-cit.ch).

A special feature for integrating SU and/or conventional sensors is the Mobius® SensorReady technology, which is implemented in Mobius® CellReady 50 L and 250 L bioreactors (see Sect. 14.3.3). It consists of an external loop that enables configurable and flexible mounting of sensors. The culture broth is pumped from and to the vessel using a Levitronix® centrifugal-type pump, operated at $3\ \mathrm{L} \cdot \mathrm{min}^{-1}$ (pump speed 2,000 rpm). CFD studies have revealed significantly lower energy dissipation rates (EDRs) inside the pump ($< 5 \cdot 10^5\ \mathrm{W} \cdot \mathrm{m}^{-3}$) than critical thresholds responsible for lethal cell responses that have been reported in the literature (Mollet et al. 2007; Godoy-Silva et al. 2009) and determined experimentally by the manufacturer for four different cell lines (Kittredge et al. 2011). Furthermore, the general applicability for microcarrier based cultivations (MDCK cultivated with SoloHill® Collagen microcarriers) has also been demonstrated (McGlothlen et al. 2013).

14.3 Often Used Instrumented Dynamic SU Bioreactors and Their Engineering Characteristics

As described in Sect. 14.2.1, the most often used SU bioreactors are mechanically driven versions, which are wave-mixed, stirred, or orbitally shaken. Furthermore, new developments, such as fixed bed bioreactors, have more recently entered the market. However, the size of SU bioreactors is still limited to approximately 2,000 L. The upper limits mainly result from manufacturing and bioengineering limitations, in particular related to mixing and mass transfer. The following sections describe selected bioreactors and their engineering characteristics that are relevant for biopharmaceutical production processes.

14.3.1 Wave-Mixed Bag Bioreactors

The first wave-mixed bioreactors that entered laboratories in the 1990s had rocker platforms. The rocker platform had a periodic, 1D oscillatory motion that moved a partially filled, pillow shaped bag (Singh 2001). The wave inside the bag is induced by the platform motion, whereas the wave characteristics depend on the bag shape/geometry (this differs according to scale), the rocking angle, the rocking rate, the filling volume and the fluid properties, i.e. liquid density and viscosity (Eibl and Eibl 2009a; Eibl et al. 2010b). Today, the 1D oscillatory concept is used in commercially available wave-mixed systems, including the Wave Bioreactor (GE Healthcare), the BIOSTAT® CultiBag RM (Sartorius Stedim Biotech), the AppliFlex bioreactor (Applikon) and the SmartRocker^{TM} (Finesse Solutions, Inc.). Major differences between these bioreactors are related to their culture bag designs (i.e. shape, dimensions, scale, film material, installations), control units, rocker platforms and instrumentation.

Both the BIOSTAT® CultiBag RM and the Wave Bioreactor Cellbags are available with optionally integrated perfusion membranes for cell retention. While the membrane in the BIOSTAT® CultiBag RM (1.2 μm and 1,070 or 1,275 cm^2 surface area) is fixed to the bottom of the bag, the Wave Bioreactor incorporates a floating filter with a flat cell-retentive membrane (0.7 μm pore size and 100 or 180 cm^2 surface area) (Tang et al. 2007). Hence, very high cell densities are achievable with 1D motion wave-mixed systems (Tang et al. 2007; Adams et al. 2011).

The fluid flow inside the 1D motion bags can be characterized by a modified Reynolds Re_{WR} number given by Eq. 14.1, which is determined using the working volume (V_L), the width of the culture bag (B), the liquid level (H), the rocking rate (k), the kinematic viscosity of the liquid (v_L), and an empirical constant that depends on the bag type (C). According to the definition provided by channel flows, turbulent conditions occur above a critical Re (Re_{crit}) of 1,000 (Eibl et al. 2010b).

$$\mathrm{Re}_{WR} = \frac{V_L \cdot k \cdot C}{v_L \cdot (2H_L + B)} \tag{14.1}$$

In contrast, the non-dimensional Womersley number (Wo), a classical non-dimensional number, and a parameter β were used to quantify the unsteady nature of the flows (Oncül et al. 2009). Wo is expressed by Eq. 14.2, where L_C denotes the characteristic length scale of the flow (i.e. liquid level in the culture bags) and ω is the angular velocity of the oscillations.

$$Wo = \frac{L_C}{2} \cdot \sqrt{\frac{\omega \cdot \rho_L}{\eta_L}} \tag{14.2}$$

The parameter β is obtained from Eq. 14.3, where Δx represents the maximum fluid element displacement in the vessel during one rocking cycle, which is hard to determine experimentally. It has been stated that turbulent conditions appear in oscillating flows when β exceeds 700 for Wo greater than 8.5 (Oncül et al. 2009).

$$\beta = \Delta x \cdot \sqrt{\frac{\omega}{\nu_L}} \tag{14.3}$$

The wave motion promotes bulk mixing, off-bottom suspension of cells and particles, bubble-free surface aeration and reduces foaming and flotation compared to stirred cell culture bioreactors (Eibl et al. 2010b). Reported k_La values are in the range of 0.5 and 24.1 h^{-1} (see Table 14.2), making them suitable for cultures with low and medium oxygen demands (Eibl and Eibl 2009a). Detailed comparisons of different cultivation bags is difficult because of non-comparable operational conditions and measurement techniques. Furthermore, it should be emphasized that the given data include limit values ($k_La < 5$ h^{-1}), which are not recommended for the cultivation of human and animal cell lines.

In general, oxygen mass transfer in a given bag geometry has been found to depend on the rocking rate, rocking angle and the filling level. Hence, Eq. 14.4 can be determined from data obtained from a BIOSTAT® CultiBag 2 L (Imseng 2011).

$$k_\mathrm{L}a \propto {V_\mathrm{L}}^{2.7} \tag{14.4}$$

It should be emphasized that this equation is only valid for this bag size. At a given volume, small changes in the rocking rate and/or rocking angle can increase the k_La more significantly than raising the aeration rate (Eibl et al. 2010b). Nevertheless, contrary data have been reported with respect to aeration rate by using the gassing-out method to determine the k_La (Singh 1999; Knevelman et al. 2002; Imseng 2011; Fietz 2013). Following the conventional definition from submerse aeration, the aeration rate is defined as the air flow rate related to the liquid volume (F_G/V_L), given in vvm (volume gas per volume liquid and minute). Air flow rate has been found to have a strong influence on the k_La value, in the BIOSTAT® CultiBag 2 L bioreactor working with different filling volumes of between 0.3 and 0.5 L up to a critical flow rate of 0.15 L · min^{-1}, corresponding to 0.3 vvm at 0.5 L, as indicated by Eq. 14.5 (Imseng 2011).

$$k_La \propto {F_G}^{1.22} \tag{14.5}$$

This correlation is consistent with measurements provided by Singh (1999), who found that the aeration rate had a significant influence in the Wave 2 L bag when filled to its maximum working volume. The $k_\mathrm{L}a$ at 30 rpm was increased from 2.0 to 2.7 h^{-1} by increasing the aeration rate fivefold (0.01–0.05 vvm), even though this results in a different exponent in Eq. 14.5. Furthermore, by correlating data reported by Singh (1999) for Wave 20 L bags, an exponent of F_G of 1.29 can be obtained. However, it should be emphasized that, following the traditional definition of the

Table 14.2 Summary of reported k_La values for selected operating conditions in 1D rocker-type SU bioreactors. The given data include limit values ($k_La < 5$ h^{-1}), which are not recommended for the cultivation of human and animal cell lines

	Working volume (L)	Rocking rate (rpm)	Rocking angle (°)	Aeration rate (vvm)	k_La value (h^{-1})	Reference
Wave Bioreactor 2 L	1	5	n.d.	0.05	2.0	Singh (1999)
	1	10	n.d.	0.05	2.1	
	1	20	n.d.	0.05	2.8	
	1	30	n.d.	0.05	2.7	
Wave Bioreactor 20 L	10	5	n.d.	0.1	0.7	Singh (1999)
	10	10	n.d.	0.1	1.4	
	10	20	n.d.	0.1	2.7	
	10	30	n.d.	0.1	3.9	
BIOSTAT® CultiBag RM 2 L (basic)	0.2	8	6	0.1	0.5	Imseng (2011)
	0.2	8	6	0.2	1.4	
	0.2	8	6	0.4	3.4	
	0.35	8	6	0.4	5.2	
	0.5	8	6	0.4	4.1	
BIOSTAT® CultiBag RM 20 L (optical)	2	27	7	0.05	19.4	Fietz (2013)
	2	27	7	0.075	17.4	
	2	27	7	0.1	18.9	
	10	6	10	0.02	1.1	
	10	6	10	0.25	1.1	
	10	30	5	0.25	15.3	
AppliFlex 20 L	2.5	16	2	0.5	5.1	Müller (2010)
	5	16	7	0.5	10.2	
	5	24	7	0.5	19.1	
	5	24	9	0.5	22	
	5	24	11	0.5	24.1	

n.d. not defined

k_La value (provided, for example, by Zlokarnik (1999)), the overall mass transfer can be expected to be limited by the resistance at the liquid side of the gas-liquid interface. It is unlikely that this resistance is influenced by the surface aeration (as long as no significant surface turbulence is induced by the air flow).

In agreement with this assumption (but in contrast to previous findings), data determined in BIOSTAT® CultiBags RM 20 L and 200 L revealed that the air flow rate had a negligible influence on the k_La value (Knevelman et al. 2002; Fietz 2013). This was demonstrated using the gassing-out method, with nitrogen, and the sulfite method, as described by (Garcia-Ochoa and Gomez 2009). It has been suggested that the apparent dependency on the air flow rate resulted from varying oxygen partial pressures (p_{O_2}) in the bag head space, after eliminating the dissolved oxygen by introducing nitrogen during the classical gassing-out method (Fietz 2013). According to Henry's law both parameters are related by the Henry coefficient H_C (see Eq. 14.6).

$$c_{O_2,L} = H_C \cdot p_{O_2} \tag{14.6}$$

A time-depended DO saturation concentration (see Eq. 14.7) can be estimated, assuming an ideally mixed bag head space. This was confirmed by measuring the gas residence time distribution using a BlueInOne gas analyzer (BlueSens).

$$\frac{dc_{O_2,L}(t)}{dt} = k_L a \cdot \left(H_C \cdot p_{O_2}(t) - c_{O_2,L}\right) \tag{14.7}$$

It should be noted, that there is doubt about whether ideally mixed conditions occur in the bag head space because of the close proximity of the gas inlets and outlets in most wave-mixed cultivation bags. Nevertheless, very good agreement between theoretical response profiles and experimental data was found for both the residence time distribution and the mean residence time (Eq. 14.8) of the oxygen used as tracer in the exhaust air.

$$t_R = \frac{V_G}{F_G} \tag{14.8}$$

This was tested for a wide range of aeration rates (0.01–0.1 vvm) and filling volumes (2–10 L) in a BIOSTAT® CultiBag RM 20 L bag (Fietz 2013). In conclusion, the experimental methods for k_La determination should be carefully taken into account when comparing literature data. Based on our experience, when using the classical gassing-out method, it is recommended, that the nitrogen in the bag's headspace, which influences the oxygen saturation concentration, should be eliminated.

According to Singh (1999), mixing times in the Wave bioreactor determined by injecting a fluorescent dye and videotaping the dispersion of the dye ranged from 5 to 10 s for working volumes of 10 L (in 20 L bags) and were up to 60 s for volumes of 100 L (in 200 L bags). These are satisfactory values for cell culture bioreactors. Using 2.5 and 5 L working volumes in an AppliFlex® bioreactor (20 L total volume), mixing times of between 4 and 14 s were determined (Müller 2010). A wider range was reported by Eibl and Eibl (2009b), where determined mixing times were between 10 s and ≈ 1,400 s in the Wave bioreactor for scales of up to 100 L with filling levels of between 40 % and 50 %. Not entirely surprisingly, the most ineffective mixing was observed at the lowest possible rocking rate, rocking angle and maximum filling level of 50 %, while the mixing could be improved by increasing the rocking rate and/or the rocking angle and by decreasing the filling volume. The most ineffective mixing (40 s to ≈ 1,400 s) was found in 20 L bags, whereas the mixing times were surprisingly similar or even longer than those at the 100 L scale. In contrast, the most effective mixing (9–264 s) was achieved in 2 L bags (Eibl and Eibl 2009b).

For these bags, the specific power inputs (P/V) were determined by calculating the momentum achieved by both analytical and graphical determination of the point of gravity of the culture bag and the surface area of the fluid (Eibl et al. 2010b).

Using the recommended minimum filling levels (0.2 L), specific power inputs of up to $\approx 560\ W \cdot m^{-3}$ were determined at a rocking rate of 30 rpm and rocking angle of 10°. However, significantly lower power inputs of $\approx 70\ W \cdot m^{-3}$ have been reported at the maximum filling level (1 L at 30 rpm and 10°). For identical operational conditions, somewhat higher values ($\approx 150\ W \cdot m^{-3}$) were predicted by Computational fluid dynamics (CFD) simulations, which, in contrast to the experimental method, take the dynamic energy of the fluid into account (Löffelholz et al. 2010). Nevertheless, both methods revealed that the power input for a given filling volume is directly proportional to the rocking rate and rocking angle. It should be noted, that operational parameters must be evaluated together. For example, by filling BIOSTAT® CultiBag RM 200 L bags to 50 %, specific power inputs of $\approx 150\ W \cdot m^{-3}$ can be achieved by either setting a rocking rate of 30 rpm and rocking angle of 6.5° or a rocking rate of 20 rpm and a rocking angle of 9° (Löffelholz et al. 2010). Interestingly, the power input levels out and may even slightly decrease for certain operational conditions (Eibl et al. 2010b).

In contrast to the bag bioreactors mentioned above, the CELL-tainer® SU bioreactor employs a 2D-motion that combines the vertical rocking motion with a horizontal translation. Even though there are some doubts on the reliability of the reported data, this combination may allow significantly higher oxygen mass transfer rates to be achieved. Thus, k_La values of up to $600\ h^{-1}$ for 15 L working volume have been reported. Even with a 150 L volume k_La values of up to $300\ h^{-1}$ have been found (Oosterhuis et al. 2013). For these operational conditions, the specific power input is about $3\ kW \cdot m^{-3}$, which is comparable to standard stirred bioreactors that are used for microbial cultures (Oosterhuis and van der Heiden 2010). Thus, the cultivation of fast-growing microorganisms with a high oxygen demand is possible in 2D wave-mixed SU bioreactors, which is a limitation of the 1D-motion rocker-type bag bioreactors. Nevertheless, it should be emphasized, that animal and human cell cultures with low oxygen demands do not require such high oxygen transfer rates (see also Sect. 14.5) and that the maximum power inputs may cause cell damage to shear sensitive cells.

An even more complex motion is performed by the XRS 20 Bioreactor System, which consists of a 3D culture bag with integrated optical SU sensors for pH and DO measurement, offering a maximum working volume of 20 L. The system uses a simultaneous bi-axial rocking motion (3D) and is designed to give the flow a tumbling characteristic. According to the manufacturer, this results in almost three-times lower mixing times ($t_m < 20$ s) than the aforementioned 1D rockers (50–98 s) at comparable maximum rocking rates of about 40 rpm. Consequently, the k_La values that can be achieved are also higher. For example, $73\ h^{-1}$ is claimed for 40 rpm and 15° on both axes (www.pall.com), however, no data can be found in the scientific literature.

14.3.2 Stirred Rigid Systems

Due to their free-standing, rigid plastic vessels, these liter scale systems (Mobius® CellReady bioreactor, UniVessel® SU bioreactor, BioBLU, CellVessel) do not require an outer support container. Furthermore, folding stress, which is likely to occur in the plastic films of bag systems and may cause film layers to break, thus leading to leaks under internal pressure from the medium, is eliminated (Gossain et al. 2010). The first stirred SU bioreactor with a rigid cultivation container was the Mobius® CellReady bioreactor (Merck Millipore). The cultivation container, with a total volume of 3 L, provides a maximum working volume of 2.4 L and a recommended minimum volume of 1.0 L (for geometrical details see Table 14.3). The bioreactor is equipped with a single marine impeller and can be aerated by open pipe and micro spargers (sintered polyethylene, 15–30 µm nominal pore size). Measuring DO and pH values is performed by electrochemical probes, which have to be pre-sterilized before being introduced into the bioreactor vessel via 12 mm screw ports in the vessel lid. This increases the risk of contaminations and requires sensor polarization and calibration prior to use. Temperature is monitored with a non-invasive Pt-100 probe that is inserted into a plastic sleeve and controlled via heating blankets.

Due to the small bubbles produced by the microsparger, k_La values of up to 35 h^{-1} can be achieved at aeration rates of 0.25 vvm and impeller speeds of 250 rpm, which corresponds to tip speeds of 1.0 $m \cdot s^{-1}$ (Kaiser et al. 2011b). The authors defined the correlation for the k_La (in h^{-1}) given in Eq. 14.9 as a function of the aeration rate Q_F (in vvm) and the impeller tip speed u_{tip} (in $m \cdot s^{-1}$).

$$k_La = 4.249 - 10.61 \cdot u_{tip} + 60.0 \cdot Q_G + 4.606 \cdot {u_{tip}}^2 - 161.7 \cdot {Q_G}^2 + 160.4 \cdot u_{tip} \cdot Q_G \quad (14.9)$$

Using the decolorization method, mixing times in the Mobius® CellReady of between 55 and 7 s were determined for a 2.0 L working volume and tip speeds of 0.2 and 1.0 $m \cdot s^{-1}$, respectively. Due to the low amount of mixing in the upper part of the vessel resulting from the single bottom-mounted impeller, the mixing time increased significantly as the filling volume increased. Mixing times (at $u_{tip} = 0.2\ m \cdot s^{-1}$) of up to 78.6 s were determined for a 2.5 L working volume (Kaiser et al. 2011b). Good correlation of the mixing time (in s) with the specific power input P/V (in W m^{-3}) was established according to Eq. 14.10. It should be noted that the exponent is similar to the third radical, which can be derived theoretically, assuming fully-turbulent conditions (see Sect. 14.5).

$$t_{m,95\%} = 26.54 \cdot (P/V)^{-0.36} \quad (14.10)$$

At tip speeds of up to 2 $m \cdot s^{-1}$, the specific power input of the marine impeller at the maximum working volume was about 187 $W \cdot m^{-3}$, based on a numerically predicted power number Ne of 0.3 (see Sect. 14.5), and was confirmed by torque

Table 14.3 Summary of geometrical details of commercially available stirred SU bioreactors with cylindrical vessels

Bioreactor	Total volume V_T (L^3)	Min./max. liquid volume V_L (L)	Vessel diameter D (mm)	Impeller diameter d (mm)	Geometric ratios (−) H/D[a]	H_L/H[b]	d/D[c]
BIOSTAT® UniVessel 2 L SU	2.6	0.6/2.0	126[d]	55	1.92	0.74	0.44
BIOSTAT® CultiBag STR	68	12.5/50	370	143	1.80	0.72	0.39
	280	50/200	585	225	1.80	0.74	0.38
	700	125/500	815	310	1.80	0.69	0.38
	1,300	250/1,000	997	379	1.81	0.76	0.38
Mobius® CellReady	3	1.0/2.4	137	76	1.82	0.80	0.55
	60	10/50	340	109	2.10	0.80	0.32
	250	40/200	540	183	2.10	0.69	0.34
CelliGEN® BLU	5	1.25/3.75	170	100	1.50	0.75	0.59
	14	3.5/10.5	214	100	2.00	0.75	0.47
	50	18/40	337	160	1.70	0.80	0.47
S.U.B. (Hyclone)	65.5	25/50	349	118	2.29	0.65	0.34
	120	50/100	438	146	2.18	0.87	0.33
	316	125/250	597	200	1.94	0.79	0.34
	660	250/500	756	251	1.93	0.78	0.33
	1,320	500/1,000	959	321	2.09	0.71	0.33
	2,575	1,000/2,000	1,194	398	1.93	0.78	0.33
XDR	n.d.	4/10	200	135	1.50	0.68	0.68
	n.d.	10/50	305	203	2.50	n.d.	0.67
	260	40/200	559	203	1.50	0.77	0.36
	560	100/500	762	254	1.50	0.89	0.33
	1,100	200/1,000	965	305	1.50	0.91	0.32
	2,200	400/2,000	1,219	406	1.50	0.91	0.33

[a]Vessel height-to-diameter ratio
[b]Normalized filling height (maximum)
[c]Impeller-vessel-diameter ratio
[d]The vessel has a slope of 1.2° resulting in a top-wards increase of the vessel diameter from 122 to 130 mm. n.d. – not defined

measurements (Löffelholz et al. 2010). This power input is within the range of typical cell culture applications, as stated by Nienow (2006). However, such high impeller speeds are neither advisable, due to vortex formation in the unbaffled vessel, nor required for most cell culture applications.

Single impellers are also used in BioBLU bioreactors (Eppendorf/New Brunswick), which are agitated by 3-blade pitched blade impellers (also referred to as 'Elephant ear' impellers). These impellers induce an upwards-directed axial flow with a clockwise rotation. The impeller blades are mounted at 45° and the impeller-to-vessel diameter ratio is 0.59 in the BioBLU 5c and 0.47 in the BioBLU 14c/50c (see Table 14.3). The power number for the impellers is Ne = 1.3 (www.eppendorf.

com), which is comparable to its conventional counterpart. Zhu et al. (2009) reported a power number of Ne = 1.7 in a baffled glass vessel (d/D = 0.45). Similar to the Mobius® CellReady 3 L bioreactor, a porous microsparger (7–12 μm pore size) is used for aeration and, therefore, comparable k_La values can be assumed at comparable specific power inputs. However, no data are available in the scientific literature.

Small scale versions of the BioBLU bioreactor, called BioBLU 0.3c, can be used in combination with a DASbox (DASGIP), representing a stackable modular system with up to 32 or more parallel cultivation vessels. The cultivation containers provide working volumes of 100–250 mL (www.dasgip.com) and are, therefore, suitable for screening experiments and process development, allowing DoE approaches and QbD compliant proceedings. Agitation is performed using magnetically-coupled top-driven 3-blade 45° pitched blade impellers that are geometrically similar to the impellers in the L-scale BioBLU bioreactors. The process critical parameters (i.e. DO, pH and optical density) are measured by optical sensors, following industry standards. Integrated dip tubes enable media addition, sampling and aeration. A special feature of the DASbox system is the liquid-free Peltier element, which controls the temperature and condensation in the exhaust air. This can be used to reduce volume loss through evaporation, which is particularly important in small scale bioreactors, where volume change may affect the process. A case study describes the successful expansion of human pluripotent stems cells, where cell yields of up to $2.3 \cdot 10^6$ cells $\cdot$ mL^{-1} were obtained in a 7-day culture (Olmer et al. 2013).

The rigid UniVessel® SU bioreactor (Sartorius Stedim Biotech) is the first commercially available rigid SU cell culture bioreactor agitated by two-stage impellers. The three elements of the segment blade impeller (SBI) are similar in shape to the 'Elephant ear' impellers of the BioBLU, but they have a lower blade angle of 30°, which results in lower power inputs. The power number above the critical Reynolds number that is required to achieve fully turbulent conditions ($Re_{crit} \approx 2 \cdot 10^4$) was determined by CFD to be Ne = 1.5, which was also confirmed by torque measurements (Löffelholz 2013). The impellers have a diameter of 54 mm and the lower one is positioned at a distance of 47 mm ($h_R/D = 0.39$) from the bottom of the vessel. The impeller distance is 70 mm ($c_s/d_R = 1.3$), which enables the individual impellers to form individual flow regions without significant interactions between the impeller discharges (Liepe et al. 1998). This was confirmed by our own CFD fluid flow analysis and predicted power inputs (data unpublished). Because of the manufacturing process, the diameter of the cylindrical vessel (D) increases towards the top (from 122 to 130 mm), but the mean vessel diameter results in a common impeller-to-vessel diameter ratio of 0.43 (see Table 14.3). Aeration is performed by a submerged L-shaped macro-sparger with small holes (0.5 mm, 14 holes) and/or via the headspace.

Comprehensive engineering characterizations of the UniVessel® SU bioreactor have been carried out in several studies (Kaiser et al. 2011a; Löffelholz et al. 2013a, b; Jossen et al. 2014). CFD simulations where the steady-state flow was predicted using an approach based on Reynolds averaged Navier-Stokes (RANS) equations

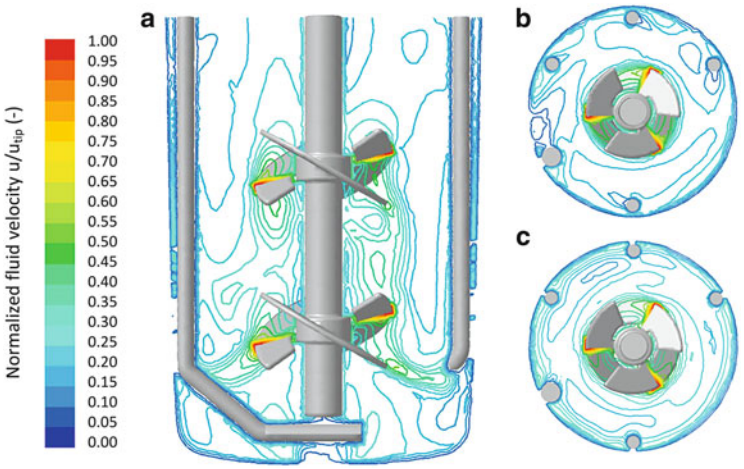

Fig. 14.4 CFD results for flow prediction in the UniVessel® SU. The fluid velocities are normalized by the impeller tip speed u_{tip}. For improved clarity the probes and part of the harvest tube are not shown. (**a**) *Front* view; (**b**) *Top* view, *upper* impeller mid-plane; (**c**) *Top* view, *lower* impeller mid-plane

with a multiple-reference-frame methodology were validated by Particle Image velocimetry (PIV) measurements, which confirmed the expected axial flow pattern of downward pumping discharges from the clockwise rotating SBIs (see Fig. 14.4). Not entirely surprisingly, the maximum fluid velocities predicted at the tips of the impellers agreed well with theoretical tip speeds (see Eq. 14.21). Considerably lower velocities ($v \leq 0.1\ u_{tip}$) were predicted near the vessel bottom, in particular below the impeller shaft, and in the upper portion of the vessel, although the effect of high filling levels was less pronounced than in the Mobius® CellReady because of the upper SBI. The discharge from the lower impeller was inclined towards the vessel wall, resulting in a separate flow loop with low axial fluid velocities near the bottom. This effect could be explained by the relative low impeller blade angle (30°) and the high off-bottom clearance ($h_R/d = 0.41$) (Jossen et al. 2014). Comparing the fluid flow pattern with the BIOSTAT® CultiBag STR pilot scale models, it was possible to establish good qualitative and quantitative agreement, indicating the scalability of the benchtop bioreactor (Kaiser et al. 2011a).

The primary and secondary axial flow numbers defined by Eqs. 14.11 and 14.12, which represent dimensionless volume flow rates through the impeller's cross-section and can be used to estimate the circulation time within bioreactors (Liepe et al. 1998), were predicted to be 0.4 and 0.77 respectively. These values are within the range of conventional stirrers, for which primary flow numbers between 0.17 and 1.27 have been reported (Patwardhan and Joshi 1999; Patwardhan 2001; Kumaresan and Joshi 2006; Ayranci et al. 2012). Furthermore, a primary flow number of 0.7 for the 'Elephant ear' impeller was obtained in a baffled tank, where the axial flow is enhanced by the lower tangential circulation (Zhu et al. 2009).

$$Fl_{z,p} = \frac{2 \cdot \pi}{N_R \cdot {d_R}^3} \cdot \int_{r=0}^{r=d_R/2} r \cdot v_z(r)\ dr \tag{14.11}$$

$$Fl_{z,s} = \frac{2 \cdot \pi}{N_R \cdot {d_R}^3} \cdot \int_{r=0}^{r=R_r} r \cdot v_z(r)\ dr \tag{14.12}$$

Similar to the Mobius® CellReady 3 L bioreactor, the mixing times in the UniVessel® SU bioreactor were found to be in a range of between 2 and 40 s, depending on the power input and filling volume ($< 250\ \mathrm{W \cdot m^{-3}}$; > 1 L). Again the slopes of the regression functions of t_m against P/V (i.e. -0.3) (Kaiser et al. 2011a; Löffelholz et al. 2013b) were close to theoretical values valid for turbulent conditions where

$$c_H = t_{m,95\%} \cdot N_R = const. \tag{14.13}$$

with c_H representing the dimensionless mixing number that defines the number of stirrer rotations required to achieve the desired homogeneity. In the case of the UniVessel® SU bioreactor a mixing number of $c_H = 18$ was predicted, which indicates that agitation is in the performance range of conventional impellers, as reported by Liepe et al. (1998).

To characterize the gas distribution and the oxygen mass transfer inside the UniVessel® SU bioreactor, CFD multiphase simulations with an Euler-Euler extended RANS approach were performed (Kaiser et al. 2011a; Löffelholz 2013) and the k_La values for a wide range of operating conditions were determined using the conventional gassing-out method (Löffelholz 2013). For the low gassing rates typically used in cell culture applications, no profound effect on the shape of the flow pattern could be identified (Kaiser et al. 2011a), which agreed qualitatively with findings of Zhu et al. (2009). However, the rising gas lowers the velocities of the downward directed fluid flow, which was also found in previous studies with conventional cell culture bioreactors (Kaiser 2009). The bubble flow around the impeller shaft resulted in lower fluid velocities and momentum exchange between the pair of impellers, and two-phase flow. Surprisingly, the gassed power input $(P/V)_g$ was found to be higher than for ungassed conditions (P/V). This is in contrast to expectations and measurements realized for the 'Elephant ear' impeller by Zhu et al. (2009), who found that $(P/V)_g$ decreased by up to ≈ 30 % when the impeller was in down-pumping mode. Nevertheless, it should be noted, that the CFD predicted power number in the UniVessel® SU also decreased (by ≈ 30 %) when critical gas flow rates were exceeded (e.g. 0.5 vvm), which may be explained by low gas dispersion (i.e. impeller flooding).

The experimentally determined k_La values for the UniVessel® SU (in $\mathrm{h^{-1}}$) could be correlated by Eq. 14.14, using the specific power input (in $\mathrm{W \cdot m^{-3}}$) and the superficial gas velocity (in $\mathrm{m \cdot s^{-1}}$). The strong influence of the superficial gas velocity is again indicated. For example, the k_La value was, depending on the

specific power input ($0.4\ \mathrm{W \cdot m^{-3}} < P/V < 150\ \mathrm{W \cdot m^{-3}}$), in the range of 10–50 $\mathrm{h^{-1}}$ for 0.01 vvm ($v_G = 2.8 \cdot 10^{-4}\ \mathrm{m \cdot s^{-1}}$) and 20–83 $\mathrm{h^{-1}}$ for 0.02 vvm ($v_G = 5.7 \cdot 10^{-4}\ \mathrm{m \cdot s^{-1}}$).

$$k_L a = 7.97 \cdot 10^3 \cdot (P/V)^{0.25} \cdot {v_G}^{0.87} \tag{14.14}$$

Other customized, rigid, L-scale SU stirred bioreactors with single or multi-stage impellers are available from Creel (Denmark). The cultivation containers have internal diameters and heights of up to 120 and 630 mm, depending on the desired total working volume. For mL scale applications the ambr 250 bioreactor (TAP biosystems) can be used. The baffled bioreactor vessels, which are equipped with two Rushton turbines, mimic classic bioreactors and, therefore, may provide good scale-down models for screening experiments. However, no engineering data have been published at the time of writing.

14.3.3 Stirred Bag Systems

In contrast to the rigid systems, stirred bag SU bioreactors require a support container to fix and shape the cultivation bag. This can either be heated electrically by heating blankets or can incorporate water-filled double jackets for temperature control. The cultivation bags need to fit perfectly into their holding devices for optimum performance, in particular with regard to heat transfer (Weber et al. 2013). Thus, cavities, pockets and folds, which can be attributed to the bag unfolding during installation, need to be prevented.

In 2006, the Thermo Scientific Single Use Bioreactor (S.U.B.), which was developed as a result of cooperation between Hyclone and Baxter (Eibl and Eibl 2011), was the first large-scale SU stirred bag bioreactor before Xcellerex launched its XDR SU stirred-tank bioreactor. Both systems are agitated by pitched axial flow impellers that are mounted off-center, eliminating the fluid vortex that is often observed in unbaffled vessels. Thus, no baffles are required in these SU stirred bioreactors. The Xcellerex bioreactor has a magnetically coupled, bottom-driven impeller, whereas the HyClone system is top-driven (Shukla et al. 2012).

The Xcellerex's XDR product family includes scales from 50 to 2,000 L and was recently extended by the XDR-10, which can handle volumes from 4.5 to 10 L (see Table 14.3). Unfortunately, only limited engineering data are available and none of them have been published in the scientific literature. According to the manufacturer, $k_L a$ values of up to 9.5 $\mathrm{h^{-1}}$ are achievable in the XDR-1000 using a specific (ungassed) power input of 5.8 $\mathrm{W \cdot m^{-3}}$ and an air flow rate of 15 $\mathrm{L \cdot min^{-1}}$ (single sparger configuration). It should be noted that, under those aeration rates, the specific power input by aeration is about 3 $\mathrm{W \cdot m^{-3}}$, assuming isothermal gas expansion.

More comprehensive characterizations of the S.U.B. Hyclone bioreactors (50 and 250 L scale) have been carried out in our own laboratories (Ries 2008; Löffelholz et al. 2010; Löffelholz 2013). CFD models for the S.U.B. 50 L, which were validated by PIV measurements (Löffelholz 2013), revealed that the pitched blade impeller generated a downward pumping axial flow pattern, where the fluid recirculated upwards along the outer walls. Below the impeller, two differently-sized flow loops are induced due to the off-center position of the impeller (Löffelholz 2013a; Löffelholz et al. 2013a). Based on the steady fluid flow pattern, a constant power number of $Ne = 1.9$ was predicted above a critical Reynolds number of $4 \cdot 10^4$ (Löffelholz et al. 2010). This gives CFD predicted specific power inputs of up to 19 $W \cdot m^{-3}$ at 50 L (with a tip speed of 3.1 $m \cdot s^{-1}$), which is about 20 % lower than the experimentally measured 24 $W \cdot m^{-3}$ (Löffelholz 2013). Reported mixing times (9–155 s) and k_La values (2–25 h^{-1}) for typical cell culture conditions are comparable to conventional and other SU stirred bioreactors at pilot scale (see below) (Ries 2008; Löffelholz et al. 2010).

Other stirred bag bioreactors are the Mobius® CellReady 50 and 200, which are agitated by bottom-mounted impellers with four blades pitched at 13°. Power numbers were determined to be $Ne = 3.2$ (50 L) and $Ne = 4.0$ (200 L), which results in specific power inputs of up to 120 $W \cdot m^{-3}$ (50 L) and 33 $W \cdot m^{-3}$ (200 L) at maximum tip speeds of 1.7 and 1.1 $m \cdot s^{-1}$ respectively. Although the impellers are mounted off-center, a single, top-mounted baffle is integrated into the bag in order to prevent vortex formation. In contrast to the Hyclone and XDR bags, the Mobius® systems have a round, rigid vessel base, which is intended to make installation easier and prevent folds in the bag.

The mixing times of the 50-L and 200-L Mobius® CellReady bioreactor were measured using conductivity probe responses at four locations (top, middle, bottom, and inside the Mobius® SensorReady loop) after adding a tracer (Dekarski 2013). Depending on the power input ($\approx$ 1.5 to 30 $W \cdot m^{-3}$) average mixing times in the range of 25 and 38 s were determined at maximum filling levels. The measured k_La values at the 50 L scale were in the range of 4 and 49 h^{-1}, using power inputs of up to 10 $W \cdot m^{-3}$ and aeration rates of between 0.0025 and 0.05 vvm. At the 200 L scale, slightly higher k_La values of up to 60 h^{-1} have been reported (Dekarski 2013), but the required power input was also higher (36 $W \cdot m^{-3}$). Surprisingly, a further increase in power input (to 51 $W \cdot m^{-3}$) did not result in higher mass transfer rates.

In contrast to the aforementioned stirred SU bag bioreactors, the BIOSTAT® CultiBag STR family (Sartorius Stedim Biotech), which offer working volumes up to 2,000 L, are agitated by two impellers mounted at a distance of $c/d_R \approx 1.3$ on the centered shaft (Noack et al. 2010). Two impeller configurations are available: two three-bladed segment impellers (SBI-SBI) and a combination with a lower-mounted six-blade Rushton turbine (disk impeller, SBI-RT). The latter is known to improve gas dispersion (Liepe et al. 1998; Zlokarnik 1999; Noack et al. 2010). The cultivation bags, which are designed very close to conventional stainless steel bioreactors in terms of vessel geometry, agitation and aeration (Weber et al. 2013), have a convex shaped bottom and have height/diameter ratios of about 1.8:1

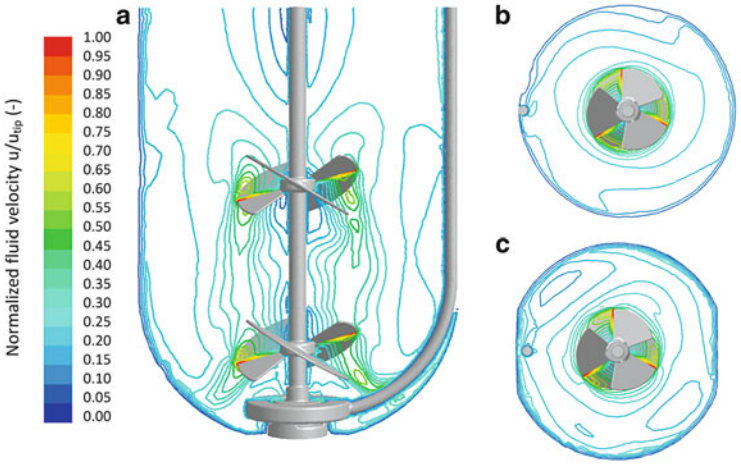

Fig. 14.5 CFD results for flow prediction in the BIOSTAT® CultiBag STR 50 L. The fluid velocities are normalized by the impeller tip speed u_{tip}. (**a**) *Front* view; (**b**) *Top* view, *upper* impeller mid-plane; (**c**) *Top* view, *lower* impeller mid-plane

(see Table 14.3). Furthermore, the impeller/vessel diameter ratio is 0.38 and the normalized off-bottom clearance *hR*/*D* of the lower impeller is 0.24, which are both within typical ranges for cell culture applications. The cultivation bags are aerated by either a classic ring sparger with 0.8 mm holes or a microsparger with 0.15 mm holes.

Comprehensive engineering characterization was carried out for fluid flow, power input, mixing times, oxygen mass transfer and microcarrier suspension at scales of up to 2,000 L for both animal and human cell cultures (Kaiser et al. 2011a; Löffelholz 2013; Löffelholz et al. 2013a, b; Jossen et al. 2014). CFD simulations, which were verified by PIV measurements, revealed the expected axial flow pattern for the impeller configuration with two SBI's, underlining the good scalability from the UniVessel® 2 L SU (see Fig. 14.5; compare Sect. 14.5). The highest fluid velocities were predicted at the blade tips and correlated well to the theoretical tip speeds ($u_{tip} = \pi \cdot d_R \cdot N_R$) for both impeller configurations. The fluid velocities decreased along the impeller discharge, whereas relative velocities ranging between 0.03 and 0.15 u_{tip} were predicted in the bulk region. Interestingly, relatively strong radial inclination of the impeller discharges of both the upper and the lower SBI were predicted, which resulted in two comparably sized flow loops. As already reported for conventional stirred vessels (Alcamo et al. 2005), the jet of the RT in the SBI-RT configuration showed a slight downward inclination, which can be explained by the absence of baffles. Nevertheless, the main body of the radial discharge from the lower RT was pumped towards the outer wall, where it impinges on the outer wall, splits, and moves up and down, forming two recirculating loops. Due to the low bottom clearance and the round-shaped bottom, the lower loop is significantly smaller than the upper. The latter reaches the liquid surface resulting in

extensive mixing of the vessel contents, while a less pronounced swirl along the bottom wall was found. In both configurations, the interaction of the two impellers can be ignored due to the high cS/dR ratio, which was confirmed by PIV and power input measurements (Löffelholz 2013).

The power input was predicted by CFD and experimentally determined by stirrer torque measurements for both ungassed and gassed conditions (Löffelholz 2013). The total power input (P/V) is usually applied to make a comparison or to scale up bioreactors, even though the effect of aeration is normally negligible, due to the low gassing rates used for mammalian cell cultures (Nienow 2006; Garcia-Ochoa and Gomez 2009). For ungassed conditions, maximum specific power inputs of approximately 86 and 240 $W \cdot m^{-3}$ were achieved at a tip speed of 1.8 $m \cdot s^{-1}$ for the SBI-SBI configuration and the SBI-RT configuration in the BIOSTAT® CultiBag STR 50 L, respectively. In contrast to expectations, an increase in the power input of 15 % was reported for an aeration rate of 0.02 vvm. Only minor differences in CFD-predicted power numbers were found for the different scales of the BIOSTAT® CultiBag STR. This can be explained by minor differences in the geometric ratios of the various size vessels and numerical uncertainties at larger scales. Power numbers of between $Ne = 1.1$ (SBI-SBI, 50 L) and $Ne = 3.1$ (SBI-RT, 50 L) were obtained. Using a tip speed of 1.8 $m \cdot s^{-1}$, power inputs of up to 48 $W \cdot m^{-3}$ (SBI-SBI) and 133 $W \cdot m^{-3}$ (SBI-RT) were achieved at the 200-L scale. This decreased even further at the 1,000-L scale to 28 $W \cdot m^{-3}$ (SBI-SBI) and 73 $W \cdot m^{-3}$ (SBI-RT).

In order to estimate potential cell damage as a result of agitation induced shear forces, mean local shear gradients were estimated. The results can be correlated by Eq. 14.15, where the P/V must be inserted in $W \cdot m^{-3}$ and the geometric parameters (d_R, D and V_L) are given in m and m^3, respectively. This equation is valid for working volumes of up to 1,000 L and, interestingly, predicted shear stresses in the Hyclone S.U.B. can also be described quite well by this correlation, even though the impeller geometry of the Hyclone S.U.B. is significantly different.

$$\dot{\gamma}_{NT} = 0.05 \cdot \left(\frac{P}{V}\right)^{1/3} \cdot \left(\frac{d_R}{D}\right)^{-2.7} \cdot V_L{}^{-0.16} \tag{14.15}$$

Depending on the scale and applied power input, shear rates between 0.1 and 30 s^{-1} were predicted in the BIOSTAT® STR bags, which corresponds to shear stresses of 10^{-4} Pa and 0.03 Pa, assuming water-like culture media (i.e. viscosity of ≈ 1 mPa · s). Significantly higher critical values (in the order of 100–300 Pa) for causing substantial cell damage have been reported (Chisti 2000). Nevertheless, physiological effects, which do not necessarily result in physical breakage of the cells, have also been observed at moderate levels of stress in the range of 0.5–5 Pa (corresponding to 500–5,000 s^{-1} in water-like culture broths) (Yim and Shamlou 2000). Even though maximum shear rates can be three to four orders of magnitudes higher than the volume-averages, it was stated that no cell damage is expected under typical cell culture conditions. This was confirmed by CHO cultivations,

where cell densities between 6 and $7.5 \cdot 10^6$ cells $\cdot$ mL^{-1} with viabilities above 96 % were achieved using chemically defined minimal medium (Löffelholz 2013).

Using conductivity methods and CFD predictions, mixing times ($t_{m,95\%}$) of between 10 and 60 s, depending on the power input (0.86–86 W $\cdot$ m^{-3}), were found at the maximum filling level for the 50-L bioreactor. Due to the larger liquid volume, between 20 and 60 s were measured in the 200-L scale (1.5–49 W $\cdot$ m^{-3}) (Löffelholz 2013). Therefore, it can be stated that the level of mixing is sufficient for cell culture applications, and the performance is comparable to conventional stirrers (Kaiser et al. 2011a). Similar results have been reported for oxygen mass transfer. In the BIOSTAT® CultiBag STR 50 L, a maximum k_La value of 35 h^{-1} was determined at an aeration rate of 0.1 vvm (Löffelholz et al. 2013b), indicating sufficient oxygen supply for cultures with a low to medium oxygen-demand. At these aeration rates, the mean bubble diameter determined by the Shadowgraphy technique was 5 mm. Not entirely surprisingly, detailed analysis indicated larger bubbles near the impeller shaft, while larger volume fractions of smaller bubbles ($d_{B,32} = 1$–4 mm) were observed near the vessel wall. This is an indication of the low gas dispersion capacity of the SBI impellers used in typical cell culture applications.

The Nucleo™ bioreactor and the ATMI Life Sciences' Integrity™ PadReactor™ have cube shaped bags with a paddle-shaped mixing element that rotates in an elliptical motion. From an engineering view point both systems are identical. Both the PadReactor™ and the Nulceo™ system include a 20 μm microsparger that is fixed to the mixing element, resulting in dynamic aeration (Rodriguez et al. 2010). As demonstrated in computational simulations, mixing in the Nucleo™ and PadReactor™ systems follow both radial and axial paths, while the walls of the cubical vessel act as baffles, preventing vortex formation (Farouk and Moncaubig 2011). Consequently, efficient mixing and solid suspension is achieved and microcarriers can be brought into suspension at impeller speeds as low as 30–50 rpm (tested with SoloHill collagen microcarriers, 125–212 μm) (Rodriguez et al. 2010; Patel et al. 2012).

14.3.4 Orbitally Shaken Bioreactors

The most often used orbitally shaken bioreactors include shake flasks, TubeSpin® bioreactors (Techno Plastic materials) and microwell plates. The latter provide miniaturization, which is advantageous for the processing of a large number of different cultivation experiments and for media optimization (Kensy et al. 2005).

TubeSpin® (also known as CultiFlask 50 disposable bioreactor from Sartorius Stedim Biotech) technology was initially developed in an attempt to provide 'bioreactor-equivalent' conditions for screening experiments at 50 mL scale (De Jesus et al. 2004; Werner et al. 2010a). The special centrifuge tubes with frusto-conical bottoms have ventilation caps for gas exchange with the incubator. Reported k_La values are up to 21 h^{-1} when operating at 150–240 rpm with

10–30 mL filling volume (Werner et al. 2010a). The experimental data could be correlated by Eq. 14.16, where V_L is the liquid volume (in L), N_R is the shaking frequency (in rpm) and d_0 is the shaking diameter (in mm).

$$k_L a = \left(1.196 + 4.089 \cdot 10^{-3} \cdot N_R - 80.3 \cdot V_L - 0.021 \cdot d_0 + 0.1663 \cdot N_R \cdot V_L + 1.252 \cdot 10^{-4} \cdot N_R \cdot d_0 + 0.317 \cdot V_L \cdot d_0\right)^2 \quad (14.16)$$

During the last decade, engineering parameters for various small scale systems, including power input, mixing time and oxygen mass transfer, have been widely reported (Büchs et al. 2000; Micheletti et al. 2006; Peter et al. 2006; Zhang et al. 2010; Tan et al. 2011; Wen et al. 2012). Furthermore, advanced parameters concerning shear stress and turbulent energy were predicted in single wells of microtiter plates (Zhang et al. 2008a), in TubeSpin® bioreactors (Werner et al. 2013), shake flasks (Zhang et al. 2005), and shaken bottles (Tissot et al. 2011b).

For unbaffled shake flasks, Eq. 14.17 was established to calculate the power input (Büchs et al. 2000), while Eq. 14.18 was proposed to estimate the achievable $k_L a$ for a wide range of operational conditions (Klöckner et al. 2013). In both equations, all parameters are to be inserted in SI units, as given in the symbol list.

$$P = 1.94 \cdot {V_L}^{1/3} \cdot \rho_L \cdot N^3 \cdot D^4 \cdot {\mathrm{Re}_{SR}}^{-0.2} \quad (14.17)$$

$$k_L a = 0.5 \cdot D^{2.03} \cdot N \cdot {V_L}^{-0.89} \cdot {\nu_L}^{-0.24} \cdot {D_{O_2}}^{0.5} \cdot g^{-0.13} \cdot {d_0}^{0.25} \quad (14.18)$$

A slightly different correlation was found for differently sized cylindrical, orbitally shaken bioreactors (also valid for the 200 L scale OrbShake™, see Eq. 14.19), which can be explained by the fact that the liquid motion is different (Klöckner et al. 2013). Again, all parameters must be inserted in SI units, as given in the symbol list.

$$k_L a = 1.06 \cdot 10^{-3} \cdot D^{4.3} \cdot N^{2.12} \cdot {V_L}^{-1.2} \cdot {\nu_L}^{-0.21} \cdot {D_{O_2}}^{0.12} \cdot g^{-0.51} \quad (14.19)$$

This is valid (with an accuracy of ±30 %) for operating conditions above critical circulation frequencies N_C that guarantee rotation of the liquid and can be calculated by Eq. 14.20.

$$N_C = \frac{1}{D^2} \cdot \sqrt{0.28 \cdot \ V_L \ \cdot g} \quad (14.20)$$

While the scaling-up of shake flasks is limited to about 1 L total volume, since the maximum working volume is only 20–30 % of the nominal volume, larger volume shaken systems with cylindrical- or cube-shaped culture containers have been developed (Muller et al. 2005; Stettler et al. 2010; Tissot et al. 2011a). Typical scales were up to 30 L, even though, the nominal volumes of some prototypes exceeded 1,000 L (Tissot et al. 2010). In addition, special constructions to improve

mixing and mass transfer have been tested, including helical tracks on the inner vessel wall. By driving the liquid onto these helical tracks, the gas-liquid interface is significantly increased. Compared to non-modified vessels, five to tenfold higher k_La values (up to 55 h^{-1}) were achieved, while cell growth was comparable to small scale TubeSpin® and 30 L stirred bioreactors (Zhang et al. 2008b).

Although none of these systems made it to market, they finally led to the development of the SU 200 L orbitally shaken bioreactor system (trade name OrbShake™ bioreactor) in 2009 (Hildinger et al. 2009). By eliminating the need for internal mixing and sparging devices, the bags are considered to be an economical alternative to stirred SU bioreactors. Furthermore, typical issues, such as foam formation, are avoided. The cylindrical cultivation container with a nominal volume of 330 L is equipped with pH and pO_2 sensors and operated using the bioreactor control system. Based on measurements in cylindrical containers agitated at ≈75 rpm with a shaking diameter of 10 cm, achievable k_La values at the maximum filling level are in the order of 8 h^{-1} (Zhang et al. 2009). Significantly higher values of up to 25 h^{-1} are reported for 100 L working volume (Anderlei et al. 2009). Under these conditions, mixing times are between 25 and 70 s, depending on the shaking frequency (50–70 rpm).

14.3.5 Fixed Bed Bioreactors

Almost no engineering data for mixing time, oxygen mass transfer (k_La) or power input are available for fixed bed bioreactors in the literature. The iCELLis bioreactor is based on a compact fixed-bed packed with macroporous, non-woven, medical-grade polyethylene terephthalate (PET) microfibers, which offer a large growth surface, depending on the fixed bed volume (Moncaubeig 2013). For all scales, the bed height is fixed at 10 cm, which provides linear scalability between the small scale iCELLis nano systems with cylindrical fixed beds and the production scale iCELLis systems containing a donut-shaped basket (Lehmann et al. 2013). In both systems, two carrier compaction levels with specific surface areas of about 13,320 and 20,000 $m^2 \cdot m^{-3}$ are available, resulting in a total growth surface area of up to 500 m^2 in only a 25 L fixed bed in the largest version (iCELLis 500/500; 660 m^2 announced).

The liquid inside the bioreactor is circulated by a built-in centrifugal-based flow impeller, which pumps the culture medium through the fixed bed from the bottom to the top. When flowing down the outer wall as a thin film, the intention is for the culture medium to be effectively oxygenated in a waterfall-like flow. To date, no k_La values or comparable data has been found in the literature. Due to the separation of the pumping impeller, which runs at high rotational speeds of up to 1,500 rpm, from the growth compartment, where linear velocities of only up to 0.05 $m \cdot s^{-1}$ were determined (Drugmand et al. 2013), the suitability of the iCELLis bioreactor for the growth of shear-sensitive, adherent cultures has been confirmed (Lennaertz et al. 2013).

Similar to iCELLis fixed-bed bioreactors, the BioBLU SU packed-bed bioreactor (BioBLU 5p, Eppendorf/New Brunswick) also employs internal recirculation. This is driven by a packed-bed basket impeller that incorporates Fibra-Cel disks as the cell attachment matrix. The two horizontally-positioned perforated screens holding the bed disks are extended to the walls of the bioreactor vessel and discharge ports positioned above the basket incorporate a central hollow tube. A low differential pressure at the base of the impeller tube is generated by the rotation of these discharge ports, which results in media circulation throughout the vessel.

The Fibra-Cel, which is fabricated according to cGMP guidelines, is composed of two layers of non-woven polyester and polypropylene that are sonicated together and electrostatically treated to attract cells and facilitate their attachment (Cino et al. 2003). The high porosity of the polymer mesh of around 90 % reduces intra-carrier diffusion limitations and provides efficient cell entrapment (Meuwly et al. 2006, 2007), which reduces cell attachment time. According to Cino et al. (2003), cells can attach within 15–60 min on the Fibra-Cel disks while it normally takes about 6 h for cells to attach to microcarriers (with a normal inoculum of $1 \cdot 10^6$ cells $\cdot$ mL^{-1}). The BioBLU 5p packed-bed bioreactor is available with a vessel volume of 5 L (3.5 L working volume) and is pre-loaded with 150 g of Fibra-Cel® disks, which offer a specific surface area of $119 \cdot 10^3$ m$^2 \cdot$ m^{-3} corresponding to 0.12 m^2 of effective surface area per gram of disks. A similar bioreactor concept is used in the CellTank^{TM} bioreactor (CerCell), which provides a non-woven polymer matrix with a surface area of about 3.6 m^2. The liquid is circulated by a centrifugal-type impeller.

An external medium recirculation system is employed in the AmProtein Current bioreactor (AmProtein), which consists of a wide-body culture vessel with an inverted frusto-conical bottom on an orbital shaker platform (Hui 2009; Jia et al. 2008). The bag is fabricated from EVA plastics, which is suggested to play a major role in oxygen mass transfer within the system. It has been shown that small bubbles are absorbed onto the plastic surface, significantly increasing the specific surface area for oxygen mass transfer (Jia et al. 2008). The periodic orbital movement repeatedly washes the exposed bubbles from the vessel wall, which is further enhanced by the inverted conical bottom. Although the applied operating conditions are difficult to compare, the shaking motion was found to provide higher oxygen transfer rates than bubbling air directly into the medium (Hui 2009).

14.4 Established and New Applications for Dynamic SU Bioreactors

Today, SU bioreactors dominate in processes based on continuous suspension cell lines, where the cells are the final product (e.g. seed or inoculum train, see Sect. 14.4.1) or high titer and high value products that are produced up to medium scale. The last mentioned products include monoclonal antibodies (mAbs)

(Sect. 14.4.2), viral vaccines (Sect. 14.4.3), virus-like particle (VLP) vaccines (Sect. 14.4.4), and viral vectors for gene therapies (e.g. adeno-associated virus, paramyxovirus, lentivirus) (Negrete and Kotin 2007). Commonly, wave-mixed systems are used for the cell expansion, whereas stirred bioreactors are preferred as production bioreactors for protein therapeutics.

However, production processes for vaccines are still often performed using adherent production cells lines (e.g. *African green monkey* kidney-derived *Vero cells*, *Madin-Darby Canine Kidney* cells or PBS-1 cells) (Josefsberg and Buckland 2012; Whitford and Fairbank 2011). Both stirred (Chaubard et al. 2010; George et al. 2010) and wave-mixed (Genzel et al. 2010; Magnusson et al. 2011) SU systems operated with microcarriers as well as fixed bed bioreactors with fiber carriers (Moncaubeig 2013) have proven themselves.

As shown in the following, their development involved the establishment of novel cultivation technologies, such as large-volume (Bögli et al. 2012) and high density cell banking (Tao et al. 2011) and XD process technology (Zijlstra et al. 2012). Furthermore, users became increasingly interested in perfusion technology (see Fig. 14.6) (Bonham-Carter and Shevitz 2011; Wang et al. 2012a). While the product quality is maintained or even increased, 10- to 30-fold higher cell densities and space-time yields of the products can be obtained in perfusion mode compared to fed-batch processes. For example, the same product amounts may be produced in 50 L SU perfusion bioreactors instead of using 1,000 L fed-batch systems (Langer 2011).

Finally, the successful application of wave-mixed, stirred, hollow-fiber and fixed bed SU bioreactors for the development of cell therapeutics has been demonstrated (van den Bos et al. 2014). Section 14.4.3 provides an overview of cultivations performed with primary human therapeutic cells in dynamic SU bioreactors and focuses on the expansion of human mesenchymal stem cells (hMSCs).

14.4.1 Modern Seed Train Production with Continuous Suspension Cell Lines

Wave-mixed bioreactors were originally designed as substitutes for spinner flasks for seed train production of animal and human continuous suspension cells. Although they were viewed skeptically in the beginning, wave-mixed bioreactors are today well-established in seed train production. As shown in Fig. 14.7, pre-cultures are commonly cultivated in shake flasks, which are inoculated from pooled cells originating from the vial-based working cell bank (WCB). Detailed descriptions of seed train production with recombinant CHO cells growing in a 10 L cultivation bag (max. working volume 5 L) to produce the inoculum for the BIOSTAT® CultiBag RM 20 are provided elsewhere (Eibl et al. 2014a). Starting with the thawing of the cells and the inoculation of the shake flasks, only 8 days are

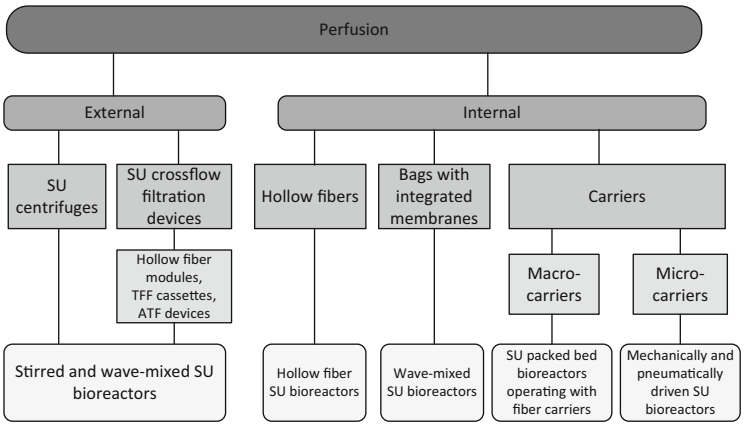

Fig. 14.6 Schematic of principal techniques applied to run SU bioreactors and devices in perfusion mode. In addition to internal perfusion (where the cells are bound on capillary fibers, membranes or microcarriers within the SU bioreactor) there is a growing interest in external perfusion. In the case of external perfusion, the cells are retained by using external SU cross flow filtration devices (e.g. Refine Technology's ATF system) or SU centrifuges (e.g. Carr UniFuge from Carr Centritech Separation Systems). If complete cell retention occurs in perfusion mode it is sensible to remove 10–20 % additional medium in order to prevent aging of the cell population. *TFF* tangential flow filtration, *ATF* alternating tangential flow

typically required to achieve medium cell densities of approximately $5 \cdot 10^6$ cells $\cdot$ mL^{-1} in the cultivation bag.

If the WCB is established using larger volumes in cryogenic bags instead of vials (Heidemann et al. 2002; Bögli et al. 2012) or using very high cell densities of approximately $1 \cdot 10^8$ cells $\cdot$ mL^{-1} in traditional vials (Alahari 2009; Tao et al. 2011), the intermediate step of shake flak expansion can be omitted. The cells of both large volume and high cell density WCBs can be generated by continuous perfusion processes in wave-mixed bioreactors (1 L working volume). Special perfusion bags with integrated, fixed or floating micro filtration membranes (see also Sect. 14.3.1) are commercially available and easy to operate (see also Fig. 14.6). By shortening the seed train production, the upstream processing of the antibody production process shown in Fig. 14.7 can be made more efficient. For example, Bögli et al. (2012) reported that it is possible to inoculate 50 L medium with a total of $5.5 \cdot 10^{10}$ with cabbage looper cells (*Trichioplusia ni; Hi-5*) after 5 days. Normally, this takes at least 10 days. The suspension cells, stored in a 120 mL cryogenic bag at −196 °C, were previously expanded in a BIOSTAT® CultiBag RM (10 L total volume) operated in repeated fed-batch mode.

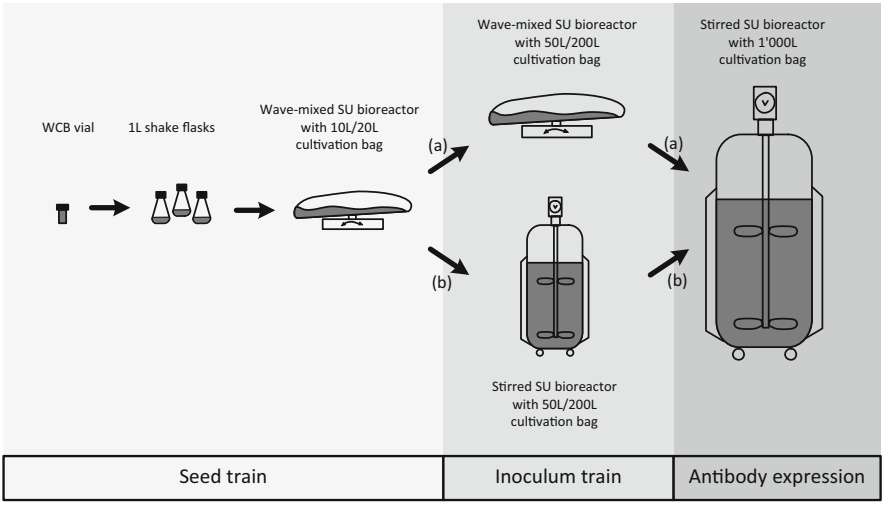

Fig. 14.7 Typical seed inoculum production and antibody expression carried out in SU bioreactors of up to 1 m^3 production scale. For the inoculum train production the user can choose between a wave-mixed and a stirred SU bioreactor system. Due to the predominance of stirred SU bioreactors in product expressions, many users switch from wave-mixed benchtop systems to stirred SU bioreactors to deliver the inoculum for the production bioreactor

14.4.2 CHO Cell-Based Production of Monoclonal Antibodies (mAbs) up to Medium Volume Scale

The great demand for therapeutic mAbs and the advantages of SU bioreactors, as described in Sect. 14.1, explain their broad usage in preclinical and clinical production processes. Meanwhile, SU bioreactors are used by many contract manufacturing organizations (CMOs) due to their scalability up to working volumes of 2,000 L. This development is supported by identical results from several comparison studies with respect to living cell density, viability profiles, expression profiles and product quality when compared to standard stainless steel vessels (Cameau et al. 2010; Diekmann et al. 2011; Smelko et al. 2011).

Continuous cell lines preferred for mAb production are nowadays genetically stable and include CHO cells, lymphoma (NS0, SP2/0) cells, human embryogenic kidney cells (HEK293), hybridomas and human embryonic retinoblast derived Per.C6 cells (Ho et al. 2013). Antibody production, which is generally carried out in fed batch mode (e.g. cells are supplemented with a concentrated nutrient solution) by using serum-/protein-free or chemically defined culture media, is often enhanced by temperature shifts (to between 28 °C and 31 °C). After 8–21 days of cultivation, the product is harvested batch wise, which leads to typical antibody titers of between 2 and 5 $g \cdot L^{-1}$ (Yang and Liu 2013).

To further increase the space-time yield of antibody production processes, SU stirred bioreactors with working volumes of up to 1 m^3 have been combined with

recently developed external SU cross flow microfiltration systems, such as the ATF module from Refine Technology. Fresh medium is continuously supplied while exhausted medium containing the product is harvested and the cells are retained inside the bioreactor. Nevertheless, the large volumes of the diluted product may complicate downstream processing.

XD technology was developed by DSM in the Netherlands in order to guarantee both high cell densities and high product titers in stirred SU bioreactors. In the case of XD technology, a cross flow filtration system is applied, which has a pore size or molecular weight cut-off that is two- to threefold smaller than the target product. Hence, not only the cells but also the product is retained in the bioreactor, which makes antibody concentrations of between 10 and 27 $g \cdot L^{-1}$ at cell densities of 10^8 cells $\cdot mL^{-1}$ realistic (Zouwenga et al. 2010).

14.4.3 Viral Vaccine and Virus-Like Particle Production

Production processes for viral human and animal vaccines differ from those of antibodies, since the production expression of viral vaccines is mostly lytic. The non-infected WCB cells are infected at the end of the growth phase by the amplified virus stock from the working virus bank (WVB). The viruses are replicated inside the cells, which leads to an increase in the cell diameter. The released viruses are processed into solutions for injections containing live (attenuated) or inactivated viruses. Because clinical doses of viral vaccines are typically smaller than those of mAbs, the production scale (100–2,500 L) is also smaller (Ball et al. 2009). However, many virus production processes require higher biosafety demands (often biosafety level 3 environments). As already mentioned, the use of animal products and in particular serum in the commercial production of viral vaccines is still relevant (Whitford and Fairbank 2011). Furthermore, there are more adherently cultivated production cell lines than in antibody production processes, including the *African green monkey* kidney-derived *Vero cells*, *Madin-Darby Canine Kidney* (MDCK) cells or C*hick Embryo Fibroblast* (CEF) cells (Chaubard et al. 2010; Hu et al. 2008). Therefore, bioreactors are used which allow 2D cell growth, such as roller bottles and multitray systems, or which operate with hollow fibers (Hirschel 2011) or microcarriers (Moncaubeig 2013). Many vaccine producers (e.g. GSK, IDT Biologica, MedImmune, Sanofi-Aventis, Virbac) have replaced roller bottles and multitray systems with microcarrier based (see overview about common microcarrier types given by Whitford and Fairbank (2011)) wave-mixed (Sect. 14.3.1) and stirred (Sects. 14.3.2 and 14.3.3) SU bioreactors. Different studies, where MDCK and Vero cells were grown on microcarriers, revealed that tenfold higher cell densities and virus titers can easily be achieved when using wave-mixed and stirred bioreactors for virus production (e.g. Influenza, Polio, mink enteritis virus) (Genzel et al. 2004, 2006; Hundt et al. 2007; George et al. 2010; Schouwenberg et al. 2010; Thomassen et al. 2012).

As an alternative to stirred and wave-mixed SU bioreactors operating with microcarriers, SU fixed bed bioreactors (Sect. 14.3.5) are used for viral vaccine production. Moncaubeig (2013) described two virus production processes based on Vero cells in an iCELLis™ system (0.53, 1, 13.2, 132, 660 m^2 fixed bed made of medical-grade polyester microfibers). Moreover, scale-up from the smallest to 660 m^2 fixed bed (prototype, no yet commercially available) was successful for a human vaccine production process. The cell growth and virus productivity were equivalent to those found in a reusable, stirred 600 L bioreactor with 6 $g \cdot L^{-1}$ Cytodex microcarriers. Using an iCELLis™ with 2.7 m^2 fixed bed and serum free medium, up to 11-fold higher productivity of paramyxovirus was achieved compared to T-flasks. Comparability of the small-scale iCELLis™ nano bioreactor (0.53 m^2, 40 mL fixed-bed) with a CellSTACK® for the production of recombinant adeno-associated viruses was also proven by Lennaertz et al. (2013). Maximum viral yields of up to $4.5 \cdot 10^8$ vector particles per $\cdot cm^2$ were achieved using HEK cells. Peak cell densities of $40 \cdot 10^6$ cells $\cdot mL^{-1}$ were achieved with Vero cells producing an undisclosed enveloped virus in serum-free conditions in a 500 mL iCELLis™ fixed-bed bioreactor (Drugmand et al. 2009).

A benchtop scale alternative is the 5 L BioBLU SU packed-bed bioreactor (see Sect. 14.3.5). It is close in design to its re-usable counterpart, which became very popular for vaccine production at laboratory scale. For example, cell densities of up to $12.4 \cdot 10^6$ cells $\cdot mL^{-1}$ were achieved in a 14 day culture of the *TE Fly* retroviral vector producer cell line, resulting in a maximum titer of 10^7 viral particles $\cdot cm^{-3}$ (Merten et al. 2001). At the time of writing, no results from vaccine production processes executed in the SU version model have been published in scientific journals, but the general applicability in CHO based protein expression has been demonstrated (Hatton et al. 2012).

Using the AmProtein Current Perfusion bioreactor fixed bed system, a total of $3.2 \cdot 10^{10}$ MDCK cells have been measured after 6 days and influenza virus production (H_1N_1) was induced with a multiplicity of infection of 0.05. The peak virus titer of about $7.68 \cdot 10^6$ hemagglutinin units per liter, which corresponds to $7.8 \cdot 10^7$ 50 % tissue culture infectious doses per mL, was obtained 3 days post infection (Sun et al. 2013). Furthermore, analysis of the cell density at different positions suggested a stable and even distribution pattern throughout the perfusion column.

Interestingly, the importance of insect suspension cells used as production organisms for vaccines has increased during recent years (Cox 2012; de Jongh et al. 2013). This concerns lytic product expressions with the baculovirus expression vector system (BEVS), which have been characterized and optimized by several authors (Kamen et al. 1996; Schmid 1996; Palomares and Ramírez 2009; Vicente et al. 2011). Fall army worm cell lines (*Spodoptera frugiperda*; *Sf-9*) as well as the cabbage looper (*Hi-5*) and its derivatives have become well established. The main advantages of insect cell and BEVS based vaccine production processes include short process time (about 5–6 days) and the inability of BEVs to infect humans (Weber and Fussenegger 2009). Thus, a biosafety level environment 1 is sufficient for the production of VLP vaccines (since they are non-replicating and

non-pathogenic due to their complete lack of DNA or RNA, but provoke high protective immunity) using insect cells and BEVS.

With respect to the quick availability of preclinical and clinical samples for seasonal and pandemic vaccine candidates, the usage of wave-mixed and stirred SU bioreactors is advantageous (Eibl et al. 2013; Hahn 2013). Currently, upstream concepts, which are entirely based on SU devices, are being realized. Eibl et al. (2013) developed an upstream concept, which is based on the wave-mixed BIOSTAT® CultiBag RM 20/50 and provided preclinical Influenza A/H_1N_1/Puerto Rico8/34 VLPs for animal studies in the single-digit $mg \cdot L^{-1}$ range. The authors used the BIOSTAT® CultiBag RM 20/50 for (1) the production of a large volume WCB of *Sf-9* cells stored in cryogenic bags at −196 °C, (2) the generation of working seed virus at −80 °C, (3) cell expansion (bag-to-bag inoculation and repeated fed batch expansion) and (4) influenza VLP stock production at 1 and 10 L scale. Due to the omission of intermediate cultivation steps in shake flaks and the increase of the scale-up rate to > 1.5 steps, time savings of about 35 % are achievable in upstream processing (USP).

14.4.4 Expansion of Human Primary Cells for Production of Cell Therapeutics

Compared to the production of vaccines and antibodies, the production of human primary cells uses small batch production processes that still take place in serum-containing media. The final products are the therapeutically relevant cells themselves, which are injected or implanted into the patient in order to treat serious disorders (e.g. cancer, myocardial, metabolic and orthopedic disorders). A distinction is made between autologous and allogeneic transplantations.

Mostly T-cells, natural killer (NK) cells, dendritic cells and hematopoietic stem cells are used for autologous transplantations, where the donors receive their own cells after biopsy, manipulation (e.g. cell activation, genetic manipulation) and cell expansion. As described by van den Bos et al. (2014), the cell amounts required for a patient's treatment can be generated in commercially available 2D SU cultivation systems, such as gas permeable bags or planar cultures (CellFactories, CellSTACK®, HyperFlask etc.). Furthermore, different published studies have revealed that clinically relevant doses of activated T-cells (mean $1.7 \cdot 10^{10}$ T cells) and NK-cells (mean $9.8 \cdot 10^9$ NK cells) can be produced in wave-mixed SU bioreactors (Wave Bioreactor) (Tran et al. 2007; Hollyman et al. 2009; Sutlu et al. 2010).

Allogeneic transplantation is characterized by the fact that the donor and the receiver of the cells are different. For clinical allogeneic therapies, the growing interest in hMSCs is obvious (Trounson et al. 2011; van den Bos et al. 2014). This can be explained by less ethical concerns and the higher safety of the hMSCs compared to human embryonic and induced pluripotent stem cells (Wang

et al. 2012b). However, the required number of therapeutic hMSCs for a single patient (70 kg) in clinical trials is in the order of $3 \cdot 10^7$ to $5 \cdot 10^8$ cells (Rafiq et al. 2013). According to Rowley et al. (2012) and van den Bos et al. (2014), clinical indications require trillions of hMSCs per year. Due to the high cell amounts, alternatives to the aforementioned 2D SU systems are still required for allogeneic therapies. Although 3D SU systems, such as SU hollow fiber bioreactors (Quantum cell-expansion system (Kilian 2013)), or SU fixed bed bioreactors (iCELLIs (Rowley et al. 2012)), offer controllable and efficient hMSC expansion, they have limited scalability. For this reason, several research groups are working on the development of scalable platforms for the expansion of hMSCs, where they are cultivated as aggregates or on microcarriers in instrumented and automated SU bioreactors. The largest working volumes have been achieved in hMSC expansions with microcarriers (cyclical perfusion or feeding mode) when stirred and wave-mixed systems have been used. After an attachment phase without agitation, mixing should be performed under low shear stress conditions in order to prevent cell damage or loss of stem cell characteristics, to achieve viable cell densities of about $1 \cdot 10^6$ cells $\cdot$ mL^{-1}. Timmins et al. (2012) succeeded in the expansion of human placental mesenchymal stem cells in 2 L wave-mixed bioreactors (0.5 L culture volume) using CultiSpher-S microcarriers over a 7 day cultivation period. Similar expansion factors were achieved for bone marrow-derived (hBM-MSCs) and adipose tissue derived mesenchymal stem cells (hADSCs), where expansion factors of between 16 and 18 (cell densities between 1.4 and $2.5 \cdot 10^5$ cells $\cdot$ mL^{-1}) were reported for 14 days of cultivation in glass spinners (Santos et al. 2011).

In a 12-day cultivation in the stirred Mobius® CellReady 3 L bioreactor using serum-supplemented culture medium (10 %) and microcarriers, cell densities of between 2.5 and $2.7 \cdot 10^5$ hBM-MSCs $\cdot$ mL^{-1} have been achieved (Cierpka et al. 2013; Jing et al. 2013). The course of a typical cultivation of hADSCs in the Mobius® CellReady 3 L (2 L culture volume), as performed in our laboratories, is delineated in Fig. 14.8a. After 6 days of cultivation, about $5.5 \cdot 10^5$ hADSCs $\cdot$ mL^{-1} were grown. Using ProNectin-F COATED microcarriers and serum-reduced Lonza medium (5 % serum), cell growth comparable to small reference scale spinner flasks was achieved. In the stirred bag bioreactor BIOSTAT® CultiBag STR 50 L we produced a total cell quantity of $1 \cdot 10^{10}$ hADSCs in a 35 L culture volume ($2.9 \cdot 10^5$ hADSCs $\cdot$ mL^{-1}) with the same microcarrier-medium combination. This number of cells is sufficient for allogeneic therapies (20 doses of $5 \cdot 10^8$ cells) for 20 patients (Schirmaier et al. 2014).

Within the scope of a collaboration project between the Zurich University of Applied Sciences, Lonza Cologne and Sartorius Stedim Biotech, the design of the stirred BIOSTAT® UniVessel SU 2 L was optimized with respect to stem cell based microcarrier suspension, in order to guarantee homogenous microcarrier-cell-aggregates and cell amounts of up to $1 \cdot 10^9$ hADSCs at high microcarrier concentrations (≥ 8 g $\cdot$ L^{-1}) (Jossen et al. 2014). Furthermore, it has been shown that direct scale-up from spinner flasks to the pilot scale BIOSTAT® CultiBag STR 50 L is possible, based on screening experiments to establish the optimal combination for the culture medium, the microcarrier type, the operational conditions and

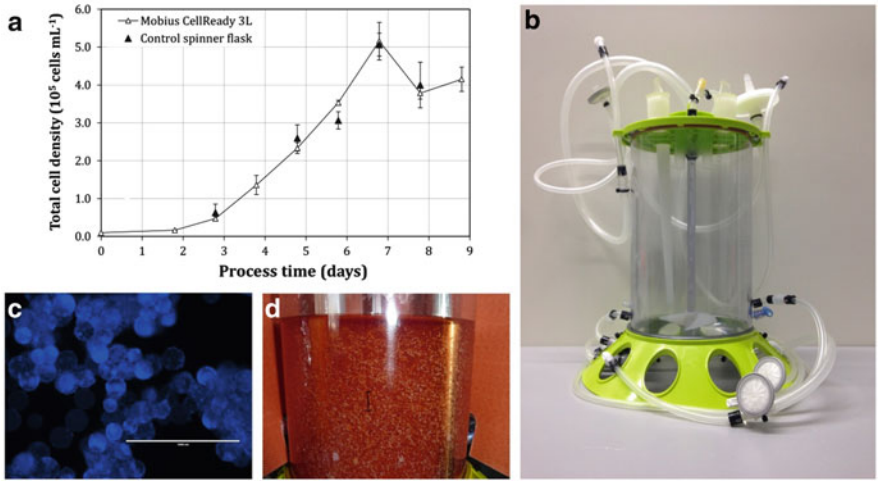

Fig. 14.8 hADSC expansion in the Mobius® CellReady 3 L (2 L culture volume). (**a**): Typical growth course (batch mode) observed in a Mobius® CellReady bioreactor compared to a control spinner flask (100 mL culture volume). (**b**): Photo of the bioreactor vessel used, which was equipped with marine impeller, microsparger and standard sensors (not shown). (**c**): DAPI colored hADSCs grown on microcarriers cultivation day 7 (metering bar = 1,000 μm). (**d**): Photo of the microcarrier-cell-suspension in the Mobius® CellReady on cultivation day 7

the most suitable engineering tools (i.e. CFD and suspension studies). Cell growth of the hBM-MSCs used was similar in the different cultivations systems (results being prepared for publishing).

14.5 Scale-Up of Processes Based on SU Bioreactors

Published case studies report the scalability of SU systems from micro- to production-scale bioreactors (Fernald et al. 2009; Legmann et al. 2009). Since scale-up principles for stirred bioreactors are generally better characterized than those for other bioreactor types, such as rocker-type wave bioreactors (Gossain et al. 2010), the main focus of this section is on stirred SU bioreactors. However, comparability with conventional bioreactors may become difficult because of factors which include eccentrically or non-vertically installed stirrers (e.g. Thermo Fisher's S.U.B., Mobius® CellReady 250), and deviation from standard impeller and sparger types used in SU bioreactors (Gossain and Mirro 2010). Not all bioreactor manufacturers meet the requirements for geometrical similarity between the scales (see Table 14.3). Nevertheless, typical height-to-diameter H/D ratios of stirred SU bioreactors are between 1:1 and 2:1, where the maximum working volume is around 70–80 % of the total volume. The impeller-to-vessel

diameter ratio is typically between 0.33 and 0.6, whereas large impellers provide efficient mixing at low impeller speeds.

The impeller diameter d_R and speed N_R determine the impeller tip speed u_{tip} defined by Eq. 14.21, which correlates well with the maximum fluid velocities and consequently the maximum shear rates within many SU bioreactors (in fact only valid for unaerated conditions) (Kaiser et al. 2011a; Löffelholz 2013).

$$u_{\mathrm{tip}} = \pi \cdot d_{\mathrm{R}} \cdot N_{\mathrm{R}} \tag{14.21}$$

Although the tip speed is often used for scaling-up in biopharmaceutical applications (Kunas and Papoutsakis 1990; Smith and Greenfield 1992; Shiragami 1997), it does not consider the actual shape of the impeller, and volume changes in fed-batch processes. Furthermore, scaling-up to larger bioreactors using a constant tip speed decreases the specific power input, as the relationship in Eq. 14.22 confirms, a relationship that was also shown for the UniVessel® SU and the BIOSTAT® CultiBag STR 50 L (Kaiser et al. 2011a).

$$P/V \propto \frac{{u_{tip}}^3}{D} \tag{14.22}$$

The most often applied scale-up approaches are based on similar specific power inputs P/V (Smith and Greenfield 1992; Al-Rubeai et al. 1995; Platas Barradas et al. 2012; Dekarski 2013), which can be predicted by Eq. 14.23 if the power number (also called Newton number, Ne) is known.

$$P/V = \frac{Ne \cdot \rho_L \cdot {N_R}^3 \cdot {d_R}^5}{V_L} \tag{14.23}$$

However, Ne is a function of the impeller type, the Reynolds number Re, the diameter ratio d/D, the bottom clearance h_R, the number of baffles etc. (Liepe et al. 1998). Reported Ne numbers for stirred SU bioreactors range from 0.3 (Mobius® CellReady, (Kaiser et al. 2011b)) to 4.2 (Mobius® CellReady). Using a constant (ungassed) specific power input as the primary scale-up criterion for three scales of the Mobius® CellReady bioreactor family (3, 50, 250 L), comparable values for cell growth (with $\mu \approx 0.0398$–$\mu \approx 0.0428\ \mathrm{h}^{-1}$), viability, and nutrient metabolism of the CHO cell line were obtained at each scale (Dekarski 2013). In addition, comparable maximum growth rates were found (0.04–0.046 h^{-1}) for *VPM8 hybridoma* cells cultivated in microwell plates and shake flasks at matched power consumptions ($\approx 40\ \mathrm{W \cdot m^{-3}}$) (Micheletti et al. 2006). This suggests that constant specific power input is, at least initially, a good basis for scale translation, even in geometrically dissimilar cultivation systems. However, it should be mentioned that almost identical growth rates (0.048 h^{-1}) and similar peak cell densities were even found in a 3.5 L stirred bioreactor in the same study, although the specific power input ($\approx 3.64\ \mathrm{W \cdot m^{-3}}$) was lower by a factor of 10 (Micheletti et al. 2006).

While no scaling-up study has been found in the literature for SU stirred systems based on mixing time, successful process transfer from the 1D rocker-type BIOSTAT® CultiBag RM to the 2D moving CELL-tainer® based on mixing time has been described for Vero cell based polio virus production (Thomassen et al. 2012). Nevertheless, based on turbulence theory, it has been suggested that mixing times in stirred systems is independent of the impeller type and inversely proportional to turbulent diffusion, as defined by Eq. 14.24:

$$t_{m,95\%} \propto \left(\frac{\varepsilon_T}{L_c{}^2}\right)^{-1/3} \tag{14.24}$$

where ε_T and L_c represent the local energy dissipation rate and the integral scale of turbulence respectively. Assuming that the integral scale is proportional to the vessel diameter, a correlation was established between mixing time and the third radical of the specific power input and the geometrical parameters (Nienow 1997):

$$t_{m,95\%} \propto \left(\frac{P}{V}\right)^{-1/3} \cdot \left(\frac{d_R}{D}\right)^{-1/3} \cdot \left(\frac{H_L}{D}\right)^{2.43} \cdot D^{2/3} \tag{14.25}$$

Löffelholz et al. (2013b) found that CFD predicted mixing times in different bioreactors from the Sartorius Stedim BIOSTAT® STR family follow the same trend (see Fig. 14.9a). However, it should be emphasized that the specific power input at larger scales increases significantly when the mixing time is kept constant during scaling-up (Junker 2004; Xing et al. 2009), which may result in unfavorable impeller speeds. Comparing different geometrically dissimilar SU bioreactors at 200 L scale (including cylindrical and cube-shaped vessels with one or two impellers), Chaubard et al. (2010) examined mixing times at constant tip speeds of between 44 and 86 s for 0.5 $m \cdot s^{-1}$ decreasing to 16 and 58 s for 1 $m \cdot s^{-1}$. In this study the BIOSTAT® CultiBag STR and the XCellerex XDR had the shortest and

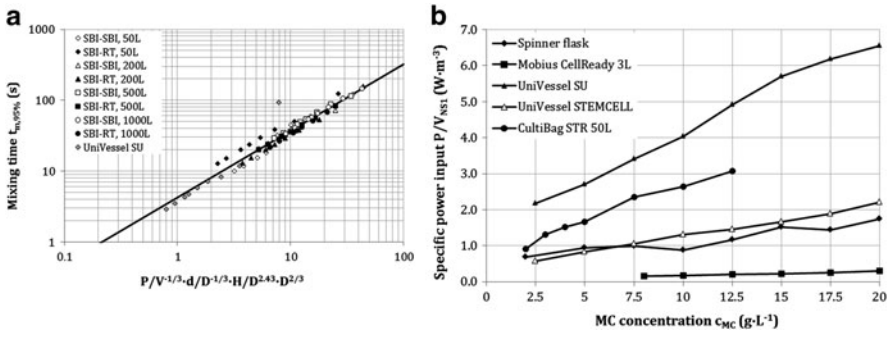

Fig. 14.9 Considerations on scale up. (**a**) Correlation of mixing times in selected stirred SU bioreactors given by Eq. 14.25 (Adopted from Löffelholz (2013)); (**b**) Specific power inputs at N_{SI} criteria for different stirred SU bioreactors

longest mixing times respectively, but there is still a lack of systematic comparisons of mixing in the SU bioreactors that consider geometrical parameters.

For microcarrier based processes, the use of mixing and suspension criteria as scale-up factors have been proposed for animal (Vorlop and Lehmann 1988; Thomassen et al. 2012) and human (Duvar et al. 1996; Hewitt et al. 2011; Kaiser et al. 2013) cells. The microcarriers have to be kept in suspension, at least for most of the batch time, if the available area is to be used effectively. The "just-fully" suspended criterion N_{S1} represents the impeller speed, where no microcarriers are located at the vessel bottom for longer than 1 s, but it does not necessarily mean that the microcarriers are homogenously dispersed throughout the bioreactor (Hewitt et al. 2011). The less common criterion N_{S1u} represents the lower limit of N_{S1}, meaning that some particles are still located on the bioreactor bottom, but none of them are at rest (Liepe et al. 1998). Although the N_{S90}, which represents the impeller speed required to lift particles up to 90 % of the filling height, is easier to determine experimentally (Zlokarnik 1999), it is not recommended for microcarrier-based processes (Kaiser et al. 2013). For several stirred SU bioreactors it was found that the N_{S1u} criterion was achieved at approximately 20 % lower impeller speeds than N_{S1} (Kaiser et al. 2013; Schirmaier et al. 2014). Typical impeller speeds for N_{S1} were between 50 and 145 rpm (corresponding to tip speeds up to 0.82 $m \cdot s^{-1}$) at benchtop scales. However, between 50 and 62 rpm were required for a microcarrier suspension in the BIOSTAT® CultiBag STR 50 L. This corresponds to specific power inputs of up to ≈ 1.8 $W \cdot m^{-3}$, depending on the microcarrier concentration (see Fig. 14.9b). Thus, N_{S1} and N_{S1u} are very sensitive to the vessel configuration, the agitator type and the microcarrier type used (i.e. density and size). As a result, these criteria have to be determined individually for every microcarrier-bioreactor combination.

Another typically applied scale-up criteria related to oxygen mass transfer is the specific liquid mass transfer coefficient, k_La. It was, for example, used as a secondary scale-up criterion in Dekarski's study (2013) to identify suitable aeration rates at larger scales. For a given bioreactor geometry and culture medium (i.e. viscosity, density, surface tension), the k_La in stirred bioreactors is mainly influenced by the specific power input and the aeration rate (see also Eq. 14.28). The latter is quantified by either the superficial gas velocity ν_G (Eq. 14.26; for cylindrical vessels) or the volume-related gas flow rate Q_G (Eq. 14.27).

$$v_G = \frac{F_G}{A} = \frac{4 \cdot F_G}{\pi \cdot D^2} \tag{14.26}$$

$$Q_G = \frac{F_G}{V_L} \tag{14.27}$$

Obviously from Eqs. 14.26 and 14.27, the gas flow rate F_G has to be further increased at larger scales if the constant Q_G is applied ($\nu_G \propto D^{-2}$;$Q_G \propto D^{-3}$). Typical k_La values in animal cell cultures are in the range of 1 and 10 h^{-1} (Henzler and Kauling 1993; Langheinrich et al. 2002). For several stirred SU bioreactors the

correlation given by Eq. 14.28 can be applied, where a, b and c are empirical constants.

$$k_L a = c \cdot (P/V)^a \cdot \nu_G{}^b \tag{14.28}$$

These not only depend on the bioreactor geometry but also on the media properties (i.e. viscosity, surface tension, antifoam concentration etc.). Interestingly, the constant b (0.6–0.9) was found to be larger than a (0.25–0.45) in most SU bioreactors operated under typical cell culture conditions (e.g. Mobius® CellReady 3 L, as reported by Kaiser et al. (2011b)). This means that the influence of the gas flow rates on the $k_L a$ is more pronounced than that of the specific power input. This is in contrast to correlations obtained by van't Riet (1979) and Nienow (2006), but can be explained by the low agitation and resulting low gas dispersion of the primarily axially pumping cell culture impellers.

Nevertheless, $k_L a$ was used as a key parameter for scaling-up between geometrically dissimilar small scale (35 mL working volume) stirred and wave-mixed (2 and 3.5 L working volume) bioreactors (Hanson et al. 2009). Based on identification of achievable $k_L a$ values as a function of impeller speed and rocking rate in both systems, similar trends were observed for the DO in Immunoglobulin G producing hybridoma cultures. This implies comparable oxygen delivery rates, assuming approximately equal oxygen uptake rates, which resulted in similar culture performance based on peak viable cell density ($1.28/1.18 \cdot 10^6$ cells $\cdot$ mL^{-1}), maximum antibody concentrations (93/92 mg $\cdot$ L^{-1}) and average growth rate (0.026/0.029 h^{-1}) (Hanson et al. 2009).

Concluding Remarks

Nowadays, SU bioreactors are used by developers as well as CMOs for the production of preclinical and clinical samples. This can be explained by their high flexibility and the reduction in time and expenses if the systems are correctly implemented (Whitford 2010). Although customers can choose between several different kinds of SU bioreactors, SU stirred bioreactors operated in fed-batch mode are preferred for animal and human cell based processes. One exception is inoculum production, where wave-mixed systems have become the standard.

The broad acceptance of stirred SU bioreactors can be explained by their scalability, the similarity of their design compared to their conventional counterparts, and the availability of engineering parameters (Löffelholz et al. 2013). Furthermore, scale-up can be achieved using classical criteria, such as the specific power input (Minow et al. 2013), or by using modern technologies (Venkat and Chalmers 1996; Letellier et al. 2002), such as PIV and CFD. By performing integrated processes, such as the combination of stirred SU bioreactors with SU cross flow filtration systems, tenfold or even

(continued)

higher increases in product titers are possible. Therefore, production processes with scales between 500 and 2,000 L are regarded as sufficient.

However, three issues associated with SU bioreactors still exist: the low degree of instrumentation and automation, the lack of standardization, and the problem of leachables/extractables. Since, the cultivation vessels are only used once, SU sensors which come into direct contact with the medium are inexpensive, but do not need a long life-time (Glindkamp et al. 2010). Furthermore, sensors that are pre-configured and integrated into the cultivation system have to withstand gamma irradiation used for sterilization. Only if this is guaranteed, critical process parameters that may influence critical attributes can be determined, as required by the Process Analytical Technology initiative of the US Food and Drug Administration. Solutions for the improved standardization and reduction of leachables/extractables are under development, which will lead to further increases in the implementation of SU bioreactors in commercial GMP production processes (Langer 2012).

There are nine fields in which the future application of SU bioreactors for animal and human cell based cultivations show clear promise: (1) large-volume and high density cell banking, (2) inoculum production, (3) production of mAbs at medium-volume scales, (4) production of personalized mAbs at small-volume scale, (5) production of viral and VLP vaccines, (6) production of viral vectors for gene therapies, (7) expansion and/or differentiation of human autologous cells for cell therapeutics at small scales and (8) expansion and/or differentiation of human allogeneic cells for cell therapeutics at medium scales.

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Chapter 15
An Overview of Cell Culture Engineering for the Insect Cell-Baculovirus Expression Vector System (BEVS)

Laura A. Palomares, Mauricio Realpe, and Octavio T. Ramírez

Abstract The insect cell-baculovirus protein expression vector system (BEVS) has gained increasing attention as more of its products are approved for human use. However, the system has been relevant for many years, being used for the manufacturing of recombinant veterinary vaccines, as a workhorse in the research laboratory, as an important tool for new drug discovery and as an important source of commercial materials and reagents for research. In this chapter, the key elements that should be considered for the design of a productive BEVS process are discussed, along with a presentation of the state of the art of the system.

Keywords Insect cells, baculovirus expression vector system • Recombinant protein production, transient gene expression

15.1 Introduction

The insect cell-baculovirus expression vector system (BEVS) is a system for the expression of recombinant proteins that consists of a continuous insect cell line (or insect larvae) that is infected with a baculovirus containing a gene of interest. The system was developed by Gale Smith and Max Summers (Smith et al. 1983). It is a highly versatile system that can be used for the production of one recombinant protein, or to simultaneously express various genes. It has several advantages that have made it the system of choice for a wide number of applications. Some of the advantages are listed below:

(a) High expression of the gene of interest. The baculovirus contains one of the strongest promoters known, the promoter of *polh*. At 48 h after infection, the

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transcripts of *polh* can constitute up to 24 % of all messenger RNA in the cell (Chen et al. 2013). The use of this promoter results in very high concentrations of the protein of interest.

(b) High versatility. A single cell line and a single baculovirus backbone can be used to express any desired gene. The BEVS is a plug-and-play expression system (Cox 2012; Buckland et al., in press). Process development for a particular gene starts with a standard process that can be easily modified for the optimal production a specific protein. In addition, several proteins can be simultaneously produced, as the baculovirus genome allows the insertion and efficient expression of one or several large genes. As a result, over 500 different functional recombinant proteins have been expressed in the BEVS.

(c) Fast. The construction of a recombinant baculovirus has been standardized and can be efficiently used to produce a recombinant protein within 5 days. High amounts of a recombinant protein can be quickly obtained.

(d) Posttranslational modifications. Insect cells can perform most posttranslational modifications present in proteins of eukaryotic origin. As a result, proteins that cannot be expressed in other systems can often be easily expressed in the BEVS maintaining their biological function.

(e) Scalability. Insect cells are readily grown in suspension, and have been cultured in tanks of 1,000 of liters, particularly for the production of biopesticides. This makes process scale up simple and fast.

(f) Safety. Processes employing BEVS have been classified as biosafety level 1, resulting in low requirements for cell and baculovirus containment.

These advantages have made the BEVS one of the most widely used for a large number of applications that include the production of recombinant proteins for research, the production of three licensed vaccines for human use and several veterinary vaccines. Table 15.1 lists some of the licensed products with pharmaceutical application that are produced in the BEVS. There are many other products of the BEVS in the market, mostly reagents for research. The BEVS is a very relevant system for the production of gene therapy vectors. The first vector approved by the FDA for gene therapy, an adeno-associated virus vector (AAVv), is produced in the BEVS (Table 15.1). The capacity of baculovirus to enter and deliver genes into mammalian cells (Hofmann et al. 1995) has been exploited to design systems for fast high throughput protein screening (Kost et al. 2005) and as a potential gene therapy vector (Luo et al. 2011; Torres-Vega et al., in press). Another interesting application is the baculovirus display technology, an analog of phage display, but with the additional ability of displaying proteins with eukaryotic posttranslational modifications (Kost et al. 2005).

As can be seen in Table 15.1, the BEVS is an ideal system for the production of recombinant vaccines. Many candidate vaccines under development are virus-like particles (VLP), conformed by a viral protein that is expressed in the BEVS and that self-assembles into a structure identical to the virus capsid (Cox 2012; Liu et al. 2013; Lua et al. 2014; Mena and Kamen 2011; Palomares and Ramírez 2009). The first BEVS product licensed for human use is a VLP (Table 15.1).

Table 15.1 Pharmaceutical products manufactured through the BEVS that have been licensed for human or animal use

Product (manufacturer)	Target	Year licensed	Protein	Cell line
Bayovac® CFS (Bayer)	CSV (vaccine)	2002	gEnv E2	Sf9
Porcilis® Pesti (Intervet-Schering-Plough)	CSV (vaccine)	2004	gEnv E2	Sf9
Porcilis® PCV (Intervet-Schering-Plough)	PCV2 (vaccine)	2004	ORF2	Sf9
Cervarix® (Glaxo Smith Kline)	HPV (vaccine)	2007	L1	High Five®
Ingelvac Circoflex® (Boehringer Ingelheim Vetmedica)	PCV2 (vaccine)	2009	ORF2	expresSF + ®[a]
Provenge® (Dendreon)	Prostate cancer immunotherapy	2010	PAP-GMCSF	Sf21
Best-H5® (Boehringer Ingelheim Vetmedica)	Avian influenza virus, A H5N1 (vaccine)	2012	HA	expres SF + ®[a]
Glybera® (uniQure)	Restore LPL enzyme (gene therapy)	2012	AAVv1	expres SF + ®[a]
Flublok® (Protein Sciences Corporation)	Seasonal influenza (vaccine)	2013	Three HA, H1, H3 and B	expres SF + ®[a]

AAVv1 adeno-associated virus vector type 1, *CSV* classic swine fever virus, *HA* influenza virus hemagglutinin, *HPV* human papilloma virus, *LPL* lipoprotein lipase, *PAP-GMCSF* prostatic acid phosphatase linked to granulocyte-macrophage colony stimulating factor, *PCV* porcine circovirus
[a]expresSF + ® is a proprietary cell line derived from Sf9 cells (Protein Sciences Corporation)

VLP can contain one or several proteins that assemble into single or multiple layered capsids, which represents an important challenge for manufacturing (Palomares et al. 2012; Castro-Acosta et al. 2014). In addition to the production of vaccines, protein assemblies produced in the BEVS have been used as scaffolds for the synthesis of nanobiomaterials (Plascencia-Villa et al. 2009, 2011; Carreño-Fuentes et al. 2013).

15.2 Producing Recombinant Proteins Using the BEVS

The BEVS is a transient protein expression system. Insect cells are grown to a desired cell density, and then are infected with a recombinant baculovirus containing the gene of interest. Soon after infection, the baculovirus takes control of the insect cell, inhibiting the transcription of the cellular genes, until the infected cell dies. Recombinant protein production with the BEVS requires two elements, a high viability insect cell culture and a highly infectious baculovirus stock (Fig. 15.1). Several steps are needed to amplify both the baculovirus and the insect cell culture to the desired production scale. In all steps for baculovirus stock

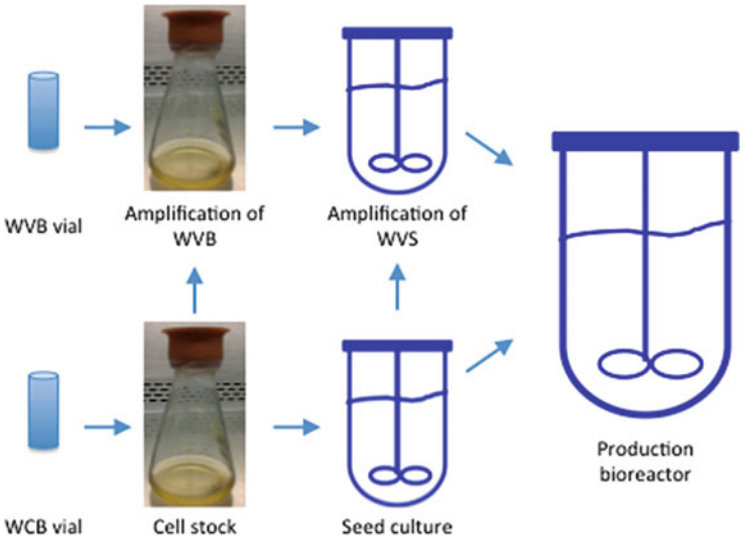

Fig. 15.1 The BEVS process. A working cell bank (WCB) vial is routinely thawed, and the insect cell culture is maintained for several passages. The cell stock is utilized to seed cultures for the amplification of the working virus bank (WVB). The amplification of the WVB can be performed as needed to obtain a working virus stock (WVS) with the volume needed to infect the production bioreactor. In parallel, the cell stock is amplified to a sufficient volume to seed the production bioreactor. Cells are grown to a set cell density and then the culture is infected with the WVS

amplification, an insect cell culture is needed. Recombinant protein production with BEVS requires process development for the amplification of the baculovirus and cell stocks, and for the production step. Process development is rarely done in a research setting. Fortunately, the BEVS is a very robust system that will yield high concentrations of recombinant proteins even in suboptimal conditions. The baculovirus virus stock amplification is an important part of the process, and is often the limiting step for process scalability.

15.2.1 Insect Cells

Insect cell culture originated with the need for studying sex-cells (Goldschmidt 1915), and continued with the interest for studying the biochemistry and physiology of insects. The first reported insect cell culture was performed using the hanging-drop method; spermatocysts from the testis of *Samia cecropia L.* were cultured in hemolymph for up to 3 weeks (Goldschmidt 1915). From the beginning of insect cell culture, it was of major interest to study the pathologies of insects, specifically those caused by virus. In 1935, Trager reported the maintenance of silkworm tissue and the propagation of a baculovirus, called at that time the virus of grasserie, in a salt solution with maltose and egg albumin digest supplemented with 10 %

hemolymph (Trager 1935). The first insect cell lines were established by Prof. Gao in China, who was also able to replicate polyhedrovirus in them using the Trager solution (Vlak 2007). Grace independently established cell lines and set important basis for the in vitro culture of insect cells (Grace 1962).

From the establishment of the first insect cell lines, over 400 continuous insect cell lines have been established (for a partial list, see Palomares et al. 2006), but only a few have gained commercial importance. Most cell lines have been obtained from undifferentiated tissues of insects in several stages of their life cycle. For the BEVS, cell lines from *Spodoptera frugiperda* and *Trichoplusia ni* have been the most well characterized and used. The Sf21 cell line, isolated from the pupal ovarian tissue of *S. frugiperda*, was the cell line used by Smith et al. (1983) when the BEVS system was originally developed. Its derivative, Sf9 (Summers and Smith 1987), is the most widely used cell line for the expression of recombinant proteins by BEVS. Most pharmaceutical products of the BEVS in the market are produced in these cell lines (Table 15.1). The other cell line of commercial importance is the High Five® insect cell line, isolated in the group of Granados in the Boyce Thompson Institute, and commercialized by Invitrogen. The first BEVS product licensed for human use, Cervarix, is produced in this cell line (Cox 2012). The High Five® cell line, derived from the egg cell line BTI-Tn5B1-4 after adaptation to serum-free suspension culture, has been reported to produce several times more recombinant protein than Sf9 cells, particularly when a secreted protein is expressed (Davis et al. 1993; Monteiro et al. 2014; Taticek et al. 2001). The use of the High Five® cell line was limited to a few companies/ commercial projects as it was protected by patents and exclusive agreements (Granados 1991; Granados and Li 2002).

Insect cells are cultured at room temperature, between 26 °C and 29 °C, and can be stored for long term in liquid nitrogen. Both the Sf and the High Five® cell lines have been adapted to grow in suspension, and are cultured in shake flasks at the laboratory scale. Typically, a 250 mL flask will contain up to 60 mL of culture. Insect cell lines are grown at larger scales in bioreactors that are designed to satisfy the oxygen demand of baculovirus-infected insect cells, at dissolved oxygen tensions between 30 % and 100 % of saturation with air (Palomares and Ramírez 1996, 1998). Insect cells also attach to a surface when cultured in static conditions. Static culture of cells is often used for baculovirus cloning or titering of virus stocks.

Initial strategies for the culture of insect cells were focused on reproducing the *in vivo* conditions for cell growth, by designing culture media that resembled the composition of hemolymph. Wyatt et al. (1956) characterized the organic components of hemolymph of two lepidopterans and one hymenopteran, and formulated a culture medium based on the hemolymph composition (Wyatt 1956). As the hemolymph, insect cell culture media have a pH between 6.2 and 6.5 and an osmolarity between 300 and 380 mOsm (Palomares et al. 2006). Early insect cell culture media were supplemented with 10 % hemolymph, and later with 10 % of fetal bovine serum (FBS). Traditional media supplemented with FBS are still used for research applications, the most popular being Grace's medium (Grace 1962) and TnMFH, which is Grace's medium supplemented with lactoalbumin and yeastolate

Table 15.2 Serum-free media (SFM) available for insect cell culture. Information collected from web sites of the manufacturers

Culture medium	Manufacturer	Cell line	Maximum cell concentration, $\times 10^6$ cell/mL
baculoGROW®	Oxford Expression Technologies	Sf9, SF21, High Five	6 (Sf9)
CCM3®	HyClone	Sf9	NA
EX-CELL® 405	Sigma-Aldrich	High Five®	3
EX-CELL® 420	Sigma-Aldrich	Sf9, Sf21	3
Express Five®	Life Technologies	High Five®	5.5
Insect-XPRESS®	Lonza	Sf9, Sf21	8
IS BAC®	Irvine Scientific	Sf9, Sf21 and High Five®	NA
PSFM®	Protein Sciences Corporation	expreSF + ®	NA
PSFM-J1®	Wako Pure Chemical Industries	Sf9 and High Five®	3-4 (Sf9)
			2–4 (High Five®)
Sf900®II	Life Technologies	Sf9, Sf21	10
Sf900®III	Life Technologies	Sf9, Sf21	10-14
SFM4Insect®	HyClone	NA	NA
SFX-Insect®	HyClone	Sf9, Sf21 and High Five®	NA
TiterHigh Sf Insect®	Sigma-Aldrich	Sf21	20 (Sf21)

NA Information not available

(Hink 1970). Since the 1990s, serum free media (SFM) for insect cell culture have been commercially available. Considerations should be taken when using a SFM, as the minimum seeding cell concentration is higher (at least 0.5×10^6 cell/mL) than for serum containing cultures (at least 0.2×10^6 cell/mL), due to the need of growth factors produced by cells (Taticek et al. 2001). Some of the SFM for insect cell culture that are on the market are listed in Table 15.2. An increase of metabolic activity when insect cells are grown in the better-formulated SFM can be seen in Table 15.3.

Insect cell media are complex, containing most if not all amino acids, trace elements, lipids, cholesterol, hormones, vitamins and undefined supplements such as yeastolate. All serum-free media contain Pluronic F-68, a non-ionic copolymer that protects cells from shear stress (Palomares et al. 2000). It should be noted that this additive has physiological effects that go beyond protection to shear. Glucose is the preferred carbon source for insect cells, although they can also consume fructose and maltose (Bédard et al. 1993; Palomares and Ramírez 1996). The metabolism of *S. frugiperda* and *T. ni* cell lines is different. The High Five® insect cell lines has a higher metabolic activity than the Sf9 cell line (Rhiel et al. 1997;

Table 15.3 Typical growth rates and metabolic coefficients for Sf9 and High Five® insect cell cultures

	Uninfected cultures		Infected cultures	
Sf9	High Five®	Sf9	High Five®	
Growth rate, h^{-1}	0.03[a]	0.03 ± 0.003[c]	–	–
	0.02 ± 0.005[b]			
Maintenance coefficient, $\times 10^{-10}$ mmol/cell h	2.53[a]	–	–	–
Glucose consumption rate, $\times 10^{-10}$ mmol/cell h	0.52–0.78[a]	1–2[a]	0.18–0.6[a]	1.6–1.8[a]
	0.86–4.89[b]	1.13–9.16[c]	41–47[d]	13.4–13.8[e]
Glutamine consumption rate, $\times 10^{-10}$ mmol/cell h	0.34–0.4[a]	0.54[a]	0.18–0.34[a]	0.6–0.8[a]
	0.4–3.6[b]	0.65–1.81[c]	2.1–2.6[d]	2.7–4.1[e]
Oxygen consumption rate, $\times 10^{-10}$ mmol/cell h	2–5[a]	2.8–5.4[a]	2–8.1[a]	3.2–5.8[a]

Updated from Palomares et al. (2006)
[a]Cultures in TnMFH medium + 10 % FBS. Data from Bédard et al. (1993); Donaldson and Shuler (1998); Kamen et al. (1996); Mendonça et al. (1999); Palomares and Ramírez (1996); Palomares et al. (2004); Rhiel et al. (1997)
[b]Cultures in Sf900II SFM (Monteiro et al. 2014) or IPL-41 SFM (Benslimane et al. 2005)
[c]Cultures in Insect Xpress SFM (Monteiro et al. 2014) or IPL-41 SFM (Benslimane et al. 2005)
[d]Cultures in Sf900II SFM (Monteiro et al. 2014)
[e]Cultures in Insect Xpress SFM (Monteiro et al. 2014)

Monteiro et al. 2014), consuming glucose at a higher rate (Table 15.3). As a consequence, High Five cells produce more by-products than Sf9 cells, such as ammonia and alanine (Benslimane et al. 2005; Monteiro et al. 2014; Rhiel et al. 1997). Different media have been developed for each cell line (Table 15.2).

Similar to mammalian cells, glucose and glutamine play a central role in cell metabolism. Palomares et al. (2004) showed that glutamine is the main energy source, as its depletion causes an abrupt cessation of respiration. In contrast, glucose depletion resulted in a decrease of the specific oxygen uptake rate that was 20-times slower than when glutamine was depleted. Glutamine limitation decreased the specific recombinant protein production rate 1.8-times. Glutamine metabolism results in the accumulation of ammonia, which can be used to synthetize glutamine by Sf9 cells (Ohman et al. 1996), but not by High Five® cells. Insect cells can metabolize ammonia and lactate (in the form of pyruvate) for the synthesis of alanine, a non-toxic by-product that accumulates in cultures (Drews et al. 2000; Mendonça et al. 1999). Lactate can also be used as a carbon source (Palomares and Ramírez 1996). Cystine and glycine are essential amino acids for Sf9 cells (Tremblay et al. 1992), and their limitation negatively impacts recombinant protein productivity.

Interestingly, it has been reported that Sf9 cells produce baculovirus titers up to 1.5 logs higher than High Five® cells, while High Five® cells are better for recombinant protein production (Krammer et al. 2010). A detailed comparison between both cell lines can be found in Drugmand et al. (2012). In an effort to

identify the metabolic characteristics that result in the higher recombinant protein productivity of High Five® cells over Sf9, Monteiro et al. (2014) analyzed their metabolism and identified by multivariate analysis the metabolic signatures characteristic of higher productivity of a recombinant protein under the polyhedrin promoter. They found higher intracellular concentration of reduced and oxidized glutathione in High Five® cells, suggesting that they have a higher capacity of handling oxidative stress than Sf9. Moreover, High Five® cells had a higher biosynthetic activity than Sf9 cells.

15.2.2 The Baculovirus

The initial interest in baculoviruses resulted from the damage they caused to colonies of silk worms, resulting in economic losses for the silk industry. Baculoviruses are double-stranded DNA viruses that have insects as hosts (Rohrmann 2013). Of the various baculovirus known, the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) is the most widely used in the BEVS. *Ac*MNPV was the baculovirus that Smith et al. (1983) selected for the initial development of BEVS. The baculovirus life cycle involves two types of virus particles (Fig. 15.2). One type is occluded within a crystalline matrix formed mostly by the protein polyhedrin. Occluded virions (OV) are only infectious when they are released from the occlusion body, or polyhedra. Polyhedrin is only soluble at high pH, similar to that present in the larvae midgut (Rohrmann 2013), and OV are protected from the environment by their occlusion into polyhedrin. The second type of capsids buds through the cell membrane during the late phase of the baculovirus life cycle, and are called budded virus (BV). Only BV are infectious in cell culture. When the baculovirus is expanded in vitro, OV are not infectious. Moreover, the protection from polyhedrin is not necessary. Therefore, the BEVS is based in replacing the *polh* gene by the gene of interest (GOI), taking advantage of the very strong promoter of *polh*. Using this strategy, large amounts of recombinant proteins can be obtained in a fast, easy, and efficient manner.

Recombinant baculovirus are constructed following the standard procedure depicted in Fig. 15.3. Several commercial kits exist for generating recombinant baculoviruses, being the most used the Bac-to-Bac® system from Life Technologies. This system utilizes a bacmid, which is a baculovirus genome with an origin of replication from *E. coli*, and was developed by Luckow et al. (1993). Another alternative is the use of a linearized baculovirus genome, that when recombined with a plasmid containing the GOI, results in a baculovirus genome that produces a viral progeny containing the GOI. Regardless of the system that is used to produce the recombinant baculovirus, the quality of the virus stock is determinant for the productivity of the system.

In addition to the promoter of *polh*, other promoters have been used to simultaneously express several genes (Palomares et al. 2002; Roldao et al. 2006), or to manipulate the concentration of a particular protein (Urabe et al. 2002). The

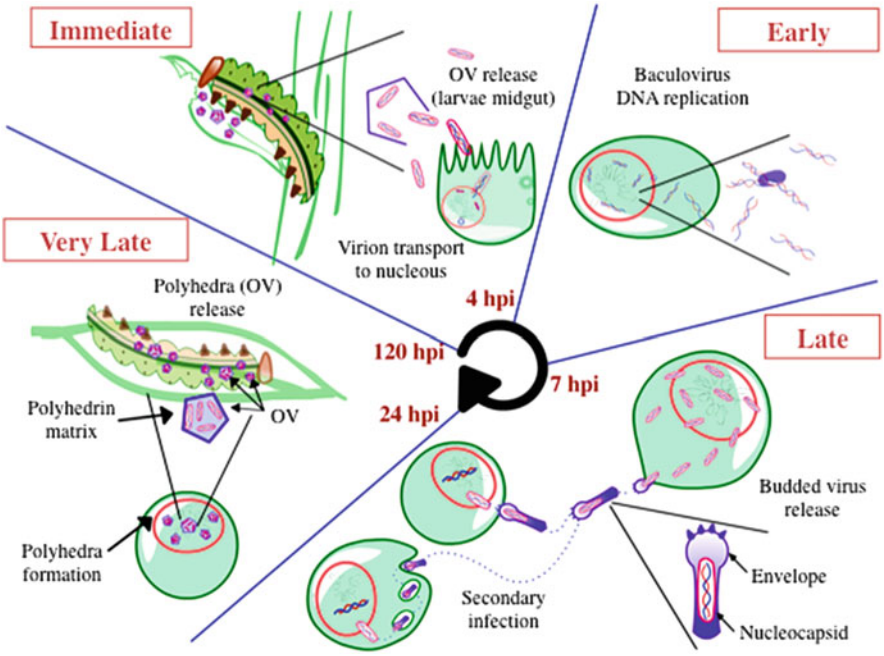

Fig. 15.2 Baculovirus life cycle. Immediate phase: Ingested polyhedra are solubilized in the insect midgut, occluded virions (OV) are released and they infect epithelial cells. Virions migrate to the cell nucleous, they are uncoated, and the transcription and translation of viral genes start. In the early phase, the cellular cytoskeleton and nucleous are rearranged, the host genome is degraded, and baculovirus DNA replication starts. In the late phase, nucleocapsid assembly occurs. Nucleocapsids bud through the cell membrane acquiring an envelope (budded virus, BV). BV spread infection throughout the insect in secondary infections. In the very late phase, polyhedrin and p10 production starts. Virions accumulate in the nucleous and are occluded into polyhedra. Polyhedra crystals accumulate until the larva disintegrates, releasing the crystals, which are consumed by other insects (Palomares et al. 2006) (Illustration by Enrique Paz)

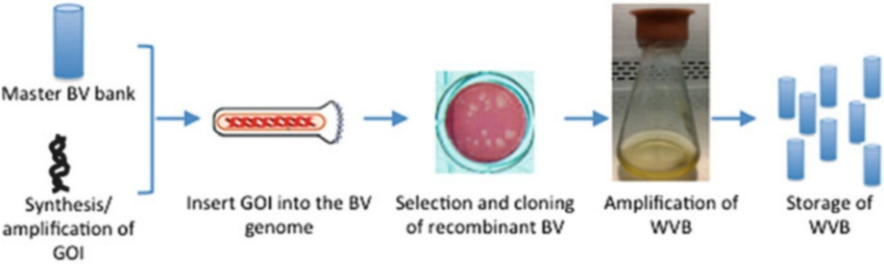

Fig. 15.3 Generation of a baculovirus (BV) working virus bank (WVB). *GOI* gene of interest (Pictures by Miranda González and Enrique Paz)

characteristics of various promoters, which can be used for expression of the GOI in the BEVS, are discussed in Palomares et al. (2006).

The amplification of the baculovirus stock is one of the most important part of the process, as a low quality stock results in very low productivities. There are two issues that can affect the quality of a baculovirus stock. The first is genetic instability, where the GOI is lost during the amplification of the stock by subsequent passaging (Kohlbrenner et al. 2005). This issue can be solved by carefully designing the vector to avoid unstable constructions. The second is the appearance of defective interfering particles (DIP), which have deletions in their genome comprising 43 % of the full genome length (Kool et al. 1991). DIP accumulate upon passaging of the virus stock, as they replicate faster than the complete baculovirus. DIP cannot replicate by themselves, as they need a complete virus to provide the missing genes. For this reason, the multiplicity of infection (MOI) has a key role on the propagation of baculovirus stocks. The MOI refers to the number of infectious particles that are added to a culture, and is expressed as plaque forming units per cell (pfu/cell). Infection of an insect cell culture follows the Poisson distribution, where the number of infectious particles that infect each cell is a function of the MOI (Fig. 15.4). DIP will only replicate if they coinfect a cell with a complete baculovirus. Therefore, to reduce the appearance of DIP, very low MOI ($\leq$0.1 pfu/cell) are recommended for the expansion of baculovirus stocks. In this way, infection of a cell with more than one virus becomes a rare event.

Even when caution is taken to reduce genetic instability and the appearance of DIP, the passage number of the baculovirus stock should be maintained as low as possible, preferentially below 6. This is specially challenging for large scale manufacturing, as the production of sufficient amounts of the baculovirus stock often needs several passages. If DIP or genetic instability are detected, a new stock can be generated by replaquing the baculovirus, screening for the high producing clones, and reamplifying the baculovirus stock.

Determining the titer of baculovirus stocks in a reproducible and precise way is a challenge. The classical method, the plaque assay, has standard deviations within the one log range. Other methods with high reproducibility and low standard deviations have been developed (Mena et al. 2003). The different methods available have been evaluated by Roldao et al. (2009). Titered baculovirus stocks can be stored at -80 °C or in liquid nitrogen and remain stable for over a year. Storage at 4 °C results in the progressive loss of titer (Jorio et al. 2006).

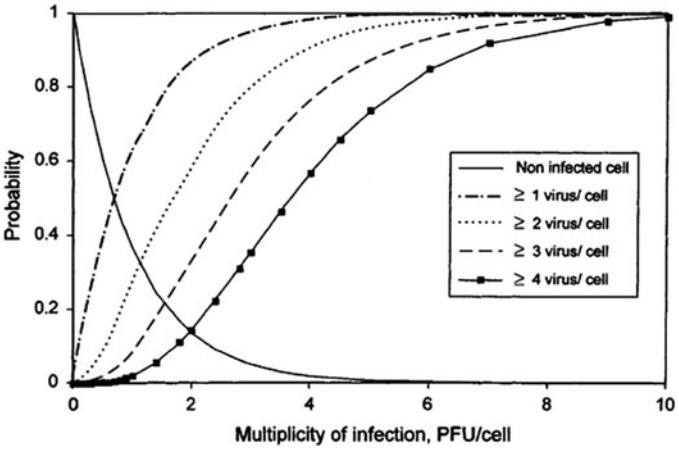

Fig. 15.4 Probability of a cell of being infected as a function of the multiplicity of infection (MOI) (Reprinted by permission from Palomares et al. (2006))

15.3 Bioprocess Engineering Considerations for the Production of Recombinant Proteins with the BEVS

After a cell line has been selected, and a recombinant baculovirus has been constructed, a process can be developed for the production of a recombinant protein by the BEVS. A general scheme of the process to produce a recombinant protein is presented in Fig. 15.1. A healthy insect cell culture, with viability above 90 %, is required at all steps. The passage number of the insect cell culture should be maintained within the range of genetic stability of the cell line, usually below 70 passages. A productive infection requires a baculovirus stock with insignificant presence of DIP, culture conditions that ensure no nutrient limitation (including oxygen), and negligible amounts of toxic by-products. The cell concentration at the time of infection (CCI) and the MOI should be taken into account to prevent both nutrient limitation and toxic product accumulation. Two strategies can be used for the production of a recombinant protein by the BEVS. They are:

(a) Low MOI (below 1 pfu/cell). Only a fraction of the population is infected by the baculoviruses added to the culture (primary infection). Uninfected cells continue growing until they are infected by the baculovirus progeny of the initially infected cells (secondary infection). All viable cells in the culture are thereafter infected by secondary infection (Mena et al. 2007). This strategy results in a heterogeneous cell population in the culture. It has the advantage of requiring lower volumes of the baculovirus stock, which is especially important as scale increases. However, this strategy is difficult to implement and can result in process variability, as small changes in the MOI, caused by inefficient stock addition or by variability of the titering methods, affect importantly the

performance of the culture, as in this range the number of initially infected cells is highly dependent on the MOI (Fig. 15.4). The low MOI strategy is usually performed at a low CCI, to prevent nutrient limitation during growth of uninfected cells and secondary infection.

(b) High MOI (above 2 pfu/cell). Results in a synchronous infection. Is highly reproducible, as the fraction of the population that is initially infected is not a function of the MOI. However, it requires large amounts of the virus stock, which can be impractical at large scales. Usually, a high CCI is used, as there will be no significant cell growth after infection.

The duration of the process and product production kinetics are a function of the strategy selected. The low MOI strategy requires an additional day of culture, which affects the productivity and can be undesirable if an unstable protein is produced.

Insect cell culture has been mostly limited to batch and fed-batch culture throughout the years, as the appearance of DIP has obstructed the use of continuous or perfusion cultures (Kool et al. 1991). Insect cell culture has also been traditionally limited to low cell concentrations by the so called "cell density effect" (Elias et al. 2000; Kioukia et al. 1995; Bernal et al. 2009), which has been partially solved by nutrient feeding (Elias et al. 2000). Bernal et al. (2009) investigated the cause of the cell density effect by metabolic flux analysis. They observed that as CCI increased, glycolysis and the TCA cycle were down regulated. As a result, the oxygen uptake rate did not increase after infection. Such a decrease of the glycolytic and the TCA activity could not be correlated to the limitation of glucose or an amino acid, or to the accumulation of lactate or ammonia. The cell density effect is probably caused by the accumulation of a signal element as cell concentration increases, or by the depletion of an unidentified trace nutrient. Therefore, the CCI should be carefully selected to maximize productivity while avoiding the cell density effect.

pH control is not traditionally performed in insect cell cultures, as under adequate aeration conditions it does not change significantly until the end of the culture (Palomares et al. 2004). However, it has been observed that controlling the pH can increase culture performance and productivity (W. Mahmoud, personal communication). Also, it has been found that a slightly higher pH (6.5) than that traditionally used for insect cell cultures increases the infection efficiency by facilitating the baculovirus entry into cells (Jakubowsla et al. 2009).

An important aspect to consider when designing a BEVS process is mass transfer. The oxygen uptake rate (OUR) after infection increases up to 60 % (Palomares et al. 2004), and specific oxygen uptake rate can be higher than for other animal cells (Lara et al. 2006). Moreover, dissolved carbon dioxide (dCO_2) can also limit insect cell growth (Mitchell-Logean and Murhammer 1997) and reduce recombinant protein yield (Garnier et al. 1996; Meghrous et al., submitted). The two elements governing mass transfer in a bioreactor are the energy dissipation rate and superficial gas velocity (Nienow 2006). The transfer area available is also important, and it can be increased by decreasing the size of the bubbles sparged to the reactor. Yet, increasing the oxygen transfer rate by increasing the agitation

speed and the gas velocity and decreasing bubble size can provoke shear damage to cells. Shear damage to insect cell cultures is prevented by the addition of Pluronic F68, which protects the cells from the shear stress produced through agitation and sparging (Murhammer and Goochee 1988; Palomares et al. 2000; Ramírez and Mutharasan 1990). Damage to insect cells by agitation is unlikely, as they withstand energy dissipation rates higher than those originating from agitation in a stirred tank (Palomares et al. 2006). Nonetheless, insect cells are usually cultured in bioreactors with impellers that are very good mixers and that cause low shear, such as pitch blade impellers or "elephant ear" impellers (Nienow 2006). Such impellers can provide shear gradients ten times lower than a Rushton turbine (Buckland et al., In press).

Sparging is the main cause of insect cell damage in bioreactors, as the energy dissipation rates caused by bubble rupture can be from 1 to 5 orders of magnitude higher than the energy dissipation rate from agitation (Garcia-Briones et al. 1994; Palomares et al. 2006). The provision of a gas current to an insect cell culture, either by sparging or through a gas overlay, is a critical parameter for a successful recombinant protein production by the BEVS (Garnier et al. 1996; Palomares et al. 2004). Gassing of the bioreactor is important for satisfying the oxygen requirement of an infected culture, but it is also very important to desorb dCO_2 produced by cell respiration (Garnier et al. 1996; Meghrous et al., submitted). As reviewed by Nienow (2006), the lethal energy liberated by bubble bursting in the liquid surface increases as bubble diameter decreases. Therefore, using small bubbles to increase the area of oxygen transfer can damage cells. Moreover, bubbles are also required for dCO_2 removal (Garnier et al. 1996; Meghrous et al., submitted). An aeration/desorption strategy that maximizes transfer but minimizes damage to cells is required.

Nienow (2006) recommends that animal cell culture is performed in a bioreactor with two axial flow impellers, where the low impeller disperses air and the upper one mixes the culture. He recommends the use of baffles and an aspect ratio around 1 and 1.3, to reduce the ratio between the area of bubble bursting (superficial area) and reactor volume. Our experience and other reports emphasize the need of a superficial gas current that strips dCO_2 (Garnier et al. 1996; Meghrous et al., submitted; Mitchell-Logean and Murhammer 1997). For the larger scales, a continuous air sparge at a low aeration rate should be maintained to strip dCO_2, and the dissolved oxygen concentration can be controlled by an intermittent pure oxygen sparge. Ideally, mass flow controllers should be used to precisely control gas composition and flow into the reactor (Palomares and Ramírez 1996; Palomares et al. 2004). Mass flow controllers are powerful tools for controlling and monitoring insect cell cultures (Kamen et al. 1996; Palomares and Ramírez 1996; Palomares et al. 2004). Other parameters that can be used for monitoring and control of insect cell cultures include the CO_2 production rate (Zeiser et al. 2000) and the cell size increase after infection, which correlates with the efficiency of virus propagation and with the amount of recombinant protein (Palomares et al. 2001; Taticek and Shuler 1997).

15.3.1 Downstream Processing and Product Quality

An important challenge for the downstream processing of proteins produced by the BEVS is the presence of the baculovirus, which is a subproduct of the system when recombinant protein production is the target. Baculovirus are present in large amounts in the supernatant of infected cultures. Several strategies have been developed to remove this contaminant. Our group has developed a flow-through anion-exchange chromatography step where the baculovirus remains bound to the column (Plascencia-Villa et al. 2011), and a two-liquid phase extraction system that removes the contaminant baculovirus with a high efficiency (Benavides et al. 2006). Such strategies are scalable and efficient. A break-through strategy was proposed by Marek et al. (2011), who created baculoviruses that lack the gene of a structural protein essential for capsid formation. Such a gene is provided in *trans* by an engineered insect cell line. Thus, when expansion of a baculovirus stock is desired, it is performed in the engineered cell line, and when recombinant protein production is desired, a non-engineered insect cell line is infected. As a result, no contaminant baculovirus particles are present in the culture producing the recombinant protein. Such a strategy is especially valuable for the production of biopharmaceuticals for human use. Further information of downstream processing of products of the BEVS can be found in Peixoto et al. (2007), Contreras-Gómez et al. (2014) and Buckland et al. (In press).

Another characteristic of proteins produced by the BEVS is their unique *N*-glycosylation pattern (Palomares and Ramírez 2002). Insects contain several β-*N*-acetylhexosaminidases that degrade chitin throughout the insect life cycle. Tomiya et al. (2006) identified and isolated a β-*N*-acetylhexosaminidase in Sf9 cultures, that has activity preferentially towards *N*-acetyl glucosamines in the *N*-glycan core. As a consequence, most *N*-glycans in proteins produced by insect cells are paucimannosidic which are truncated forms with three or less mannose residues that cannot be further processed (Palomares et al. 2006). Moreover, a large number of glycans are not processed in the Golgi, resulting in high mannose forms. The characteristic *N*-glycosylation profile of proteins produced by BEVS can have important effects on their function. Proteins that must contain complex or sialylated glycans to be functional should be expressed in a mammalian system. For other proteins, the typical high-mannose/paucimannose *N*-glycosylation profile of proteins produced by insect cells may or may not be relevant. The role of insect-like *N*-glycosylation of recombinant influenza virus hemagglutinin (rHA) in its receptor affinity and immunogenicity has been studied and compared to mammalian *N*-glycosylation. It was found that deglycosylated rHA or rHA with paucimannose or high mannose glycans (produced in Sf9 or *Drosophila* s2 cells) induced lower titers of hemagglutination neutralizing antibodies in mice than rHA with complex *N*-glycosylation (de Vries et al. 2012; Lin et al. 2013). Immunization with rHA with complex *N*-glycosylation induced better protective immunity than rHA produced in Sf9 cells (Lin et al. 2013).

15.4 Perspectives

The BEVS system has proven its value as an expression system for pharmaceutical and commercial use. The four products that have been licensed for human use and a similar number of veterinary vaccines have opened a vast number of possible applications for the system. BEVS is especially suitable for the production of viral vaccines, either subunits vaccines or virus-like particles. It can be expected that an increasing number of recombinant vaccines will be produced in the BEVS system in the years to come. As a result, more interest in process development for the BEVS can be anticipated. Expected improvements are the development of totally defined media that sustain infection at high cell concentrations, novel baculovirus vectors, and process that take the BEVS systems to productivities in the range of grams/l.

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Chapter 16
Metabolic Flux Analysis: A Powerful Tool in Animal Cell Culture

João V. Sá, Tiago M. Duarte, Manuel J.T. Carrondo, Paula M. Alves, and Ana P. Teixeira

Abstract Metabolic flux analysis (MFA) is being increasingly applied to animal cells as a tool for better understanding their metabolism in culture. The generated knowledge can be used to improve the productivity of biopharmaceutical processes, by optimizing feeding regimes, media formulation or engineering cell targets. Furthermore, biomedical research has also benefited from flux analysis studies by phenotyping diseased cells leading to the identification of therapeutic targets. Moreover, as drug-induced changes on cell metabolism can be readily inspected by MFA, this tool can help saving money and time in drug development. Nevertheless, comprehensive reviews with instructive guidance on the application of MFA to animal cell cultures can be scarcely found in the literature. Herein, different techniques and experimental settings for MFA studies will be addressed, including recent advances and overall trends, supplemented with relevant examples in several animal cell systems. A general picture on the subject will be depicted aiming at the design and development of new techniques for tackling prevailing challenges that need to be overcome in biopharmaceuticals production, biomedical research and toxicology.

Keywords 13C metabolic flux analysis • Animal cell culture • Metabolism • Optmization of cell culture processes • Biomedical research • Toxicology

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16.1 Introduction

The metabolism of the majority of mammalian cell lines used to produce therapeutic proteins is characterized by high glucose and glutamine uptake rates, combined with high rates of ammonium and lactate secretion (Ahn and Antoniewicz 2012). Significant accumulation of these by-products can induce the transition from growth to stationary phase and can have deleterious effects on cell viability, productivity and product quality (Ahn et al. 2008; Cruz et al. 2000; Lao and Toth 1997). This macroscopic characterization of cellular metabolism and physiology has supported media optimization as well as the design of fed-batch and perfusion processes which have contributed to significant improvements in culture performance over the last years, with some monoclonal antibodies being produced at over 10 g/L (Kelley 2009). However, despite the intense research on producer animal cell lines, relatively little is known about their metabolism under industrially relevant culture conditions, limiting the potential of applying metabolic engineering to further improve product yield and quality (Ahn and Antoniewicz 2012; Carinhas et al. 2012).

Framed in a systems biology view, the fluxome (the collection of metabolic fluxes inside the cell) reflects the integrated response of molecular interactions at the genome, transcriptome, proteome and metabolome levels, including their interregulatory mechanisms (Sauer 2006) (Fig. 16.1). Such comprehensive studies of metabolism can provide valuable information to effectively optimize biopharmaceutical processes. Likewise, differences of cell metabolism observed under pathological conditions or under drug administration can provide important insights for biomedical research and toxicology. Studies of disease phenotypes have traditionally focused on different "omic" layers independently of each other, but current trends show a more integrated view of disease, which can be best reported by a study of the cell fluxome. As for the toxicology area, the substantial amount of resources and time spent on drug development before reliable results are obtained could be obviated by the direct inspection of the response of the cell fluxome to a given drug, expediting the process of drug development.

Determining in vivo fluxes provides a quantitative description on the degree of engagement of various metabolic pathways in the overall cellular metabolism (Bonarius and Schmid 1997; Moxley et al. 2009; Crown et al. 2011). During the past two decades, metabolic flux analysis (MFA) has become the preferred technique for obtaining quantitative information on in vivo fluxes (Ahn and Antoniewicz 2013), in particular to validate metabolic pathways and uncover its regulation (Crown et al. 2011; Moxley et al. 2009), to identify bottlenecks in product formation and gain fundamental understanding of the balance between catabolic and anabolic processes, and homeostasis in general (Wiechert 2001). Overall, a solid systems understanding gained by MFA will assist in rewiring metabolic fluxes towards increased product yields and better quality attributes in biopharmaceutical production. On a cautionary note, however, important issues are still waiting to be solved, including how to handle metabolite compartmentalization

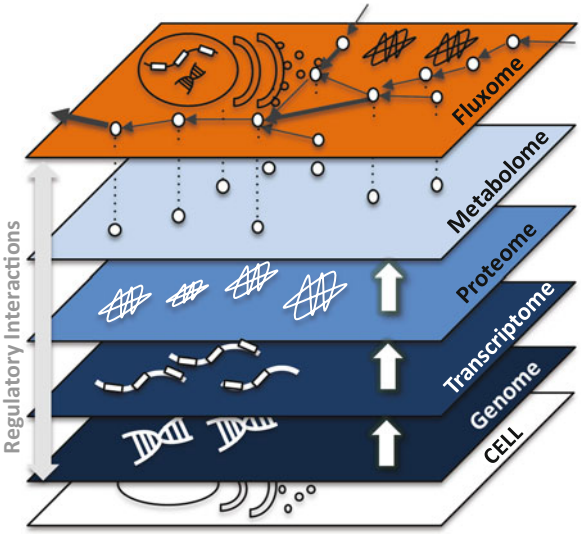

Fig. 16.1 The fluxome, being the layer closer to the cell phenotype, integrates information from interactions between all other layers of cellular components

in eukaryotic cells or the utilization of multiple substrates which increases the number of compounds that need to be experimentally measured (Niklas and Heinzle 2012).

16.2 Metabolic Flux Analysis in Practice

16.2.1 Metabolic Network Setup

A key step in MFA studies is the assembling of the metabolic network representing the metabolism of the cells under study. The main focus is generally on central metabolism, comprising glycolysis, pentose phosphate pathway, TCA cycle, as well as amino acid and fatty acid metabolism (Fig. 16.2). Additional intracellular reactions and transport rates can be included upon reconciliation of all available experimental measurements. Concerning cofactors that contribute to energy balancing (e.g. ATP) or redox balancing (e.g. NADH and NADPH), they can be omitted from the model to ensure that their difficult-to-quantify balancing does not unduly bias the overall flux estimation (Zamboni et al. 2009).

The stoichiometry of biochemical reactions can be retrieved from online databases such as the Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG; http://www.genome.jp/kegg/), BioCyc (http://biocyc.org/), ENZYME (http://enzyme.expasy.org/) or BRENDA (http://www.brenda-enzymes.info/), each cataloguing the metabolisms of several species.

Animal cells are compartmentalized in different organelles and there is metabolite flow between the different compartments (Niklas et al. 2010). For the analysis of

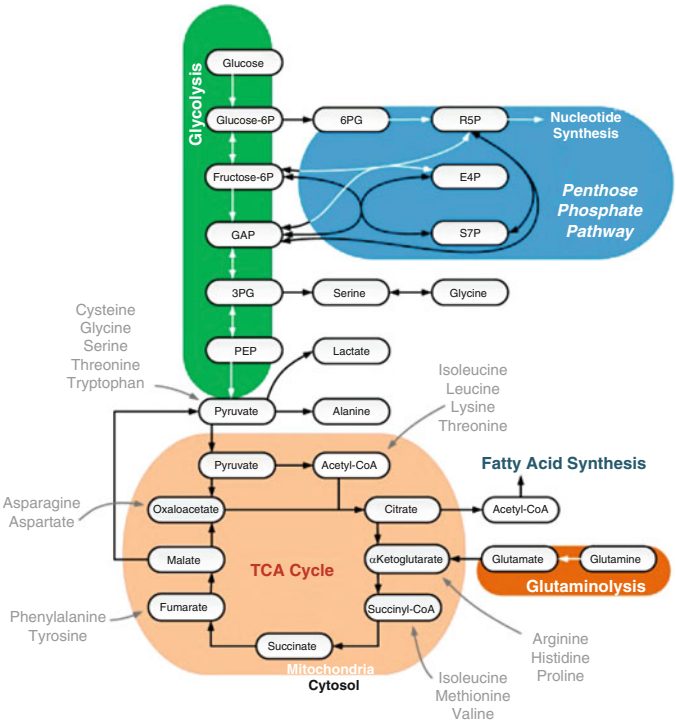

Fig. 16.2 Overview of a typical metabolic network for metabolic flux analysis in animal cell cultures

central metabolism, the cytosolic and mitochondrial compartments are normally considered, with metabolite pools of the cytosol distinguished from those residing in mitochondria (Fig. 16.2).

16.2.2 MFA Methods

As opposed to metabolite concentrations, the intracellular reaction rates (or fluxes) are not directly measurable; in vitro, the rate of a reaction is determined by monitoring the concentration profiles of both substrates and products through time. This method can be extended to sequential reactions in a network of metabolites; mass balances are written for each metabolite of the network, resulting in the following matricial equation: $\frac{dX}{dt} = S \cdot v$, where X is the vector of metabolite concentrations, S is the stoichiometric matrix (with dimensions of $m \times n$, m metabolites and n reactions) and v is the vector of reaction fluxes. The stoichiometric matrix is mainly sparse, meaning that most of their coefficients are zero, since most metabolites participate in just a small number of reactions. Assuming the

pseudo-steady state hypothesis, which is normally valid during the exponential growth phase, the pools of intracellular compounds are constant for the time interval under study and a homogenous system of linear equations is obtained: $S \cdot v = 0$. This system has $f = n - m$ degrees of freedom, meaning that f fluxes have to be measured so that all the remaining can be estimated. If the system is overdetermined (number of flux measurements higher than f), a complete set of fluxes can be estimated representing a least squares solution of the system. In this case, it is also possible to verify if experimental measurements are consistent with the network stoichiometry if allowing accurate balancing of each metabolite.

The original and most simple method of MFA uses only measured metabolite transport rates in and out of the cells as experimental data, which are then balanced in the reaction network to provide estimates of intracellular fluxes. Despite the simpleness, it is still the most applied method for the analysis of metabolism in animal cells (Quek et al. 2010), with recent applications to hepatocytes (Nault et al. 2013), Chinese Hamster Ovary (CHO) (Carinhas et al. 2013), and human neural AGE1.HN (Niklas et al. 2013) cells. A key limitation of this classical MFA method is that it cannot resolve fluxes through parallel pathways (e.g. glycolysis vs. pentose phosphate pathway) and cyclic pathways (e.g. pyruvate cycling).

To address this limitation, isotopic tracer-studies, usually using ^{13}C substrates, have been applied to complement extracellular data with isotopic labeling information obtained by nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) (Choi and Antoniewicz 2011; Choi et al. 2012; Jeffrey et al. 2002; Szyperski 1995; Wittmann 2007). When ^{13}C containing nutrients are added to cell cultures, the label propagates through the network as a function of metabolic activity, producing labeling patterns in the backbone of metabolic intermediates over time. Mathematical models describing label propagation are then used to calculate the in vivo fluxes from measured isotopic patterns and extracellular transport rates. A more realistic representation of the cell metabolism is attained and more subtle differences in flux phenotypes can be identified (Niklas and Heinzle 2012). Recent studies applying ^{13}C substrates revealed that several assumptions used in previous metabolite balancing MFA studies may not be valid, especially regarding the activities of the oxidative pentose-phosphate pathway and anaplerosis at the pyruvate node (Goudar et al. 2010; Sengupta et al. 2011; Ahn and Antoniewicz 2011). Thus, future studies on animal cells should therefore use isotope tracers to better constrain flux solutions and validate other modeling assumptions (Ahn and Antoniewicz 2012).

The simpler version of ^{13}C-based flux analysis is the isotopic stationary ^{13}C-MFA method, which assumes an isotopic steady-state that is reached when all intracellular isotopomer species are constant over time; this can be checked by plotting the label incorporation of each metabolite throughout time and verifying if label incorporation reaches a plateau (Fig. 16.3). The major advantage of this method is that it simplifies the experimental design and saves computational power. However, in some cases the ^{13}C-labeling incorporation in intracellular metabolites of animal cells is slow due to: (i) utilization of multiple non-enriched

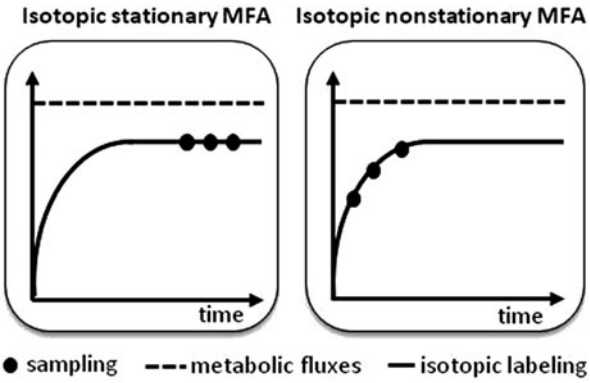

Fig. 16.3 Isotopic stationary versus nonstationary metabolic flux analysis. When isotopic steady-state is reached during the metabolic steady-state under study, the former methodology is preferentially applied due to the lower experimental and computational burden

carbon sources that dilute labeling of intracellular metabolites, (ii) existence of large intracellular metabolite pools that act as buffers for labeling incorporation, and (iii) metabolism compartmentalization. Moreover, different intermediates show distinct isotopic dynamics depending on their concentration, fluxes and pathway positioning in relation to the source of label. As a result, the requirement for isotopic steady-state imposes severe limitations on the minimum duration of a labeling experiment, with implications on the feasibility of maintaining metabolic steady-state until isotopic steady-state is reached. For example, Sengupta et al. (2011) did not detect any significant labeling in the tricarboxylic acid cycle (TCA) metabolites of CHO cells, 6 h after administration of ^{13}C-glucose, and Ahn and Antoniewicz (2011) reported that at least 24 h were needed to accumulate ~10 % labeling in TCA cycle metabolites in a fed-batch culture of CHO cells. This means that the fluxes will have poor temporal resolution, and therefore the pseudo-steady state assumption may not be valid for long labeling times (Ahn and Antoniewicz 2013).

In case of slow labelling dynamics, isotopically nonstationary MFA (INST-MFA) is preferentially applied to estimate the fluxes, using data from the transient period of label incorporation into intracellular pools (Nöh et al. 2006; Noh and Wiechert 2006) (Fig. 16.3). Then, an iterative optimization procedure fits the mass balance equations describing the labeling of each isotopomer specie to the measured time profiles, by adjusting the flux values (Maier et al. 2008; Amaral et al. 2011a). However, the application of INST-MFA implies higher experimental effort as well as higher computational power to solve the system of differential equations, which becomes prohibitive for larger scale network models.

As hinted before, the issue of metabolic compartmentalization poses a significant limitation to the ability of accurately resolve metabolic fluxes. While some metabolic pathways are restricted to a single compartment, e.g., pentose-phosphate pathway in the cytosol, other pathways span multiple compartments. However, the analytical procedures available today only allow measuring the combined mitochondrial and cytosolic pools (Zamboni 2011). Experimental data suggest that some of these pools are differently labeled and have different labeling time scales

in vivo. For example, Munger et al. (2008) showed differential labeling patterns for metabolically related metabolites using uniformly labeled glucose and glutamine tracers, providing strong evidence of compartmentalized metabolism in fibroblast cells. Lu et al. (2002) also suggested the existence of two pyruvate pools based on ^{13}C NMR measurements in INS-1 β cells.

To address the resolution of fluxes between compartments, a technique based on Fluorescence resonance energy transfer (FRET) has been proposed to measure conformation changes in certain protein sensors upon metabolite binding (Niittylae et al. 2009). FRET sensors permit the analysis of dynamic animal cell cultures with high temporal and subcellular resolution, by calculating intracellular fluxes for the sensed metabolite (Okumoto et al. 2009). Several sensors are available, from carbohydrates to amino acids (Wiechert et al. 2007). For example, Kaper et al. (2007) detected the accumulation of tryptophan in the cytosol of mammalian cells, and the amino acid was trapped inside the cell even after removal of tryptophan from the perfusion medium. Furthermore, the authors observed that tryptophan effluxed from the cells only after addition of an exchange substrate, such as histidine or kynurenines (the degradation products of tryptophan). Another example of FRET ability for compartment resolution was shown by Takanaga et al. (2008), where the authors found that the kinetics of import and export rate across the endoplasmatic reticulum membrane are asymmetric at lower intracellular glucose concentrations. Thus, combining this method with ^{13}C-MFA can be very promising for tackling the compartmentalization issues that affect the current MFA methods, as it will help to differentiate compartment pools of some metabolites.

Finally, all MFA methods described before are based on the assumption of metabolic pseudo-steady state. In reality, a true steady state may not ever be reached in animal cells due to constant fluctuations in media components and the adapting response of cellular metabolism to varying substrate concentrations (Sanfeliu et al. 1997). During the past years, metabolic flux dynamics in batch and fed-batch cultures of animal cells was described in several studies using a combination of stationary MFA with a kinetic model (Provost and Bastin 2004; Provost et al. 2006; Goudar et al. 2006; Teixeira et al. 2007; Zamorano et al. 2010; Nolan and Lee 2011; Naderi et al. 2011). For instance, Nolan and Lee (2011) used a kinetic modeling approach to trace changes in intracellular and extracellular metabolic fluxes in fed-batch cultures of CHO cells. With this in mind, it is expected in coming years the appearance of more research in animal cells where MFA is used in dynamic culture states, as well as an evolution of those methods which are still underdeveloped.

16.2.3 Experimental Design of ^{13}C Studies

The precision of estimated fluxes is heavily dependent on experimental conditions: the isotopic tracer(s) applied, the timing of the measurements (for transient isotopic methods), and the metabolites that are measured. For the latter, the predominant

philosophy is to measure as many metabolites as possible that are relevant to the pathways of interest. Caution should be taken when measuring certain metabolites that are prone to natural degradation, or more generally consider the increase in metabolite concentrations due to evaporation. Ideally, experiments without cells should be performed in parallel to estimate the natural decomposition rates of compounds in the culture medium, such as glutamine (Ozturk and Palsson 1990), and to estimate the increase of metabolite concentrations due to evaporation (Ahn and Antoniewicz 2011). Additionally, in some animal cell processes, the medium is supplemented with protein hydrolysates that contain amino acids used as substrates by cells; these must be accounted for to provide accurate metabolic flux estimates (Nyberg et al. 1999).

The choice of labeled tracers determines which metabolic pathways are resolved. Multiple tracers may be chosen in order to amplify the scope of observed metabolic pathways, especially relevant for the complex networks of animal cells. These pathways are disclosed through analysis of mass isotope distributions (MID) for each labeled metabolite. The sensitivity of each MID to changes in metabolic fluxes becomes as important as the flux itself since it unveils specific pathways. Thus, it is essential to wisely evaluate the choice of tracer(s) as well as optimizing measurements for flux determination (Metallo et al. 2009; Antoniewicz 2013). Efficient algorithms have been developed for this purpose combining different tracers, namely glucose and glutamine, aiming at a more detailed view of metabolism in cancer cells (Walther et al. 2012). Furthermore, a recent open source software for assisting the choice of tracers is available – IsoDesign (Millard et al. 2013). In order to fully exploit the use of multiple tracers, parallel experiments using different labeled substrates or multiple isotope types of the same substrate can be particularly beneficial. This has already started to be tested in animal cells (Ahn and Antoniewicz 2013), with a thorough description of the advantages and disadvantages of this approach exposed in Crown and Antoniewicz (2013). Parallel experiments can better clarify the structure and function of metabolic pathways, eliminating inconsistencies in a hypothesized network. However, inherent to parallel experiments is, of course, the increase of time and resources spent for its completion and for data analysis (Crown and Antoniewicz 2013).

The impact of the timing of measurements on the accuracy of flux estimation is particularly relevant for INST-MFA. Instead of collecting a single sample at isotopic steady state, multiple samples must be collected over time to obtain accurate profiles of ^{13}C-labeling dynamics. A careful selection of sampling time points is required, for which several computational tools have been developed, including parameterized sampling and *a posteriori* ranking of measurement time points (Nöh et al. 2006; Noh and Wiechert 2006).

Another important aspect of the design of ^{13}C experiments is that the standard culture media should be replaced by customized media with reduced concentrations of the metabolites which will be added as tracers, so that their total concentrations remain identical to the standard media. Furthermore, replicate experiments without tracers should always be performed in parallel to (i) accurately determine metabolite uptake/secretion rates, which normally require longer time courses than

isotope incorporation (Jazmin and Young 2013), and (ii) validate natural isotope abundances of measured intracellular metabolites to determine the real label incorporation from the added tracer (Ahn and Antoniewicz 2013). As an option, the open source software IsoCor (Millard et al. 2012) is able to correct mass isotopomer data for the contribution of all naturally abundant isotopes based on the tracer applied in the experiment and the derivatization reagents used for MS analytics.

16.2.4 Metabolomics Techniques

The quantification of metabolite levels in the supernatants of animal cell cultures and/or intracellularly is essential for estimation of flux models of cell metabolism. For classical MFA, monitoring extracellular metabolite concentrations is required to determine macroscopic fluxes of substrate uptake and product formation. The most used techniques are automated biochemistry analyzers (e.g. YSI and BioProfile) and High Performance Liquid Chromatography (HPLC), which are sufficient to measure the essential carbon and nitrogen sources in animal cells and their by-products, providing an overall snapshot of metabolic activity. For more comprehensive profiling, MS (Dettmer et al. 2007) and NMR spectroscopy, mainly ^{1}H-NMR and ^{13}C-NMR (Duarte et al. 2014; Khoo and Al-Rubeai 2009) can be used, allowing the identification of larger metabolic networks.

When resorting to isotope tracer methods, the analysis of intracellular metabolites is crucial for their full exploitation to resolve metabolic fluxes that cannot be discriminated otherwise. While monitoring isotopic fractions of each metabolite is necessary to determine flux distributions in both types of ^{13}C-MFA methods, the quantification of absolute intracellular metabolite levels is determinant only for flux estimation in transient isotopic studies. In either case, since intracellular metabolites are usually present in very small quantities and have short turnover rates, quenching of metabolism should be done immediately after sampling. Isotopic labeling analysis can be performed through NMR and/or MS, the latter coupled with gas chromatography (GC) or liquid chromatography (LC) for peak separation (Choi and Antoniewicz 2011; Goudar et al. 2010). Despite the lower sensitivity of NMR for absolute metabolite quantification, it provides complete information on the labeling pattern for each detectable metabolite. MS, on the other hand, is a highly sensitive method but gives limited information on the position of carbon-labeled atoms. To tackle this limitation, tandem MS is starting to be used to obtain additional labeling information on key metabolites (Choi and Antoniewicz 2011; Choi et al. 2012).

As the number and precision of the measured metabolites determines the quality of the estimated metabolic fluxes, the choice of the technique and metabolites to be measured should be carefully chosen during experimental design. Ideally, the goal is to measure all metabolite concentrations and isotopic fractions of the chosen metabolic network, if it was not for the incurred cost, time-consuming procedures and the unavailability of effectively measuring some metabolites.

16.2.5 Flux Estimation Algorithms for Isotope Models

The first proposed model for deriving material balances in carbon labeled experiments was based on atom-mapping matrices (AMM) (Zupke and Stephanopoulos 1994). This approach allowed following the label in each atom through the entire metabolic network. The AMM was built on the notion of a metabolite vector containing all fractional enrichments at individual carbon atom positions and considering all possible enzymatic reactions for each metabolite. This framework was later generalized by Schmidt et al. (1997), constructing a new model considering the possibility of each carbon atom to be in a labeled (^{13}C) or unlabeled state, enabling its representation as a binary code of zeros and ones. The conversion to decimal numbers provided a unique way of ordering labeling patterns. Thus, in a similar way to AMMs, this model defines isotopomer mapping matrices (IMM) where each vector is based on the isotopomer distribution for each metabolite in the network (Schmidt et al. 1997). Here it became evident the need for powerful computation to solve the isotopomer balance equations analytically due to their nonlinear structure and high dimensionality. In this respect, the concept of cumulative isotopomers (cumomers) was introduced, representing a variable transformation which allows to explicitly solving isotopomer balances (Wiechert et al. 1999). More recently, the concept of elementary metabolite units (EMUs) allowed reducing the total number of the balanced equations in a typical ^{13}C-labeling system by one order-of-magnitude, while significantly reducing the computation time required for flux estimation without loss of information. This approach uses an algorithm that allows identifying the minimum amount of information needed to simulate the measured isotopomer species within a metabolic network based on the knowledge of atomic transitions (Antoniewicz et al. 2007b). A number of user-friendly software for flux estimation based on carbon labeling experiments is currently available, such as ^{13}CFlux (Wiechert et al. 2001), Metran (Yoo et al. 2008), OpenFlux (Quek et al. 2009) and INCA (Young 2014).

16.3 Optimising Animal Cell Cultures Processes

MFA can be instrumental in providing a deeper understanding of the physiology of biopharmaceuticals producer cells at a global metabolic level. In the context of recombinant protein production, the main applications of MFA-based tools to probe the metabolism of animal cell lines include the comparison of growth versus non-growth phases (Ahn and Antoniewicz 2013; Sengupta et al. 2011; Ahn and Antoniewicz 2011; Templeton et al. 2013), of producer versus parental cell lines (Niklas et al. 2013), the effect of butyrate treatment (Carinhas et al. 2013) or the induction of recombinant protein expression when controlled by inducible promoters (Sheikholeslami et al. 2013). In what follows, key contributions based on MFA studies in different animal cell models of industrial relevance will be summarized.

CHO cells are the preferred animal host for production of protein biopharmaceuticals, being the prevalent biological system studied with MFA methods under different culture conditions. Several results on medium design and favourable fed-batch strategies for CHO cells were published (Altamirano et al. 2001, 2004, 2006). Metabolic plasticity due to growth phase transition holds also considerable attention. For instance, Sengupta et al. (2011) has shown, during the late non-growth phase in a CHO cultivation, that almost all consumed glucose is channelled through the pentose phosphate pathway (PPP), in shear contrast with what happens in the growth phase of CHO cells or in other cell lines (Maier et al. 2008; Goudar et al. 2010; Metallo et al. 2009; Mancuso et al. 1994; Lee et al. 1998; Vo and Palsson 2006). Similar results were obtained in a recent study, where metabolic fluxes of the exponential growth phase and the early stationary phase were compared (Ahn and Antoniewicz 2013), revealing significant rewiring in many pathways, namely PPP, anaplerosis, amino acid metabolism and fatty acid biosynthesis. Recently, the increase in metabolic activity in response to butyrate treatment in a culture of glutamine synthetase (GS)-CHO cells was shown, correlating with increasing antibody productivity (Carinhas et al. 2013).

A vast amount of studies have been also performed in hybridoma cell lines, whose industrial interest concerns antibody production. Multiple steady states were observed in hybridoma continuous cultures by applying MFA, which indicates cell performance could be improved by inducing specific metabolic shifts leading to favourable flux distributions (Follstad et al. 1999). In a study comparing three different physiological states of hybridoma cells (Gambhir et al. 2003), amino acid metabolism was found to be very important for reducing lactate production. The cellular response to oxidative and reductive stress was also analyzed by Bonarius et al. (2000) through MFA, observing that particularly NAD(P)H-dependent dehydrogenase reactions were decreased under oxygen limitation. More recently, Omasa et al. (2010) used metabolic flux estimations to design supplementation schemes of the intermediates pyruvate, malate and citrate leading to increased ATP and antibody production. MFA has also been incorporated into mathematical models used for prediction of cell and metabolite concentrations along culture time in hybridoma batch and fed-batch cultures (Dorka et al. 2009).

Other animal cell lines that produce recombinant antibodies have also been investigated through MFA. For instance, Niklas et al. (2013) studied the metabolic burden on human AGE1.HN cells imposed by the production of recombinant α1-antitrypsin by comparing a selected high-producing clone with the parental cell line, in an effort to identify potential targets for cell engineering. Although the results did show overall similarities in energy metabolism, increased C1-units, nucleotides and lipid precursors demand was observed for the producer cells, matching observations of higher RNA, lipid, and phosphatidylcholine fractions in these cells.

The impact of viral infection on cellular metabolism has also been appreciated in recent years and assessed by MFA methods to optimize viral vector or vaccine production processes. The aim is to compare infected vs. non-infected cells, infection at different cell concentrations, early vs. late stages of infection or even

infection of different cell lines with the same virus, ultimately to increase understanding of the host-virus relationship and enable targeted optimization. For example, Wahl et al. (2008) investigated an influenza vaccine production process in MDCK cells using a segregated growth model for distinct growth phases. The observed metabolic fluxes were compared with theoretical minimum requirements and revealed large optimisation potential for this process. Using HEK-293 cells, Henry et al. (2005) showed that MFA can provide a basis to develop a feeding strategy for a perfusion process for adenovirus vectors production. In the same biological system, Martinez et al. (2010) separated HEK-293 cell growth from adenovirus production and compared its metabolic states in order to optimise the medium according to cellular demand, achieving increased cell densities and adenovirus production. Finally, an *Sf*9 insect cell line producing baculovirus was also analyzed by an hybrid MFA methodology, combining stoichiometric and statistical constraints (Carinhas et al. 2011). Through this hybrid approach, the authors found a strong association between TCA cycle and mitochondrial respiration with virus replication. This new approach might serve in the near future as a valuable complement to metabolic studies in bridging the metabolic state with improved cell culture performance.

Overall, these studies have been contributing to the progress in understanding the causes and consequences of metabolic shifts and inefficiencies in animal cell cultures. The quantitative information given by MFA studies will eventually allow a finer control and manipulation of animal cell culture needed to optimize biopharmaceutical processes.

16.4 Applications in Biomedical Research and Toxicology

The ability to quantify metabolic fluxes in animal cells, in particular mammalian cells, has opened the door to elucidate the dynamics of health and disease at the level of metabolic network operation (Sauer and Zamboni 2008). In principle, diseases or genetic alterations generating certain physiological responses should be reflected on specific flux patterns. In the area of cancer metabolism, Forbes et al. (2006) observed through MFA that breast cancer cells are dependent on PPP activity and glutamine consumption for estradiol-stimulated biosynthesis, concluding that these pathways may be possible targets for estrogen-independent breast cancer therapy. Furthermore, in breast cancers with amplified phosphoglycerate dehydrogenase activity, MFA allowed to estimate that 8–9 % of the glycolytic flux was directed towards serine biosynthesis (Possemato et al. 2011), confirming previous suppositions that functionality of the serine biosynthesis pathway is required for breast cancer proliferation (Keibler et al. 2012). In order to globally understand cancer metabolic phenotypes, Gaglio et al. (2011) applied ^{13}C-MFA in mouse fibroblasts and human carcinoma cell lines to analyse the metabolic alterations induced by the oncogene K-*ras*. An enhanced glycolytic flux, decreased TCA cycle activity and increased glutamine utilization for anabolic synthesis were

observed, providing evidence for a role of that oncogene in the metabolic reprogramming of cancer cells. ^{13}C-MFA proved also valuable in elucidating a novel oncogenic metabolic phenotype by confirming the reductive carboxylation of α-ketoglutarate to isocitrate under conditions of mitochondria stress (Metallo et al. 2012). A metabolic characterization of the effect of cancer cell detachment from the extracellular matrix, required for metastatic tumor invasion, was investigated by Grassian et al. (2011) showing decreased glycolytic, TCA cycle and PPP fluxes due to a substantial decrease in pyruvate dehydrogenase (PDH) activity. Interestingly, the authors assessed the influence of ErbB2 overexpression (which is amplified in about one quarter of breast tumors) and found that the PDH flux was partially rescued in breast cancer cell lines.

As another application in biomedical research, MFA has also been used to identify metabolic targets for antiviral therapy (Munger et al. 2008). The impact of human cytomegalovirus infection on mammalian fibroblasts was assessed by ^{13}C-MFA, and a significant increase in TCA cycle activity and fatty acid biosynthesis was observed. The latter pathway was then pharmacologically inhibited, successfully suppressing virus replication. As well, brain function and particularly the physiological and pathophysiological regulation of neural metabolism were also investigated using MFA methods. Teixeira et al. (2008) investigated the metabolism of primary cultures of astrocytes, integrating ^{13}C NMR data from secreted glutamine with transport rates in and out of the cells. In a following study, metabolic alterations induced by ischaemia in astrocytes were analysed, identifying a significant enhancement in branched-chain amino acids catabolism (Amaral et al. 2010). Neurons subjected to hypoglycemia were also studied by MFA, indicating glutamine as an important energy substrate and a significant activation of the pyruvate recycling pathway during recovery (Amaral et al. 2011b).

The analysis of drug effects on cellular metabolism is another very promising application of MFA highly relevant for toxicological research. Drug toxicity is one of the leading causes of attrition at all stages of drug development (Kramer et al. 2007) and is often detected late in the process implying larger economic costs (Kola and Landis 2004). Earlier identification of drug toxicity by assessing global metabolic alterations would save money and expedite the drug development process. In Niklas et al. (2009), subtoxic drug effects of three hepatotoxic compounds were analysed in Hep G2 cells. Several changes in metabolism could be detected upon exposure to subtoxic drug levels, in particular an increase in TCA cycle activity. Also applying MFA to Hep G2 cells, Srivastava and Chan (2008) observed that free fatty acid toxicity is associated with the limitation of cysteine import causing reduced glutathione synthesis. In primary rat hepatocytes, the effect on hepatic cholesterol synthesis of therapeutic doses of atorvastatin, a hypolipidemic drug, was studied by transient ^{13}C-MFA by Maier et al. (2009). Despite the potential of these studies in toxicology research, large scale toxicity screening usually requires a high-throughput format which was not yet been implemented for MFA studies. Moreover, improved analytical methods are needed to facilitate more detailed fluxome resolutions.

Conclusions and Perspectives

During the past two decades, MFA has emerged as a powerful and convenient tool to characterize the metabolism in living cells (Boghigian et al. 2010; Antoniewicz et al. 2006, 2007a, b, 2011; Choi and Antoniewicz 2011). The application of MFA in animal cells allows identification of intracellular metabolic bottlenecks at specific stages of culture, eventually leading to novel strategies for improving cell performance towards optimized biopharmaceutical processes (Niklas et al. 2010). These process optimization strategies can be implemented either by medium engineering (Xing et al. 2011; Niklas and Heinzle 2012) or genetic manipulation of identified cellular pathways (Henry and Durocher 2011; Niklas and Heinzle 2012). Furthermore, in the field of process control, MFA was successfully integrated with kinetic models to predict fed-batch culture profiles (Naderi et al. 2011; Nolan and Lee 2011; Teixeira et al. 2007).

Moreover, MFA can be combined with transcriptome and proteome profiling to provide an enlarged systems view of cell states. Nonetheless, MFA methods and associated experimental constraints are still evolving with refined techniques for flux estimation at the same time as solutions to important bottlenecks are being pursued. For example, Goudar et al. (2009) described a procedure for determining error propagation in flux analysis from extracellular metabolite measurements. To allow estimation of fluxes in underdetermined systems, Llaneras and Picó (2007) and Zamorano et al. (2010) proposed an extension to classical MFA for calculating upper and lower bounds of unresolved fluxes. Concerning intracellular compartmentalization, however, current extraction techniques are still not well suited to separate different intracellular metabolite pools without significant leakage. Advances in metabolite extraction techniques would greatly increase the potential of using ^{13}C-MFA to resolve compartment specific fluxes in animal cells. Furthermore, developments in detection and quantification of new metabolites would allow expanding the scope of metabolic networks used for MFA studies, thus harnessing the full power of flux analysis in animal cells.

Overall, promising new mathematical approaches as well as new experimental designs have been established during the past years. These are on the way to enable broad application and in-depth insights into the function and control of metabolic networks in animal cells in the near future.

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Chapter 17
Cell Immobilization for the Production of Viral Vaccines

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Abstract Innovative vaccine production platforms are needed to efficiently generate countermeasures against (re)emerging diseases or pandemic outbreaks such as the Influenza pandemic H1N1 in 2009. Traditional viral vaccines manufacturing platforms such as embryonated eggs and conventional/classical cell substrates no longer satisfy the needs in terms of production capacity and speed to market and therefore require urgent replacement (Hess et al. 2012).

Well-established mammalian cell substrates such as MRC-5, MDCK or VERO have been used for many years for viral vaccine production. However, there is a major limitation in using these cells; they are anchorage-dependent and require a matrix to adhere and grow in stirred tank bioreactors, often used for large scale vaccine manufacturing. Moreover, the majority of the virus production processes are lytic to the host cells posing extra challenges, namely to preserve as much as possible cell viability (i.e. cell adherence) during virus replication phase; their tumorigenic and/or oncogenic nature are also of concern.

Microcarrier technology or cell aggregation strategies are the most common approaches for mass production of anchorage-dependent cells in large-scale bioreactors. Hollow fibers and cell encapsulation in biocompatible polymers are also alternatives. Efforts were made during the last two decades to adapt some of these cells to grow as single cells in suspension, but often this approach compromise cell specific productivities and product quality.

These and other issues related with viral vaccine production, e.g. the selection of the cell line, type of bioreactor and culture mode, the use of adherent or suspension cultures, and cell immobilization techniques are presented in this chapter. In addition, alternative cell substrates for vaccine production, namely insect cells, and an overview of the viral vaccine production market are discussed.

Keywords Vaccines • Virus • Cell culture • Microcarriers • Encapsulation • Hollow fibers • Adherent cells • Suspension cells

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17.1 Introduction

The first rational vaccination procedure dates back to 1796 when Edward Jenner used Cowpox to protect humans against Smallpox. Upon the establishment of cell culture technology in 1950s, animal cell cultures gradually replaced live animals and eggs in the preparation of some viral antigens used as vaccines. The observation by Enders and co-workers (1949) that non nervous tissue culture could be used to replicate and produce poliovirus, paved the way to large-scale production of vaccines. This, together with the discovery that poliovirus could be attenuated by serially passing it in cell culture, presumably by fortuitous selection of mutants better adapted to replication in vitro than in living hosts, were two major breakthroughs that gave rise to the first commercial vaccine generated using mammalian cell cultures. Following the same strategy, numerous attenuated viral vaccines were developed during the period of 1950–1980, such as measles (Kawana et al. 1970), rubella (Peetermans and Huygelen 1967), mumps (Hosai et al. 1970) and varicella (Trimble 1957) vaccines. Tremendous advances in vaccine technology, immunology and genetics were made since then, and vaccines became safer, more efficient and most importantly mass-produced. This has led to eradication of smallpox and the elimination of poliomyelitis and measles from large parts of the world, saving millions of lives. The use of vaccines has also contributed to promote animal health by preventing disease outbreaks with devastating impact on animal production, e.g. eradication of Rinderpest in 2011 (Mariner et al. 2012). Today, more than 120 vaccines are commercially available worldwide, generating revenues close to 30B$ which represent 2–3 % of the global pharmaceutical drug sales (WHO 2014). Merck & Co, GlaxoSmithKline, Sanofi Pasteur, Sanofi Pasteur MSD, Pfizer, and Novartis are the main blockbusters holding up to 80 % of market share (WHO 2014). Considering the discovery of new vaccine markets, the increased demand from population growth in developing countries and the advances in genetics and immunology, the global vaccine market is expected to grow in the next 5 years at an annual rate of around 10–15 % (WHO 2014).

Viral vaccines can be generated using one of the following strategies: (1) live viruses in an attenuated form (live/attenuated vaccines), (2) inactivated viruses using chemicals, heat or other procedures (inactivated/killed vaccines), (3) segments of the pathogen (subunit/conjugate vaccine), (4) recombinantly produced viral proteins, including virus-like particles (VLPs), and (5) genes that code for important antigens (DNA vaccines). Many of the vaccines in the market or in the R&D pipelines are based on virus production (live/attenuated and inactivated/killed vaccines), traditionally performed in anchorage-dependent cell lines. Although developmental work can be done in monolayer cultures using either tissue culture flasks or roller bottles, scale-up is extremely labor intensive and susceptible to contamination since it is achieved by increasing the number of flasks or bottles (scale-out). Modern facilities had overcome this problem by using robots. However, these culture systems do not allow for a continuous monitoring and control of

process parameters such as pH or dissolved oxygen levels that can affect product quality.

Prompted by increasing safety demands from regulatory authorities and technological breakthroughs in bioprocess and biomaterial engineering, viral vaccine production in anchorage-dependent cells rapidly progressed towards the use of environmentally controlled bioreactors, mostly stirred tanks with cells immobilized in microcarriers. Today and despite the significant advances achieved with novel equipment, new materials and better knowledge on process engineering, efforts to obtain more robust processes for viral vaccine production in stirred tanks are still pursued. These and other issues related with viral vaccine production such as the selection of the cell substrate, cell immobilization technique, bioreactor type and culture mode will be discussed in this chapter.

17.2 Cell Substrates in Viral Vaccine Production

Primary monkey kidney cells were used in the production of the first cell culture-based vaccines. However, these cells enclosed several drawbacks such as the relatively high risk of contamination with adventitious agents (contamination by various monkey viruses), shortage of donor animals at risk of extinction and limited expansion of anchorage-dependent cells (Stones 1976; van Wezel et al. 1978; van Steenis et al. 1980; Beale 1981). The first continuous mammalian cell line was established a few years later and was derived from African green monkeys, named VERO cells. This is currently the most widely accepted cell line by regulatory authorities for vaccine development mostly because VERO-derived human vaccines have been around for nearly 30 years. Other cell lines traditionally used in the vaccine field include human diploid fibroblasts cells, WI-38 (Hayflick and Moorhead 1961) and MRC-5, first applied for the production of vaccines against rabies virus (Wiktor et al. 1964), and baby hamster kidney cells (BHK-21 (C13)), first applied for the production of a foot-and-mouth disease vaccine (Capstick et al. 1962). Nowadays, several of the licensed human viral vaccines are produced using cell substrates (Table 17.1), and many others are in clinical development such as Influenza vaccines based on virus replication in Vero (Howard et al. 2008; Barrett et al. 2009; Ehrlich et al. 2012) or MDCK (Halperin et al. 2002; Liu et al. 2009) cells, amongst others. Novel cell substrates of human or avian origin may prove to be a highly replication-proficient way of producing live viral vaccines such as Influenza A viruses. Examples of human cell substrates being considered in the vaccine field include HEK 293 cells, PER.C6 cells and CAP cells. These cell lines were developed by transfecting human embryonic kidney cells (HEK 293) (Graham et al. 1977), human embryonic retinal cells (PER.C6) (Fallaux et al. 1998), and primary human amniocytes (CAP, from CEVEC) with the early region 1 (E1) of adenovirus type 5 (Ad5) (Schiedner et al. 2000, 2008).

Examples of novel cell substrates of avian origin include the duck embryonic stem cell line EB66 (from Vivalis) (Brown and Mehtali 2010), AGE1. CR® (from

Table 17.1 Licensed human viral vaccines produced in cell culture

Vaccine name	Manufacture	Tradename	Cell line
Hepatitis A vaccine, inactivated	GlaxoSmithKline Biologicals	**Havrix**	MRC-5
Hepatitis A vaccine, inactivated	Merck & Co., Inc.	**VAQTA**	MRC-5
H5N1 pandemic influenza vaccine, inactivated	Baxter AG	**Preflucel**	Vero
Influenza vaccine	Novartis	**FLUCELVAX**	MDCK
Japanese encephalitis vaccine, inactivated, adsorbed	Intercell Biomedical	**IXIARO**	Vero
Measles virus vaccine live	Merck & Co., Inc.	**ATTENUVAX**	Chick embryo cell
Measles, mumps and rubella virus vaccine, live	Merck & Co., Inc.	**M-M-R II**	Chick embryo cell
			Chick embryo cell
			WI-98
Measles, mumps, rubella and varicella virus vaccine live	Merck & Co., Inc.	**ProQuad**	Chick embryo cell
			Chick embryo cell
			WI-98
			MRC-5
Poliovirus vaccine inactivate	Sanofi Pasteur, SA	**IPOL**	Vero
Rabies vaccine	Sanofi Pasteur, SA	**Imovax**	MRC-5
Recombinant influenza hemagglutinin	Protein Sciences	**Flublok**	*Sf*-9
Recombinant L1 protein of human papillomavirus	GlaxoSmithKline	**Cervarix**	High Five
Rotavirus vaccine, live oral	GSK	**Rotarix**	Vero
Rotavirus vaccine, live oral	Merck & Co, Inc.	**RotaTeq**	Vero
Smallpox (vaccinia) vaccine, live	Acambis Inc.	**ACAM2000**	Vero
Varicella virus vaccine live	Merck & Co, Inc.	**Varivax**	MRC-5
Zoster vaccine, live	Merck & Co., Inc.	**Zostavax**	MRC-5

Adapted from: Roldão et al. (2011)

Probiogen) or CR (Cairina Retina) cell lines derived from muscovy duck retinal tissue, recently evaluated for influenza and modified vaccinia Ankara virus production (Lohr et al. 2009). Besides vaccines based on the replication of live viruses in mammalian cell cultures, also vaccines based on viral proteins recombinantly produced in cells, by expression of one or more structural genes of the virus (antigens) are extensively represented in the development pipeline. In some cases these proteins self-assemble in multi-protein structures (VLPs), resembling the virus particle, thus potentially inducing strong immune responses. Insect cell lines have played a major role in the development of this type of recombinant vaccines. Currently, there are two licensed human vaccines produced in insect cell platforms, namely Cervarix® (GlaxoSmithKline), a human papillomavirus (HPV) VLP

vaccine produced in *Trichoplusia ni* BTI-Tn5B1-4 cells, and Flublok (Protein Sciences), an hemagglutinin-based influenza vaccine produced in *Spodoptera frugiperda Sf-9* cells (Table 17.1). The most widely adopted insect cell expression system is based on baculovirus infection, which carries the gene(s) coding for the protein(s) composing the vaccine to be expressed in the nucleus of the host cell (revised in Fernandes et al. 2013). Recently, acknowledging the increasing relevance of insect cells in vaccine production and bypassing the lytic baculovirus infection, stable insect cell lines have been developed using recombinase-mediated cassette exchange (RMCE) technology (Fernandes et al. 2012; Vidigal et al. 2013). Besides competing with the well-established baculovirus technology in terms of protein production speed and yield, these RMCE-stable cell lines allow wider room for improvement through cell growth optimization (Fernandes et al. 2014).

17.3 Cell Immobilization and Entrapment

Cell immobilization was first described as the act of restricting the mobility of a cell by chemical or physical methods while conserving its catalytic activity (Carrel 1923). A simple classification of cell immobilization techniques has been previously proposed and includes adsorption to neutral or charged supports (e.g. microcarriers), aggregation, entrapment in natural or synthetic polymers (e.g. encapsulation and hollow fibers), covalent coupling and containment (Tampion and Tampion 1988; see Fig. 17.1). Table 17.2 depicts examples of viral vaccines generated using immobilization production systems. The first step for cell immobilization is adhesion to the support surface, which involves initial contact of the cell with the surface, cell spreading on the surface and finally cell growth. Since the proliferation of anchorage-dependent cells can only occur after adhesion to a suitable culture surface, it is important to use surfaces and culture procedures that enhance all steps involved in adhesion. In cell culture, two factors are essential for cells adhesion onto surfaces: divalent cations and protein(s) in the culture medium or adsorbed to the culture surface. For instance, while certain cell

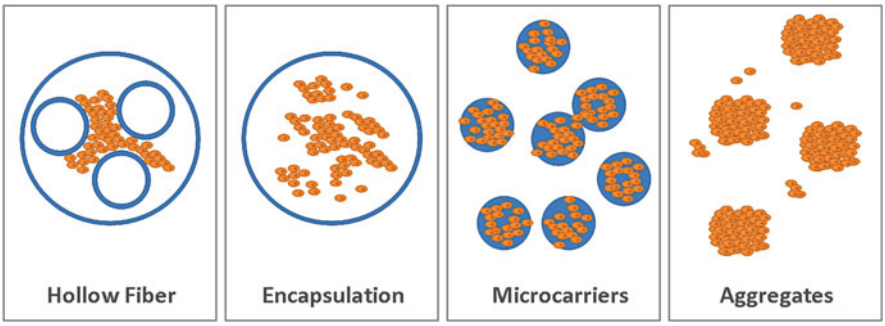

Fig. 17.1 Cell culture immobilization techniques

Table 17.2 Virus/vaccines generated through immobilization production systems

Virus/Vaccine	Cell line	System	Mode of operation	Reference
Adenovirus vectors	HEK293 cells	Aggregates	Perfusion	Liu et al. (2009)
Bovine parainfluenza 3 virus	MDBK cells	Cytodex 1	Perfusion	Conceicao et al. (2007)
Bovine rhinotracheitis virus	MDBK cells	Gelaspher M	NA	Lesko et al. (1993)
Chemically inactivated Parapoxvirus ovis	Bovine kidney cell line	Cytodex 3	Volume-expanded-fed batch	Pohlscheidt et al. (2008)
Dengue virus	MRC-5 cells and Vero	Cytodex 1	Batch	Liu et al. (2008)
Foot-and-mouth disease virus	BHK 21 C 13 cells	DEAE sephadex A50 beads	NA	Spier and Whiteside (1976)
Hepatitis A virus	Vero cells	Cytodex 1	Perfusion	Sun et al. (2004)
Hepatitis A virus (Attenuated)	MRC-5 cells	Collagen-coated Cytodex 3	Batch	Junker et al. (1992)
HIV-1 inactivated vaccine candidate	Human lymphocytes	Hollow-fiber bioreactor	Perfusion	Leong et al. (2007)
Human influenza A virus	Adherent and suspension MDCK cells	Single-use hollow fiber bioreactor	Perfusion	Tapia et al. (2014)
Human influenza A virus (H1N1)	Vero cells	Cytodex 3	Batch	He et al. (2011)
Inactivated enterovirus type 71 vaccine	Vero cells	Cytodex 1	Fed batch	Wu et al. (2004)
Influenza virus	MDCK cells	Cytodex 1/wave bioreactor	Batch/ medium exchange at infection	Genzel et al. (2006b)
Japanese encephalitis virus	Vero cells	Packed-bed BelloCell/ BioNOCII carrier	Fed batch	Toriniwa and Komiya (2007)
Live attenuated influenza	MDCK cells	Cytodex 3	Batch	Aggarwal et al. (2011)
Measles virus	MRC-5 cells	Cytodex 1	Perfusion	Trabelsi et al. (2012)
Marek's virus		Cytodex 3 and DE-53 granular	NA	Fiorentine et al. (1985)
Newcastle disease virus	Vero cells	Cytodex 1	Batch	Arifin et al. (2010)

(continued)

Table 17.2 (continued)

Virus/Vaccine	Cell line	System	Mode of operation	Reference
Parvovirus	PK-15 cells	"Microcarrier"	NA	Rivera et al. (1986)
Peste des Petits ruminants vaccine	Vero cells	Cytodex 1	Batch	Silva et al. (2008)
Poliovirus	Vero cells	"Microcarrier"	NA	Montagnon (1985)
Rabies vaccine (Veterinary)	BHK-21 cells	Cytodex 3	Perfusion	Kallel et al. (2003)
Rabies virus	Vero cells	Cytodex 1	Recirculation culture	Trabelsi et al. (2005)
Rabies virus	Vero cells	Cytopore and gelatin-Cultispher G	Batch	Yokomizo et al. (2004)
Rabies virus	Vero cells	Cytodex 3	Batch	Frazatti-Gallina et al. (2004)
Replication deficient influenza vaccine candidate	Vero cells	Cytodex 1	Batch	Chen et al. (2011)
Respiratory syncytial virus	Diploid bovine nasal mucosa cells	Cytodex 3	Batch	Hayle (1986)
Retrovirus vectors	psi2-VIK packaging cells	Encapsulated in microporous polyether-sulphone hollow membranes	NA	Martinet et al. (2003)
Rotavirus	Vero cells	Cytodex 1	NA	Zhang et al. (2011)
Vaccinia virus	HeLa cells	Cytodex 3	Perfusion	Bleckwenn et al. (2005)
Yellow fever virus	Vero cells	Cytodex 1	Batch	Souza et al. (2009)

NA information not available/not applicable

types can secrete sufficient fibronectin to ensure that cell adhesion occurs (Grinnell et al. 1977; Grinnell and Feld 1979), many established and transformed cells require surface coating or medium supplementation with adhesion-involved proteins or serum (Grinnell et al. 1977; Hughes et al. 1979). Therefore, immobilization materials, such as microcarriers, present several options in what regards surface chemistry and coating (Table 17.3).

Table 17.3 Types of microcarriers used in cell culture

Type/brand		Manufacture/supplier	Matrix/surface chemistry/geometry	Diameter (μm)	Area (cm^2/g)	Density (g/cm^3)
Nonporous	Nunc 2D MicroHex	Thermo Fisher Scientific	Polystyrene Nunclon® surface; 2D hexagon	125L × 25D	760	1.05
	HyQ Sphere	Hyclone/Thermo Fisher	Cross-linked polystyrene; collagen-coated, Animal Derived Component Free (ProNectin® F, a recombinant fibronectin) or cationic charged; 3D sphere	125–212/ 160–180	NA	1.02/ 1.11
	Collagen, gelatin	SoloHill Engineering	Cross-linked polystyrene; type I porcine collagen (gelatin); 3D sphere	90–150/ 125–212	480/ 360	1.02–1.04
	Plastic Plus	SoloHill Engineering	Cross-linked polystyrene; cationic; 3D sphere	90–150/ 125–212	480/ 360	1.02/ 1.04
	FACT III	SoloHill Engineering	Cross-linked polystyrene; cationic type 1 porcine collagen (gelatin); 3D sphere	90–150/ 125–212	480/ 360	1.02/ 1.04
Microporous	Hillex II	SoloHill Engineering	Modified polystyrene; cationic trimethyl ammonium; 3D sphere	160–200	515	1.11
	Cytodex 1	GE Healthcare/Sigma	Cross-linked dextran; charged throughout matrix; 3D sphere	131–220	4,400	1.03
	Cytodex 3	GE Healthcare/Sigma	Cross-linked dextran; acid-denatured porcine collagen; 3D sphere	131–220	2,700	1.04
	Global Eukaryotic Microcarrier (GEM)	Global Cell Solutions Inc.	Alginate with magnetic particles; multiple; 3D sphere	75–150	342	NA

Macroporous	Cytoline 2	GE Healthcare	Polyethylene and silica; slight negative charge; 3D sphere solid; pore diameter 10–400 μm	Average 230	>1,000	1.03
	Cytopore 2	GE Healthcare	Cross-linked cotton cellulose; hydrophilic DEAE exchanger (positive charge, 1.8 meq/g density); 3D sphere; 30-μm average pore diameter	Average 230	11,000	1.03
	Cultispher G	Percell Biolytica AB/Sigma/Thermo Fisher	Cross-linked gelatin (original bead); porcine gelatin; 3D sphere	130–380	NA	1.02–1.04
	Cultispher S	Percell Biolytica AB/Sigma/Thermo Fisher	Cross-linked gelatin; porcine gelatin; 3D sphere	130–380	NA	1.02–1.04

NA information not available

17.3.1 Microcarriers Technology

Anchorage-dependent cell lines grown in monolayers commonly reach higher specific productivities than those adapted to grow in suspension (Iyer et al. 1999). However, they generally require large surface areas to generate sufficient biomass to meet product needs, thus making adherent culture systems difficult to scale-up. Cell factory systems and roller bottles can be multiplied in number to reach larger surface areas for cell growth without compromising specific productivities (Okada et al. 2005). In particular, roller bottles have been used for many vaccine products namely VERO cells-derived rabies virus (Jagannathan et al. 2009), PPR virus (Hegde et al. 2008), amongst others.

A major breakthrough for the establishment of agitated cultures for anchorage-dependent cell substrates was achieved with the development of microcarriers (van Wezel 1967). Microcarriers are support matrixes that, while being able to be kept in suspension under agitation, they allow the adhesion and growth of anchorage-dependent cells. The idea of culturing anchorage-dependent animal cells on a small spherical support matrix (microcarrier) was first conceived by van Wezel (van Wezel 1967, 1971). Indeed, microcarriers represent the simplest way of transfer anchorage-dependent cells to stirred cultures, aiding process scale-up. Microcarriers are mainly used for manufacturing vaccines (Table 17.2 and references therein), viral vectors for gene therapy (Wu et al. 2002; Fernandes et al. 2013), and proteins (Giard et al. 1979; Giard and Fleischaker 1980; Shirokaze et al. 1995) but recently their use for expansion of stem cells in bioreactors has also been reported (reviewed in Serra et al. 2012). Large-scale vaccines production processes using microcarriers is widely described and includes poliovirus, rubella, rabies, Influenza, and many other vaccines (Table 17.2). In addition, several authors describe increased virus productivity using microcarriers with Sindbis virus (Sinskey et al. 1981), Poliovirus (Mered et al. 1981), and foot-and-mouth disease virus (Spier and Whiteside 1976), when compared to other cell culture methods.

Aiming at efficient cell attachment, there are several types of microcarriers commercially available made from different materials (gelatin, glass, collagen, cellulose, silicon and others) and with different surface materials, porosity, size and shapes (Wang et al. 1996; Berry et al. 1999; Yokomizo et al. 2004). Different types of microcarriers commercially available are presented in Table 17.3.

The immobilization in microcarriers depends on the ability of cells to adhere and expand on their surface (Fig. 17.2). Therefore microcarriers must be selected according to specific cell line adhesion characteristics (highly dependent on the cell type) and the product quality (QA/QC) requirements for final product. For instance, Cytodex 3 microcarriers, covered with a collagen layer, are designed to improve cells adhesion and cells recovery after detachment. For adhesion of primary cultures or diploid cells there are specific coatings recommended by different manufacturers. Given the distinct demands of cells to adhere, it is necessary to screen a set of different microcarriers for each cell line and culture system. Typically, serum containing medium enhances cell adhesion to microcarriers.

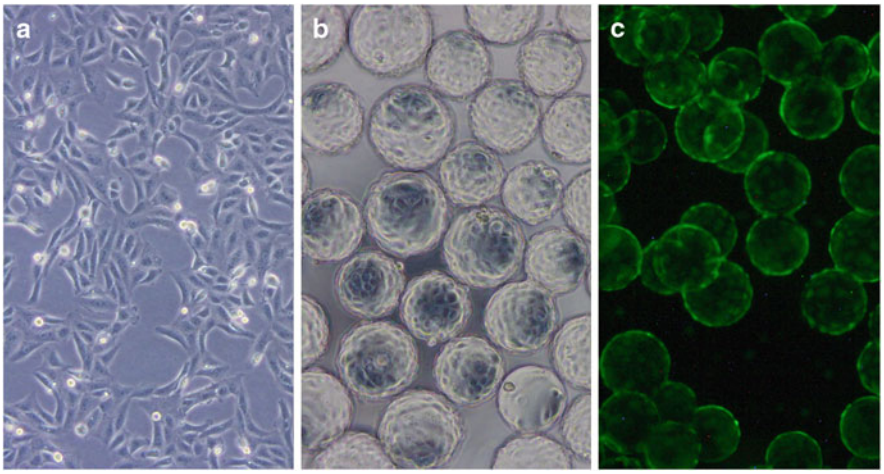

Fig. 17.2 Phase contrast images of cells in culture. (**a**) Vero cells in T-flask cultures, (**b**) Vero cells in Cytodex-1 microcarriers, (**c**) MDCK E1 cells infected with an canine adenovirus (CAV-2), 12 h post-infection

However, due to regulatory and safety concerns serum-free and animal-free components in the culture medium should be avoided; removing serum usually represents a drawback in assuring cell adhesion and usually results in extended lag phases and limited cell growth (Fernandes et al. 2013; Genzel et al. 2006a). To overcome this drawback several strategies have been attempted and are reviewed in the literature, e.g. supplementing medium with Ca^{2+} might improve cell attachment as observed for MDCK cells (Genzel et al. 2006b) or coating microcarriers with synthetic adhesion molecules (Simao et al. 2011).

Another drawback of microcarriers technology is the impact that collisions between microcarriers can have on cell viability, compromising productivity and product quality. Extensive literature from the 1980s–1990s from Croughan and Wang's lab address this issue, proposing strategies to diminish the impact of shear stress and collisions in bioprocess productivity yields (reviewed in Croughan and Hu 2006; Croughan et al. 2006).

An alternative to "protect" cells from collisions and shear stress is the use of porous microcarriers. However, porous microcarriers inoculation is a challenging step; to ensure that cells migrate and adhere inside the microcarriers is not straightforward. Often cell growth occurs only at the microcarrier periphery (donut-like growth) due to inefficient inoculation procedures. In addition, even in well agitated stirred tank bioreactors, gradients can be formed inside the microcarriers thus forming necrotic areas where oxygen supply, nutrients diffusion and removal of inhibitory product of cell metabolism (e.g. lactate and ammonia) are inadequate. Efficient and synchronized virus infection is also more difficult when porous microcarriers are used. Thus, typically, for viral vaccines production non-porous microcarriers are selected over porous microcarriers (Table 17.2).

Before inoculating and immobilizing cells, microcarriers must be sterilized, hydrated and incubated with culture medium and/or adhesion factors. Depending on the cell and microcarrier type, the optimum microcarrier concentration (microcarriers per volume of culture in bioreactor) and inoculum concentration (cell per microcarrier at inoculation) should be optimized.

Microcarriers cost, the need of optimizing initial culture steps and the need of high cell inoculums for scale-up (that also need to be produced adherently) represent the main drawbacks for the use of microcarrier technology. Cell inoculums required for larger scales can be prepared by growing the cells in microcarriers in smaller scale bioreactors, harvested, enzymatically detached and used, as single suspensions, to inoculate production scale bioreactors. Alternatively, and whenever cells present characteristics of migration from confluent carrier to empty carrier, this strategy can be used for inoculation, as reported for VERO (Wang and Ouyang 1999) and bovine kidney cells (Pohlscheidt et al. 2008). Moreover, several larger scale bioprocesses include cell inoculum preparation steps in roller bottles, cell-factories and hyperflasks.

Once the initial adhesion steps are accomplished and hydrodynamic parameters optimized, microcarrier based bioprocesses are robust and allow for high cell densities and enhanced productivities. Moreover, the relatively fast sedimentation of microcarriers once agitation is stopped, facilitates culture medium exchange and the reduction of bioreactor volume at infection time, often performed in viral vaccines production processes, maximizing virus infection and productivity. In addition, this property can also be used to separate intracellular and extracellular fractions and concentrate bioreactors bulk. This, being feasible also for viruses produced intracellularly, represents a very appealing feature to reduce working volume and alleviate the subsequent downstream process (Altaras et al. 2005).

17.3.2 Hollow Fibers

A number of entirely new approaches for vaccine production have been attempted based on novel materials and/or production technologies. The hollow fiber bioreactor (HFBR) is one example.

The first description of hollow-fiber systems for cell culture was described by Knazek et al. (1972) who sought a method to grow cells at densities similar to those found in vivo, i.e., 10^8 cells/mL or higher, as opposed to the 10^6/mL or so achieved by standard cell culture techniques. Hollow-fiber modules can provide a tremendous amount of surface area in a small volume, as much as 200 cm^2 per mL. This allows a large number of cells to attach in a very small volume and a extremely efficient exchange of nutrients and waste products occurs across the fiber wall. A wide range of materials, e.g., polysulfone and cellulose derivatives, can be used for the hollow fibers. Molecular weight cutoffs begin at 5 kDa and go up to virtually any desired upper limit. The fiber materials can vary in such properties as percent porosity, molecular weight cut-off and hydrophilicity, and they can be further

modified during either manufacturing or their actual application to introduce defined functionalities onto their surfaces (Whitford and Cadwell 2009).

In the last decades, hollow fiber bioreactors have proved is capacity to support the production of highly concentrated biologics such as monoclonal antibodies (Evans and Miller 1988; Heifetz et al. 1989; Handa-Corrigan et al. 1992; Lowrey et al. 1994; Jackson et al. 1996; Kessler et al. 1997; Dowd et al. 1999; Nilsang et al. 2008) and recombinant proteins (Marx et al. 1993; Ala-Uotila et al. 1994; Inoue et al. 1999; Yazaki et al. 2001; Jardin et al. 2008). The HFBRs have also been widely used in tissue engineering (Jasmund et al. 2002; Meng et al. 2004; De Bartolo et al. 2009; Shipley et al. 2011; Marx et al. 1993). Significant effort is being put on improving the hollow fiber bioreactor system to support the demand for production of many other high molecular weight and highly processed proteins in cell lines such as hybridomas (Kessler et al. 1997), CHO (Hessing et al. 1992), HEK293 (Jardin et al. 2008) and HepG2 (Palakkan et al. 2013) . In addition, the availability of chemically defined and animal product free media formulations specifically designed for high-density perfusion applications will further promote the use of these bioreactors for the production of secreted biologics (Whitford and Cadwell 2009).

The use of hollow-fibers for viral vaccines production is negligible and to our knowledge no vaccine in the market is produced using these bioreactors. There are, however, several reports on the literature describing their use for a wide range of virus types infection studies including hepatitis C virus (Aly et al. 2009), baculovirus (Shimoizu et al. 1993), vaccinia (McSharry et al. 2009), adenovirus (Gardner et al. 2001), retrovirus (Pan and Whitley 1999) and human immunodeficiency virus (Leong et al. 2007; Whitford and Cadwell 2009). Noteworthy, hollow-fibers bioreactors have been recently proposed as the new single-use platform for production of high-titer influenza A virus (Hirschel et al. 2011; Tapia et al. 2014).

17.3.3 Cell Microencapsulation

Cell-encapsulating strategies offer the opportunity to customize and design a scaffold using specific biomaterials, e.g., alginate, agarose, polylactic-coglycolic acid, poly-l-lactic acid, hyaluronic acid, among others (Zimmermann et al. 2007; Khetan and Burdick 2009). This strategy promotes cell-cell communication at inoculation and decreases the negative impact of shear stress to the cells, but often presents the disadvantages of porous microcarriers described above (gradients within the capsule).

Encapsulating cells has been used extensively for cell transplantation (Stange and Mitzner 1996; Kizilel et al. 2005; Teramura and Iwata 2009; Lee et al. 2012), production of cell-derived products (Nilsson et al. 1983; Scheirer et al. 1983; Jarvis et al. 1986), clonal selection of desired cell phenotypes (Pueyo et al. 1995), in vitro culture of primary cells highly dependent on cell–cell contact for improved

functionality (Tostoes et al. 2011), cell cryopreservation (Malpique et al. 2010; Mazzitelli et al. 2011; Yuan Ye et al. 2011), cytotoxicity testing (Goguen and Kedersha 1993; Yang et al. 2013), virology fundamental studies (Yang et al. 2013) amongst others. More recently, this cell immobilization technique has become relevant for Stem Cells applications. Reports on encapsulation strategies for expansion and differentiation of different stem cells types show that the microenvironment attained inside the microcapsules can enhance self-renewal (Serra et al. 2011) and differentiation of stem cells (reviewed in Serra et al. 2012).

To our knowledge, for "traditional" viral vaccine production processes cell microencapsulation have not been used for any product in the market. However, for the gene and cell therapy fields, microencapsulation "offers" unique advantages: (1) "avoiding" the host immune response, (2) allows for a 3D "more like tissue" cell-cell organization that can impact virus transduction efficiency (Neumann et al. 2013), (3) allows the encapsulation of packing cells for continuous production of recombinant viruses, as nicely shown for retroviral vectors-secreting cells that remained viable and releasing virus both in vitro (Saller et al. 2002) and in vivo (Armeanu et al. 2001).

17.3.4 Cell Aggregates

Cell aggregation is another alternative to grow anchorage-dependent cells in suspension. Cell aggregation strategies has been used for several types of cells ranging from traditional cell lines such as BHK cells (Moreira et al. 1995a, b; Alves et al. 1996) to stem cell cultures (review Serra et al. 2012) and for mimicking microtissues in vitro, e.g., human liver cell (Tostoes et al. 2012) and central nervous system (Brito et al. 2012). Cell aggregates are not traditionally used for virus vaccine production as this strategy present some of the disadvantages discussed above for porous microcarriers, hollow-fibers and microencapsulation approaches, i.e. an homogeneous and synchronized virus infection is not easy to achieve.

Some anchorage-dependent cell lines can be adapted to growth as aggregate cultures but care must be taken to control the cell aggregates size (Moreira et al. 1995b) and avoid gradients within the aggregate structure as this can lead to necrosis at the aggregates center (see also porous microcarriers – Sect. 17.3.1 above). Work by our group and others on strategies to control aggregate size in stirred tank bioreactors is reported in the literature (e.g. Moreira et al. 1995a, b). However for several traditional viral vaccine cell hosts, namely VERO and MRC-5, it is difficult to control aggregate size and avoid aggregate clumping and fusion, even when high agitation rates in stirred tank bioreactors is used; the formation of large aggregates with necrotic centers cannot be circumvented compromising cell viability and process production yields.

It is worth to refer that studies reporting the use of HEK 293 cell aggregates for production of adenovirus mention that this "cell immobilization approach" facilitates bioreactor perfusion, resulting in increased virus production yields when

compared with traditional batch cultures (Liu et al. 2009). In a second example, MDCK cells have been adapted to grow in suspension as cell aggregates for Influenza virus production, thus being a promising alternative to microcarrier cultures (Lohr et al. 2010).

17.4 Bioreactors for Viral Vaccines Production

The most common bioreactors used for vaccine production are stainless steel stirred-tank bioreactors (STB). Depending on the cell growth strategy (single cells in suspension, non-porous and porous microcarriers, capsules or aggregates) different type of impellers can be selected for STB, the most commonly used being Rushton and marine impellers. During the last 15 years, efforts have been made by equipment suppliers to develop disposable (single use) bioreactors to produce biopharmaceuticals, namely viral vaccines. Nowadays there are several commercially available disposable bioreactors, at different scales and in all the formats mentioned above.

Bioreactor types such as the rotating-wall vessel (RWV), hollow fiber bioreactors (HFB), wave induced-motion bioreactors (WB) and air-wheel bioreactors (AW) can also be used for virus production using animal cells.

RWV bioreactors were specially designed to obtain laminar flow and thus reduce the shear stress induced to cells in culture (Saarinen and Murhammer 2000; Hammond and Hammond 2001). In HFB, culture medium is perfused through semi-permeable hollow fibers and cells grow to high concentrations in the fibers outside space (see Fig. 17.1). A recent publication describes the use of single-use HFBs for MDCK growth and production of influenza virus (Tapia et al. 2014).

WB are disposable plastic bags mounted on a rocking platform that generates waves (Singh 1999). These motion waves are responsible for keeping cells in suspension and for distributing oxygen and nutrients to the cells; being blade- and bubble-free, the shear stress to cells is lower when compared to stirred-tanks. However, these bioreactors still present some limitations in what concerns scalability. There are several reports in the literature describing the use of WB for viruses and VLPs production, process optimization schemes. However these bioreactors still present some limitations in what concerns limited scalability. An example of WB application for vaccines is the mink enteritis virus (MEV) production in adherent embryonic feline lung fibroblasts (E-FL) grown on microcarriers; a ten-fold higher virus production yield (maximum viral titers of $10^{6.6}$–$10^{6.8}$ $TCID_{50}$/mL) was obtained when compared to roller-bottle based production processes (Hundt et al. 2007).

The introduction of WB in Pharma and Biotech industry paved the way for the development of other types of single-use bioreactors and nowadays several companies commercialize single-use stirred tank and wave bioreactors at different scales. Recently “less traditional” bioreactor formats are also available in the

market. Examples are compact fixed-bed pre-packed (with proprietary micro-carriers) bioreactors and air-wheel bioreactors.

For viral vaccines production, bioreactors can be operated in batch, fed-batch and perfusion. The best operation mode is cell- and product- dependent. Cell nutritional requirements during growth and virus infection and replication phases, and most importantly virus characteristics (infection kinetics, replication, product quality requirements) are determinant for selecting the bioreactor operation mode (Table 17.2). Cell-specific uptake rates, calculated for key cell nutrients, can be used to estimate optimal bioreactor feed rates (e.g. high-cell density microcarrier cultures of MDCK and high influenza H1N1 volumetric yields could be obtained for fed-batch and perfusion cultures) (Bock et al. 2011).

For fed-batch and perfusion operation mode, cell retention devices are needed; cells are retained inside the bioreactor while virus is harvested from the cell supernatant for further downstream processing. Perfusion is particularly useful for bioprocesses were the virus are highly susceptible to culture conditions (e.g. pH, temperature, proteases). The major advantage of perfusion is that higher cell concentrations and productivities can be achieved in relatively small-size bioreactors when compared to batch/fed-batch (Voisard et al. 2003). Several reviews and reports on cell retention devices can be found in the literature (Tokashiki and Takamatsu 1993; Castilho and Medronho 2002; Scheirer 1998; Langer 2011/2012), in particular for large scale stirred tank bioreactors. The most commonly used are cyclones, settlers, centrifugation based (e.g. Centritech) and alternating tangential flow devices (ATF). Details on the virus production process using perfusion are not disclosed even though these technique are used by many companies.

Concluding Remarks

Compelled by the demands from the regulatory authorities and the technological developments in bioprocessing and biomaterials engineering, cell based viral vaccines production platforms using stirred tank and single use bioreactors became a reality. Aiming at generating rapidly countermeasures against (re)emerging diseases or pandemic outbreaks, such as the Influenza pandemic H1N1 in 2009, and at developing novel vaccines, scientists, clinicians, engineers, technologists, materials/equipment manufacturers and regulatory bodies have to join efforts to conjugate disposable equipment systems, process intensification through the integration of up- and down-stream bioprocessing in a continuous or semi-continuous setting, and the implementation of systems biotechnology and scale down models for cell culture characterization under PAT and QbD initiatives. Further advances in genetics and immunology will lead to the identification of new targets for vaccine research that certainly will improve human health by protecting us against avoidable disease.

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Chapter 18
Cell Engineering for Therapeutic Protein Production

Eric Baek, Che Lin Kim, Jin Hyoung Park, and Gyun Min Lee

Abstract In order to meet the ever-growing demand for therapeutic proteins, high therapeutic protein productivity in mammalian cell culture is necessary. Cell engineering is one of the most effective and powerful ways to improve the production of therapeutic proteins. This chapter describes various strategies of engineering biotechnologically important mammalian cell lines, mainly Chinese hamster ovary (CHO) cells, to achieve high therapeutic protein productivity.

Keywords Chinese hamster ovary cells • Therapeutic proteins • Cell engineering • Specific productivity • Time integral of viable cell concentration

18.1 Introduction

The era of biopharmaceuticals began as the human plasminogen activator (tPA), the first therapeutic protein from recombinant mammalian cells, was approved in 1986 (Wurm 2004). The field of biotechnology and biopharmaceutics has been expanding since. The economic success of therapeutic proteins from mammalian cells has been recognized as the sales of biologics reached $120 billion in the US in 2012 and are expected to increase to $150 billion by 2015 (Butler and Meneses-Acosta 2012). In order to satisfy the ever-growing demand for therapeutic proteins and to maximize biopharmaceutical manufacturing, the establishment of high and stable producers and the optimization of culture conditions is certainly important.

Among the 58 biopharmaceuticals approved from 2006 to 2010, 32 were produced from mammalian cells, such as NS0, baby hamster kidney (BHK), human embryo kidney-293 (HEK-293), and Chinese hamster ovary (CHO) cells (Kim et al. 2012). Among them, CHO cells are the most widely used mammalian host due to several advantages: CHO cells are safe hosts without any adverse effects. They accomplish high specific productivity (q_p) through the gene amplification system, encompass efficient post-translational modification suitable for human therapeutics, and easily adapt to growth in a serum-free suspension culture. All of

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these features have contributed to the popularity of CHO cells and their domination in the commercial production of therapeutic proteins. For a few decades, more than 100-fold yield improvement of titers in CHO cell culture was achieved as a result of a tremendous amount of studies and efforts. From cell line development to an omics-based approach, there have been numerous innovative strategies aimed to optimize the culture condition and maximize the production. Among those strategies, this chapter will primarily focus on genetic engineering in CHO cells and discuss current achievements and potential ways to increase q_p through genetic engineering.

18.2 Cell Engineering Strategies

For the commercial production of therapeutic proteins, including monoclonal antibodies and erythropoietin, an achievement of high volumetric productivity and/or high product titer is important to meet profitable standards. A variety of novel and innovative strategies have been designed, manipulated, and combined to optimize the process of mammalian cell culture and to maximize the economical profits of producing therapeutic proteins. The rationale behind the strategies is quite simple: to increase the time integral of viable cell concentration (TIVCC) and/or increase q_p. Correspondingly, cell engineering has been applied to various intracellular mechanisms, such as cell death, metabolism, and cell cycle. Cell engineering has been striving to improve culture characteristics as shown in Fig. 18.1.

Figure 18.1 schematically depicts two variables, q_p and Xv × dt, that contribute to the final product titer. The increase in Xv × dt means that cells that produce therapeutic proteins have remained viable at a higher cell concentration for a longer culture period. Along with, the increase in q_p means the rate at which cells are

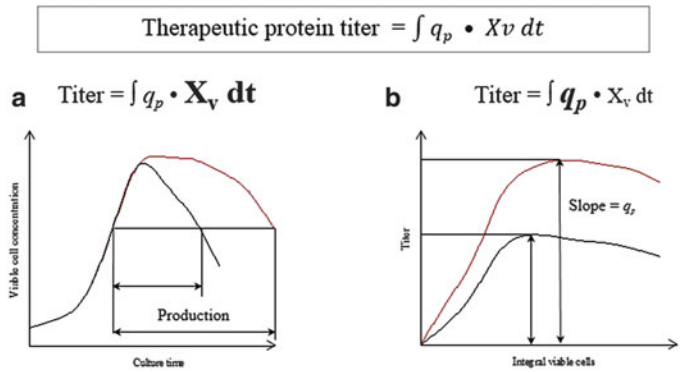

Fig. 18.1 The engineering scheme of CHO cells for improved therapeutic protein production. (**a**) Engineering for improved Xv × dt. (**b**) Engineering for improved q_p. Xv and dt represent viable cell concentration and culture time, respectively

producing therapeutic proteins has increased. These two variables are the keys to achieve a cost-effective level of production. Cell engineering strategies aiming to increase these two variables are discussed in this chapter.

18.3 Improving Viable Cell Concentration

One of the main challenges facing the optimization of therapeutic protein production in mammalian cell cultures is to increase TIVCC. Many studies have revealed various strategies to increase TIVCC by enhancing culture longevity, improving the specific growth rate (μ), and increasing the maximum viable cell concentration. These strategies primarily aim to expand the culture duration, providing more time for mammalian cells to produce therapeutic proteins, thereby maximizing the production in a given time. Many factors are involved in TIVCC in mammalian cell culture. However, programmed cell death (PCD), cell proliferation, and metabolic engineering are three main areas that researchers have been focusing on.

18.3.1 Anti-cell Death Engineering

Cell death is undoubtedly the most important issue in mammalian cell culture. During cell culture, cell death is caused by various stresses such as nutrient depletion, accumulation of toxic by-products, hypoxia, and shear stress (Kim et al. 2012). Cell death occurs in two general forms: necrosis and PCD (Arden and Betenbaugh 2004). Necrosis is a sudden and passive form of cell death that is characterized by the distortion and degradation of organelles and cellular swelling (Danial and Korsmeyer 2004). In this sense, necrosis is a type of cellular injury, while PCD, as the name refers, is tightly programmed under a genetically controlled mechanism. There are two types of PCD: apoptosis and autophagy (Hwang and Lee 2008). Both types of PCD are intimately regulated by active cellular signaling, which is able to be engineered and interfered (Broker et al. 2005). Consequently, many studies have targeted PCD to overcome a variety of stresses and to increase culture longevity.

18.3.1.1 Apoptosis

The term ‘apoptosis’ was first proposed in 1972, in which ‘apoptosis’ was derived from the Greek word apo- (from), ptosis- (falling off) (Kerr et al. 1972). Apoptosis was first observed as a structural breakage and burst of the cell into membrane-bound fragments. It is also further characterized by chromatin condensation and nucleosomal DNA fragmentation (Kerr et al. 1972; Wyllie 1980). Until now, due to its special and significant implications in medical fields such as cancer and

neurodegeneration, apoptosis and its mechanisms have been well studied (Mohan et al. 2009). In the field of therapeutic protein production, however, apoptosis and its implications in cell culture have gained attention in the last few decades. Various commercial cell lines used for large-scale production of therapeutic proteins have shown to undergo apoptosis during their cultivation (Cotter and Al-Rubeai 1995). In the case of CHO cell culture, it is known that the primary cause for the inability to maintain a viable cell culture is apoptosis. In particular, the removal of serum or nutrient deprivation at the end of the cell culture has led cells to undergo apoptosis (Goswami et al. 1999; Singh et al. 1994). Therefore, preventing or alleviating apoptosis is crucial for maintaining a high viable cell concentration throughout the culture.

Mechanism of Apoptosis

Apoptosis is an outcome of the caspase-dependent cascade. Although the cascade and participating pathways are complex, they have been well studied and genetically defined. Consequently, researchers have targeted specific genes involved in apoptotic pathways and limited the activation of the cascade in order to prolong culture longevity and enhance the production of therapeutic proteins. Nevertheless, a lot of undiscovered effects of apoptotic targetable sites have yet remained to be analyzed for the optimization of CHO cell cultures. Therefore, understanding the apoptotic pathway is the key to its further application in CHO cell culture. Two major signaling pathways of apoptosis, intrinsic and extrinsic pathways (Fig. 18.2), are briefly discussed in this section.

Intrinsic apoptotic pathway is activated via non-receptor-mediated intracellular signals inside the cell in response to stress, such as DNA damage, lack of nutrients, hypoxia, and detachment from the extracellular matrix (Fulda and Debatin 2006). These kinds of pro-apoptotic stress signals induce mitochondria to release proteins into cytosol to activate a caspase proteolytic cascade in the cytoplasm (Degterev et al. 2003). Cytochrome c, located in the mitochondrial intermembrane space, is one of the important proteins that are released for the initiation of the apoptotic program (Yang et al. 1997). When cytochrome c is released, it triggers a procaspase-activating adaptor protein called Apaf1 to oligomerize into a heptamer called an apoptosome, which then recruits procaspase-9 through a caspase recruitment domain (CARD) (Fulda and Debatin 2006). Eventually, the executioner caspase-3 is activated by apoptosome and cleaves major substrates to run apoptosis, which leads to the destruction of the cell. The fate of the intrinsic pathway lies between the pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins, which regulate the permeability of the mitochondrial membrane by balancing the release of cytochrome c to the cytosol (Mohan et al. 2009). The Bcl-2 family consists of three families: anti-apoptotic Bcl-2 proteins, pro-apoptotic Bcl-2 homology (BH) 123 proteins, and pro-apoptotic BH 3 only proteins. BH123 proteins include Bax and Bak, and pro-apoptotic BH3 only proteins include Bad, Bim, Bid, Puma, and Noxa (Hengartner 2000). Anti-apoptotic proteins, i.e., Bcl-2

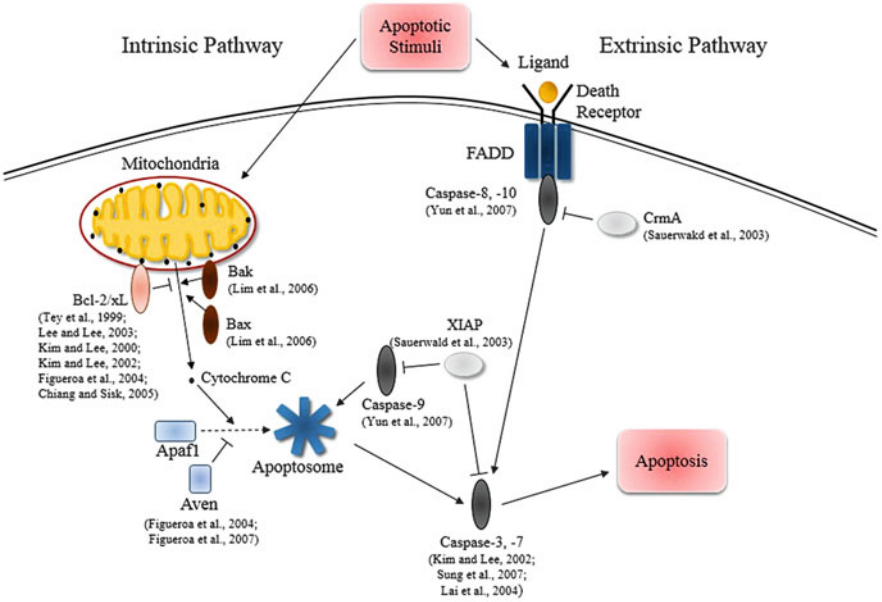

Fig. 18.2 Apoptotic pathway and various strategies for anti-apoptosis cell engineering. *CrmA* cytokine response modifier A, *FADD* Fas-associated death domain, *XIAP* X-linked inhibitor of apoptosis

and Bcl-xL, located on the mitochondrial membrane, not only prevent the release of cytochrome c, but also inhibit the activities of pro-apoptotic proteins.

The extrinsic pathway is regulated within the cell plasma membrane by the binding of ligands to death receptors. These ligands include Apo2L/TRAIL and CD95L/FasL (Ashkenazi 2002). The binding of ligands to death receptors promotes the recruitment of the protein Fas-associated death domain (FADD), which then assembles initiator procaspases, i.e. procaspase-8 and procaspase-10, to form a death-inducing signaling complex (DISC) (Fulda and Debatin 2006). In turn, DISC initiates caspase cascades and executes PCD by eventually activating the downstream effector class of caspases-3, -6, and -7 (Arden and Betenbaugh 2004).

Apoptosis Engineering

Although only the major regulators of apoptosis are described above, there are a lot of key genetic factors that determine either pro- or anti-apoptotic signals. This fact provides us a wide range of cell signals that could be used for genetic modification. However, for therapeutic protein production, the genetic modification to prevent or alleviate apoptosis has mainly focused on the overexpression of anti-apoptotic genes, down regulation of caspases, and the down regulation of pro-apoptotic genes.

The effect of the overexpression of Bcl-2 and Bcl-xL, the anti-apoptotic genes, on CHO cell cultures are the most widely studied topic in the anti-apoptosis approach. In batch cultures, the CHO cell line producing a chimeric antibody with enhanced levels of Bcl-2 showed a 75 % increase in maximum viable cell concentration compared to control cultures. Bcl-2 overexpressing CHO cells also showed better resistance to ammonia toxicity and growth arrest using thymidine (Tey et al. 2000a). The establishment of Bcl-2 overexpressing the dhfr-CHO cell line as the host cell line expedited the developmental process of establishing apoptosis-resistant CHO cell lines, and increased the productivity of therapeutic proteins as well (Lee and Lee 2003). The overexpression of Bcl-2 was also seen in cultures with the addition of sodium butyrate (NaBu). NaBu is known to enhance the expression of foreign genes. Thus, NaBu can improve the level of expression of therapeutic proteins. Despite its positive effect, NaBu has a drawback of causing the cytotoxic effect and inducing apoptosis during cell culture. However, the CHO cells overexpressing Bcl-2 suppressed the NaBu-induced apoptosis and yielded a greater monoclonal antibody concentration than non-overexpressing cells (Kim and Lee 2000). Furthermore, CHO cells overexpressing Bcl-2 showed greater resistance to hyperosmotic stress-induced apoptosis (Kim and Lee 2002a).

The beneficial effect of overexpression Bcl-2 on culture longevity was also seen in other mammalian cell lines, such as NS0 and Burkitt's lymphoma (BL). The overexpression of Bcl-2 in an NS0 cell line suppressed apoptosis and resulted in an approximately 20 % increase in maximum viable cell number. The Bcl-2 transfected NS0 cell line also showed great resistance to nutrient limitation and cytostatic agent, i.e. thymidine (Tey et al. 2000b). In the case of BL cell lines, Bcl-2 transfected cells exhibited a better protection from apoptosis in batch culture as well as in culture under glutamine deprivation (Singh et al. 1996).

Bcl-xL, another widely studied anti-apoptotic gene, also showed positive results in CHO cell culture with its overexpression. The overexpression of Bcl-xL protected cells from apoptosis, and exhibited an increased cell survivability and titer of the products (Figueroa et al. 2004; Chiang and Sisk 2005). Moreover, the overexpression of Bcl-xL suppressed apoptosis upon nutrient depletion in the later stage of batch culture (Kim et al. 2009). The overexpression of Mcl-1, another member of the Bcl-2 family, in CHO cells also showed increased viabilities, suggesting an alternative cell engineering strategy (Reynolds et al. 1994; Majors et al. 2009).

The combinatorial anti-apoptosis engineering of Bcl-2/Bcl-xL with other apoptotic-related genes was investigated. It was found to have displayed synergistic effects against apoptosis. The co-overexpression of Bcl-xL and Aven, a gene known to inhibit the activation of caspases, showed better protection against apoptosis (Figueroa et al. 2004, 2007). The overexpression of Myc-c, a gene related to cell proliferation, with Bcl-2 overexpression resulted in higher proliferation rates and maximum cell concentrations as well as a decreased apoptosis (Ifandi and Al-Rubeai 2005). The co-overexpression of Bcl-2 and Beclin-1, an autophagy inducing gene, yielded a synergistic effect on anti-cell death compared to a single overexpression of Bcl-2 (Lee et al. 2013).

Caspases are members of a family of cysteine protease, which plays a central role in the caspase-cascade system of the induction, transduction, and amplification of intracellular apoptotic signals (Degterev et al. 2003). Thus, the suppression of caspases is a promising strategy of anti-apoptosis engineering. While there are 15 mammalian members of the caspase gene family, genetic engineering in CHO cells has been attempted on two sub-groups of caspases: initiator and effector caspases. Dominant negative mutants of caspase-8 and -9, the initiator caspases, in CHO cells showed an enhanced viability in both batch and fed-batch cultures (Yun et al. 2007). By using antisense RNA and small interfering RNA of caspase-3 and -7, CHO cells were found to be more resistant to NaBu-induced apoptosis (Kim and Lee 2002b; Sung et al. 2007). The cleaved caspase-3 by U6 snRNA promoter-driven ribozyme also resulted in enhanced cell viability and the production of interferon-beta (IFN-β) in low serum cultures (Lai et al. 2004). Another strategy targeting the activity of caspases was using genetic caspase inhibitors, such as X-linked inhibitor of apoptosis (XIAP) and cytokine response modifier A (CrmA). XIAP and CrmA are known to inhibit caspase-9,-3,-7, and caspase-8, respectively. A stable CHO cell line expressing XIAP and CrmA was investigated and showed increased and prolonged viability (Sauerwald et al. 2003).

The down-regulation of caspases may be a beneficial strategy, but a close consideration of the apoptotic pathway and strategy of usage is necessary. Caspases are activated in the downstream of the apoptotic pathway, which is regulated by the upstream release of cytochrome c in mitochondria. Although cells do not cross apoptosis by inhibiting caspase activation in the downstream, cells may compensate for the lack of some effectors by up-regulating the others. Hence, even with the inhibition of caspases, the upstream depolarization of the mitochondrial membrane and the release of cytochrome c may be unavoidable (Sung et al. 2007). Therefore, a meticulous investigation on the interrelationship between apoptotic signals is essential.

Down regulation of pro-apoptotic genes, such as Bax, Bak, Alg-2, and Requiem, was also applied to anti-apoptosis engineering in CHO cells. A stable CHO cell line producing IFN-γ with Bax and Bak genes knocked down displayed an extended culture longevity and higher viable cell densities in fed-batch cultures (Lim et al. 2006). Using small interfering RNA technology, two stable CHO cell lines of Alg-2 and Requiem-silenced successfully yielded higher viable cell densities. In particular, the titer of IFN-γ increased by 2.5-fold in these cell lines (Wong et al. 2006).

Cell engineering on genes that are not directly related to apoptosis, such as telomerase reverse transcriptase (TERT), has been also investigated to enhance cell growth and viable cell density. TERT, which is one of two subunits in telomerase, is responsible for a proper cell division by maintaining the structural integrity of a chromosome and ensuring complete replication of the extreme ends of chromosome termini. The TERT-transfected adherent CHO cell line revealed an increase in telomerase and a better resistance to apoptosis, presumably through a process of healing of DNA breaks (Crea et al. 2006). The role of TERT in producing collagen fibers suggests that the overexpression of TERT strengthened the ability of cells to

adhere to plastic in serum free media and to overcome apoptotic cell death (Crea et al. 2006).

There is no doubt that genetic engineering on apoptosis is beneficial on culture longevity and cell viability. However, a major problem lies with the compensation between the cell survivability and q_p of the therapeutic proteins. In other words, apoptotic engineering does not guarantee its beneficial effects on q_p (Chiang and Sisk 2005; Lee and Lee 2003; Meents et al. 2002a; Tey et al. 2000a). Although there have been many studies that reported positive effects of anti-apoptosis engineering on q_p, there have also been many studies that found no effect on q_p. The clonal and cell line variability is the most reasonable explanation for this variance on q_p. Consequently, the anti-apoptosis engineering is accentuated along with q_p-enhancing factors, such as NaBu and hyperosmolality, even though these factors induce apoptosis. In this regard, the anti-apoptosis engineering, along with q_p-enhancing factors, produces synergistic effects by overwhelming apoptosis induced by q_p-enhancing factors and improving q_p simultaneously.

18.3.1.2 Autophagy

Autophagy, classified as PCD type II, is an evolutionarily conserved catabolic process through the lysosomal-mediated degradation pathway. The term, autophagy, originated from the Greek words meaning 'to eat oneself' (Levine 2005). Upon various stresses, such as nutrient depletion, hypoxia, reactive oxygen species, and DNA damage, the role of autophagy is to commit 'suicide' or to degrade long-lived cytoplasmic organelles to release substrates for biosynthesis and energy generation in order to endure such stress (Kroemer et al. 2010; Rabinowitz and White 2010). Unlike apoptosis, the involvement of autophagy in PCD has been controversial, whether cell death or cell survival, even though opinions are leaning toward a cell survival. While autophagy has been observed in dying cells, the idea of compensation for energy loss suggests that autophagy is a survival mechanism.

Mechanism of Autophagy

Multiple autophagic pathways are involved to form the double-membrane vesicles called autophagosomes, which fuse with lysosome later in the process for the degradation of cytoplasmic organelles and proteins (Fig. 18.3). Many stress sensors are activated through various stresses in order to initiate autophagy.

AMP-activated protein kinase (AMPK) is an energy sensor that is activated following nutrient deprivation, and that triggers autophagy. Activated AMPK phosphorylates the ULK complex, which plays an important role in initiating the autophagic cascade (Egan et al. 2011). The phosphorylated ULK complex dissociates from mTORC1, which is an autophagy inhibiting complex, and forms phagophores, which are the premature forms of autophagosomes (Jimenez-Sanchez

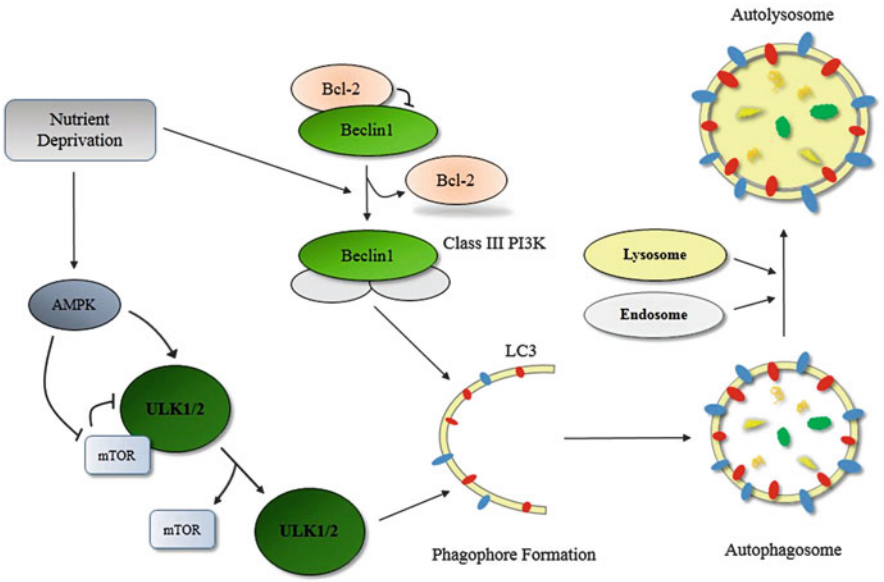

Fig. 18.3 A simplified autophagic pathway from induction by nutrient deprivation to formation of autolysosome. *AMPK* adenosine monophosphate-activated protein kinase, *LC3* microtubule-associated protein light chain 3, *mTORC* mammalian target of rapamycin complex, *PI3K* phosphatidylinositol 3-kinase, *ULK* unc-51-like kinase

et al. 2012). The formation of phagophores requires another critical complex called the Class III phosphatidylinositol 3-kinase (Class III PI3K), while the key protein in the Class III PI3K is Beclin-1, which is responsible for promoting phagopore maturation. The interesting aspect of Beclin-1 is that it is closely related to the anti-apoptotic protein Bcl-2. Under normal conditions or when conditions of nutrient deprivation is not occurring, the binding of Bcl-2 to Beclin-1 inhibits autophagy induction. But under nutrient deprivation, Beclin-1 dissociates from Bcl-2 to induce autophagy (Kroemer et al. 2010).

Autophagy-related proteins (Atgs) undergoes ubiquitin-like reactions to elongate and maturate phagophores to form autophagosomes. Another important protein in the process of autophagosome formation is the microtubule-associated protein 1 light chain 3 (LC3). LC3-I is converted to LC3-II and recruited to phagophore during the maturation of the phagophore. Eventually, LC3-II remains on the autophagosomal membrane until fusion with a lysosome. Thus, the level of LC3-II serves as the bone fide marker for the presence of autophagosome and the level of autophagy (Shvets et al. 2008). The autophagosome undergoes fusion with endosomes and lysosomes. Then, the cytoplasmic contents inside the autophagosome are degraded and the breakdown products are released into the cytosol where the cell utilizes them in various metabolic pathways (Kroemer et al. 2010).

Autophagy Engineering

The role of autophagy has been studied in CHO cell culture for only a few years. The occurrence of autophagy in batch/fed batch cultures and under stressful conditions was observed in 2008 (Hwang and Lee 2008). When glucose and glutamine were limiting towards the end of batch culture, autophagy was observed by the accumulation of LC3-II and TEM images of autophagic vacuoles containing cytoplasmic materials.

In an effort to improve therapeutic protein production, various approaches implying an autophagy pathway have been attempted. These approaches have included using chemical inducers and inhibitors of autophagy, and genetic engineering of core autophagy-related genes. Chemical autophagy inducers and inhibitors appear to exert only partial effects on autophagy and affect a wide range of cellular responses by targeting other cellular pathways. Thus, they are prone to impact the product quality and safety (Kim et al. 2013; Mizushima and Klionsky 2007; Jardon et al. 2012). Therefore, genetic engineering of autophagy has become a promising strategy to modulate autophagy and enhance the production of therapeutic proteins in CHO cell cultures. However, the genetic approach of autophagy and its effect on therapeutic proteins production has not been widely studied yet.

In 2012, four core autophagy pathway genes (ULK1, Beclin1, Atg7, and Atg9A) were investigated in CHO cell cultures (Lee and Lee 2012). The changes in the mRNA and protein expression of these genes were observed, in which Atg9A was the only gene showing decreased levels of mRNA and protein simultaneously at the late period of the culture. However, the overexpression of Atg9A did not significantly influence the autophagy induction and culture longevity, which suggests that combinatorial regulations of the genes, such as Ulk1 and Beclin-1, would be effective in producing therapeutic proteins (Lee and Lee 2012). Meanwhile, Beclin-1 was co-overexpressed with Bcl-2, which resulted in a more efficient protection of cells from the stressful culture conditions than Bcl-2 alone (Lee et al. 2013). This result provided a potential application of using the synergistic effect of pro-autophagy together with anti-apoptotic engineering in CHO cell cultures.

Although the role of autophagy in CHO cell culture has not been clearly understood yet, the management of autophagy by genetic engineering is promising and opens up novel ways to improve the production of therapeutic proteins (Kim et al. 2013).

18.3.2 Cell Proliferation Engineering

The specific growth rate (μ) of CHO cells is definitely an important feature that needs to be considered when improving TIVCC. Many studies have been targeting genes and chemicals related to cell proliferation in order to increase μ and the maximum viable cell concentration. Although the increase in μ might boost the

maximum viable cell concentration, it is not always the case. The extent of increase in one factor does not correlate with that of the other (Kim et al. 2012).

Cell growth is a thoroughly coordinated process of cell division, which consists of four phases: G1 (growth), S (synthesis), G2 (gap2), and M (mitosis) (Majors et al. 2008). The most important regulators of this pathways are cyclin and cyclin-dependent kinases (CDK), and their role is to drive the transition between each phase of cell cycle (Fussenegger et al. 1998). Many studies have aimed to regulate the process of cell cycle by media modification, feeding materials in a fed-batch culture, and genetic engineering (Altamirano et al. 2000; Bibila and Robinson 1995).

The genetic engineering of cell proliferation in CHO cells has been conducted with genes such as cyclin-dependent kinase like 3 (cdkl1), E2F-1, and cyclin E (Jaluria et al. 2007; Majors et al. 2008; Renner et al. 1995). The enhanced expression of cdkl3, which is involved in G1 to S phase transition, elevated cell proliferation in CHO cells as well as other mammalian cell lines such as HeLa and HEK-293 (Jaluria et al. 2007). The overexpression of E2F-1, which is an important link between phases in the cell cycle, also increased the viable cell concentration in a batch culture of the CHO cells. However, there were no significant changes in monoclonal antibody production (Majors et al. 2008). The high expression level of Cyclin E also stimulated cell proliferation in CHO cells (Renner et al. 1995). A typical oncogenic protein, c-myc, was also found to have positive effects on cell proliferation and, consequently, enhanced μ and the maximum viable cell concentration (Kuystermans and Al-Rubeai 2009). Many other potential engineering targets of genes contributing to the enhancement of cell proliferation, such as valosin-containing protein, requiem, Alg-2, and malate dehydrogenase II, have been discovered via the omics-based approach (Chong et al. 2010; Doolan et al. 2010; Wong et al. 2006).

In an attempt to increase μ in CHO cell culture, the ironic relationship between μ and q_p was found. Although it is difficult to evaluate the definite relationship between μ and q_p, q_p seems to unexpectedly decrease in high μ in CHO cells. Therefore, it is important to interpret the results of cell proliferation engineering with consideration of other unanticipated factors.

18.3.3 Metabolic Engineering

Metabolic engineering is a challenge to indirectly improve the cell growth and TIVCC by mitigating the adverse effects of toxic metabolic by-products, i.e. ammonia and lactate. Ammonia, a product of cellular metabolism and the chemical decomposition of glutamine in the medium, accumulates during the culture. The high level of ammonia accumulation causes the inhibition of cell growth and a decline in TIVCC (Yang and Butler 2000). Lactate, another major waste in CHO cells, is also a troublesome metabolic product during culture. CHO cells have the disadvantage of being unable to completely oxidize glucose to CO_2

and H_20. Therefore, most of the glucose is oxidized to pyruvate, which is eventually converted to lactate by lactate dehydrogenase (LDH) (Kim and Lee 2007a). The accumulated lactate causes acidification, which inhibits cell growth and decreases TIVCC.

In order to reduce the accumulation of waste products, genetic engineering has been used to modify energy metabolism and to redirect cells into pathways utilizing energy more efficiently. In 2006, Zhang et al. investigated metabolism in CHO cells expressing glutamine synthetase (GS), which catalyzes glutamate with ammonia to yield glutamine. When glutamate, a less ammoniagenic substrate, was substituted for glutamine, the ammonia accumulation was reduced (Zhang et al. 2006). With a similar approach to reduce ammonium accumulation, carbamoyl phosphate synthetase I (CPS I) and ornithine transcarbamoylase (OTC) were overexpressed. These genes are related to the urea cycle that eliminates and converts ammonia into urea. The overexpression of CPS I and OTC successfully enhanced cell growth and alleviated the adverse effects of ammonia (Park et al. 2000).

In an attempt to reduce lactate accumulation, pyruvate carboxylase was expressed in CHO cells. Pyruvate carboxylase catalyzes the adenosine triphosphate (ATP)-dependent irreversible carboxylation of pyruvate to form oxaloacetate by bypassing lactate production. Compared to control cells, CHO cells expressing pyruvate carboxylase had 21–39 % decrease in lactate production and a higher cell viability (Kim and Lee 2007b).

Lactate dehydrogenase, which is a major pro-regulator of the conversion between pyruvate and lactate, is definitely a promising target in metabolic engineering. Accordingly, the down-regulation of lactate dehydrogenase-A (LDHa) by siRNAs reduced lactate production with no impairment in cell proliferation and therapeutic protein production (Kim and Lee 2007a). Similar results were also seen in co-knocked down of LDHa and pyruvate dehydrogenase kinases (PDHKs) by siRNAs. The down-regulation of LDHa and PDHKs reduced lactate production, while it increased q_p and volumetric monoclonal antibody production (Zhou et al. 2011).

The effect of fructose-specific transporter (GLUT5) was also investigated in order to reduce lactate accumulation. GLUT5 expression allowed the utilization of fructose as an alternative to glucose and avoided the overflow of excess carbon to lactate by supplying sugar to cells at a more moderate rate. Although an appropriate and precise expression level of GLUT5 is needed to reduce lactate production, the metabolic engineering of transporters for the uptake of nutrients may be a strategy to reduce lactate (Wlaschin and Hu 2007).

Throughout the cultures, ammonia and lactate have always been inconvenient by-products that inhibit cellular growth and apoptosis. The strategies that manipulate the metabolism of CHO cells and reduce toxic waste products demonstrate an alternative way to enhance TIVCC. Rather than targeting cell death or proliferation that might directly influence the TIVCC of CHO cells, metabolic engineering aims to indirectly improve TIVCC by targeting the metabolic characteristics of CHO cells.

18.4 Improving Specific Productivity

To achieve a high product titer in CHO cells, there have also been many attempts to increase q_p as well as to increase TIVCC. The q_p is a key indicator of the high producer cell lines. In accordance with an improved ability to isolate high producers, the typical q_p has increased fivefold over the last decade (Butler and Meneses-Acosta 2012). Along with the enhanced culture longevity, an increased q_p has resulted in at least 20-fold higher product titer over the last two decades (Lim et al. 2010).

Generally, it has been observed that μ and q_p are inversely related. CHO cell cultures under a low culture temperature (Yoon et al. 2003) or hyperosmotic pressure (Kim and Lee 2002a; Lee and Lee 2000) have resulted in an enhanced q_p accompanied by a retarded growth rate. The basis of the relationship between μ and q_p remains elusive because of their effects on multiple cellular processes. For example, the adaptation of CHO cells to a low culture temperature was expected to have increased q_p and μ, but resulted in a decrease in the q_p (Yoon et al. 2006). Generally, high producer cell lines have slow growth rates due to the additional metabolic burden thrust on them (Mohan et al. 2008).

The relationship between cell size, cell cycle and q_p has also been studied. The cell clones expressing a high level of thrombopoietin (TPO) were larger in size and had morphologies resembling non-transfected dhfr-CHO parental cells (Chung et al. 2000). The studies of centrifugal elutriated fractions of a particular cell cycle phase revealed that the cell size is the major cellular determinant of recombinant protein productivity (Lloyd et al. 2000). Recently, a strong positive correlation between cell size and productivity has been reported by transcriptomic and proteomic analysis of CHO cell lines producing monoclonal antibodies (Kang et al. 2013).

With an aim to improve q_p, the bottlenecks in the process of protein secretion need to be identified and resolved. The components in the regulation of cell cycle, folding, secretion, and transport are considered as key regulators. This section will describe these four engineering approaches to resolve those bottlenecks.

18.4.1 Cell Cycle Engineering

A common feature of q_p-enhancing conditions, such as low culture temperature, hyperosmolality, and chemical (NaBu) treatment, is cell cycle arrest (Sunley and Butler 2010). Based on this consensus, one of the strategies to increase q_p in CHO cells is to control proliferation. The proliferation of mammalian cells is controlled by a series of checkpoints that are regulated by a complex network of signaling molecules to ensure that events of cell division do not occur prior to the completion of necessary preceding steps.

The cell cycle itself can be divided into two different phases: an interphase, in which cells are growing and accumulating the nutrients for mitosis and DNA

duplication, and an M-phase, in which cells are split into two daughter cells. The interphase has three sub-divisions, which are G1, S, and G2, and the transitions between the sub-divisions are tightly regulated (Fig. 18.4). During the S phase of interphase, the amounts of DNA in the cells are duplicated for cell division. G1, the first phase within the interphase, and G2, directly after S phase, are called gap preparing for either the replication of DNA (S phase) or cell division (M phase), respectively. The M phase, which consists of mitosis and cytokinesis, is the process of cell separation into two daughter cells that start the cell cycle again. Non-proliferating cells, which are arrested and remained for a long period of time in the G1 phase, may enter the quiescent G0 phase. These cells are maintained in G0 because of the phosphorylation of key cell cycle regulators (Afshari and Barrett 1994). However, they can re-enter the cell cycle process if provided with an appropriate condition.

To enhance the therapeutic protein production in CHO cells, the genetic manipulation of the cell cycle related genes, especially G1/S cell cycle arrest, was actively studied in biotechnology industries. While cell cycle arrest can also occur in the G2/M phase, the induction of arrest in the G1/S phase is more common (Kumar et al. 2007), as summarized in Table 18.1. It has been observed that cell cycle arrest at G1 phase was usually more metabolically activated, increased the cell size, and actively expressed many genes related to ribosome biosynthesis (Bi et al. 2004; Carvalhal et al. 2003; Dez and Tollervey 2004). Genetic targets that induce cell growth arrest have included members of the cyclin-dependent kinase inhibitor (CKI) family such as $p21^{Cip1}$ and $p27^{Kip1}$.

Cyclin dependent kinases (CDKs) are central components of the pathway that regulate cell cycle transition (Sugimoto et al. 2002) and therefore are an important target to attain cell cycle control. CDK activity was directly influenced by cyclins, which are phosphorylated and formed ternary complexes with CKIs such as $p21^{Cip1}$

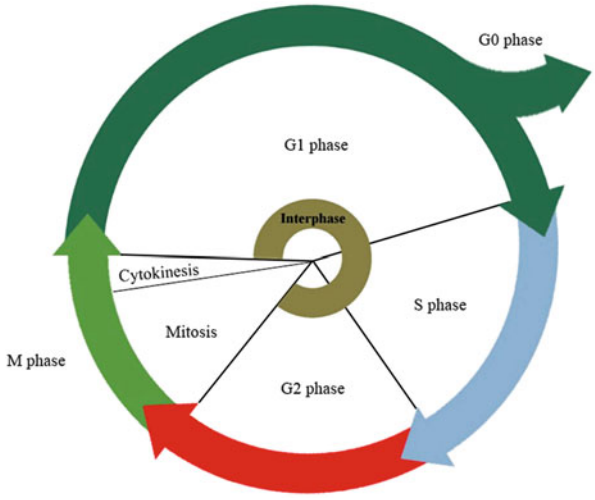

Fig. 18.4 The phases of the mammalian cell cycle, including three sub-divisions of interphase (G1-, S-, G2-phases) and M-phase

and $p27^{Kip1}$ (Grana and Reddy 1995). Based on this consensus, $p21^{Cip1}$ and $p27^{Kip1}$, the cell cycle regulating factors, have been used as engineering targets of mammalian cell culture to obtain a similar effect. The overexpression of the CKIs ($p21^{Cip1}$ and $p27^{Kip1}$) induced growth arrest through the inhibition of CDK activity, blocking the downstream phosphorylation of the Rb protein and consequently arresting the cells in the G1 phase of the cell cycle. Engineering for growth arrest, however, allows only for the overexpression of a few targeted genes such as p53, $p21^{Cip1}$, and $p27^{Kip1}$.

Transiently introducing $p21^{Cip1}$, $p27^{Kip1}$, or p53175P (a p53 mutant showing specific loss of apoptotic function) into secreted alkaline phosphatase (SEAP) producing CHO cell lines achieved approximately fourfold greater productivity than that of the unmodified cell line by G1 phase cell cycle arrest (Fussenegger et al. 1997). The use of G1 cell cycle arrest in CHO cells by stably overexpressing $p27^{Kip1}$ showed a dramatic increase of q_p, for not only SEAP (Carvalhal et al. 2003; Mazur et al. 1998), but also for soluble intracellular adhesion molecule-1 (sICAM) (Meents et al. 2002b). The overexpression of $p21^{Cip1}$ induced the cell cycle arrest and led to about a fourfold increase in q_p of IgG4 producing CHO cell lines (Bi et al. 2004). Similarly, IPTG-inducible expression of $p21^{Cip1}$ resulted in fourfold higher q_p in IgG4 producing NS0 myeloma cells (Watanabe et al. 2002). The combinatorial strategy with $p21^{Cip1}$, anti-apoptotic protein, Bcl-xL, or differentiation factor, CCAAT/enhancer binding protein α (C/EBPα), resulted in a further increase of cell growth and about 10–15 times increase in SEAP production in CHO cells (Fussenegger et al. 1998). Also, an attempt to engineer the combination of $p21^{Cip1}$ and another representative anti-apoptotic protein, Bcl-2, resulted in a greater enhancement in q_p of CHO cells and NS0 cells (Astley and Al-Rubeai 2008; Ibarra et al. 2003). Moreover, it has been suggested that $p21^{Cip1}$-induced cell cycle arrest can shorten the time required for adaptation of cell lines to suspension and protein-free environments (Astley et al. 2007).

The overexpression of transcription factor c-Myc has been recently investigated to enhance the proliferation of CHO cells (Kuystermans and Al-Rubeai 2009). This finding that the therapeutic protein production was increased through enhanced cell growth rather than cell growth arrest showed another target for proliferation engineering, as predicted through a study of histone deacetylase (HDAC)-inhibited growth arrest (Jiang and Sharfstein 2008). However, the effect of genetic engineering for inducing cell growth arrest is hindered by the intrinsic complexity to the biological system. A high-throughput combinatorial screening of cell cycle regulating genes would provide significant benefits to target gene selection for cell engineering by determining gene sets that are able to repress apoptotic triggers in dysregulated cell cycle control.

Table 18.1 Effect of genetic engineering for G1-phase arrest on q_p in mammalian cells

Engineered gene	Cell	Therapeutic protein	Expression system	Effect on q_p	References
$p21^{Cip1}$	CHO	SEAP	Transient	4.6-fold increase	Fussenegger et al. (1997)
$p27^{Kip1}$	CHO	SEAP	Transient	3.9-fold increase	Fussenegger et al. (1997)
p56175P (p53 mutant)	CHO	SEAP	Transient	3.9-fold increase	Fussenegger et al. (1997)
$p27^{Kip1}$	CHO	SEAP	Stable	4-fold increase	Carvalhal et al. (2003)
$p27^{Kip1}$	CHO	sICAM	Stable	5-fold increase	Meents et al. (2002b)
$p21^{Cip1}$	CHO	IgG4	Stable	4-fold increase	Bi et al. (2004)
$p21^{Cip1}$	NS0	IgG4	Controlled[a]	4-fold increase	Watanabe et al. (2002)
$p27^{Kip1}$ and p53175P (p53 mutant)	CHO	SEAP	Stable	10–15-fold increase	Mazur et al. (1998)
$p21^{Cip1}$ and C/EBPα	CHO	SEAP	Controlled[b]	10–15-fold increase	Fussenegger et al. (1998)
$p27^{Kip1}$ and Bcl-xL	CHO	SEAP	Controlled[b]	30-fold increase	Fussenegger et al. (1998)
$p21^{Cip1}$ and Bcl-2	CHO	Monoclonal Antibody (mAb)	Stable	2-fold increase	Astley and Al-Rubeai (2008)
$p21^{Cip1}$ and Bcl-2	NS0	IgG4	Controlled[a]	4-fold increase	Ibarra et al. (2003)

C/EBP α CCAAT/enhancer binding protein α, *SEAP* secreted alkaline phosphatase, *sICAM* soluble intracellular adhesion molecule

[a]IPTG-inducible Lacswitch II system was used

[b]Tetracycline-regulated coexpression system was used; the multicistronic expression unit which is driven by the tetracycline-repressible promoter was used so that gene expression could be simultaneously induced upon withdrawal of tetracycline from the cell culture medium

18.4.2 Chaperone Engineering

Since the therapeutic proteins are secreted proteins, the proteins resident in the endoplasmic reticulum (ER) that has an essential role in the secretory pathway are thought to be a good resource for cell engineering. It has been reported that the amount of heterologous protein secretion does not increase proportionally with the gene copy number or even the intracellular amount of heterologous protein (Schröder 2007). Therefore, the rate-limiting steps in enhancing q_p are believed to be translational or post-translational processes. Some studies have demonstrated that the up-regulation of the ER-resident proteins can increase the production of therapeutic proteins.

Among the ER-resident proteins that have a major role in protein folding, the chaperones machineries are mainly targeted to enhance q_p. The molecular chaperones are ubiquitous proteins that assist polypeptides to reach a proper conformation or cellular location without affecting folding rates or becoming part of the final structure (Baneyx 2004). The heat shock protein (Hsp) family, protein disulfide isomerase (PDI) family, and lectin binding enzymes are three of the common molecular chaperones. Members of these families take part in various functions of the cells.

Numerous studies dealing with chaperone engineering have revealed that the effects of molecular chaperones on protein production are dependent on several factors, including the chaperones concerned, the target therapeutic proteins, and expression systems (Mohan et al. 2008). For example, the effects of PDI overexpression have been mixed, resulting in either enhanced, decreased, or even unaffected q_p. It was discovered that controlled overexpression of ERp57, an isoform of PDI, showed a twofold increase in q_p (Hwang et al. 2003). In another study, PDI overexpression had no effect on the q_p for an interleukin-15 producing cell line, whereas PDI overexpression appeared to negatively affect the q_p for a tumor necrosis factor receptor:Fc fusion protein (TNFR:Fc) producing cell line due to its co-localization and intracellular retention (Davis et al. 2000). It was also observed that the controlled overexpression of PDI showed no effect on the q_p for the TPO producing cell line, while it only moderately enhanced the q_p in a monoclonal antibody producing cell line (Mohan et al. 2007). PDI is the most widely studied chaperone for CHO cell engineering and could enhance q_p, depending on the target therapeutic protein. Recently, it has been reported that the transient expression of PDI family proteins, PDI, ERp72, or pancreatic PDI (PDIp), did not show any improvement in the q_p (Hayes et al. 2010).

Other chaperones have also been studied and showed their relation to increase the q_p. The overexpression of immunoglobulin heavy chain-binding proteins (BiP) appeared to be positively related to an increased productivity in mammalian cells (Jones et al. 2005). However, BiP overexpression in combinations with PDI resulted in a decreased q_p (Borth et al. 2005) and its down-regulation rather than its overexpression, resulted in an improved q_p (Dorner et al. 1988). Simultaneous overexpression of both calnexin and calreticulin, which are lectin binding chaperones, resulted in an enhanced q_p with no inhibiting effect on the cell growth (Chung et al. 2004).

Employing an inducible chaperone expression system seems superior to the constitutive overexpression of chaperones for determining the effect of chaperone expression on q_p, because the occurrence of clonal variability is excluded (Mohan et al. 2008). Fundamentally, the effects of chaperones on protein production depend on several factors such as the employment of an expression system, the target therapeutic proteins, and the overexpressed chaperones. This can be described by previous studies shown in Table 18.2.

Targeting a single component of the secretory pathway may not be the best strategy for increasing the q_p since the gene regulation system in mammalian cells is complicated. A detailed understanding of protein folding in the ER should be

Table 18.2 Effect of chaperone engineering on q_p of various target proteins produced in CHO cells

Chaperone	Expression system	Therapeutic protein	Effect on q_p	Reference
PDI	Overexpression	mAb	Positive	Borth et al. (2005)
PDI	Overexpression	Interleukin-15	No effect	Davis et al. (2000)
PDI	Overexpression	TNFR:Fc	Negative	Davis et al. (2000)
PDI	Controlled expression[a]	Thrombopoietin	No effect	Mohan et al. (2007)
PDI	Controlled expression[a]	mAb	Moderate effect	Mohan et al. (2007)
BiP	Overexpression	mAb	Negative	Borth et al. (2005)
BiP and PDI	Overexpression	mAb	Negative	Borth et al. (2005)
ERp57	Controlled expression[a]	Thrombopoietin	Positive	Hwang et al. (2003)
Calnexin and calreticulin	Controlled expression[a]	Thrombopoietin	Positive	Chung et al. (2004)

BiP immunoglobulin heavy chain-binding protein, *mAb* monoclonal antibodies, *PDI* protein disulfide isomerase, *TNFR: Fc* a tumor necrosis factor receptor: Fc fusion protein
[a]Doxycycline-regulated expression system (Tet-Off system) was used

preceded so that a more delicate chaperone regulation could enhance the productivity. An overexpression of several chaperones, co-chaperones, holdases and/or foldases, along with a functionally meaningful ratio to modulate the folding environment might be a better approach (Mohan et al. 2008).

18.4.3 Secretion Engineering

Secretion of therapeutic proteins in mammalian cells is mediated by membrane-bound transport vesicles. An effective regulation of this complex trafficking machinery might be one of the strategies to achieve an increased secretion and ultimately improved productivity. To do that, detailed understanding of the membrane fusion between transport vesicles and the target membrane is necessary. In vesicle trafficking, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that are anchored to both transport vesicles and their target membranes trigger membrane fusion (Jahn and Scheller 2006). In the process of SNARE-mediated membrane fusion, the interaction of Sec1/Munc18 (SM) family proteins with SNAREs is essential.

In order to increase q_p by relieving the bottleneck in secretion, key components of membrane fusion events have been introduced for cell engineering. Stable expression of SNAREs and SM protein in mammalian cells increased the production of therapeutic proteins. While other SNAREs related to exocytosis have had no effect or a negative effect, SNAP-23 and VMAP8 specifically showed a positive effect on the q_p when ectopically and stably expressed in mammalian cells (Peng et al. 2011). Also, Sly1- and Munc18-based vesicle traffic engineering resulted in an increased secretory capacity of different therapeutic proteins in CHO cells. Sly1 and Munc18 emerged as SM proteins modulate ER-to-Golgi- and Golgi-to-plasma membrane-addressed exocytosis (Peng and Fussenegger 2009).

While a lot of efforts have been made to develop transcription- and translation-based engineering strategies for improved production of therapeutic proteins, little work has been done on the posttranslational capacity of mammalian cells (Barnes and Dickson 2006; Fussenegger and Hauser 2007; Weber and Fussenegger 2007; Wurm 2004). As some of the strategies have targeted the posttranslational process, boosting the secretion by up-regulating proteins involved in trafficking machinery is a novel engineering strategy to increase q_p.

18.4.4 Unfolded Protein Response-Based Engineering

In mammalian cells, secreted therapeutic proteins are post- or co-translationally translocate into the lumen of the ER for protein folding and maturation. The ER provides an oxidizing environment and enzymes required for protein modification (Ellgaard and Helenius 2003). In the ER, a protein must have reached a correctly folded conformation. If the folding and maturation process fails, the protein is not transported to its final destination, and is eventually degraded.

When the ectopic expression of therapeutic proteins reaches the ER protein-folding capacity, unfolded proteins are accumulated in the lumen and cause ER stress. This leads to the activation of intracellular signal transduction pathways for an unfolded protein response (UPR) to maintain cellular homeostasis (Ron and Walter 2007). In mammalian cells, the UPR signaling pathway activates four processes: (i) attenuation in protein translation (Harding et al. 1999; Ron 2002), (ii) induction of chaperone expression (Haze et al. 1999; Mori 2000), (iii) ER-associated degradation (ERAD) of misfolded proteins (Hosokawa et al. 2001; Yoshida et al. 2003), and (iv) apoptosis (McCullough et al. 2001; Fig. 18.5). The regulation of components in the UPR signaling pathway has been suggested to increase the secretory capacity of cells.

X-box binding protein 1 (XBP-1) has been widely studied in UPR-based engineering. The sliced form of XBP-1 (XBP-1s) functions as a transcription activator and up-regulates many ER chaperones. It has been revealed that the overexpression of XBP-1 can increase the q_p of various therapeutic proteins, whereas XBP-1u has no effect on the q_p (Tigges and Fussenegger 2006). Also, in fed-batch cultivation, the heterologous expression of XBP-1 led to an increase in ER content and

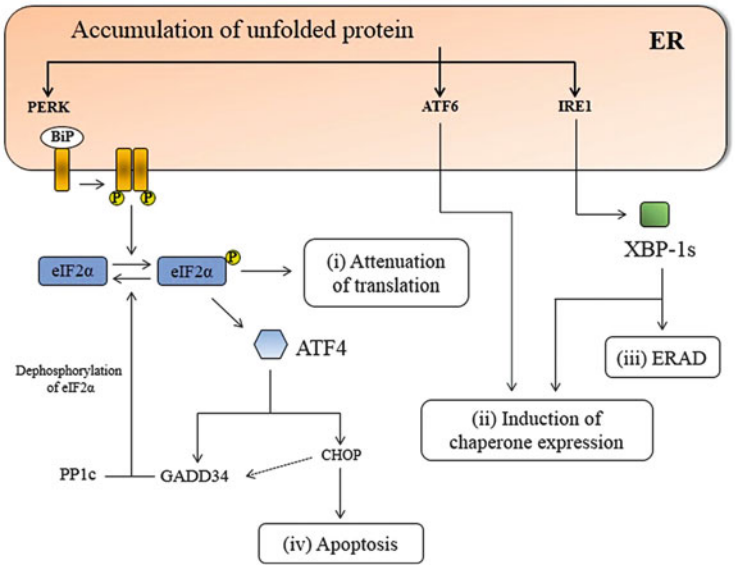

Fig. 18.5 Three different unfolded protein response (*UPR*) signaling pathways that are mediated by inositol-requiring protein-1 (*IRE1*), activating transcription factor-6 (*ATF6*) or protein kinase RNA (*PKR*)-like ER kinase (*PERK*). *ERAD* ER-associated degradation, *XBP* X-box binding protein

enhanced q_p in monoclonal antibody producing cells (Becker et al. 2008). In another study, it has been suggested that the effects of the overexpression of XBP-1 on q_p depends on the expression levels of recombinant proteins (Ku et al. 2008). The overexpression of XBP-1 was particularly effective in CHO cells experiencing secretion bottleneck.

Another strategy in UPR-based engineering was to restore the attenuation in protein translation. Activating transcription factor (ATF4), one of the key regulators in UPR system, releases the translational attenuation by the dephosphorylation of eukaryotic initiation factor-2α via growth arrest and DNA damage inducible protein 34 (GADD34) (Ron 2002). The overexpression of ATF4 and GADD34 showed an enhanced q_p of recombinant antithrombin III (AT-III) (Ohya et al. 2008; Omasa et al. 2008).

Conclusions

We have discussed the two keys to achieve a cost-effective level of production: the specific productivity and time integral of viable cell concentration. Many different cellular mechanisms and characteristics of CHO cells including cell death, metabolism, cell cycling, and protein folding are involved to increase these two variables. Therefore, understanding these mechanisms is

(continued)

certainly important to magnify the benefits of genetic engineering and to propose additional applications. Primarily by expressing specific targeted genes, many studies have increased q_p and TIVCC. Recently, not only the productivity of therapeutic proteins is a significant issue in CHO cell cultures, but also the quality of them is of great concern. With a clear evaluation of the elusive characteristics of CHO cells, CHO cell engineering will efficiently maximize the production of therapeutic proteins.

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Chapter 19
Proteomics in Cell Culture: From Genomics to Combined 'Omics for Cell Line Engineering and Bioprocess Development

Kelley Heffner, Christian Schroeder Kaas, Amit Kumar, Deniz Baycin-Hizal, and Michael Betenbaugh

Abstract The genetic sequencing of Chinese hamster ovary cells has initiated a systems biology era for biotechnology applications. In addition to genomics, critical 'omics data sets also include proteomics, transcriptomics and metabolomics. Recently, the use of proteomics in cell lines for recombinant protein production has increased significantly because proteomics can track changes in protein levels for different cell lines over time, which can be advantageous for bioprocess development and optimization. Specifically, the identification of proteins that affect cell culture processes can aid efforts in media development and cell line engineering to improve growth or productivity, delay the onset of apoptosis, or utilize nutrients efficiently. Mass-spectrometry based and other proteomics methods can provide for the detection of thousands of proteins from cell culture and bioinformatics analysis serves to identify and quantify protein levels. Optimizations of sample preparations and database development, including a detailed CHO proteome now available, have improved the quantity and accuracy of identified proteins. The applications are widespread and expanding, thus suggesting numerous applications of proteomics and combined 'omics experiments in coming years.

Keywords Proteomics • Genomics • Transcriptomics • Metabolomics • Bioprocess development • Cell line engineering • Bioinformatics

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19.1 Introduction

Chinese hamster ovary (CHO) cells are the production host of choice for many recombinant proteins. Their growth in suspension cell culture is scalable for high-density production of biotherapeutics. As mammalian cells, CHO cells provide glycosylation processing that is typically compatible with humans. In 2012, the majority of top biologics were produced in CHO cells as shown in Table 19.1 (Lawrence and Lahteenmaki 2014). The top biotherapeutic is Humira, produced using the CHO expression system for the treatment of rheumatoid arthritis (Lawrence and Lahteenmaki 2014). Characteristics such as human-compatibility and manufacturing scalability help explain CHO cells' dominating use in biotechnology applications.

Table 19.1 Top selling drugs of 2013

Drug	Company	Indication	Production host
Humira (adalimumab)	AbbVie	Rheumatoid arthritis (RA), juvenile rheumatoid arthritis, Crohn's disease, psoriatic arthritis (PA), psoriasis, ankylosing spondylitis, ulcerative colitis (UC), Behçet syndrome	CHO
Enbrel (etanercept)	Amgen	RA, psoriasis, ankylosing spondylitis, PA, juvenile rheumatoid arthritis	CHO
Lantus (insulin glargine)	Sanofi	Diabetes mellitus type 1	E. coli
Rituxan (rituximab)	Roche	RA, chronic lymphocytic leukemia/small cell lymphocytic lymphoma, non-Hodgkin's lymphoma, antineutrophil cytoplasmic antibodies associated vasculitis, indolent non-Hodgkin's lymphoma, diffuse large B-cell lymphoma	CHO
Remicade (infliximab)	J&J	RA, Crohn's disease, psoriasis, UC, ankylosing spondylitis, Behçet syndrome, PA	CHO
Avastin (bevacizumab)	Roche	Colorectal cancer, non–small cell lung cancer, renal cell cancer, brain cancer (malignant glioma; anaplastic astrocytoma, glioblastoma multiforme)	CHO
Herceptin (trastuzumab)	Roche	Breast cancer, gastric cancer	CHO
Gleevec (imatinib)	Novartis	Chronic myelogenous leukemia, gastrointestinal stromal tumor, acute lymphocytic leukemia, hypereosinophilic syndrome, mastocytosis, dermafibrosarcoma protuberans, myelodysplastic syndrome, myeloproliferative disorders	Small molecule
Neulasta (pegfilgrastim)	Amgen	Neutropenia/leukopenia (NL)	E. coli
Copaxone (glatiramer acetate)	Teva	Multiple sclerosis	Small molecule
Revlimid (lenalidoamide)	Celgene	NL	CHO

An improved understanding of CHO cell physiology resulted from the recently completed genome sequence of CHO cell lines and hamsters (Xu et al. 2011; Lewis et al. 2013; Brinkrolf et al. 2013). From this information, a variety of methods have been used to quantify the genome, transcriptome, proteome, and metabolome. These data sets offer new insights into cell physiology. To complement these studies, Baycin-Hizal et al. completed the proteome of the CHO cell line, including information on intracellular, secreted, and glyco-proteins (Baycin-Hizal et al. 2012). This study complemented the results from the CHO genome (Xu et al. 2011) and provided a codon frequency analysis of the differences between CHO cells and humans for improved expression of therapeutics (Baycin-Hizal et al. 2012). Additionally, this study integrated proteomic and transcriptomic data to analyze pathway changes, such as enrichment of protein processing and apoptosis, and depletion of steroid hormone and glycosphingolipid metabolism (Baycin-Hizal et al. 2012). The complete CHO proteome will enhance our capacity for bioprocess development by increasing knowledge of the most-widely used production host.

Proteins have diverse functions in the cell and are involved in growth, signaling, regulation, and metabolism. Their rapidly changing levels provide important information about subtle changes in the cell that may not be detected at the transcriptome or genome level. A variety of methods are used to generate large, complex data sets, which require processing and analysis to reveal useful information about the cellular phenotype. Production of biologics, such as monoclonal antibodies (mAbs), for therapeutic use requires development of a scalable and consistent bioprocess, ensuring high yield and purity of the biotherapeutics. Quantification of protein levels provides a clearer understanding of cell physiology and can lead to improvements in cell culture for biotechnology applications. Proteomics can also be used during development in order to identify proteins that affect cell culture growth, apoptosis, recombinant protein productivity, and product quality. This information can suggest methods to improve the bioprocess through cell line engineering and rational media formulation.

Recently, combined 'omics approaches have become more widespread in application. These approaches provide useful insights because direct one-to-one correlations between approaches rarely exist. The combination improves the reliability and accuracy of the results compared to either approach alone.

This chapter highlights how both proteomics and genomics can be used for cell culture applications. Proteomics can provide identification and quantification of thousands of cellular proteins and can be used to increase the understanding of production hosts such as CHO cells. In recent years, the number of published proteomic data sets has fluctuated but expanded gradually as shown in Fig. 19.1. Due to the availability of the CHO genome and proteome and introduction of new techniques to increase protein identification and accuracy, the number of applications of CHO proteomics is likely to grow even larger in coming decades.

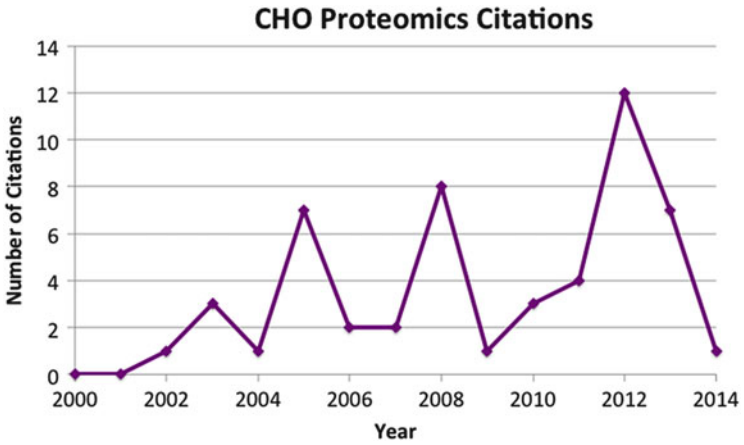

Fig. 19.1 Increase in the number of PubMed citations over time. The number of citations annually is determined by searching "Chinese hamster ovary proteomics" for the specified year (search performed February 2014)

19.2 Genomics

Compared to the genomes of several other mammals such as human (Lander et al. 2001), mouse (Waterston et al. 2002) and rat (Gibbs et al. 2004), the first draft of the CHO-K1 genome was not publicly available until recently (Xu et al. 2011). The sequence revealed 24,383 predicted genes associated with 21 chromosomes with a total of 2.45 gigabytes of genomic sequence (Xu et al. 2011). To facilitate accessibility of the genomic sequence the online database www.CHOgenome.org has been created (Hammond et al. 2012). Before the first publicly available CHO genome came out, the microRNA transcriptome (Hackl et al. 2011) and first transcriptome (Becker et al. 2011) were published as well as several papers using microarrays from mouse without knowledge of the genetic modifications found in hamster yielding suboptimal results (Baik et al. 2006; Yee et al. 2008; Tabuchi et al. 2010; Hernandez Bort et al. 2012). With the genomic sequence available for the CHO-K1 cell line it has now been possible to identify miRNAs (Hackl et al. 2012) and use the sequence for siRNA design (Fischer et al. 2013) and genome editing using Zing finger nucleases (Gaj et al. 2012). One of the main advantages of expressing heterologous protein in CHO has been deciphering the similarity of post-translational modifications of proteins between human and CHO (Kim et al. 2012). Analysis of the CHO-K1 genome revealed that homologs existed for 99 % of the genes in the human genome associated with glycosylation. Of these genes 53 % were detected as transcribed. Furthermore, analysis of the transcriptome revealed that numerous genes associated with viral entry were not expressed, thus explaining the resistance of CHO cells to viral infection (Xu et al. 2011).

In 2013, two groups (Lewis et al. 2013; Brinkrolf et al. 2013) published the genomic sequence of the Chinese hamster (*Cricetulus griseus*) that the CHO cell line was originally extracted from (Puck et al. 1958). The data from the two independent sequencing efforts are presently being merged into a single well-characterized genome, which will be used as the standard reference for the future study of CHO cells lines. The genomic sequence from a number of CHO cell lines (CHO-S, DG44, serum free CHO-K1, CHO protein-free, and C0101) were also published in 2013 (Lewis et al. 2013). Analysis identified more than 3.7 million point mutations in these cell lines compared to the Chinese hamster, highlighting the mutagenesis that has occurred in the process of creating the various cell lines.

19.3 Proteomics

The applications of proteomics in cell culture applications are now widespread. However the use of proteomics for cell culture has also coincided with advances in methods, such as the optimization of sample preparation, digestion, labeling, mass spectrometry (MS), and bioinformatics. Proteomics is increasingly applied to understand cell lines and aid in cell line engineering and process optimization efforts to increase cell growth, increase recombinant protein productivity, and maintain high product quality.

19.3.1 Optimization of Proteomics Methods

Following the initial proteomics experiments in CHO cells, there have been considerable refinements in the methods in order to improve the recovery of cell proteins and enhance their identification. The ability to elucidate increasing numbers of proteins with high accuracy is dependent on optimized sample preparation methods. Proteomics methods include extraction, reduction, alkylation, digestion, and peptide fractionation prior to quantification with MS as shown in the workflow in Fig. 19.2. After cell culture pellets are generated, the cells are lysed and proteins extracted. Following reduction, alkylation, digestion, and fractionation, the individual peptides can be identified. This requires the use of search engines and databases to match the mass spectra to specific peptide sequences and finally proteins. In recent years, there have been numerous improvements to proteomic methods including the optimization of sample preparation, digestion, labeling, and mass spectrometric analysis.

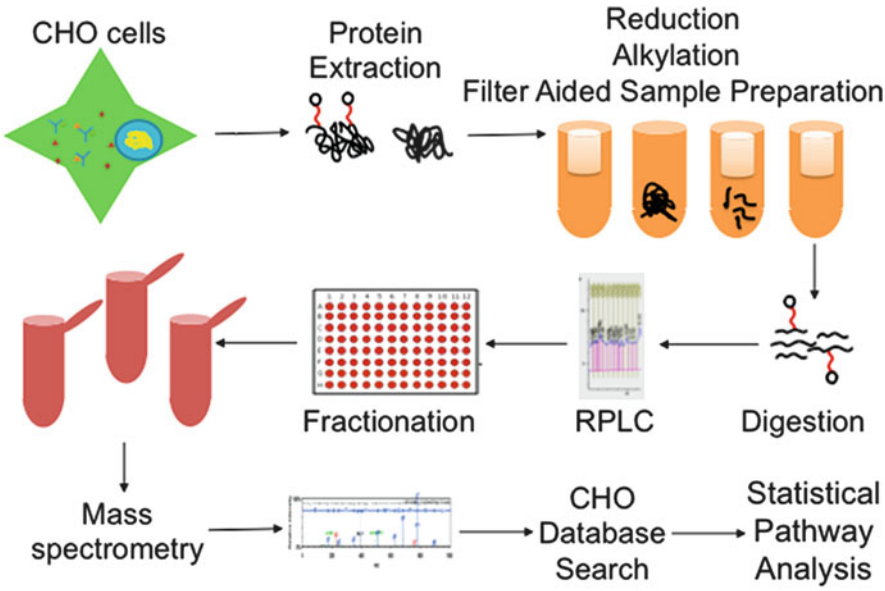

Fig. 19.2 Overview of proteomics workflow. After proteins are extracted from cell culture, sample preparation involves reduction, alkylation, filter aided sample preparation, and fractionation. Peptides are injected into the mass spectrometer and the resultant peaks are analyzed. Proteins are identified and quantified using CHO-specific databases

19.3.1.1 Sample Preparation Methods and Improvements

Initial sample preparation involves using different extraction and digestion techniques. One method involves the recovery of proteins from two-dimensional (2D) gels which serve as an initial separation technique. In order to improve protein recovery, the concentration of solubilizers such as urea, DTT, CHAPS, and SDS have been optimized (Valente et al. 2012). Maintaining solubility enables recovery of proteins with diverse physical and chemical properties. The optimum solubilizing factors for CHO cells include DTT, urea-DTT cross-interaction, and urea-CHAPS cross-interaction for improved protein recovery (Valente et al. 2012). A final solution composition of 8 M urea, 32.5 mM DTT, and at least 2 % CHAPS was selected for maximal recovery of CHO cell lysates (Valente et al. 2012).

Besides the extraction efficiency, digestion efficiency is another criteria for increasing the number of proteins identified. In-gel and in-solution digestion methods have separate and distinct advantages. In-gel digestion involves solubilizing proteins with detergent and separating proteins by gel electrophoresis. Isolated proteins are then digested from the gel and quantified by MS. In-solution digestion involves extracting proteins with strong reagents and digesting in the solution. There are advantages and disadvantages of each method. In-gel digestion protects

against impurities but the protein yield is typically poor in comparison to in-solution digestion. On the other hand, in-solution digestion is easier to implement but there is greater risk of impurities or incomplete solubilization. Sodium dodecyl sulfate (SDS) is one of the principal detergents used for full extraction of the cell lysates including insoluble membrane proteins; however, SDS has to be removed prior to MS analysis. Filter aided sample prep (FASP) was developed to remove the SDS prior to digestion and MS (Wisniewski et al. 2009). The addition of this step allows for more complete coverage of the proteome, and the inclusion of FASP was used to maximize the protein recovery during the CHO proteome analysis prior to trypsin digestion (Baycin-Hizal et al. 2012).

Both in-gel and in-solution digestions continue to be used for biotechnology applications and there are various examples. In-gel digestion was used to identify a number of proteins during cell line engineering efforts (Baik et al. 2008, 2011; Van Dyk et al. 2003) and also aided in quantifying differences between cell lines (Kuystermans et al. 2010; Beckmann et al. 2012). In-gel digestion was also used to identify phosphorylated proteins from CHO-K1 cell culture (Hayduk et al. 2004) and to elucidate the proteome of the CHO DG44 cell line (Lee et al. 2010).

In-solution digestion can help to address some of the limitations of in-gel digestion such as difficulties in separating proteins with low molecular weight, high molecular weight, or hydrophobic properties. Meleady used in-solution digestion to profile protein levels in cell lines with or without miR-7 overexpression (Meleady et al. 2012a). In-solution digestion was also used to identify secreted proteins from the CHO-S and CHO DG44 cell lines (Slade et al. 2012).

In other experiments, a combination of both in-gel and in-solution digestions are used. Both digestion techniques were used to prepare protein fractions prior to MS analysis (Baycin-Hizal et al. 2012) and were also used directly prior to LC/MS injections (Meleady et al. 2012a). Although digestion methods can vary, these can be important for preparing peptide samples for MS injection. An optional step that is now widely used for comparative proteomics is the labeling of digested peptides as will be discussed in more detail below.

Following digestion, fractionation can be applied to enhance protein identification. In one example, basic reversed phase liquid chromatography was used to separate samples into 96 fractions that were then combined into 48 fractions for MS analysis (Baycin-Hizal et al. 2012). For some applications, proteins related to a specific organelle or intracellular and extracellular compartments can be targeted. Two recent examples include the secretome and mitotic spindle, as discussed next.

The secretome includes host cell proteins (HCP) that must be removed prior to formulation of the final drug product. Identification of secreted proteins is often limited by their low abundance. A design of experiments approach was used to optimize sample preparation methods and to increase protein recovery for HCP identification (Valente et al. 2014). Precipitation parameters such as the precipitant chemical, precipitant concentration, and incubation length, were evaluated for both gel-based and shotgun proteomics (Valente et al. 2014). The results were used to optimize a method for identification of HCPs, which differ in physical and chemical properties, as well as their physiological function; this method used methanol

precipitation to identify 178 HCPs, including clusterin, beta-actin, glyceraldehyde-3-phosphate dehydrogenase, and immunoglobulin superfamily member 8 (Valente et al. 2014). Optimization of sample preparation is critical to maximize the recovery of low abundance secreted proteins.

The mitotic spindle proteins were also characterized in CHO cells (Bonner et al. 2011). Cell division is an important event to study, as it relates to the growth of cell lines. Isolation of the spindle aids in identification of factors affecting division and thus growth. After synchronizing all cells in metaphase, over 1,100 proteins were identified, of which 239 proteins were cell division factors and 841 proteins were involved in early stages of division (Bonner et al. 2011). Of the proteome, 11 % of proteins localized to the membrane, 7 % were associated with microtubules, and 3 % were associated with actin (Bonner et al. 2011). Identification of cell division factors may aid bioprocess development due to their importance in cell growth and ultimately recombinant protein yields.

19.3.1.2 Protein Labeling

Labeling strategies are used in proteomics to provide relative quantification in addition to the identification of proteins. Recent methods for labeling proteins include stable isotope labeling with amino acids in culture (SILAC), isobaric tags for relative and absolute quantification (iTRAQ), and tandem mass tags (TMT). Various labeling methods as well as label-free methods are now used for relative quantification.

In SILAC, proteins are labeled when amino acids from the culture medium are incorporated into the cell. Incorporation of amino acid labeling into proteins can be detected by MS. SILAC experiments involve cell adaptation to labeled media, cell growth, protein identification by MS, and data analysis (Harsha et al. 2008). Both in-gel and in-solution digestion can be used to extract proteins prior to MS. SILAC labeling experiments provide information on protein dynamics because they quantitate the incorporation of labeled amino acids. However, this method introduces experimental errors, such as incomplete amino acid isotope incorporation and sample mixing errors (Park et al. 2012). To reduce error, label-swap replication experiments were used to average ratios of individual replicates and validated with a triplet experiment (Park et al. 2012). The approach corrected for incomplete labeling and arginine to proline conversion, thus providing consistent experimental ratios (Park et al. 2012).

An alternative labeling method is iTRAQ, which is used to label peptides before running the sample on MS. The N-terminus and primary amine groups of digested peptides are covalently labeled in this technique (Wiese et al. 2007). The isobaric mass design of the labeling reagents provides the quantification in the MS/MS spectra with tag-specific reporter ions (Wiese et al. 2007). The benefit of this method is that cells do not require adaptation to labeled medium because the labeling is performed on protein extracts. It is important to equalize masses of iTRAQ reagent added across samples because the quantification is relative.

Besides iTRAQ, the mTRAQ technique can allow direct quantification at MS1 level by providing mass differences in precursor ions (Mertins et al. 2012). A comparison of iTRAQ versus nonisobaric labeling (mTRAQ) revealed that iTRAQ labeling identifies over double the total number of proteins and approximately triple the phosphopeptides as compared to mTRAQ (Mertins et al. 2012). Additionally, kinase identification was significantly increased using iTRAQ, thus improving knowledge about protein-protein interactions involved in recombinant protein production (Mertins et al. 2012). In addition to iTRAQ, TMT is another widely used isobaric labeling technique, which provides the measurement of the intensities at the peptide fragmentation level. The greatest advantage of these labeling techniques is they can provide multiplexing of up to eight samples (iTRAQ) and ten samples (TMT), which significantly decreases not only LC-MS/MS run time but also the variations between the samples in the whole process (Megger et al. 2013). All of these proteomics experiments can yield novel insights into CHO cells that lead to a better understanding of their use as biopharmaceutical production hosts.

19.3.1.3 MS

High quality digested peptides are injected into the MS for protein identification. Components of the MS instrument include the source, which produces gas phase ions from the sample; mass analyzer, which resolves the ions based on mass to charge ratio; and detector, which detects ions that have been resolved by the analyzer. Both tandem MS and matrix-assisted laser desorption/ionization time of flight (MALDI TOF) MS have been used to identify and quantify proteins in cell lines with different growth or productivity characteristics (Doolan et al. 2010; Beckmann et al. 2012; Van Dyk et al. 2003; Baik et al. 2008, 2011; Lee et al. 2010; Hayduk et al. 2004).

Peaks from the MS/MS spectra are next identified as specific peptides that are ultimately attributed to specific proteins. Proper identification requires the use of search engines and databases. Different search engines such as TagRecon and MyriMatch (Baycin-Hizal et al. 2012) have been combined to match the CHO genome database. Currently, many open source search engines such as X!Tandem and OMSSA, as well as many proprietary identification programs such as Mascot and SEQUEST, exist for matching the identification and quantification of MS/MS spectra. Due to algorithm differences, identifications from each search engine may show slight variances; for that reason coupling of multiple search engines can increase the confidence levels.

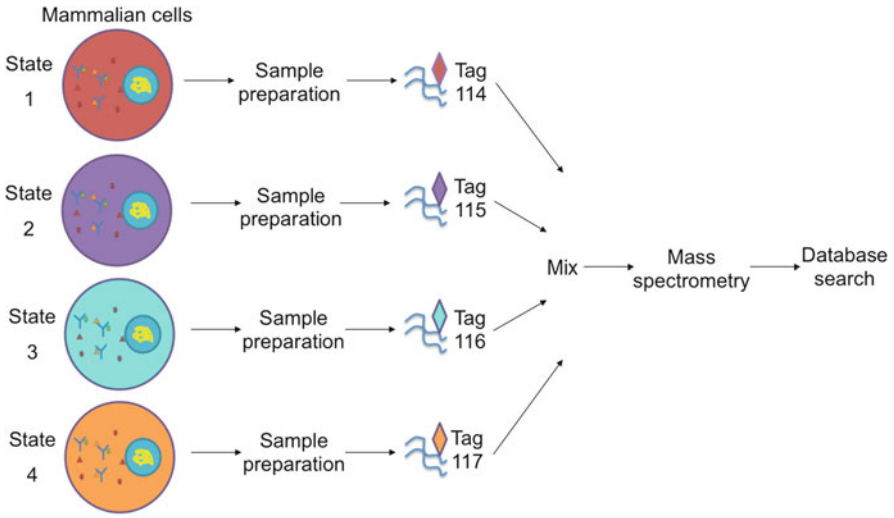

Fig. 19.3 Overview of comparative proteomics. Comparative proteomics can be used to identify differences in protein expression levels between culture conditions. Following sample preparation, peptides are labeled separately with unique tags and then mixed in equal amounts prior to MS injection. Peptides are identified and quantified using CHO-specific databases

19.3.2 Proteomics for Bioprocess Development

Recently, proteomics has been applied to identify proteins that play key roles in recombinant protein production for use in bioprocess optimization. A comparison of high and low expressors as shown in Fig. 19.3 can provide significant insights and understanding of cell properties that are important for improving product yields.

In recent years, comparative and label-free proteomics have been used in a number of studies to gain insights about CHO cells used in bioprocessing. Table 19.2 summarizes some of the recent publications in this area.

19.3.2.1 Proteomics Analysis to Increase Cell Growth Rate and Viable Cell Density

A critical bioprocess development goal is to maximize growth rate in order to increase the amount of biotherapeutic produced over a given time period. An approach combining transcriptomics and proteomics was enacted to identify candidates for a high growth phenotype (Clarke et al. 2012). The proteomics results indicated that 285 proteins were differentially expressed between cell lines with fast and slow growth rates (Clarke et al. 2012). Benefits of combining the 'omics approaches include accounting for low abundance protein expression and

Table 19.2 Summary of CHO proteomics for bioprocess development

Reference	Purpose	Method	Conclusion
Baik et al. (2008)	To determine differences in proteins between CHO cell cultures with or without sodium butyrate supplementation	Used in-gel digestion for proteins. Used MALDI TOF MS and MS/MS to identify proteins	Identified increased levels of GRP78 and peroxiredoxin following treatment with sodium butyrate. Phosphopyruvate hydratase levels decreased with sodium butyrate treatment
Baik et al. 2011	To determine differences in protein levels during adaptation to serum-free medium	Used in-gel digestion for proteins. Used MALDI TOF MS and MS/MS to identify proteins	Identified increased levels of HSP60 and HSC70 in serum-free media. Subsequent overexpression resulted in improved cell concentration during serum-free adaptation
Baycin-Hizal et al. (2012)	To identify the proteome, secretome, and glycoproteome of CHO-K1 cell line	Used SPEG to generate glycoprotein fractions and in-gel and in-solution digestion for proteins. Used MS/MS to measure proteome, secretome, and glycoproteome	Identified important proteins in CHO-K1 cell line and combined proteomic and transcriptomic data for improved analysis
Carlage et al. (2009)	To compare the proteomes of high and low producing cell cultures over time	Proteins were digested and identified by LC-MS	Found differentially expressed proteins between cultures. Eukaryotic translation initiation factor 3 and ribosome 40S were upregulated and vimentin, annexin, and histones were downregulated in the high producer
Carlage et al. (2012)	To determine the changes in the proteome over time for a CHO cell culture overexpressing Bcl-xL	Used iTRAQ labeling and LC-MS to identify proteins	Identified proteins with changing levels over time from exponential to stationary transition and related the differences to cell growth and apoptosis
Clarke et al. (2012)	To determine important proteins that elucidate how miRNAs affect CHO cell growth	Used LC-MS to identify proteins	Compared miRNA, mRNA, and protein expression levels and identified processes

(continued)

Table 19.2 (continued)

Reference	Purpose	Method	Conclusion
			regulating cell growth, such as ribosome synthesis, translation, and mRNA processing
Doolan et al. (2010)	To combine transcriptomics and proteomics to identify important proteins that relate to high growth rate	Used in-gel digestion for proteins. Used MALDI TOF MS to identify proteins	Identified valosin containing protein as important regulator of cell growth. Overexpression resulted in improved growth with no decrease in viability
Dorai et al. (2013)	To identify proteins that affect high productivity in bioreactor cell cultures	Collected spent media samples for host cell proteins. Used in-gel digestion for proteins. Used LC-MS to identify proteins	Comparison of high and low expressing clones revealed 180 differentially expressed proteins. Identified proteins related to cytoskeletal organization, protein synthesis, metabolism, and growth
Kang et al. (2013)	To identify differences between cell lines that affect antibody production	Used LC-MS/MS shotgun proteomics to identify protein expression differences between cell lines	Identified proteins with positive correlation to productivity, including DHFR, adaptor protein complex subunits AP3D1 and AP2B2, DNA repair protein DDB1, and ER translocation subunit SRPR
Kim et al. (2011)	To determine the effect of hydosylate supplementation on antibody productivity in serum-free cell cultures	Used 2D gel electrophoresis combined with nano LC-ESI-QTOF MS/MS to identify proteins	Found significant changes in protein expression in serum-free medium supplemented with hydrosylates, such as upregulation of metabolic, cytoskeletal organization, and growth regulated proteins
Kuystermans et al. (2010)	To determine the effect of cMyc on proteome	Used in-gel digestion for proteins. Used MS/MS to identify proteins	Found increase in nucleolin and decreased in regulation of proteins related to matrix and cell adhesion. Also found increased ATP synthetase and mitochondrial protein levels

(continued)

Table 19.2 (continued)

Reference	Purpose	Method	Conclusion
Lee et al. (2010)	To identify the proteome of CHO DG44 cell line	Used in-gel digestion for proteins. Used MALDI TOF MS and MS/MS to identify proteins	Improved protein identification by enrichment of medium and low abundance proteins. Most identified proteins function in energy metabolism
Lim et al. (2013)	To identify growth factors that can be used as supplements in serum-free cell cultures to improve antibody production	Used MS shotgun proteomics to identify proteins in spent medium	Identified 290 secreted proteins from CHO cell culture including 8 novel growth factors. Used growth factors as medium supplements to increase cell growth rate
Meleady et al. (2011)	To compare the proteomes of high and low producing cell cultures over time	Used in-gel digestion for proteins. Used LC-MS to identify proteins	Identified 89 proteins with differential expression between high and low producer cell lines
Meleady et al. (2012a)	To determine the effect of miR-7 overexpression on proteome	Used in-solution digestion for proteins. Used label-free LC-MS to identify proteins	Identified 93 decreased proteins and 74 increased proteins resulting from miR-7 overexpression. Decreased proteins related to protein translation and DNA/RNA processing. Increased proteins related to protein folding and secretion
Meleady et al. (2012b)	To improve identification of proteome by using multiple databases	Used in-gel and in-solution digestion for proteins. Used MALDI TOF MS and electrospray ion trap MS to identify proteins	Improved protein identification by 40–50 % through multiple CHO-specific databases
Slade et al. (2012)	To develop a method for identifying secreted proteins	Cell culture medium supplemented with GalNAz and enriched by copper catalyzed click chemistry. Used iTRAQ labeling and MS to identify proteins	Identified differences between CHO-S and CHO DG44 secreted proteins. Found 70 % similarity between cell lines
Van Dyk et al. (2003)	To identify important proteins that affect protein productivity in butyrate and zinc treated culture	Used in-gel digestion for proteins. Proteins identified with MALDI-TOF MS	Identified increased expression of GRP75, enolase, and thioredoxin in response to media supplements

(continued)

Table 19.2 (continued)

Reference	Purpose	Method	Conclusion
Wei et al. (2011)	To identify the proteome of CHO cells during prolonged cultivation	Used in-gel digestion for proteins. Electrospray ionization tandem MS used to identify proteins	After prolonged cultivation, identified 40 proteins with different expression levels related to cytoskeletal proteins, chaperones, and metabolic enzymes

identifying mRNA post-translational processing, thus reducing error (Clarke et al. 2012). The most significantly enriched gene ontology terms were translational elongation, translation, generation of precursor metabolites and energy, oxidation-reduction, and aerobic respiration (Clarke et al. 2012). Combining 'omics strategies improves the confidence in the findings from either data set and provides useful information about the culture conditions that improve growth.

Another study used a combined transcriptomics and proteomics approach to compare cell lines with fast and slow growth (Doolan et al. 2010). Valosin-containing protein (VCP) was shown to have a significant effect on cell growth and viability. This finding was confirmed by silencing VCP expression, which resulted in decreased viable cell density and viability (Doolan et al. 2010).

Proteomics was also used to compare CHO cell lines with and without the cMyc gene, which had been previously shown to improve cell growth rate and concentration (Kuystermans et al. 2010). Over 100 proteins were differentially expressed between cultures. Culture performance improved as measured by the increase in nucleolin (important for growth, preventing apoptosis, protein productivity, and energy utilization) and decreases in regulation of adhesion proteins (Kuystermans et al. 2010). Specific components of ATP synthetase were up-regulated, which may indicate a change in energy utilization to release more ATP in cMyc cultures (Kuystermans et al. 2010). These identified proteins may now be further investigated through hypothesis-driven research approaches.

One way to understand cell death is to elucidate what happens at the transition of cell culture from exponential growth to stationary phase. Comparative proteomics, using iTRAQ labeling, identified 59 proteins with significantly different protein expression levels between the exponential and stationary phases (Carlage et al. 2012). Some of these proteins included binding immunoglobulin protein, protein disulfide isomerase, DNA replication licensing factors MCM2 and MCM5, transglutaminase-2, and clusterin (Carlage et al. 2012). These time points were compared in order to identify proteins associated with cell growth and apoptosis. By classifying differentially expressed proteins, it was found that both growth and apoptotic proteins are expressed highly during the stationary phase (Carlage et al. 2012). Results from this study will facilitate a better understanding of the dynamic changes that occur during the different stages of cell culture and may be

used to prolong cell growth and protein production during the later stages of a bioprocess.

Another proteomics study examined protein samples collected throughout the cell culture process (Wei et al. 2011). This study also identified 40 differentially expressed proteins between exponential and stationary phases (Wei et al. 2011). The results indicate that over time, apoptosis occurs due to the initiation of the unfolded protein response (Wei et al. 2011). Thus, delaying apoptosis can increase protein production. This was validated in other studies including one in which co-expression of the anti-apoptotsis gene Bcl-xL increased membrane protein expression in CHO cells (Ohsfeldt et al. 2012).

19.3.2.2 Proteomics Analysis to Increase Recombinant Protein Production

An important goal in bioprocess development is to maximize the recombinant protein yields. In one study, proteomics was used to identify proteins related to improved protein productivity observed following supplementation of butyrate and zinc sulfate to the culture medium (Van Dyk et al. 2003). Increased expression was observed for metabolic and chaperone proteins including GRP75, enolase, and thioredoxin (Van Dyk et al. 2003). These proteins were thus identified as potential cell engineering targets for improving recombinant protein production.

Another study compared high and low producing cell cultures. Proteomics analysis showed that over 30 proteins were differentially expressed between high and low producing cultures (Carlage et al. 2009). In the high producing cell line, eukaryotic translation initiation factor 3 and ribosome 40S were upregulated, whereas vimentin, annexin, and histone H1.2/H2A were downregulated (Carlage et al. 2009). Additionally, the chaperone binding immunoglobulin protein was upregulated in the high producing cell line to suggest that the unfolded protein response occurs as a consequence of endoplasmic reticulum stress (Carlage et al. 2009).

A combination of proteomics and transcriptomics was used to determine the effect of low temperature and sodium butyrate on recombinant protein production in CHO cells (Kantardjieff et al. 2010). This approach relied on the transcriptomic information to identify hundreds of differentially expressed genes between treatments. From this data, proteomics was used to further identify different protein levels. Butyrate treatment and low temperature were shown to improve recombinant protein production rates by improving cell secretory capacity (Kantardjieff et al. 2010). Enriched pathways included Golgi processing, cytoskeleton binding, and GTPase mediated signal transduction (Kantardjieff et al. 2010).

In more recent years, the development of the CHO genome database has led to improvements in protein identifications. Different cell culture conditions were used for proteomics experiments in order to compare high and low producing cell lines (Dorai et al. 2013). From 180 differentially expressed proteins, 12 proteins exhibited differential expression levels over the culture duration in bioreactors,

including ADP-ribosylation factor protein, V-type proton ATPase, colony stimulating factor 1, and angiopoietin 4 (Dorai et al. 2013). These differentially expressed proteins included functions related to growth, metabolism, organization, and protein synthesis (Dorai et al. 2013), which can be used for process optimization by genetic engineering or media supplementation strategies.

Meleady also investigated differences in protein levels between high and low producing cultures (Meleady et al. 2011). Results identified 89 differentially expressed proteins between the high and low producing cultures (Meleady et al. 2011). In particular, 12 proteins were shown to differ in expression level in the same direction, including aldose reductase-related protein 2, annexin, eukaryotic translation initiation factor, glucose-6-phosphate 1-dehydrogenase, endoplasmin, and nuclear migration protein (Meleady et al. 2011). Proteins that were expressed at higher levels in the high-producing cell line included proteins involved in translation and folding (Meleady et al. 2011), which are associated with recombinant protein production.

Overexpression of miRNAs may also improve process development by regulating protein productivity. A proteomic comparison of a cell line overexpressing miR-7 compared with a control cell line revealed differences in protein expression that contributed to higher productivity in the miRNA-engineered cell line (Meleady et al. 2012a). Overexpression of miR-7 resulted in 93 downregulated proteins and 74 upregulated proteins (Meleady et al. 2012a). Proteins with decreased levels were involved in protein translation and DNA/RNA processing and those with increased levels were related to protein folding and secretion (Meleady et al. 2012a), representing potential cell line engineering targets. In addition to protein production, overexpression of miRNAs can affect other processes such as cell growth and apoptosis (Druz et al. 2011, 2013).

The genetic factors contributing to high productivity of a cell line were studied by maintaining the same process conditions (Kang et al. 2013). Results from proteomics indicated a positive correlation between productivity and expression of dihydrofolate reductase, adaptor protein complex subunits, DNA repair proteins, and the endoplasmic reticulum translocation complex components (Kang et al. 2013). Both transcriptomics and proteomics data were combined in order to improve understanding of the high production phenotype (Kang et al. 2013). Differences in expression suggest important roles for these proteins in high producing clones and targets of opportunity for cell line engineering.

19.3.2.3 Proteomics to Optimize Media Formulations

The growth and productivity of a cell line is highly dependent on the media composition. Media formulation is a key component of process development and methods to adjust the media in order to improve product yields and quality are highly sought. The formulation of cell culture medium can have a significant effect on cell growth, viability, and protein production rates. Proteomics analysis can

provide detailed information about cell protein levels that can aid in subsequent medium development.

Recently, proteomics has been used to profile differences between medium formulations in order to correlate improved cell growth with protein expression levels. In one case, proteomics was used to help identify proteins helpful for adaptation from serum-bearing to serum-free media (Baik et al. 2011). Results indicated that two molecular chaperones and four de novo nucleotide synthesis related proteins were significantly increased in the serum-free cell culture (Baik et al. 2011).

Subsequently, two of the chaperones identified (HSP60 and HSC70) were overexpressed and this increased the cell growth rate up to 15 % and decreased adaptation time up to 33 % (Baik et al. 2011). Thus, quantification of protein levels for different media formulations can provide insights into cell line engineering strategies to improve cell growth and medium adaptation.

The secretome consists of extracellular proteins processed through the secretory pathway. These extracellular molecules are in low abundance, but may be involved in diverse biological processes. In one approach, secreted proteins from conditioned media samples were identified in order to develop a serum-free media formulation (Lim et al. 2013). Supplementation of identified growth factors, such as fibroblast growth factor 8, growth regulated alpha protein, hepatocyte growth factor, and macrophage colony stimulating factor 1, to the serum-free cloning media formulation led to increased cell growth (Lim et al. 2013).

Analysis of the secretome can also aid in the identification of proteins that accumulate in the medium over time. N-azido-galactosamine labeling was used to tag the mucin-type O-linked glycans of secreted proteins in order to enable their identification in cell-conditioned media (Slade et al. 2012). This method helped to identify secreted proteins in low abundance and minimize the number of background proteins (Slade et al. 2012). The secretomes of CHO-S and CHO DG44 cell lines were compared and it was observed that 171 proteins were identified in both cell lines (Slade et al. 2012). Close to 70 % of the proteins identified were the same between the CHO-S and CHO DG44 cell lines (Slade et al. 2012). However, there were also 96 proteins unique to CHO DG44 and 85 proteins unique to CHO-S (Slade et al. 2012). Proteins observed at different levels were related to adhesion, cell growth, and proteases (Slade et al. 2012). Important secreted proteins may be investigated as medium supplements in the future. It is also critical that secreted proteins are identified and removed from the final biotherapeutic drug product.

In another experiment, label-free comparative proteomics was used to identify differences between cells cultivated in serum-free medium formulations with or without hydrosylates (Kim et al. 2011). The changes in protein expression upon addition of hydrosylates, containing blends of peptides, free amino acids, vitamins, and trace elements, helped to explain the increased recombinant protein expression (Kim et al. 2011). Proliferative proteins were upregulated whereas pro-apoptotic proteins were downregulated in the culture containing hydrosylates (Kim et al. 2011).

Proteomics can thus be used to identify differences in protein expression between media formulations and to help optimize formulations for high growth and recombinant protein production. These studies can also help elucidate cell engineering targets as well as potential novel media supplements.

19.3.2.4 Systems Biology

A full characterization of the proteome significantly aids efforts to understand cell physiology through the analysis of metabolic pathways. The complete proteome was recently elucidated for the CHO-K1 cell line (Baycin-Hizal et al. 2012). Analysis of genomics, transcriptomics and proteomics data at the systems biology level using KEGG pathway analysis revealed that pathways for protein processing and apoptosis were enriched in CHO-K1, whereas pathways for steroid hormone and glycosphingolipid metabolism were depleted (Baycin-Hizal et al. 2012). The complementary glycoproteomics analysis elucidated major cell adhesion and membrane proteins, which are difficult to identify with intracellular proteomics approaches. Furthermore, Baycin-Hizal et al. used both genomics and proteomics information to generate the codon usage preference tables for CHO cells.

In another experiment, various databases were used to improve the confidence of identified CHO cell proteins (Meleady et al. 2012b). By using multiple databases, it was possible to increase the number of identified proteins by over 40 % (Meleady et al. 2012b). Different methods were used across locations and the results were compared to improve confidence. These studies are facilitating the development of a reliable proteomic profile of CHO cell physiology going forward.

19.3.3 Database Development

In recent years, CHO-specific databases have been developed to improve sequence information availability for protein identification. Prior to this, CHO-specific sequences were not publicly available, requiring researchers to rely on cross-species information. Following the completed genome of the CHO-K1 cell line, the draft Chinese hamster genome was published (Xu et al. 2011; Brinkrolf et al. 2013; Lewis et al. 2013). The published data sets from these genomes served to provide unifying information about the genes and variations between CHO cell lines. Such genomic data sets are now available online and have been compiled into databases such as www.chogenome.org as shown in Fig. 19.4. Similar efforts have been made to establish large-scale proteomic databases for CHO cells, including http://chogenome.org/proteome.php, as shown in Fig. 19.5. This database can be used to find detected proteins and associated accession numbers which includes 6,163 entries. The proteins of interest can be found either by protein name or the accession number, as reported in the work by Baycin-Hizal et al. 2012. Upon clicking on the accession number, users can find

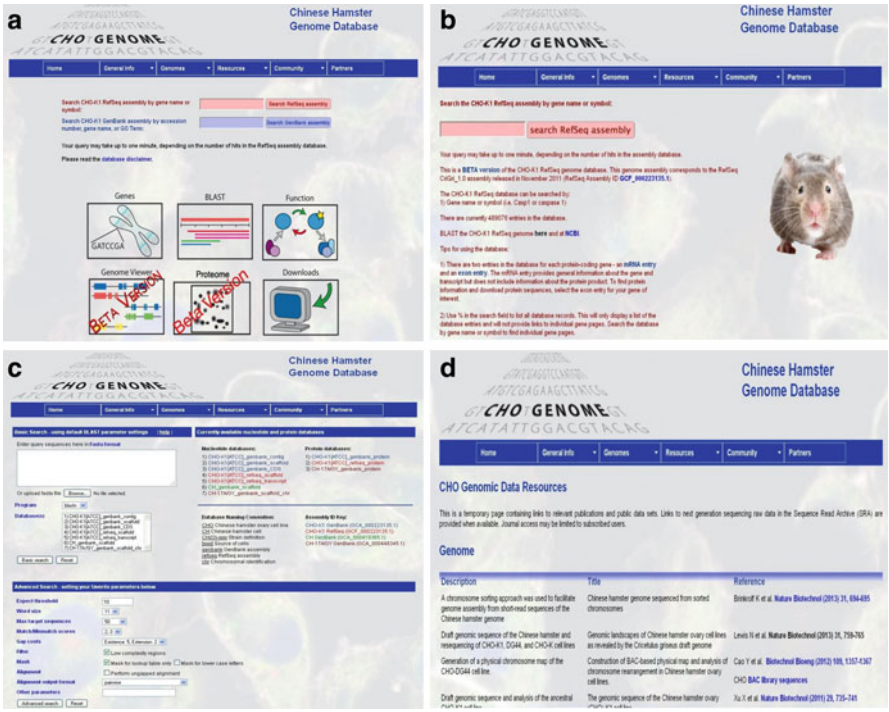

Fig. 19.4 Screenshots of www.chogenome.org, (**a**) shows the home page of the website with links to various other pages, (**b**) shows the genes page where user can search the RefSeq assembly, (**c**) shows the BLAST tool implemented in the website using which user can run CHO specific BLAST queries, and (**d**) shows the webpage from where user can download public datasets and can find links to the relevant publications

more detailed information on the webpage, such as the protein's SwissProt annotation, GO annotation, Kegg annotation, identified peptides sequences, false discovery rate, and more (Baycin-Hizal et al. 2012). The widespread accessibility and ease of use makes the CHO genome website a useful tool for proteomics analysis.

An evaluation based on the CHO databases increased protein identification by 282 proteins, a 40–50 % increase in the total number of proteins identified at that time (Meleady et al. 2012b). CHO sequence information was combined from the SwissProt database, CHO-K1 draft genome (Xu et al. 2011), and the Bielefeld-BOKU-CHO database (Meleady et al. 2012b). Because more peptides matched, there was increased confidence in the results. The increased proteins identified were related to protein translation and energy metabolism, both important for bioprocess development (Meleady et al. 2012b). The compilation of data sets from proteomics experiments around the world allows for data sharing and database generation. Multiple groups can use data to improve proteomics results.

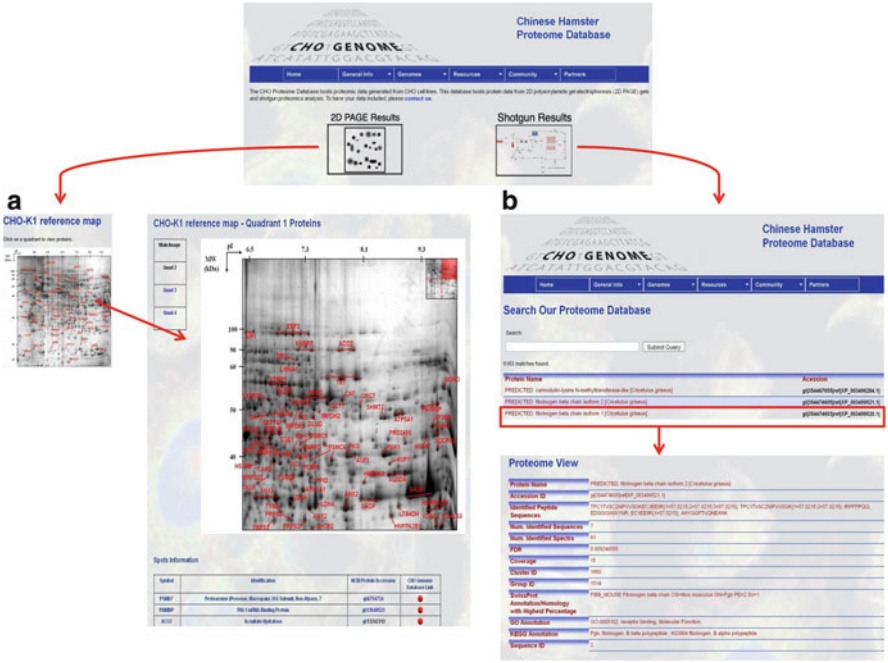

Fig. 19.5 An example output for CHO proteome portion of the www.chogenome.org website. It provides identified proteins in CHO-K1 proteome by 2D polyacrylamide gel electrophoresis as well as by shotgun proteomics. (**a**) Example output for CHO-K1 gel and identified proteins. (**b**) The protein name and accession number after searching for a protein name which can be used to view experimental details about the corresponding protein

19.3.4 Combined Metabolomics and Proteomics

In addition to combined genomics and proteomics or transcriptomics and proteomics, there have recently been examples of combined metabolomics and proteomics. In this case, protein identification confidence increases as protein expression is correlated to changes in metabolite pools. A combination of proteomics and metabolomics was used to increase understanding of CHO cells during prolonged cultivation (Beckmann et al. 2012). The observation that prolonged culture results in increased cell growth was related to an increase in adenylate energy charge (AEC) and differential protein expression (Beckmann et al. 2012). The increased AEC relates to a high energetic state of the cell. Additionally, 43 differentially expressed proteins were identified (Beckmann et al. 2012). Some of the proteins, including phosphoglycerate mutase, phosphoglycerate kinase and pyruvate kinase isozymes, were associated with the improved growth rate (Beckmann et al. 2012). Other differentially expressed endoplasmic reticulum stress proteins were related to cell robustness to changing environmental conditions (Beckmann et al. 2012). The

combined approach was useful for determining correlations between the different protein and metabolite levels and the observed cell phenotype.

Conclusions

Current developments in biotechnology rely on the wealth of information provided by the genome, transcriptome, proteome, and metabolome. Protein levels provide clear information about cell physiology; thus, proteomic methods are important for hypothesis-driven research and development. In recent years, sample preparation methods for proteomics have been optimized in order to obtain the maximum number of correct protein identifications. This has enabled more developed databases and identification of proteins localized to cell membranes, the cytoplasm, or other organelles. A variety of different digestion techniques and proteomics techniques have been developed for CHO proteomics. In addition to this, a variety of labeling techniques such as SILAC, iTRAQ and TMT have been used to study differences between cell lines and analyze important pathways such as apoptosis, growth, and protein production. In bioprocess development, applications of proteomics include identification of accumulating or depleting protein levels important in recombinant protein production. From this information, cell line engineering approaches or medium formulation developments help to increase cell growth, prevent apoptosis, improve recombinant protein productivity, or enhance utilization of nutrients. Thus, 'omics approaches enable research that delves deeper into the workings of the cell and improve its performance for biotechnology applications.

In summary, proteomics has followed genomic investigations and surged to the front of analytic methodologies to characterize and drive strategies for improving mammalian, and especially CHO, cell performance. This information will help to improve the manufacture of biotherapeutics at large scale and with high product quality, lowering operating costs and ultimately reducing overall health care costs. In the future, proteomics analysis will become a standard yet essential element in the arsenal that biotechnologists use for understanding and optimizing bioprocesses.

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Chapter 20
Metabolomics in Animal Cell Culture

Miroslava Čuperlović-Culf

Abstract Metabolomics is defined as a global quantitative assessment of metabolites within a biological system. Metabolic profiling of cell cultures has many potential applications as well as advantages to currently utilized methods for cell-line testing. Metabolite concentrations represent sensitive markers of genomic changes and responses of cells to external stimuli. Effects of drugs or toxins on cell cultures can be observed through the changes in metabolite concentrations. When cell cultures used for production of various biomolecules metabolomics can aid in optimization of cell growth. Metabolomics can also be used as a method for routine monitoring of extracellular metabolic changes in real time measurements. Nuclear magnetic resonance spectroscopy and mass spectrometry are major analytical platforms used for metabolomics measurements. These methods provide detailed, non-biased and highly complementary chemical analyses of metabolic changes within cells (fingerprint) and in excreted metabolites (footprint). This chapter provides review of current applications of metabolomics in cell cultures with an overview of experimental and data analysis methodologies.

Keywords Metabolomics • Nuclear Magnetic Resonance (NMR) • Mass spectrometry (MS) • Phenotype analysis • Metabonomics • Production systems optimization • Feature selection • Qualitative data analysis • Quantitative data analysis • Unsupervised analysis • Supervised analysis

20.1 Introduction

Metabolite can be defined as any substance produced by metabolism or by a metabolic process. Strictly, metabolites are compounds found in cells, recognized and acted upon by enzymes, with products that have to be able to enter subsequent reactions. Metabolites have to have a finite half-life and they should not accumulate in cells. Finally, metabolites must serve some useful biological function in the cells including regulating the pace of metabolism (Harris et al. 2003). At the same time,

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small molecules found in extracellular medium as well as by-products of drugs or nutrients can have significant influence on biological systems and need to be considered. Hence, in a broader definition, metabolome comprises all molecules in a biological system, other than proteins, DNA and RNA that can be sampled or profiled in different parts of the system, at various time points and/or following various stimulations. Metabolomics is generally defined as the study of the repertoire of these small molecules in biological systems. Similarly to the related fields of genomics, transcriptomics, proteomics etc. Metabolomics is a data driven approach where measurement is performed on all (or as many as possible) molecules and hypotheses are derived from the data. Metabolomics holds the greatest potential in the analysis of biological systems for a number of reasons. Metabolites show the phenotype defining processes that have actually happened. Small changes in the activity or expression of a gene or a protein often create a much larger change on the metabolite level. Finally, metabolite changes are directly responsible for cell and tissue behavior whilst also influencing the proteome and transcriptome. On the analysis side, metabolomics does not require specific instrumentation; instead it utilizes classical analytical chemistry techniques. Metabolomics analysis is also the least costly of all omics measurement. Furthermore, analytical methods can be easily transferred between organisms and species and results can be directly compared over different conditions and cell types. Hence, metabolomics provides major data about the system and the phenotype defining biological processes giving information that is in many ways superior to information derived from other omics data. As a conformation, it has been shown recently on bacterial model system that enzyme concentrations cannot explain metabolic fluxes (Chubukov et al. 2013) due to the major role of allosteric regulation and enzyme modifications. Instead, metabolic flux and metabolic processes can only be derived from measurement of metabolite concentrations.

Metabolic flux analysis can provide information about specific metabolic reactions. Metabolomics, however, does not deal with a specific reaction. Instead, it provides high-throughput information about metabolites' concentrations resulting from activity of the whole metabolic network. Because of that, metabolomics does not require extensive prior knowledge of the structure and regulation of metabolic pathways of investigated systems and can be used to monitor transient metabolic conditions (Chrysanthopoulos et al. 2010). In the studies of effect of different substances on cells, metabolic profiles provide direct measure of changes anywhere in metabolism.

Analysis of groups of metabolites in cells, tissues and biological fluids was performed long before the sequencing of DNA and the start of the era of high throughput analysis of biomolecules – "omics". But, in late 1990s metabolic profiling has been combined with chemometrics analysis and systems biology leading to establishment of metabolomics and metabonomics areas. There is a semantic as well as an application difference between *metabolomics* and *metabonomics* as well as more recently introduced *fermentonomics*. Metabolomics (Fiehn 2001; Oliver et al. 1998) is the study of an extensive collection of metabolites present in a cell, tissue or organism under certain conditions generating a biochemical profile. Metabonomics (Nicholson et al. 1999) represents the same

profiling but in response to some stimulation (e.g. drug, toxin, genetic defect) leading to the prediction of metabolic pathways induced by stimulation (Kuchel 2010). Fermentonomics is metabolomics analysis applied specifically to exploring mammalian cell cultures feed components (i.e. extracellular medium) for cell culture optimization (Bradley et al. 2010). These terms are nowadays often used interchangeably and in this text metabolomics will be used to represent all three. In this chapter we will view metabolomics as an area of science (rather than an analytical approach) that characterizes a metabolic phenotype under a specific set of conditions (including exposure to treatments and different feeding regimes), which link these phenotypes to their corresponding genotypes (Khoo and Al-Rubeai 2007; Villas-Boas et al. 2005).

Modern metabolic profiling, i.e. metabolomics, has similar aims as the metabolic profiling of the past with some significant innovations. Firstly, technologies used for metabolite profiling nowadays allow the determination of many more metabolites from dissimilar chemical groups in large number of samples quickly and accurately while striving towards analysis of all metabolites present in the system (although as of today this is still not technically possible). Methods such as Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS) and related Liquid chromatography–mass spectrometry (LC/MS), Gas chromatography–mass spectrometry (GC/MS), Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) are available in many research centers and can be used for metabolomics analysis. Secondly, superior computing power is now readily available and has made possible to collect and organize data from a larger number of samples followed by highly advanced statistical and informatics analyses. Thirdly, interest in observing biological processes on a system level and availability of other types of high throughput molecular data made information about metabolic profiles an important component in this "polyomics" analysis.

Metabolomics is being fruitfully used for the analysis of biological system's response to various conditions with applications in agriculture, industrial biotechnology, medicine or environmental sciences. With many applications of cell lines in all areas of life science research and development, metabolomics analysis of cell cultures also has numerous possibilities.

Cell cultures determine complex, controlled conditions where cells are grown outside of their natural environments. Culturing of animal, plant, fungal and microbial cells is possible although "cell culture" usually refers to growing of specific cells from multi-cellular animal. Cell culture metabolomics has been used for optimization of cell growth and increase of cells' productivity, investigation of cellular responses to stimulants, drugs or toxins or genetic changes as well as investigation of cellular metabolism in distinct species and phenotypes. Any type of cell culture, from any species can be tested using metabolomics analysis. At the same time any experimental and analytical method used for metabolomics can find its place in cell culture analysis. Cell line measurements of metabolites can be directly correlated with genomics or proteomics data. Metabolomics data independently or in conjunction with other information can be used for the development of models of biological pathways and networks. Such a vast number of possible

applications cannot be covered fully in one chapter. Instead, we will provide a brief outline of some published applications of cell culture metabolomics with outline of currently utilized procedures for metabolomics experimental and data analysis as applied to mammalian cell cultures.

Major steps in a metabolomics experiment are well established although novel applications and methods for the analysis of cell culture metabolic profiles are still under fast development (Fig. 20.1). Overall, steps in a metabolomics analysis are the same regardless of the sample source although cell culture metabolomics analysis has some specific applications, unique possibilities in experiment design and some specific features in sample processing. Cell culture metabolomics also opens up some unique possibilities in Data Processing and Analysis and Result Validation. Measurement methods are the same regardless of the source of metabolites and thus will not be explored here in any detail. In the following we will follow items shown in Fig. 20.1 and point out general approaches and specific features in cell culture applications.

Metabolomics of cell cultures can provide valuable information in all areas of cell culture application. Most examples of metabolomics use were thus far in the mammalian cell cultures and in the areas of cancer research and cellular bioreactor production. The primary focus in this text will be on these areas, with applications of metabolomics in cultures of mammalian cell and with majority of examples from

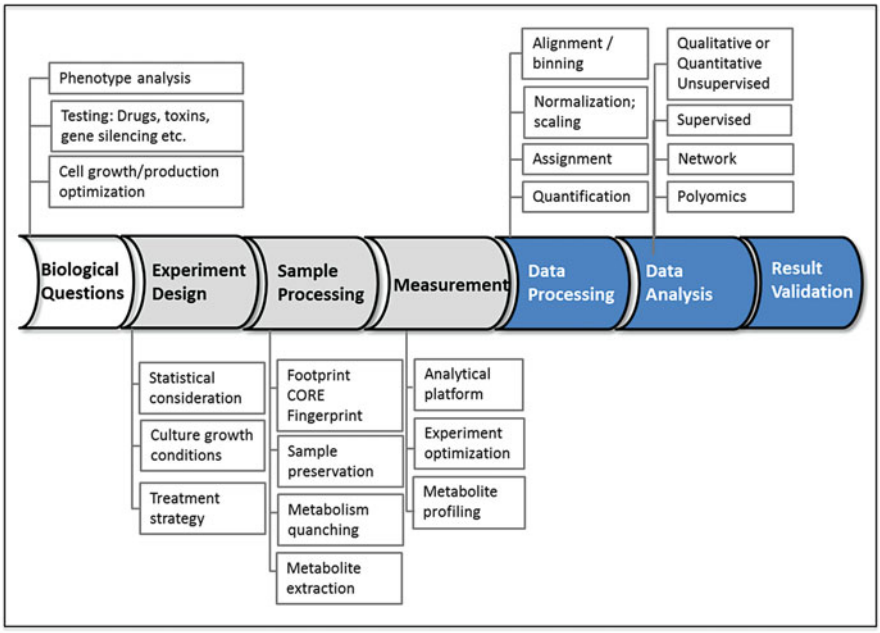

Fig. 20.1 General items (*steps*) in a metabolomics experiment. Three groups of steps including biological question development (*white*), experimental methods (*grey*) and data analysis (*blue*) should be performed in close collaboration

cancer research and cellular bioreactor optimization. It should be kept in mind however, that several extremely interesting examples of cell culture metabolomics use in other systems such as the analysis of bacterial (Villas-Boas et al. 2006) and yeast (Allen et al. 2003) cultures have also been presented.

20.2 Biological Questions

Cell culture provides a controlled model of functions and characteristics of a specific cell type. With this role in mind, metabolomics analysis of cell cultures offers a unique view on the action and behavior of a particular cell type under different conditions. Cells can additionally be synchronized and controlled for homogeneity, neither possible when studying whole organisms. They can be exposed to highly specific conditions and variety of targeted or non-targeted treatments where metabolomics can be used to quickly obtain molecular level information. With these different options there are many possible applications and questions that can be answered with metabolomics analysis of cell cultures. Application areas published thus far are listed in Fig. 20.1 with many examples of each application shown in the following.

20.2.1 Phenotype Analysis

Variety of distinct phenotypes is represented by already established cell cultures including healthy cells from numerous of tissues and organs as well as many representatives of diseased cell states. Analysis of distinct cellular phenotypes can be done for different reasons from basic investigation of phenotype characteristics all the way to more directly applied goals such as discovery of diagnostic markers. Metabolomics as the closest molecular representative of the cellular phenotype (Dunn 2008) can find its place in all of these highly diverse applications.

The first cultured cancer cell line – HeLa – has been derived over 60 years ago (Scherer et al. 1953). Since then, cell cultures from numerous types and subtypes of cancers have been developed. Not surprisingly, cell culture metabolomics has been used extensively in cancer research. With an increasing understanding of the significance of the altered metabolic phenotype in tumors' development and progression, metabolic profiles have become crucial pieces of information. Understanding of cancer metabolic phenotype development and its relation to other hallmarks of cancer has potential in diagnostics and in drug discovery. Establishment of possible metabolic markers of cancers and cancer subtypes is highly important in devising novel biomarkers for diagnosis and treatment planning and follow-up.

Large majority of metabolomics studies of cancer and cancer metabolic phenotype have been performed on cell culture models. Some notable, early examples of comparative metabolic analysis of cell cultures for cancer diagnostics is the

analysis of primary glioblastoma cultures presented by Florian et al. (1995, 1997). In this early work authors used statistical methods to determine major metabolic differences between brain tumour subtypes. Focusing on truly metabolomics applications one of the earliest examples have been the analysis of breast cancer cells relative to normal human mammary epithelial cell lines using NMR, GC/MS and isotopic labelling (Yang et al. 2007). Several authors (Rainaldi et al. 2008; Milkevitch et al. 2005; Halama et al. 2011; Griffin and Shockcor 2004) have shown a very interesting application of NMR metabolomics of cell culture in determining metabolic differences between apoptosis and necrosis in cells as well as determining metabolic markers of apoptosis. Aspartate and glutamate changes have been indicated as early markers of apoptosis however with many different factors that can lead to changes in concentrations of these two amino acids further work is necessary before they can be considered biomarkers. Other authors have used NMR metabolite profiling to get biomarkers of apoptosis in different types of cells. In HL-60 cells increase in fructose 1,6-biphosphate, as well as increase in mobile lipids and decrease in choline, glutathione and taurine; in CHO-K1 decrease in phosphocholine; in DU145 cells increase in mobile lipids and SHOV-3 and OVCAR-3 cells decrease in glucose uptake were indicative of cell death through apoptosis (Williams et al. 1998; Milkevitch et al. 2005; Rainaldi et al. 2008; Egawa-Takata et al. 2010). Chong et al. (2011) have identified oxidized glutathione, AMP and GMP as apoptosis-inducing metabolites. Clearly, with different metabolites determined as apoptosis markers across distinct cell cultures there is still need to better determine relationship between metabolic network and apoptosis and also further establish standard protocols for experimentation and analysis.

Majority of previously mentioned cancer cell culture metabolism studies explored only the intracellular metabolic profiles. Recently, Jain et al. have provided an extensive analysis of extracellular metabolites and the use of metabolites by different cancer cells included in the NCI60 cell panel (Jain et al. 2012). In this work authors used LC/MS to create cellular consumption and release (CORE) profiles for 219 metabolites representing major pathways of intermediary metabolites. Analysis was done on NCI60 cell panel – collection of 60 primary human cancer cell lines derived from nine common tumor types (Shoemaker 2006). CORE profiling provides quantitative information about the cellular metabolic activity by showing consumption of metabolites from the extracellular medium. CORE investigation has shown several metabolites that are specifically used or produced by particular cancer types. Also, this study has shown metabolic pathways that are significant for survival of highly prolific cancer only. CORE data set is publicly available and will be used in this chapter to illustrate some data analysis procedure in Sect. 20.6.

Cells in the in vivo cell culture can be synchronized allowing investigation of metabolome at particular stage of cell growth rather than, usually observed, average pool of heterogeneous cell mixtures. Maddula and Baumbach (2010) have measured metabolome of synchronized colon cancer cell cultures. From the obtained metabolic concentrations authors have proposed that during G1/S transition of the cell cycle energy comes mainly from glycolysis. During the late S phase majority of energy was produced through glutaminolysis by channeling of glutamine towards

lactate through reverse TCA cycle. In the S phase glucose and glycolysis was mostly used to produce precursors for biosynthetic pathways. This type of analysis provides highly relevant information and is possible only in cell cultures.

Work in cell cultures has also allowed control of use and production of gases (for example oxygen) while studying changes in both volatile and non-volatile metabolites. Frezza and co-workers (2011) have used controlled oxygen environment for cancer cell culture growth in studying hypoxia. Authors combined biochemical, microscopic and metabolomics analysis of colorectal cancer cells (line HCT116) growing under reduced oxygen conditions. The metabolic profiles of cells grown in normoxia and hypoxia are significantly different. Metabolomics analysis has unveiled shift towards catabolic reactions induced by hypoxic conditions. Furthermore, under hypoxic conditions, in addition to glycolysis, autophagy appears to play a significant role in supporting ATP production. Recently Tian et al. (2013) explored the effect of hypoxia on intracellular metabolic profiles of pancreatic β-cell line INS-1, in order to determine potential biomarkers for the prediction of hypoxia-induced cell death. 1H NMR metabolomics have shown changes in creatine-containing compounds at the early stage and taurine-containing compounds following prolonged oxygen deprivation. This information can lead to biomarkers of hypoxic β-cell damage following for example islet transplantation.

Diagnostic applications of metabolomics have largely been focused on urine and blood samples as easy, clinically available patient samples. In the clinical application these samples are certainly advantageous in spite of difficulties in interpreting the actual causes for observed changes. However in some cases such as cancer or infectious diseases, specific cell analysis is highly relevant and often can be the only way to provide accurate diagnosis. In these cases cell cultures provide a good model system for testing observable differences between different phenotypes that can subsequently be explored on the organism level. Comparative metabolomics of different cell cultures does not suffer from possible errors due to confounding factors. Although it cannot directly, without further validation, provide diagnostic markers it can certainly suggest possible markers for investigation on clinical samples. As an example of such effort in our group we have combined 1H NMR metabolomics with gene expression data to determine molecular differences between glioblastoma cell lines, providing possible metabolic biomarkers of distinct subtypes (Cuperlovic-Culf et al. 2012) that will be validate on clinical samples in the future. Several works on breast cancer cell lines have shown metabolic difference between subtypes. General metabolomics analysis have shown difference between ER+ and ER- cells (Cuperlovic-Culf et al. 2011). Metabolomics profiling of lipids (lipidomics) have shown significant changes in breast cancer cell lines and human mammary epithelial cells. Phosphatidylethanolamine content was relatively highest in non-malignant cells, while phosphatidic acid showed higher relative abundance in metastatic cells (Doria et al. 2012, 2013). Phenotype analysis by metabolomics of cell cultures provides valuable leads for further assessment, testing and validation on tissues ex vivo or in vivo.

20.2.2 Testing: Drugs and Toxins

Changes in the cellular metabolome resulting from the biological activity of a drug can provide information about direct as well as indirect effects of drugs. Metabolomics can thus help in determining drug target as well as off-target effects and significance or effectiveness of a drug molecule in particular phenotype. Metabolic profiles can also indicate possible metabolic transformation of a drug by different cell types. Finally, metabolomics can provide markers for follow-up analysis of drug response. Varieties of cell cultures that are currently available allow controlled testing of the effect and metabolism of drugs or toxin in models of different human tissues, there-by adding important data to explorations done in animal models. Metabolomics addition to drug discovery is expected to have significant influence in structure-pathway-activity analysis and to allow dosage and metabolic effects characterization (Cho et al. 2006; Khoo and Al-Rubeai 2007). In fact, USA FDA has already acknowledged the need for utilization of omics and particularly metabolomics methods in manufacturing and clinical trials of drug production through cell cultures – biologics (Kozlowski and Swann 2006).

Almost two decades ago El-Deredy et al. (1997) have presented a very interesting application of high throughput metabolic profiling using NMR for cell culture testing of drug effects. This early publication has shown that NMR metabolic profiling combined with pattern recognition analysis can be a useful pre-treatment predictor of drug response. Since this early example, many more advanced data analysis and metabolomics experimentation methods have been introduced, leading to more innovative applications of cell culture metabolomics in drug testing.

Tiziani et al. (2009) have used NMR metabolomics to measure changes in acute myeloid leukemia (AML) cells in response to bezafibrate (BEZ) and medroxyprogesterone acetate (MPA) treatments. The downstream effect of reactive oxygen species generated by the action of these drugs was observed through altered concentrations of Krebs cycle metabolites and pyruvate. These observed changes correlated with the metabolic changes caused by exposure of cell extracts to H_2O_2. Qualitative analysis presented in this work also shows differences in metabolome between AML subtypes as well as cells treated by MPA or BEZ alone or in combination.

Metabolic effects of the cancer cell culture treatment with a standard chemotherapy agent – cisplatin – were analyzed by Pan et al. (2011). Authors were able to show quantitative changes in uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) in glioblastoma cells treated with cisplatine while at the same time determining general metabolic changes from metabolomics NMR analysis. This analysis performed on cell cultures has significant implication in treatment follow-up using UDP-GlcNAc.

Metabolomics was also used to investigate the effect of acyclic retinoid (ACR) on hepatocytes and hepatocellular carcinoma (Qin et al. 2013). ACR is a chemical derivative of retinoid that has been shown as a promising chemo-preventative agent for hepatocellular carcinoma. Results of combined NMR and CE-TOFMS metabolomics analysis have suggested that ACR may suppress the enhanced energy metabolism of hepatocellular carcinoma cells without affecting normal hepatic

cells. Suggested avenue for this effect is through cancer-selective enhancement of pyruvate dehydrogenase kinase, isozyme 4 (PDK4). Metabolomics has in this case provided important clues about the function of ACR and also possible leads for combined therapy.

Bioactive natural products often target several different cellular processes and thus metabolomics analysis, showing an overview of the whole system, can provide very interesting, unbiased clues about their overall activity. Massimi et al. (2012) have shown an example of metabolomics analysis of the effect of resveratrol on human hepatoblastoma line, HepG2 cells. Resveratrol, a polyphenol found in several plants displays multiple protective and therapeutic effects. Metabolomics foot-printing have shown that cells treated with resveratrol experience metabolic shift from preferentially glucose and amino acid utilization to fat utilization for the production of energy. Authors have suggested that this effect can possibly come through the inhibition of sirtuin class of histone deacetylases. However, this effect could be cell type specific and thus still need to be further investigated on different cells.

Metabolomics, as a fast and inexpensive method can be very useful in high throughput testing of drug libraries and/or testing of drug effects on library of cell cultures. Tiziani et al. (2011) have presented an interesting methodology for application of NMR metabolomics in high-throughput drug testing. In their approach metabolic changes were observed in combined extra- and intracellular extracts following treatment with drugs from a large library of compounds. Authors have shown utilization of this method for testing of a library of kinase inhibitors; however this approach can be applied to testing of any drug library. NMR based-screening of the metabolic response to drug treated cells in a 96-well format can be automated and made extremely fast. Obtained measurement of overall, combined extra- and intracellular metabolic changes following treatment is a good indicator of drugs that are inducing observable metabolic shift and that need to be further explored in more detail. This method can be used for screening large number of individual or combinatorial drug libraries on different immortalized cell cultures as well as possibly primary cell cultures.

Drug resistance is another significant problem in disease treatment that is at least in part affected by cellular metabolism and that can be monitored by metabolomics. Metabolomics analysis and comparison of resistant and treatment susceptible lung cancer cell lines was presented by Gottschalk and Ivanova (2008). Authors have identified metabolites that show significant difference between resistant and susceptible cell lines suggesting possible markers of resistance. Cao et al. (2013) used metabolomics for the analysis of pharmacodynamics of drug Adriamycin and analysis of the markers of resistance to this drug in breast cancer cells. Authors have shown significantly different metabolic response to the drug treatment in sensitive and resistant cell lines. Greater metabolic shift was observed in drug sensitive cells. From metabolomics analysis authors were able to propose an avenue for the action of this drug. This paper thus shows another example of significant power of metabolomics as a tool for screening drug candidates.

Addition of molecular profiling technologies such as metabolomics, to toxicity testing in novel cellular models provides possibility for the determination of both

toxic agents, their modes of action as well as biomarkers of toxin exposure. Toxicity testing in human derived cell lines allows screening of potentially toxic chemicals that does not require cross-species corrections, does not raise ethics issues and can be done at a low cost. Toxicity testing can in this scenario be performed on cells originating from different tissues under possibly distinct conditions and media exposure. Extracellular or intracellular metabolomics analysis provides an excellent way to pin point the cellular pathways that are directly or indirectly affected by a toxin. A very interesting example of metabolomics, cell culture toxicity study was presented by Ellis et al. (2011). Metabolomics profiling of cells following exposure to low concentrations of toxins allowed determination of affected pathways. Authors have hypothesized that if treatment with low dose of tested compound causes perturbation of multiple metabolic pathways compound is likely toxic to the examined cell culture. Furthermore, if compound leads to increased proliferation and survival of cells while at the same time changing mitochondrial metabolism it can be expected to be carcinogenic. Therefore, cell culture metabolomics can be the first, controlled, fast and inexpensive screen for compounds' general or organ specific toxicity as well as carcinogenesis.

20.2.3 Production Systems

Cells are becoming important hosts for the production of biopharmaceuticals or biofuels. Improved proteins, vaccines or metabolites production by cell cultures is expected to both lower production cost and increase flexibility (Bradley et al. 2010; Dietmair et al. 2012b). Cell cultures provide highly promising systems for faster and more flexible vaccine production (reviewed in Thompson et al. 2013); monoclonal antibody generation (reviewed in Li et al. 2010) and biologics and biosimilars (Zhou and Kantardjieff 2014). Either large-scale, fed-batch or high-density perfusion cultivations require optimization of conditions for highest productivity and consistent product quality. Mammalian cell culture monitoring has been traditionally based on a small set of variables including growth rate, cell density and viability, product quality and production rate, substrate consumption and lactate production (Goudar et al. 2009; Vernardis et al. 2013). Bioreactor monitoring and process improvements are mostly based on cell growth, metabolic activity for few major metabolites (e.g. lactate or ammonia) and productivity. Quasi real-time metabolic flux analysis has been suggested as an additional approach for more robust characterization of the cellular physiological state (Goudar et al. 2006; Konstantinov 1996). Global perspective awarded by metabolomics analysis has great potential in optimizing the productivity and growth of these systems (Chrysanthopoulos et al. 2010).

Khoo and Al-Rubeai (2009) have shown one of the first uses of metabolomics towards relating physiological state and productivity of mammalian cells in biopharmaceutical production. NMR metabolomics was utilized to characterize hyperproductive state of NS0 myeloma cell line producing antibodies, striving towards

development of cell state with consistent higher productivity. The hyper-productive state was achieved by arrest in proliferation of cells. Results have indicated that fatty acid degradation, rather than glucose, was a main source of energy in these cells in proliferation arrested state (Khoo and Al-Rubeai 2009).

Vernardis et al. (2013) have developed method for time course analysis of cell metabolism in the reactors using GC/MS method. Time course analysis provides valuable information about the physiology of cells in any cell culture production scale. In the production chain time course metabolomics analysis can provide early warnings about possible problems in the manufacturing process. In the work of Vernardis et al. analysis was performed on intracellular metabolites with 68 metabolites quantified using GC/MS data. From this analysis authors have observed cell age related metabolic changes independent of bioreactor scale, suggesting this methodology as possible approach for monitoring cell physiology in perfusion cultures and as method for monitoring effects of bioreactor operating conditions.

Currently, Chinese hamster ovary (CHO) cells are the major producers of biopharmaceuticals particularly due to their ability to ensure correct folding and post-translational modifications of protein products (reviewed in Kildegaard et al. 2013). Metabolic characterization of CHO cells was initially focused on the profiling of the culture medium (Chong et al. 2009, 2010, 2011; Bradley et al. 2010) with intracellular metabolic profiling introduced only recently (Selvarasu et al. 2012; Dietmair et al. 2012a). Metabolomics analysis has been used to design optimized feeding regimes towards enhancing recombinant proteins production (Sellick et al. 2011a), identifying metabolites, from studies on different feeding media, that correlate with growth rates and maximal cell density (Dietmair et al. 2012a; Zang et al. 2011) and also to determine possible targets for gene engineering that can reduce bottlenecks in production (Chong et al. 2010). Apoptosis inducing metabolites have also been determined in CHO cells, specifically oxidized glutathione, AMP and GMP (Chong et al. 2011).

Metabolomics has been used to determine optimal feeding regimes for enhanced recombinant protein production in by CHO cells (Sellick et al. 2011b). Metabolites associated with high monoclonal antibody productivity in CHO cells have also been investigated (Chong et al. 2012). Cellular growth rate and maximal viable cell density was also correlated with metabolite profiles of media (Dietmair et al. 2012a). In CHO cells metabolomics was used to identify possible metabolic engineering targets such as enzyme bottleneck of TCA cycle – malate dehydrogenase II (Chong et al. 2010). Through overexpression of malate dehydrogenase II authors were able to increase cellular ATP and NADH concentrations and to improve integral viable cell number.

Amongst many factors that can be varied during culturing of cells is the temperature. Wagstaff et al. (2013) have shown that NMR metabolic profiling of extra- and intracellular metabolites shows significant changes as a response to cold-shock and subsequent recovery when returning to normal temperature. In addition to observing interesting temperature effects, in this example authors have shown how NMR metabolomics can be used as a method for routine monitoring of extracellular metabolic changes. Analysis of extracellular medium can be performed in a flow-through regime providing real time measurements.

Non-mammalian cultures are also extensively used in production and testing and metabolomics can certainly find its place in these applications as well. In a recent example presented by Creek et al. (2013) metabolomics have been used as a rational, quantitative method for simplification and optimization of cell culture medium for drug screening against parasite Trypanosoma brucei. In this example analysis of fresh and spend media revealed major nutritional requirements and allowed authors to significantly simplify medium while providing even better growth of the parasite culture.

Metabolomics measurement, particularly NMR of extracellular medium can provide very fast, inexpensive method for determination of metabolic profiles for approximately 50 small molecules in the medium. This measurement can help in determining excesses, redundancies or shortages that cells experience. It can also provide information about production and accumulation of possible cell toxins in the medium. Several authors (Bradley et al. 2010; Aranibar et al. 2011) have provided a rapid and robust NMR method for monitoring mammalian cell cultures involving separation of extracellular medium, buffering and referencing. This rapid measurement method can be added to any cell culture protocol for monitoring cell growth, age, toxin production or possible lack of nutrients.

20.3 Experiment Design

General idea of cell metabolomics experiment is very straightforward: (a) cells need to be grown under controlled conditions (with or without treatments), (b) sufficient replication has to be performed; (c) sample preparation has to be done using well defined, standard operating procedures; and (d) measurement has to be accomplished using the best available method with sufficient referencing. Control of the cell growth conditions can be achieved and therefore major confounding factors often threatening accuracy of metabolomics analysis in organism or populations can be avoided. In order to avoid possible technical differences and errors, experimental design must still incorporate randomization, replication and local control in a manner similar to approaches developed for clinical trials.

Randomization protects an experiment against extraneous factors of chance, such as different person performing experiments, variations in the temperature or humidity in the room etc. It is crucial to understand all possible sources of variability and minimize their effects by randomizing the samples accordingly and by introducing quality control standards.

Replication is utilized in order to increase statistical accuracy of the results. It is crucial to include biological replicates and, also if possible, technical replicates. Technical replicates represent different aliquots of a sample or of a batch of samples. Technical replicates show the consistency of the experimentation. Cell culture experiment has to include several repeats of growth and treatment for all explored cell types (biological replicates). The required number of technical and biological replicates depends on the variability between samples, the expected

range of variability, variance between observed groups (window of effect) and the power of the test being performed. The number of replicates will therefore need to be determined for each specific experiment from preliminary measurements.

Finally, *local control* requires that the known sources of variability are either completely removed (if possible) or are deliberately made to fluctuate widely (by collecting many samples with large coverage of difference sources of variability) so that their effects can be measured and eliminated, i.e. averaged out, from experimental error.

A major attention in the cell culture metabolomics experiment should be placed on the selection of culture growth conditions and medium. Cell growth conditions and medium can vary in temperature, gas mixture, pH, glucose concentration, growth factors and presence of other nutrients. Cells density as well as feeding strategy also significantly influences the metabolic profiles. Finally, passage number and cell growth stage can have significant effect as well. Metabolomics can be used to monitor these changes but at the same time, in the design of metabolomics experiment all of these factors have to be considered and controlled.

Recent review dealing with experimental design in metabolomics has been published by Suhre and Gieger (2012). Although most of the focus of the review is on clinical applications of metabolomics, many issues relate directly to cell culture applications as well. Experimental design issues and solutions specific to cell culture metabolomics and applications presented in this chapter are provided in Fig. 20.2.

Experimental step ➡	Consideration ➡	Solutions
Study design	Cell type Study type Sample type	Cell culture type has to be well defined For example: phenotype analysis or medium optimization Extra- or intra-cellular; hydrophilic and lipophilic
Sample collection	Standard operating procedure Medium use and addition Sample quantities	Ensures compatibility between centers and during study Fed batch v.s. profusion Number of cells as well as amount of material
Sample storage	Temperature; Replicates	-80°C, liquid nitrogen Amount stored; avoid thawing cycles
Sample preparation	Metabolite extraction Extracellular or intracellular Derivatization pH optimization	SOP should be in place; type of metabolites selected Changing of biochemical properties for measurement
Sample analysis	Optimal analytical method Identification Referencing	NMR, LC-MS/MS, GC-MS/MS or another Qualitative or quantitative analysis; Standards and internal or external references
Data analysis	Statistical Unsupervised Supervised System Biology	Correlation; fold changes Clustering or visualization Major features; classification; Metabolic network; polyomics
Data interpretation	Functional Biochemical	Correlation with other data and system properties Pathways analysis

Fig. 20.2 Major steps in cell culture metabolomics experiments including main issues and possible solutions

20.4 Sample Processing

For the complete cell culture metabolomics analysis it is necessary to measure both extracellular (footprint) and intracellular (fingerprint) metabolic profiles (Fig. 20.3). Metabolic footprinting or exometabolomics is technically much less involved, as it requires only the separation of culture media from the cells by, for example, centrifugation prior to the analysis. Cellular footprint shows metabolites that have been either exported by the cell or that have not been imported into the cell from the cellular medium. Footprinting provides complementary information to fingerprinting regarding perturbations of metabolites and also unique view on processes such as degradation of complex substrates (Villas-Boas et al. 2006). Metabolic fingerprinting, albeit much more technically challenging, provides necessary information about cellular metabolic processes with information about the concentrations of metabolites that stay within cell walls. Metabolic fingerprinting can be done on whole cells or on cell extracts.

For extraction of intracellular, fingerprinting, metabolites the major steps are:

- metabolism quenching;
- cell disruption;
- separation of hydrophilic and hydrophobic metabolites;
- removal of any solvent that can disrupt the analysis.

In metabolome sample collection it is crucially important to stop cellular metabolism quantitatively and rapidly in order to avoid loss of metabolites caused by residual enzyme activity or quenching conditions. Furthermore, cells need to be

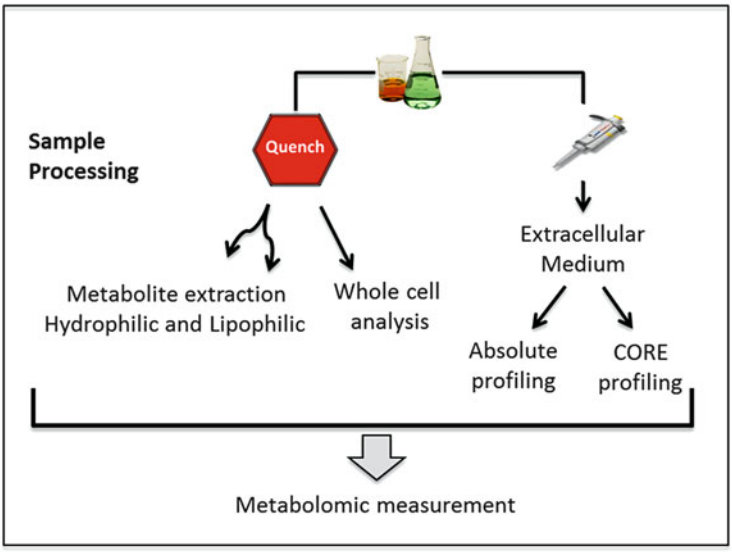

Fig. 20.3 Experimental steps in the cell culture metabolomics experiments

separated from the medium and thoroughly washed in order to remove any residual extracellular components.

Two basic systems exist for cell culture growth – monolayers on an artificial substrate – adherent cells; or as free-floating in the culture medium – suspension culture. Somewhat different procedure is needed for metabolite sample preparation for these distinct cell culture types. Over the last few years several protocols have been developed for the extraction of intracellular metabolites all claiming optimization of some aspect of extract quality and/or coverage of metabolite types (Sellick et al. 2009; Teng et al. 2009; Duarte et al. 2009; Yuan et al. 2008; Ritter et al. 2008). Methods have been presented for intracellular metabolite extractions for adherent (Martineau et al. 2011; Danielsson et al. 2010; Ritter et al. 2008) and free-floating cells (Sellick et al. 2011a; Halama et al. 2013; Duarte et al. 2009). It is very difficult to determine optimal combination of quenching and extraction procedures. Recently, an extremely valuable study appeared in the literature that compared different metabolite extraction protocols primarily aimed towards mammalian cell culture applications (Dietmair et al. 2010). In this work, the authors compare 12 different extraction methods that are most often quoted in the literature. According to the results of this study the extraction in cold 50 % aqueous acetonitrile was superior to other methods. Dietmair et al. have tested these 12 extraction methods on a known mixture of a large number of standard metabolites rather than directly of the cells. In this way the authors were able to very precisely determine the ability of different procedures to completely recover metabolites of different types. Furthermore, the authors have tested several quenching procedures on CHO cells where they have used fluorescence staining to determine whether tested cell quenching methods induced metabolite leaking through the membrane. From this analysis authors have concluded that the only acceptable quenching method for fragile animal cells was the application of cold 0.9 % (w/v) NaCl. All the other tested methods have rendered cell membranes prone to leaking thus possibly leading to loss of some metabolites. The protocol tests performed by Dietmair et al. were followed by a proposal of a very simple extraction protocol which leads to the highest level of metabolite extraction across different molecule groups and the lowest level of metabolite leaking across membrane wall.

Recently, Bi et al. (2013) compared sample preparation methods for metabolomics analysis using MS. Specifically; authors have performed optimization of harvesting, extraction and analysis for LC/MS metabolomics of adherent mammalian cell cultures. From the compared methods for cell harvesting authors concluded that direct scraping after flash quenching with liquid nitrogen provides best method that allows storage of samples while causing less metabolite leakage compared to trypsinization. Methanol/chloroform/H2O (mixture with 75 % methanol/chloroform to water and 9:1 methanol to chloroform) was the most optimal extraction solvent mixture in these tests insuring highest recovery of metabolic features and the best reproducibility.

In the analysis of cell cultures it is also very important to keep in mind possible dependence of metabolic profiles induced by differences in growth medium formulation and additives used in cell culturing. Thus, whenever possible it is

important to grow cells under the equalized growth conditions. This can easily be achieved when for example metabolic profiles are compared in the same cell lines before and after some stimulation. If this is not possible – for example in the comparison of different cell types – it is still recommended to try to equalize growth conditions during the whole growth period or at least during a short period of time prior to quenching. Finally, "omics" experiments by-and-large suffer from insufficient number of replicates for obtaining statistically relevant conclusions. Robotic systems can be employed for cell growth and manipulation as well as metabolite extraction making collect larger number of samples possible. At the same time, more strict control of cell cultures can lead to removal or at least reduction of confounding factors and errors and therefore, smaller numbers of replicates usually suffice.

20.5 Measurement

Major requirements in any metabolomics analysis are (based on Khoo and Al-Rubeai 2007):

- to provide an instantaneous measure of all metabolites in a given system;
- to utilize analytical methodologies that provide high recovery, robustness and reproducibility while at the same time insuring high resolving power and high sensitivity and universality;
- to provide unambiguous identification and quantification of metabolites;
- to insure ability to determine in the same analysis major distinguishing features and also use all data as part of biological network and systems investigation.

Although no method can fully satisfy all of these requirements several modern analytical instruments and methods can provide sufficiently accurate measurements. Major metabolomics measurement methods are nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) with few examples of applications Fourier Transform Infra-Red spectroscopy (FTIR) and Raman Spectroscopy. Both NMR and MS offer many choices from overall screening to more detailed, focused analysis. There are numerous reviews describing variety of applications of these methods in metabolomics (Dunn 2010; Kaddurah-Daouk et al. 2008; Cuperlovic-Culf 2013). Major advantage of MS based methods compared to NMR is their higher sensitivity. However, in order to achieve this high sensitivity MS methods require much more complex sample preparation opening possibilities for experimental errors and introduction of variances and confounders in the resulting data set. NMR based methods require no or minimal sample preparation and are thus more efficient and less prone to errors; however they can only currently quantify less than 50 metabolites. Both MS and NMR require standard information for assignment and quantification (although determination of novel metabolites is possible with both methods with additional experimentation). NMR methods are highly reproducible across instruments whereas in

MS batch effects present a problem. MS methods provide possibility for quantification of larger numbers of metabolites however standards for all metabolites, measured on the given instrument under the same conditions, are required. Application of these methods to cell culture samples is not any different than any other metabolomics analysis.

NMR measurements of whole cells have in the past provided information about both free and bound metabolites but were hampered by broad lines of molecules caused by their restricted movement in the cells (Bloom et al. 1986). Recently, particularly good results for whole cell measurements have been provided by high-resolution magic angle spinning (HRMAS) NMR (Bayet-Robert et al. 2010; Griffin et al. 2006; Shi et al. 2008; Peet et al. 2007; Borel et al. 2009; Morvan et al. 2003). At the same time liquid state NMR is extensively used for measurement of cellular metabolic extracts. Both HRMAS of whole cells and liquid state NMR of metabolite extracts have their advantages and disadvantages. Recent work has attempted to establish optimal cell manipulation, freezing and storage conditions for HRMAS NMR procedures (Duarte et al. 2009). The efforts of Duarte and co-workers have shown that although cell integrity is generally well preserved in both fresh cells and in cells frozen in cryopreservative, higher degree of membrane degradation is observed in fresh cells. Furthermore, cells lysed by mechanical methods or by freeze-thaw cycling without cryopreservative have shown significant change in lipid profiles relative to intact cells. Thus for studies of cellular lipids HRMAS analysis of whole cells appears to be a more accurate method than extraction of metabolites (Duarte et al. 2009). At the same time, liquid state NMR as well as MS of cellular extracts provides more information about the intracellular metabolites. Analysis of metabolite extracts is, additionally, unhindered by possible binding of metabolites to larger molecular systems in cells that can lead to signal broadening or even signal loss. Furthermore, metabolite extraction from cells allows separation of metabolite molecule subtypes.

An interesting approach for high throughput amino acids measurements in cell samples was the application of Newborn Screening (NBS) assay initially developed for quantification of amino acid and acetylcarnitines in blood samples. Halama et al. (2011) used NBS method to measure highly quantitatively 42 amino acids and acylcarnitines in cells following treatment. Metabolites are in this approach measured using FIA-MS/MS where authors used 4×10^5 cells, but possibly lower number of cells can also be measured. Detailed, recent review focused on LC/MS metabolomics analysis of cells has been provided by Leon et al. (2013).

20.6 Data Analysis

20.6.1 Qualitative and Quantitative Analysis

Measurement results obtained either from NMR or MS or any other instrument initially require extensive pre-processing which includes phase and baseline

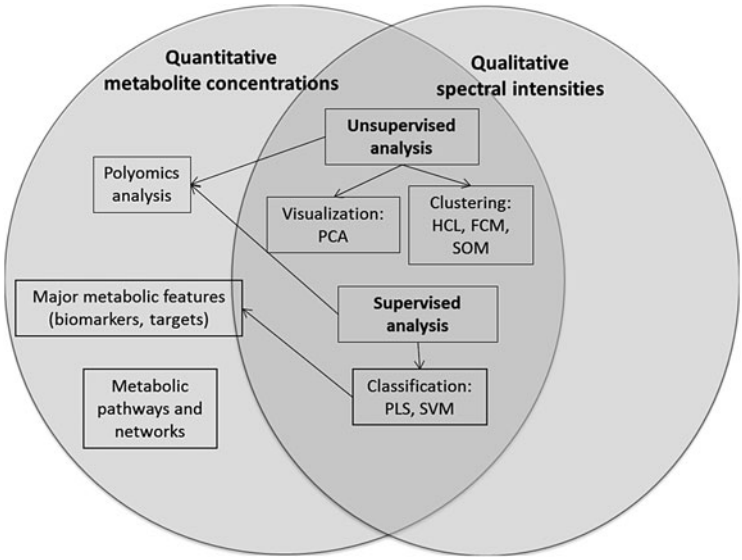

Fig. 20.4 Two distinct data analysis strategies in metabolomics study. Analysis can be performed directly on instrument measurements, qualitatively or on quantified metabolic data – quantitatively. Two major options for analysis present different possibilities and options in further analysis and interpretation

correction, referencing as well as normalization and scaling. All these steps are the same in all metabolomics applications and have been explored in detail for each specific methodology elsewhere. Following pre-processing data can be analyzed directly, without metabolite quantification in the qualitative analysis or following quantification of metabolites – leading to quantitative analysis. Different types of analyses that can be performed in the qualitative and quantitative sense are outlined in Fig. 20.4.

If metabolites are not identified and quantified, spectral patterns and intensities are statistically compared. This approach termed qualitative or chemometric method can lead to the identification of differences between samples or the determination of relevant spectral features that distinguish sample classes. Metabolites leading to the obtained major features can be subsequently assigned and quantified (Wishart et al. 2007; Tulpan et al. 2011).

Alternatively, in the approach called quantitative metabolomics (targeted profiling or magnetic resonance diagnostics) compounds are identified and quantified initially. Metabolite concentrations and properties can be de-convoluted from results and quantified by comparison to the standard spectra of pure molecules of interest provided in reference libraries or by using spiking of sample with known quantities of standards. Regardless of the type of metabolomics measurement, quantification of metabolites is performed using different mathematical methods for comparison with measurements of standards. Aranibar et al. (2011) have shown

that iteratively calculating and fitting the whole NMR spectrum using the quantum mechanical total line shape algorithm (QMTLS) produces the most accurate quantification results particularly from spectra with large overlaps. QMTLS method (available in the commercial software PERCH, from PERCH Solutions Ltd. and Bruker) utilizes user provided starting values for chemical shifts, protein-protein couplings and individual line widths (from standard measurements) which are used to iteratively optimize the fit to experimental data. This type of analysis provides quantitative information for 30–40 metabolites from standard 1D NMR measurements. Similarly, metabolites from MS measurements are quantified from comparison with standard measurements performed on the same instrument. Useful resources in metabolite quantifications are standard databases such as HMDB (www.hmdb.ca; Wishart et al. 2007), which contains both MS and NMR data for large number of metabolites; Biological Magnetic Resonance Data Bank – BMRB (www.bmrb.wisc.edu) containing various NMR data for metabolites; METLIN (Tautenhahn et al. 2012). Data for standards can be used for quantification of metabolic profiles using software tools such as Perch (Perch Inc), Chenomx (Chenomx Inc) or XCMS (Smith et al. 2006). Also, using measurements of standards from the databases it is possible to develop user specific regression algorithms for quantification as presented in (Cuperlovic-Culf et al. 2012).

Once the metabolites are assigned and quantified this data is used for further analysis including sample comparison or feature selection or metabolite clustering. Qualitative approach has its place in fast, overall separation of samples and this can be used in for example determination of subtypes (Cuperlovic-Culf et al. 2012). However, determining concentration changes for specific metabolites opens opportunities for much more in-depth analysis of the biological characteristics of cells and is highly advantageous in majority of applications.

20.6.2 Unsupervised and Supervised Analysis

Both spectral (qualitative) and concentration (quantitative) data can be further analyzed in an unsupervised and supervised sense.

Unsupervised analysis approaches are used for grouping of features (samples, metabolites or spectral points) according to the measurements and without external, supervisor input. This type of data analysis includes the visualization (projection) and clustering methods. The unsupervised methods are most appropriate for the examination of similarities and differences between samples based on metabolic profiles or between spectral features or metabolites over time or phenotypes. Unsupervised analysis of samples can be performed based on spectral and concentration data. Unsupervised analysis of quantitative data and the corresponding spectral data should give identical or comparable results making unsupervised analysis a good approach for validation of metabolite quantification procedures. Unsupervised analysis of samples provides unbiased grouping that can be used to either confirm known sample types or to determine groups of samples based on

metabolic profiles. Furthermore, unsupervised analysis across samples can provide information about the grouping of spectral features or spectral peaks in order to identify structurally related features that result either from the same metabolite or from metabolites that are closely associated across biological pathways. Grouping of metabolites based on concentration changes across samples can be utilized for the exploration of possible biological co-regulation of metabolites. These distinct applications present range of challenges from very large number of features relative to the number of samples (in qualitative analysis) to considerable range in data value and non-normal data (in both cases). Only a limited number of methods have been utilized for unsupervised analysis in metabolomics thus far. Still the most popular is the Principal Component Analysis (PCA) – projection method that provides visualization of major differences between features. PCA can also be used to determine outliers and erroneous results. Through dimension reduction, PCA provides improved visualization of the major characteristics of the data. PCA makes extraction and display of systematic variations in the data possible (Trygg 2007) while at the same time providing clear and easy-to-interpret graphical representation of data groups. However PCA groups data only by major variances. Clustering methods on the other hand use all the data for feature (usually sample) grouping and thus can be advantageous in some cases. Any clustering method can in principle be used for determining groups of similar samples or metabolites from metabolomics data. Methods such as hierarchical clustering (HCL), fuzzy K-means and Self-organizing map (SOM) have been extensively used in metabolomics and can be used for cell culture analysis as well. In determining groups of similar cell types clustering methods can provide unbiased information about the similarities in addition to providing powerful visualization. Grouping of metabolites based on the behavior across samples can indicate involvement in the same pathways or co-regulation. A demonstration of results obtained using the most popular unsupervised methods: PCA, fuzzy K-means, HCL and SOM are shown Fig. 20.5. Figure 20.5 shows analysis of breast cancer cell lines measured in the CORE, NCI60 experiment (Jain et al. 2012). All four methods show grouping of replicates (2 done for each cell line) and also grouping of some of the cell lines however different methods suggest different groups in this particular case. Thus, it is always important to use more than one method for unsupervised analysis in order to prevent erroneous results.

When class labels of all data are available this information can be used to When class labels of all data are available this information can be used to determine class pure clusters, i.e. features that provide the largest and the most complete separation of samples by groups. Statistical and supervised methods can in this case provide the most accurate results for, respectively, ranking of features and selection of features for predictor development. In the cell culture applications determination of the most significantly different features can be used for example in marker or target determination of for determination of significantly changing features for cell culture medium optimization.

Statistical methods use information about the classes for comparison of features for selection of the most significantly different ones (similarly to supervised

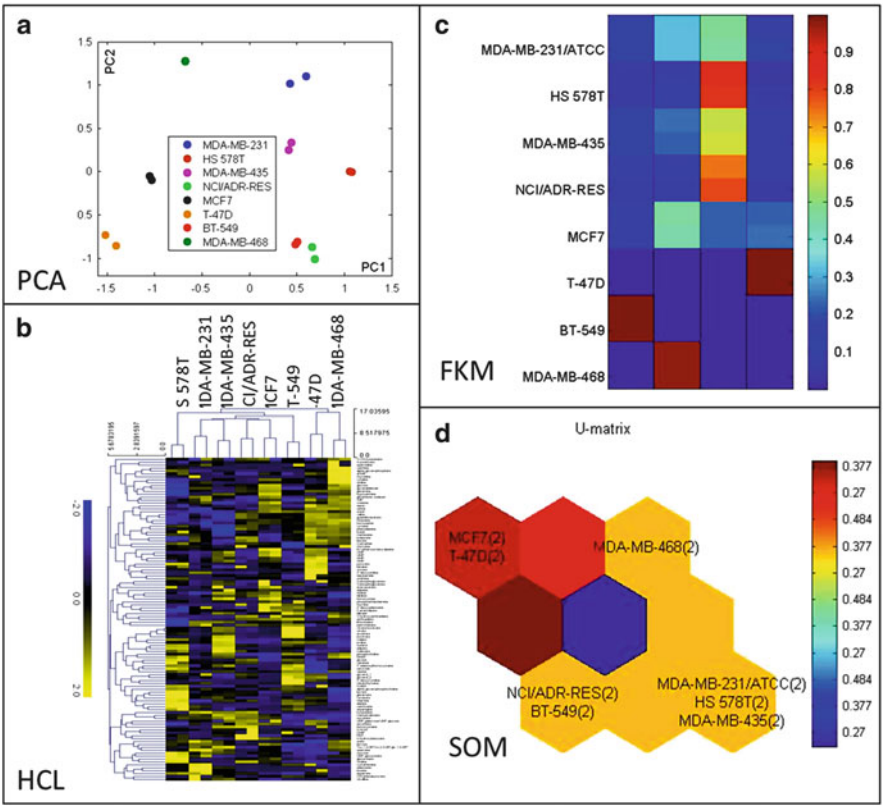

Fig. 20.5 Different methods for unsupervised data visualization and clustering previously used in cell culture metabolomics. Shown are examples on the breast cancer subset of CORE NCI60 data (Jain et al. 2012). Analysis is performed on normalized data. (**a**) Principal Component Analysis (*PCA*) shows projection of major variances in the data; (**b**) Hierarchical clustering (*HCL*) present hierarchical clusters of samples and metabolites using all the data and grouping each feature to one cluster; (**c**) *FKM* – fuzzy clustering graphs shows the memberships of each sample to each of the user set four clusters, membership values are represented by heat plots where 1 show completely belonging; (**d**) Self-Organizing Map – *SOM* results are presented by U graph showing mapping of samples to the grid where the Euclidean distance between the vectors of neighboring neurons is shown by *color*

methods). However, they do not create predictors for classes. Statistical methods provide ranking of feature relevance and this is used for feature selection. Of real statistical methods null hypothesis testing has been extensively utilized for feature selection. These include for example, unpaired methods: two sample t-test and Mann-Whitney-U test as well as paired methods (for dependent samples), paired t-test and Wilcoxon signed-rank test. Null hypothesis testing provides a well-known p-value as an evaluation of the discriminatory ability of variable. It is appropriate for two class problems; however, p- value is highly sample size dependent (Baumgartner 2011; Lehmann 2005). T-test and related Student t-test are highly

popular statistical methods. However, they operate under the assumption of normal distribution which is rarely the case in biological data. Many alternatives to t-test have been proposed in different applications including methods developed for omics data analysis such as Welch statistics or Significance Analysis for Microarrays (SAM) (Tusher et al. 2001). SAM method has found its uses both in feature selection of major spectral features as well as metabolites (Cuperlovic-Culf et al. 2011, 2012; Morin et al. 2013). The basic statistical procedure used in SAM is similar to a t-test. SAM determines the difference between means, in units of standard deviations. A weighting term is added to the pooled standard deviation in SAM preventing overestimates of the significance of features with a small variance. Significant features search is performed by first ranking features by the magnitude of the relative difference t. Then a relative difference is recalculated for each permutation of sample labels giving p-values, tp. The expected relative difference tE is obtained as the average of all tp values for the feature. Feature with significant changes in expression are finally determined from the scatter plot of t versus tE as the features with values displaced from the linear function. SAM can be used for two or multi-condition feature selection. An example of features selected as significant for separation of two groups of breast cancer CORE-NCI60 profiles is shown in Fig. 20.6a.

Multivariate learning algorithms in supervised data analysis, such as Partial Least Squares regression (PLS) and related method Partial Least Squares Discriminant Analysis (PLS-DA) provide avenues for discrimination between different classes of observation as well as determination of most significant features for the sample classification according to supervisor data. PLS-DA is a feature selection method that uses PLS regression method for construction of predictive models. PLS-DA is robust to standard problem of qualitative metabolomics such as larger number of features than samples and correlation of many features. PLS is able to extract a set of latent variables that model the data and are also highly correlated to the class membership vector. From PLS model it is possible to calculate variable influence on the projection (VIP) and rank features according to VIP scores. PLS-DA feature selection is highly influenced by scaling method and the features can be hard to interpret and connect to individual metabolites (Alsberg 1998; Bryan 2008). An example of feature selection from breast cancer CORE-NCI60 data is shown in Fig. 20.6b.

Random forest method first proposed by Breiman (2001) provides an alternative to PLS-DA that is much less sensitive to scaling of the data. In the Random Forest method features are assigned importance values in relation to their influence or importance in the classification accuracy of the forest and this can be used in feature selection. The importance of a feature is calculated by random permutation of a feature over samples in each test set. Samples are then reclassified using the Random forest. The importance value for a feature is calculated as the difference between the number of correctly classified samples in the initial data and the permuted data divided by the number of trees in the forest (Bryan 2008). An example of feature selection in metabolomics data with Random Forest method has recently been shown (Huang et al. 2013).

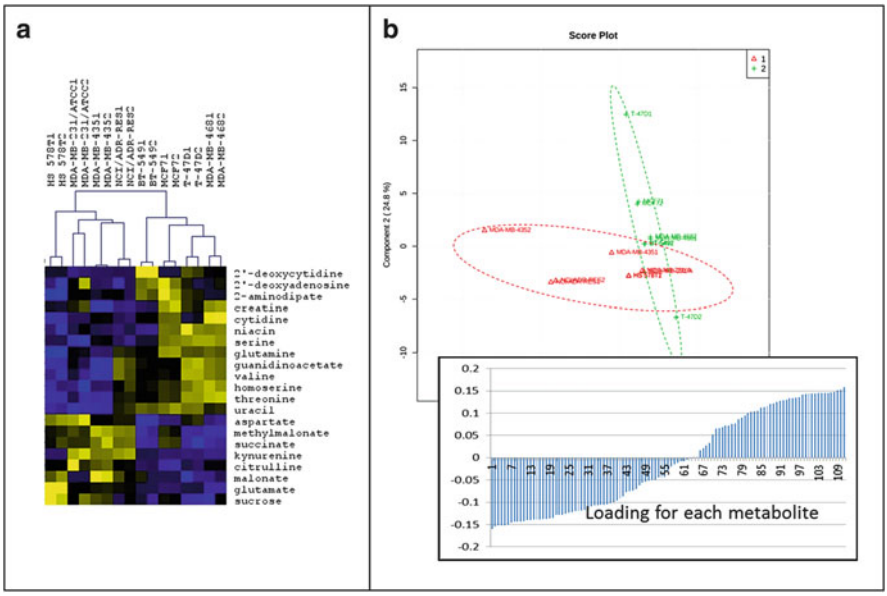

Fig. 20.6 Feature selection by two examples of supervised methods used for the selection of most differently concentrated metabolites in two user defined groups of breast cancer CORE samples (from NCI60 dataset, Jain et al. 2012). (**a**) Statistical "significance analysis for microarrays" SAM method (Tusher et al. 2001) where user defines groups and the most significantly different features are determined statistically; (**b**) Partial Least Squares Discriminant Analysis (*PLS-DA*) showing sample separation in score plots and metabolite significance through loading values. PLS-DA is a classical PLS regression where the response variable indicates the classes of samples. Loadings plot shows the relevance of each metabolite in the sample separation shown in score plot

A subgroup of multivariate methods – the machine learning – is used for building predictive models on data. Some examples of machine learning methods include discriminant analysis methods (e.g. linear discriminant analysis or logistic regression analysis), decision trees, the k-nearest neighbor classifier (k-NN) – an instance-based learning approach; the Bayes classifiers – a probabilistic methods; support vector machines – method that uses a kernel technique to apply linear classification techniques to nonlinear classification problems or artificial neural networks. Few examples of the use of these methods in metabolomics have been presented (Mahadevan et al. 2008; Issaq 2009; Brougham et al. 2011) although further acceptance of more advanced methods in analysis is still needed.

Vernardis et al. (2013) have shown an interesting example of the application of unsupervised, qualitative data analysis as well as supervised, marker analysis from quantified metabolite data in bioreactor analysis. Unsupervised analysis was performed using Hierarchical Clustering and Principal component analysis (PCA). Biomarker determination was performed using significance analysis for microarrays (SAM). HCL and PCA analysis have shown physiological state shifts over the duration of the perfusion cell culture and the analysis of significant

concentration changes over time have indicated specific metabolic pathways that are preferentially utilized if different phases.

20.6.3 System Biology and Cell Culture Metabolomics

Controlled, possibly homogeneous cell populations in cell cultures provide Controlled, possibly homogeneous cell populations in cell cultures provide base for exploring specific biochemical functions as well as metabolic network in great detail. Metabolomics data provides deeper insight into the system level response and information about the cellular metabolic network. Correlation between metabolites does not necessarily mean that they are neighbors in metabolic network; however existing metabolic networks can be combined with metabolic data to show actual relationships between metabolites. Mathematical modelling approaches can be used to investigate metabolic reactions or metabolic networks (Kitano 2002) and are generally combined with flux metabolic data (see Chap. 20 in this volume or Cuperlovic-Culf 2013). Biochemical systems theory and system networks of reactions are being developed to provide general model for metabolic network without detailed knowledge of reaction equations. Detailed metabolic network for human cells is available (Thiele et al. 2013) and can be used to model or explore metabolomics data. Metabolic network for CHO cells has also recently been provided (Smallbone 2013: ChoNet is available from http://cho.sf.net/ and Selvarasu et al. 2012). Innovative methods for optimization of biochemical networks structure and regulation from time-series metabolic profiles have been proposed (Marino and Voit 2006). The availability of detailed cellular metabolic networks for number of species provides an initial map for the metabolomics system level analysis of cells. Kotze et al. (2013) have recently used network-based correlation analysis of metabolomics data from cell cultures to explore metabolic changes in hypoxia. From metabolomics measurements on many replicated cell cultures it was possible to determine correlation between metabolites in different environments and to determine across the network connection between correlated metabolites. Work with cell cultures in this case makes it possible to also explore effects in different organelles in terms of, for example, metabolic network. Combining metabolomics and other omics data in the case of cell culture analysis can also greatly benefit from the network correlation approach.

Selvarasu et al. (2012) have presented and integrated approach for combining intra- and extracellular metabolomics profiles obtained at different time points in CHO fed-batch cultures with in silico models to investigate metabolic state and cellular physiology during culture. Detailed metabolomics analysis has contributed to the identification of additional metabolites, instigating inclusion of related enzymatic reactions into CHO network. Combining metabolomics data with the metabolic network map allows more in-depth study and determination of key metabolites and processes for cell growth, death and production. From this analysis authors determined that glycolysis is the main energy-producing pathway in CHO

cells. Decrease in intracellular oxidized and reduced glutathione and accumulation of extracellular glutathione disulfide was suggested as a sign of oxidative stress causing cellular apoptosis and growth limitation in these fed-batch cultures. Glycerophospholipid metabolism has also been associated with growth limitation with depletion of plasma membrane constitutes and reduced fluxes in late exponential and stationary phases of cell culture.

Metabolic network analysis applications currently mostly focus on: (a) finding enzymes that can produce a desired product; (b) determination of pathways that lead to a maximum yield; (c) obtaining information about the non-redundant pathways and enzymes as targets for drug design; (d) comparison between genome metabolic network and metabolic data in order to identify missing genes and pathways (Tomar and De 2013; Bower and Bolouri 2001; Schuster et al. 2000).

Logical, first step in the interpretation of metabolic results is to map and visually inspect identified metabolites on metabolic pathways or networks. This type of analysis can quickly provide information about relationships between observed metabolites through pathways or through common regulators. Several software tools allow this type of analysis in addition to many databases and tools that allow visualization of metabolic networks and pathways. Free tools such as Cytoscape (http://www.cytoscape.org/) and VANTED (http://vanted.ipk-gatersleben.de/) as well as several commercial software packages (such as Pathway Studio) can be used for this task. Enrichment analysis can be also used to test whether in an experiment list of metabolites determined as interesting has statistically significant belonging to some pathway. Examples of tools that provide metabolite enrichment analysis are MetaboAnalyst (www.metaboanalyst.ca) and MBRole (Chagoyen and Pazos 2011; http://csbg.cnb.csic.es/mbrole/). Recent review of these and other tools for functional interpretation of metabolic experiments has been provided by Chagoyen and Pazos (2013) and Booth et al. (2013).

20.6.4 Metabolomics in Polyomics of Cell Cultures

Metabolomics provides valuable information on its own, however even greater statistical and information power can be gained by integration of various omics data into a single model of the system. A very interesting publically available dataset for this type of analysis is the CORE dataset and microarray data for NCI60 tumor cell line panel. NCI60 is the panel of 59 tumor derived cell cultures that represent nine different tissue types, all grow under the same conditions and with comparable growth rate. NCI60 panel has be devised in 1990 by US NCI to screen for new anticancer drugs (Shoemaker et al. 1988). As an example of polyomics analysis of NCI60 data, Cavill et al. (2011) have utilized transcriptomics and metabolomics measurements for NCI60 cell line panel in order to determine biochemical pathways that might be associated with chemo-sensitivity to platinum-based drugs. Also, Boccard and Rutledge (2013) have recently shown a use of consensus orthogonal partial least squares discriminant analysis (OPLS-DA) strategy for

multi-block Omics data fusion. In this model individual data types are fused through application of association matrices leading to the combined data matrix that can be analyzed for consensus scores in the PLS sense as well as contributions of individual blocks. Consensus OPLS-DA method has been used for analysis of genomics, proteomics and metabolomics data for NCI-60 cell line panel.

Multiomics analysis was also performed on Hek293 cells (Dietmair et al. 2012b). The goal of this study was to understand molecular differences between stable, recombinant protein producing cell line and its parental non-producer cell line. In this example metabolomics data was combined with transcriptomics and fluxomics data. Interestingly, producer cell lines show reduced glucose up-take, glycolysis and lactate production while also having a reduced TCA and oxidative phosphorylation. This is partially regulated at the enzyme (rather than transporter) transcription levels. At the same time producer cells show increased uptake rates for many amino acids regulated by transporters up-regulation. This multiomica analysis helped identify number of features that are relevant for producer cells possibly providing targets for enhancement of production abilities of these cells.

Molecular cascade leading from oncogenic pathways to metabolic switch in cancer development have been explored through combined metabolomics and proteomics analysis (Shaw et al. 2013). In this example authors have derived from normal breast cell line (MCF10A) different tumor grades through stimulating action of an oncogene. They have shown that relative organelle specific expression of enzymes critical for lactate and pyruvate metabolism correlate with extracellular acidification profiles in different tumor grades. Organelle specific measurements show evidence that some oncogenic proteins are increasingly transported to the mitochondria. Work of Shaw et al. shows a very interesting method for parallel proteome and metabolome measurement and shows the power of this combined approach for analysis of metabolic dysfunction in cancer but possibly in other cell culture models.

Conclusion

Although improvements and standardization of all steps of cell culture metabolomics experiment are still underway, there are already many examples of highly fruitful use of this application. Cell cultures are finding use in many different areas from life science basic research to applied biotechnology. Metabolomics of cell cultures can contribute further understanding of biological processes in cells and also resolve problems and issues in cell culture applications. Metabolomics measurements can be performed in high-throughput as well as in real-time; on intra- and extra-cellular metabolites. Obtained data can be analyzed independently or as part of system biology investigation. With these many possibilities and options metabolomics analysis should become a standard method in any cell culture application.

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Chapter 21
Process Analytical Technology and Quality-by-Design for Animal Cell Culture

Stephen Craven and Jessica Whelan

Abstract Process Analytical Technology (PAT) is an area of intense research and interest currently. The interest in and applications for PAT span many industries: petrochemicals, bulk chemicals, food, pharmaceuticals and biopharmaceuticals amongst others. Adoption in the biopharmaceutical industry is in its infancy but is being driven by both regulatory demand and the business case. Ultimately, both motivations stem from the fact that effective application of PAT to bioprocesses increases process understanding and process control, mitigating the risk of sub-standard drug products to both the manufacturer and the patient. In order to realise the value that PAT can offer, all aspects of the PAT system must be considered and appropriately chosen. These include the PAT instrument, data analysis techniques, control strategies and algorithms and process optimization. It is only by the clear definition of the objective for the PAT system and the selection of suitable elements that the value may be realised. This chapter will discuss the instruments, techniques and strategies of relevance to animal cell culture currently.

Keywords PAT • Quality by design • Bioprocess control • Multivariate data analysis • Modelling • Spectroscopy

21.1 What Is Process Analytical Technology and Quality-by-Design and Why Is It of Value?

In 2004, the United States Food and Drug Administration (FDA) defined process analytical technology (PAT) as a mechanism to design, analyse, and control pharmaceutical manufacturing processes through the measurement of Critical Process Parameters (CPP) which affect Critical Quality Attributes (CQA) (FDA 2004). The philosophy behind the FDA PAT initiative is that the CQAs of a product are directly determined by the CPPs. Therefore, the delivery of the desired CQAs can be ensured if the CPPs are identified, the nature of their relationships to the CQAs understood and appropriate control strategies then applied to guarantee a high

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quality, reproducible output from the process. PAT has four main facets: **measurement** of the CPPs and/or CQAs, **analysis** of process data to translate it to process information and onward into process understanding, **control** of the CQAs using control strategies based on knowledge of the relationships between the process levers and product quality and **optimization** of the process as a result of design space mapping and process knowledge.

The PAT initiative is fundamentally linked to the regulatory move from Quality-by-Inspection to Quality-by-Design (QbD) (Read et al. 2010a, b). Traditionally, regulatory filings for pharmaceutical products were based on empirical data. The filing referenced a fixed manufacturing process with tight ranges on the measurable parameters and product quality was ensured by avoiding deviations from these ranges and with final product testing. This approach to drug manufacture is fundamentally flawed. It is not possible to test quality into a product. The output of any process is a function of the inputs to the process coupled with the trajectory of the CPPs through the process. Firstly, the raw material inputs to a process will have some inherent level of variability. Therefore, if the manufacturing process is fixed within tight ranges, the attributes of the product outputted will fluctuate because there is no facility to adjust the process in order to compensate for the varying inputs. Secondly, the format of the traditional filings did not ensure that all CPPs were identified and their relationships with the CQAs understood. As a result, the process parameters and ranges specified in the filing may or may not have been those required to ensure a high quality product. In animal cell culture in particular, the process parameters measured are those for which robust sensors exist, such as pH and dissolved oxygen (DO), rather than those that are of fundamental importance in determining the CQAs.

The lack of understanding of the process and product interdependencies results in manufacturing challenges. Often there is an inability to predict process performance on scale-up. Root cause analysis for manufacturing failures may not be definitive or satisfactory as the primary process levers, the CPPs, may not be fully identified or understood. At the extreme, this may result in worldwide drug shortages if issues with manufacturing prove difficult to resolve. The manufacturing process is often operating in a suboptimal area of the design space resulting in extended cycle times, excessive raw material and utility requirements, elevated numbers of process or product failures which together culminate in a high cost of manufacturing. The nature of the filing also presents a roadblock for process improvements and innovation. As technology advances, the manufacturer is often unable to capitalise on it as the restrictive nature of the filing may prevent adoption without a costly and time consuming refiling.

In contrast, QbD filings seek to have "a maximally efficient, agile, flexible pharmaceutical manufacturing sector that reliably produces high quality drug products without extensive regulatory oversight" (Janet Woodcock, MD, Director of the Center for Drug Evaluation and Research in the FDA). QbD is a scientific, risk-based, proactive approach to drug development that looks to have a full understanding of how the process and product are related (Rathore and Winkle 2009). The QbD workflow for drug approval integrates the product specifications

Fig. 21.1 The Quality-by-Design system (FDA 2004)

and process design (Fig. 21.1). The first step is to define the desired product performance and hence, the CQAs. The process is then designed to deliver the necessary CQAs. During process development, the impact of raw material attributes and process parameters on the CQAs are investigated, resulting in the identification of the CPPs and a mapping of the design space. Strategies to compensate for the raw material and process variability are then implemented to deliver a more uniform final drug product. Once in manufacture, continual process and product monitoring ensures that any process drift is recognized and appropriate actions taken to ensure product quality is maintained.

The QbD drug approval process mitigates against many of the challenges of the traditional filings. The integration of fundamental understanding of the links between CPPs and CQAs results in a better product and process design with less manufacturing issues and less waste. The freedom to adjust the process within the mapped design space facilitates continuous process improvement and the implementation of new technologies over the life cycle of the drug. The comparison between traditional and QbD filings is summarized in Table 21.1.

The PAT required to support a QbD product may be simple or complex depending on the nature of the product and process used for its manufacture. Generally speaking, animal cell culture processes and their products are complex and hence, there are many challenges to implementing a PAT/QbD strategy.

Table 21.1 Comparison of traditional and QbD drug filings

	Traditional	QbD
Pharmaceutical development	Empirical, univariate	Systematic, multivariate, development of design space
Manufacturing process	Fixed	Adjustable within the design space
Process controls	In process checks, offline analysis, cycle time impacts	PAT used for in/at-line measurements with the potential for real-time release
Lifecycle management	Reactive to deviations and OOS	Continual improvement within the design space

The first step in implementing PAT is the selection of a suitable measurement instrument. For an effective PAT strategy, the instrument selected should characterize the CPPs and/or CQAs for the given process or process step. The instrument chosen must be capable of quantifying the parameters of interest with a suitable level of accuracy and precision. The frequency of measurement must also be appropriate for the application when compared to the process kinetics and rate of change expected for the given unit operation. These requirements are difficult to fulfil for animal cell culture.

Mammalian cells grow in a complex, multiphase environment. The bioreactor environment contains three phases: the cells, the medium and the gas bubbles. This multiphase environment presents difficulties whether an instrument is in situ or at-line. For online instruments, the different phases may affect the measurement. For example, in the case of spectroscopy, the presence of bubbles in the path may reduce the intensity of the signal acquired and hence, the concentration of the analyte reported. The solid phase may foul inline probes. At-line instruments require a sample to be removed from the reactor. It may be difficult to remove a representative sample from the system, particularly where the volume is to be minimized, because of inhomogeneity due in part to the multiphase nature of the system and imperfect mixing environment. The measurement device may require sample preparation such as phase separation to remove the liquid from the cells or sample conditioning. The at-line PAT instrument and its associated sampling system may suffer from fouling of flow paths, bubble build up in tubing and optical surfaces etc.

The media used to support growth and protein production in animal culture are extremely sophisticated mixtures, often containing in excess of 80 different species, almost all of which are at very low concentrations when compared with small molecule production. Many of the species are similar, such as the 20 or so amino acids used by mammalian cells. The low concentrations combined with the structural similarities of multiple species means that finding an instrument with suitable sensitivity and specificity is non-trivial. Research and development of such instruments is currently an area of intense interest. At-line HPLC (Rathore et al. 2008) or flow cytometery (Al-Rubeai et al. 2012; Sitton and Srienc 2008) and in-line spectroscopy coupled with chemometric interpretation of the signal (Whelan

et al. 2012a; Roychoudhury et al. 2007; Foley et al. 2012) are currently showing potential.

In addition to these considerations, any instrument and sampling system used must be capable of satisfying the need for sterility, whether by autoclaving in the process development laboratory or by steam-in-place (SIP) in the pilot or production plant. The system needs to be robust enough to ensure that exposure to the conditions required for sterility as well as the environmental conditions such as fluctuating temperature and mechanical vibrations will not affect performance.

Given that the aim of PAT is to control the product CQAs, it is necessary to identify the CPPs, to elucidate their relationship to the CQAs and to define a control strategy for each of the CPPs that will ensure reliable delivery of the required CQAs. While the concept is simple, the body of work required to achieve this for mammalian cell culture derived products should not be underestimated and currently, is generally aspirational. Animal cell culture processes are highly non-linear and multivariate in nature. The effect of many process parameters are often so interrelated that understanding the true cause and effect of any variation is difficult. There are a number of tools now employed with increasing frequency in development to help unravel these dependencies. Design-of-Experiments (DoE), multivariate data analysis (MVDA), process modelling and advanced control algorithms are increasingly common. The integration of these skills into animal cell culture development and manufacture is vital in order to transform the ever-increasing volume of process data generated into useful process information and then to take appropriate actions based on the learnings it facilitates.

While the application of PAT to animal cell culture processes presents numerous challenges, the potential benefits coupled with regulatory pressures are driving a strong interest in and an increased level of adoption in the biopharmaceutical industry. Biological processes are known to have a much higher level of variability than their chemical counterparts, typically 20 % versus 5 % respectively. PAT and QbD offer an avenue to better understand and control this variability, which has implications for the process economics, control and final product quality. The complex, multivariate nature of animal cell culture often means that root cause analysis of any deviations or process drifts is difficult. PAT offers a window into the process, with greater visibility and the resultant process understanding facilitating the determination of the root cause and allowing meaningful remedial actions to be taken to prevent reoccurrence. The improved efficiency of the bioprocess is indicated by a reduction in the number of batch failures and hence, a decrease in the waste of raw materials and utilities. The additional process insight that PAT offers is of value, not just during excursions from desired process performance, but also during routine manufacture. Coupled with a QbD approach, continuous improvement becomes a realistic possibility. The enhanced bank of process data collected may be exploited to increase understanding and identify adjustments that may be made to increase quality or quantity of the product or reduce process variability.

The adoption and integration of PAT in animal cell culture is in its infancy when compared with the small-molecule drug space. This is due mainly to the challenges and workload involved in applying such technology to the biological space.

However, the academic and industrial trend is an increased level of interest and effort in applying PAT in this space, both in development and manufacturing across all facets: measurement, analysis, control and optimization.

When implementing PAT, all elements of the system required to measure, control and analyse the relevant parameters should be considered, including: instruments, sampling assemblies, MVDA and chemometrics, models and control strategies. Therefore, to truly discuss PAT, it is necessary to consider all aspects of its multifaceted nature. In the remainder of this chapter, the most common and useful PAT tools for animal cell culture will be discussed with reference to both published and commercially available applications.

21.2 Measurement: Acquisition of Process Data

Upstream bioprocess steps can be subdivided into cell expansion and product production. While each step in the upstream process from thaw through to production contributes to process performance, the final or N stage reactor step has the greatest impact because the final product is produced at this stage. Additionally, many processes have shown that high quality product may be produced at the N stage despite being exposed to suboptimal conditions during expansion. Therefore, for a typical upstream process, many of the CPPs are identified at the production bioreactor stage. Application of multivariate, statistically designed experiments is valuable for identifying CPPs and understanding the variability that may be expected from the manufacturing-scale process.

PAT measurements should be focussed on CPPs and CQAs as by their nature, these factors are the most informative and crucial in determining process performance. The CQAs for a biological drug substance include: identity (primary sequence), concentration, potency, post-translational modifications (e.g. glycosylation, phosphorylation, glycation or methylation), product related impurities (e.g. deamidation, aggregation, oxidation, C-terminal lysine and misfolding) or process related impurities as HCPs, DNA and endotoxins. The production stage reactor plays a pivotal role in determining the CQA profile of the final drug substance as it is at this stage that the cell, under the influence of the bioreactor environment, produces these large, complex molecules. If a poor quality product is produced at this stage, the final drug substance will be majorly impacted.

The identification of upstream CPPs which determine the CQAs is non-trivial and has been the subject of much research. They may include but are not limited to: nutrient and by-product concentrations, cell growth rates and specific productivity rates, temperature, pH, DO and pCO_2 concentration, gassing rates, the hydrodynamic environment within the reactor and so on.

Traditionally, temperature, pH and DO are the main parameters measured and controlled online due to the availability of traditional, robust sensors. However, as technology advances, other process parameters such as cell density, cell viability,

substrate concentrations, product and by-product concentrations, pCO_2 and biomarkers can now be measured and analysed in real-time in an automated manner, although not yet routinely. The availability of real time process information is of particular value in addressing the variability and unpredictability of animal cell cultures as it opens up the possibility of implementing advanced control strategies capable of directly impacting CPPs and CQAs.

Measurement can be performed in situ, at-line or offline. In situ measurement techniques require the measurement device to be installed in the reactor. At-line and off-line measurements are performed on a sample removed from the bioreactor environment, with at-line measurements typically being automated, connected to the reactor and performed almost immediately and off-line measurements being completed at some time after sampling at another location such as in a laboratory. In situ and at-line measurements are more useful as once established, they are less labour intensive and can also be exploited for control purposes.

The pros and cons of the in situ and at-line approaches are summarised in Table 21.2. In situ instruments allow for real-time continuous measurements to be made without loss of reactor volume through repeated sampling and without the associated risk of contamination. However, designing an instrument with the necessary accuracy, precision and specificity and that is robust enough to withstand sterilization and long intervals between calibrations is challenging. Biological components such as enzymes or antibodies that are often used to ensure specificity cannot withstand the conditions required to sterilize the system. Fouling and the multiphase environment affect many in situ devices.

At-line instruments can exploit a wider range of measurement principles and can be calibrated more frequently because they are external to the reactor and do not need to be sterilized. However, there are challenges related to the removal of samples. It can be difficult to remove a sample that is representative of the bioreactor, the sample volume is lost to the system and there is a potential risk of contamination. Also, the measurement interval is typically significantly longer because it is not desirable to lose a significant volume of cell culture while operating the process. The at-line PAT instrument and its associated sampling system may also suffer from fouling of flow paths, bubble build up in tubing and optical surfaces.

Table 21.2 Comparison of in situ and at-line measurement instruments

	In situ	At-line
Sampling	No sampling required	Requires sampling
Sterilization	Probe needs to be sterilizable	Sampling port needs to be sterilizable, Analytical equipment may not require sterilization
Calibration	Calibration is often process specific and completed during bioreactor set up	At a frequency as required

21.2.1 PAT Instruments

There is an ever-expanding list of instruments that have been proposed as suitable PAT technologies for animal cell culture. These have been the subject of a number of review papers (Becker et al. 2007; Beutel and Henkel 2011; Vojinović et al. 2006). Briefly, the methods include inline and at-line techniques: biosensors and chemosensors, spectroscopy techniques, in situ microscopy, capacitance probes, software sensors, flow injection analysis, and high performance liquid chromatography (HPLC). Here, the discussion will be focussed on the most common or promising measurement techniques. The instruments suitable for in situ or at-line measurement of CPPs and CQAs relevant to animal cell culture can be categorized based on their underlying measurement principle: electrical measurements, optical measurements and chromatographic measurement.

21.2.2 Electrical Measurements

Electrical based measurements can be categorised into the following three categories: electrochemical probes; capacitance probes and multifunction analysers.

21.2.2.1 Electrochemical Probes

The traditional PAT applied to bioprocesses are temperature, pH and DO probes. These three probes are based on measuring an electrical response. The classic PT100 temperature probe is a resistance thermometer which correlates the resistance of an element to temperature. Traditional pH probes measure the electrochemical potential between the reference liquid within the electrode and the bioreactor environment. Standard DO probes are based on the Clark polarographic electrode, which is an amperometric electrode consisting of an oxygen-permeable membrane and a platinum cathode.

In addition to these traditional electrochemical probes, pCO_2 probes are now emerging that use potentiometric CO_2 measurements based on the Severinghaus principle. It utilizes a CO_2-sensitive glass electrode in a surrounding film of bicarbonate solution covered by a thin plastic membrane, which is permeable to carbon dioxide but impermeable to water and electrolyte solutes. The carbon dioxide pressure of a sample gas or liquid equilibrates through the membrane and the glass electrode measures the resulting pH of the bicarbonate solution. Their results have been found to correlate well with data derived from other methods, including off-line chemical analysers, mass spectrometry (MS), and on-line IR vent gas analysers. pCO_2 is generally uncontrolled in cell cultures as the presence of an in situ pCO_2 probe is not common. Control is either absent or limited to a periodic stripping of pCO_2 based on an offline pCO_2 measurement. Typically, the range of

acceptable pCO_2 levels is broad during production; however, very high CO_2 concentrations, approximately in excess of 150 mmHg for CHO cultures, can impact the product quality (Gray et al. 1996). The ability to monitor the dissolved CO_2 concentration in real-time is a valuable asset in upstream bioprocessing.

21.2.2.2 Capacitance Probes

Capacitance based probes have been used for in situ cell density determination. The capacitance probe operates by polarising only viable cell membrane surfaces using an electric field. The cell membrane controls the flow of ions in a live cell. The polarised cell then acts as a capacitor. Measured capacitance is a function of the viable cell density present in the bioreactor. The capacitance method has proved to be robust, easy to scale up and insensitive to gas bubbles or debris with cells in suspension or attached to inert carriers (Carvell and Dowd 2006). Real-time viable cell density measurements are valuable. The viable cell is the production unit within the reactor. The nutrient requirements are strongly dependent on the cell number. Once again, the ability to determine cell density in real-time and with a short sample interval increases the wealth of the process data set collected, allowing greater process insight and also opens more viable process control strategies. Lu et al. (2013) demonstrated automated cell culture control strategies based upon on-line capacitance measurements where cultures were fed based on an on-line calculation involving growth and nutrient consumption rates. Significant increases in titre were reported for the two cell lines studied, with the largest increase being reported as an increase from 4 to 9 g/L.

21.2.2.3 Multifunction Analysers

Multifunction analysers such as the Nova Bioprofile 400 (Nova Biomedical, UK) and the Yellow Springs Instrument's, YSI 2700, operate at-line and are also based on electrical measurement principles. They are capable of the simultaneous at-line analysis of various nutrients (e.g., glucose, glutamine, glutamate, sodium and potassium), metabolites (e.g., lactate and ammonium), and gases (e.g., pO_2 DO and pCO_2) as well as parameters such as pH and osmolality in cell culture media. Osmolality and pH are important cell culture process parameters that are often examined and optimized to improve the productivity of cell-culture–based bioprocesses. pH, pCO_2, ammonia, sodium and potassium are measured using potentiometric electrodes. Potentiometric electrodes measure charged ions and have a sensing membrane that is selective to the ion being measured. They develop a voltage proportional to the concentration of the measured ion. The DO, glucose, lactate, glutamine and glutamate are measured with amperometric electrodes which develop a current proportional to the analyte. Glucose, lactate, glutamine and glutamate biosensors are amperometric electrodes that have immobilized enzymes in their membranes. The at-line Nova Bioprofile FLEX (Nova Biomedical, UK)

was used by Lu et al. (2013) to provide automated glucose measurements to a closed loop feedback control strategy where cultures were fed to maintain a target glucose level.

21.2.3 Optical-Based Instruments

Here, optical-based measurements discussed include spectroscopy techniques, analysis of microscope images, fluorescence and focussed beam reflectance measurement (FBRM).

21.2.3.1 Spectroscopy

Cell density, substrate and products are valuable targets for on-line measurement. The media used to support growth and protein production in animal culture are extremely sophisticated mixtures, often containing in excess of 80 different species, almost all of which are at very low concentrations when compared with small molecule production. Many of the species are from similar families, such as the 20 or so amino acids used by mammalian cells. The low concentrations combined with the structural similarities of multiple species means that finding an instrument with suitable sensitivity and specificity is non-trivial.

One promising means of biomass, substrate (glucose, glutamine, glutamate), by-product (lactate, ammonia) and end-product monitoring uses spectroscopic sensors based on near infrared (NIR), mid infrared (MIR) or Raman spectroscopy. These optical methods have many desirable attributes for bioprocess monitoring: they are non-invasive and non-destructive, do not consume the analyte or require sampling, are capable of monitoring several analytes simultaneously, provide continuous real-time measurements, no additional reagents are required and they do not interfere with cellular metabolism or the bioreactor environment. However, there are also challenges associated with the implementation of spectroscopic monitoring: chemometric or multivariate data analysis techniques are generally required for the interpretation of spectra for complex systems, the presence of bubbles, cells, and cell debris may interfere with the measurement, fouling of the window can occur over the relatively long process run times, and optical techniques have high measurement noise when compared with measurements based on alternative principles.

Figure 21.2 presents the raw and preprocessed Raman spectra from a complete bioreactor run. Chemometric analysis is necessary to translate such spectral data into the nutrient, metabolite and biomass concentration values of interest. Multivariate partial least squared (PLS) regression models are developed from in-line NIR, MIR or Raman spectra, which are correlated to standard reference measurements performed at-line. Individual calibration models are created for each constituent.

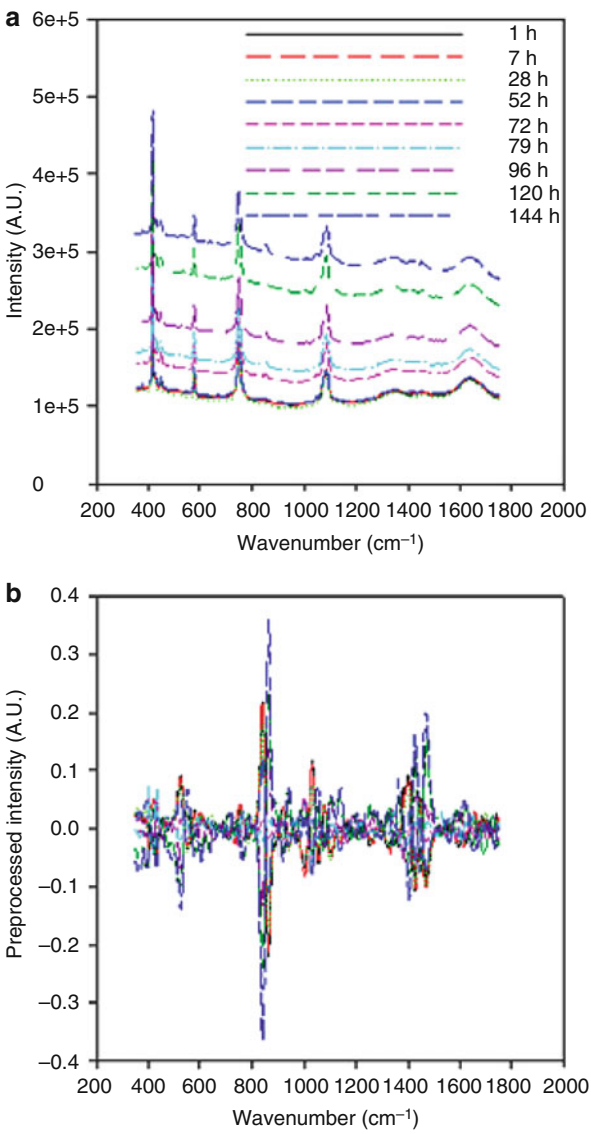

Fig. 21.2 (**a**) Raw and (**b**) preprocessed Raman spectra over the course of a bioreactor run (Whelan et al. 2012a)

The development of chemometric models will be discussed in the subsequent section on analysis.

Both NIR and MIR spectroscopy have been extensively applied for monitoring a variety of bioprocesses (Foley et al. 2012; McGovern et al. 2002; Roychoudhury et al. 2007; Sivakesava et al. 2001b). While measurement is fast and easy with NIR (780–2,526 nm), the resultant peaks are broad and overlapping, making interpretation of spectra difficult. Absorbance in the mid-infrared region (2.5–25 μm) is stronger and yields more distinct spectral features; however, strong interference

from water can make measurement in aqueous systems difficult. Raman spectroscopy measures the amount of light scattered inelastically at different frequencies by molecular vibrations. This results in very detailed molecular fingerprints with high chemical specificity. In addition, Raman measurements can be applied to aqueous systems with ease. Because of this, Raman has been used extensively for a wide variety of applications (Whelan et al. 2012a; Abu-Absi et al. 2011), and appears to be the most promising spectroscopic method for in-line analysis of complex cell culture systems detecting some analytes to levels as low as 0.3 mM. Whelan et al. (2012a) investigated the feasibility of using Raman spectroscopy for monitoring glucose, glutamine, glutamate, lactate, ammonia, total cell density (TCD), and viable cell density (VCD) in real-time. Individual chemometric partial least squares (PLS) models were built for each analyte of interest using three batches and the results were validated on a fourth batch.

21.2.3.2 Fluorescence

Many biological compounds such as proteins, enzymes, coenzymes, pigments, and primary or secondary metabolites emit characteristic fluorescent light after excitation by light from the visible or near UV region. Fluorescence is the emission of light from a molecule after it has absorbed radiation, with a spectral shift toward a longer wavelength. Generally, fluorescence emission occurs very rapidly after excitation (10^{-6} to 10^{-9} s). Molecular emission is a particularly important analytical technique because of its extreme sensitivity and high specificity. This specificity is due to the fact that the signal is measured over a zero background in comparison with the spectrophotometric method, where the signal measured is the difference between the incident and the transmitted light.

Fluorescence-based sensors for measuring DO, pH, and pCO_2 are available for in-situ applications, both as probes and as sensor spots. Each uses proprietary dye chemistries for which the level of chemiluminescence is proportional to the parameter of interest. Fluorescence-based sensors have particular appeal for miniaturized and single-use bioreactor formats because they require minimal maintenance and are simpler to use than electrochemical probes as there are no membranes to replace and no electrolyte solution needed. Online fluorescence spectroscopy sensors have also been developed and used to directly measure nicotinamide adenine dinucleotide phosphate (NADPH) among such other biogenic fluorophores such as proteins, coenzymes, and vitamins (Rao et al. 1994). NADPH is an important cellular compound since it plays a role in the metabolism of the cell. Generally, fluorescence-based sensors are non-invasive and the response times are nearly instantaneous. The use of glass fibre technology makes these sensors small, robust and reduces their cost. However, fluorescence probes are prone to sources of drift which include dye decomposition, leaching of the immobilized dye, changes in the ionic strength of the medium and temperature fluctuations. Probe fouling may also occur.

Flow cytometry is an optical-based, biophysical technology which analyses cells by suspending cells labelled with target-specific fluorophores in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multi-parametric analysis of the cell, such as cell viability, levels of apoptosis, cell cycle, mitochondrial activity, presence of specific biomarkers etc. Modern flow cytometers usually have multiple lasers and fluorescence detectors. Increasing the number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotypic markers. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface of cells.

Traditionally, flow cytometry is an off-line tool but recently, the application of automated at-line flow cytometry systems for bioreactor monitoring have been reported (Broger et al. 2011; Brognaux et al. 2013; Kuystermans et al. 2012). These systems have been configured to automate sampling, sample preparation and processing. They are capable of measuring multiple biological attributes such as cell concentration and cell viability as frequently as every 30 min. Results have been shown to correlate well with traditional haemacytometer counts. Automated flow cytometry can also measure several physiological parameters of many cells, enabling the assessment of population heterogeneity by many criteria. Such data, if of sufficient quantity and timeliness, may lead to new approaches in modelling and control applications.

21.2.3.3 Image Analysis

Two of the most important parameters for reliable fermentation control are cell density and cell viability. Commercially available, automated microscopes such as the Roche Cedex Hi-Res and Beckman Coulter Vi-Cell XR are commonly used for their analysis. While routinely used offline, it is possible to use them at-line by combining them with an automated sampling system. They both use image analysis algorithms to determine cell density and viability using trypan blue dye exclusion and a light microscope. In addition, to cell number and cell viability they also provide information about cell morphology and cell size.

Determination of cell density and viability would ideally be online. Currently, a number of instruments based on turbidity, conductivity, optical measurements or fluorescence have been employed to this end (MacMichael et al. 1987; Wu et al. 1995; Zhou and Hu 1994). Generally, the online measurements are still verified using traditional offline techniques. This in-process re-calibration or check is necessary, as the described online systems are affected by sensor drift over time or changes in the physical or chemical environment. A promising concept that eliminates the problems of existing systems is in situ microscopy, which could acquire images of cells directly inside a bioreactor. Process relevant information is extracted from the images by digital image processing and analysis software. Joeris et al. (2002) successfully tested an in-situ microscope in a CHO-cultivation and the results correlated very well with the results of haemocytometer hand counts.

The system also showed good stability and proved to be robust enough for the application in an industrial process.

21.2.3.4 Focussed Beam Reflectance Measurement

Focussed beam reflectance measurement (FBRM) is a another optical technology with the potential to provide non-destructive, non-invasive and continuous real-time monitoring of biomass concentration. It is a particle characterisation tool based on the analysis of the backscatter of laser light from particle suspensions. It can provide information on the number, nature, shape and size of the particles. It is currently used extensively in the chemical and pharmaceutical industries. It is a probe-based solid particle monitoring technique which operates by projecting a highly focussed, monochromatic laser beam (of wavelength 790 nm) into the suspension through a set of rotating optics and robust sapphire window (Fig. 21.3). The laser light backscatter is then translated into a chord length distribution (CLD) in real time. The CLD is a function of particle size and number.

Whelan et al. (2012b) showed that FBRM has potential as an on-line technology for tracking changes in cell density, concentration and morphology. Its capacity for in-situ sterilization and its ease of use makes FBRM a viable system for in-process application. It has the potentail to significantly reduce the need for sampling and off-line analysis. In addition, its ability to provide a simple fingerprint of process behaviour can form the basis for an effective control strategy.

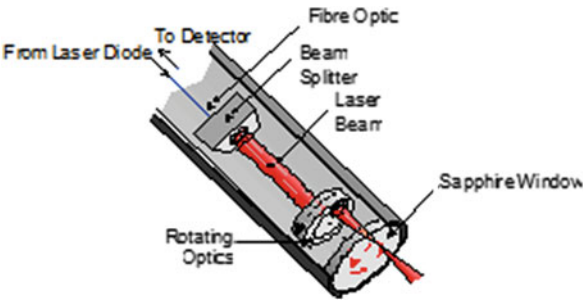

Fig. 21.3 Cutaway schematic of FBRM probe tip showing the internal arrangement and the laser lightpath (Whelan et al. 2012b)

21.2.4 Chromatographic Techniques

High-performance liquid chromatography, HPLC, is a technique used to separate the components in a mixture, to identify each component, and to quantify their concentrations. It relies on pumps to pass a pressurized liquid solvent containing the sample through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column. Derivatisation is a commonly used technique to augment chromatography analysis. It requires efficient and effective reagents that can modify the behaviour of complex compounds and allow their detection in chromatographic analysis.

HPLC is used routinely as an off-line method for analysis; however, combined with an automated bioreactor sampling system, at-line HPLC is an analytical technique that is uniquely suited to the challenges of bioreactor monitoring. HPLC has the capability of directly analysing these complex mixtures for many of their important components, such as amino acids and carbohydrates in cell culture media.

Significant advances have also been made allowing such techniques to be considered for process control loops. Advancements in detection tools now allow multidimensional analysis, which provides remarkably increased separation power and eliminates the need for sample derivatisation. Such developments are increasing the speed, resolution, range of application, and level of automation available for even complex samples. The automation of at-line HPLC has been reported by a number of authors (Kurokawa et al. 1994; Larson et al. 2002; Zhou et al. 1995), with the measurements gained being used for the development of feeding strategies in some cases. One of the future aspects of on-line liquid chromatography is to produce enough data for on-line batch analysis in combination with other on-line measurement tools. Analytical information can also be utilised as inputs to mathematical models which should be able to generate the values of the critical variables from the modelled biological mechanisms (Warth et al. 2010).

21.2.5 Single Use Technologies

Single-use bioreactors have become well established in the industry, and single-use sensing technologies are becoming more common in controlling critical parameters within them. Such sensors often provide advantages over traditional sensors by reducing risk of contamination caused by aseptic or sterile connections required to interface reusable sensors with a single-use system, reducing or eliminating the need for user-performed calibration and preventing product loss due to failures associated with reusing traditional sensors. In addition single-use sensors also will solve technical problems sometimes raised by using plastic bags in single-use

bioreactors, which can interfere with the functioning of stainless steel sensors because of static electricity problems, and therefore, cause sensor drift, especially for pH measurement. Common applications for single-use sensors include measurements of temperature, pressure, DO, and pH. Additional applications that are gaining acceptance include measurements of dissolved carbon dioxide, glucose, lactate, and other key metabolites (Spichiger et al. 2010). Some examples of the commonly used single-use sensors include PreSens and Polestar pH and DO sensors which are based on the fluorescent technologies discussed above.

21.2.6 Automated Sampling and Sample Preparation

Sampling and sample preparation are essential to the application of automated at-line measurement approaches. There are several considerations that should be taken into account when designing or selecting a sampling system: (1) does the sampling pose any risk of contamination (aseptic sampling)?, (2) how representative is the sample of the bulk batch?, (3) how much sample volume and dead volume are removed at each sample point?, (4) can the sampling system be automated and integrated with the at-line instruments?, (5) if sample preparation is necessary prior to analysis, can the sampling system reliably automate these operations?

Various automated sampling systems are commercially available, such as the Seg-Flow by Flownamics and the BaychroMAT from Bayer. One key aspect of these systems is the sampling valve assembly which is automatically sterilised before a sample is withdrawn from the bioreactor. After the sterilisation sequence, the withdrawn sample can be prepared and transferred to an integrated at-line instrument for analysis or to a sample tube for later off-line analysis. Chong et al. (2013) used the BaychroMAT to support robust automated sampling and analysis of mammalian cell bioreactors. High frequency automated samples provide a higher resolution description of the dynamics of cell density change compared to less frequent manual sampling and frees up personnel to carry out other tasks. These systems are also suited for use with disposable bioreactors.

21.3 Analysis: Transforming Process Data into Process Knowledge

With the ever-increasing volumes of process data acquired through new sensor technologies and high-throughput systems, data analysis is now a pivotal element in ensuring that the effort and costs associated with deploying a PAT strategy are beneficial to delivering a high quality product in an economical manner. Collecting the data is the first step. It must be translated into process information and further

transformed into knowledge of the process and design space in order to be of value and repay the investment.

Data analysis can falls into three areas: overview of the data, prediction and classification. Each of these areas has an application to PAT for animal cell culture. At an instrument level, it is necessary to process the signal to extract the information relating to the process parameter of interest. This may range from simple two-point pH probe calibrations to the application of chemometric techniques to determine a concentration from an IR or Raman spectrum.

At the outset of mapping the design space, it is important to implement a formal DoE in order to be certain that the investment in time and resources will result in a data set, which can be collected systematically and analysed to reveal the relationships between the process inputs and outputs. If a robust DoE is not applied from the outset, it may not be possible to determine the mechanistic nature of these relationships. Traditionally, developmental experiments have been executed for a particular purpose. They are usually one-factor-at-a-time (OFAT) style experiments. For example, an experiment to examine the effect of pH on product titre is typically conducted by running a number of parallel cultures at varying pH set-points. Animal cell culture experiments are costly and time and labour intensive to run. Every effort should be made to extract all the available knowledge from the data set. Often, however, the experimental results are inspected to answer the primary question and then archived. This is a missed opportunity. There is usually additional process knowledge available in these data sets that can be accessed with further analysis. In this example, the relationship between pH and specific glucose consumption could be investigated which could impact the feeding strategy. Furthermore, animal cell culture is complex and multivariate in nature which means that the true links between cause and effect can be obscured and misidentified. Is the observed difference in titre due to pH or a correlated parameter like dissolved carbon dioxide or osmolarity? A combination of modelling and MVDA of the experimental outcomes of a well-designed DoE addresses these challenges and are both areas of significant interest across research and development.

These same tools, modelling and MVDA, can also be applied to the manufacturing space, albeit with a different focus. In production, the value proposition is around process monitoring and management and on-line fault detection.

21.3.1 Multivariate Data Analysis

MVDA has many benefits to offer to both R&D and manufacturing: enhanced process understanding, improved process consistency, reduction in the number of failed batches, identification for potential process improvements and as a tool for root cause analysis. Furthermore, the regulatory authorities are expecting manufacturers to move toward MVDA. As the number of sensors and PAT instruments increases, so too does the volume of process data collected. Process data sets can consist of many streams of data: readings from online sensors such as temperature,

pH, DO, flowrates and so on, data associated with the control actions required to maintain a setpoint, for example, cooling water flowrates and temperatures, online or at-line PAT instruments, in process check (IPC) data from offline analysis of samples and data pertaining to process inputs such as raw materials. It becomes increasingly difficult to extract the pertinent process information from the large data sets and the potential for deeper process insight offered by PAT can be obscured.

MVDA enables the management and visualization of large data sets and has many applications to animal cell culture. These include:

- Multivariate calibration
- Batch analysis
  - Process characterization and design space mapping
  - Development of scale up/scale down models and in tech transfer
  - Batch consistency/root cause analysis
  - Online process monitoring and fault detection
  - Soft sensor/predictive monitoring

21.3.1.1 Multivariate Calibration

All measurement signals require some degree of processing to interpret them in terms of the measurement target. A PT100 temperature probe is calibrated to convert the electrical resistance of its element to temperature. A polarographic dissolved oxygen sensor must be calibrated to translate the current that results from the reduction of oxygen at the cathode into an oxygen concentration. HPLC methods typically use a standard curve to convert peak height or peak area for a chromatogram into a concentration. Instruments such as flow cytometers often have proprietary software packages attached which the user parameterises to calibrate the instrument for their particular cell line and reagents.

As discussed previously, a number of spectroscopies such as Raman, NIR and MIR, are showing promise as PAT tools for animal cell culture (Whelan et al. 2012a; Sivakesava et al. 2001a; Roychoudhury et al. 2007; McGovern et al. 2002). They offer many benefits such as being non-invasive, non-destructive and capable of analysing multiple species simultaneously. It is often necessary to employ chemometric techniques to extract the desired information, especially in animal cell cultures. The reactor environment consists of multiple phases: cells, medium, gas bubbles and other solids. The medium itself is composed of many chemical species, all of which are at relatively low concentrations in water. Not all species are well defined or quantifiable and many have common functional groups, such as the amino acids. For an instrument to be of value, it needs to be precise, accurate, sensitive and specific to the component(s) of interest. To reliably extract information from the spectra collected, be they NIR, MIR or Raman, chemometric techniques are often required.

Chemometrics is the use of mathematical and statistical analysis to extract information from systems by data-driven means. In the case of spectroscopy,

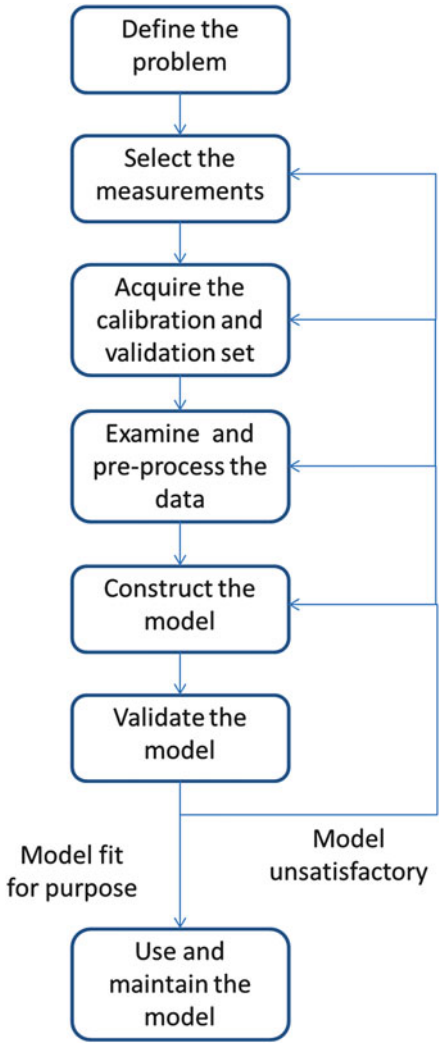

Fig. 21.4 Multivariate calibration model construction and validation

chemometrics seeks to correlate patterns in the spectra with the CPPs or CQAs and thereby, calibrate the spectral response to variations in the parameter of interest. Multivariate calibration is one of the cornerstones of chemometrics. Typically, multivariate calibration models are necessary in animal cell culture as there is rarely a clear, single peak related to the component to track due to the complex formulation and low concentrations involved. Multivariate calibration uses more than one x variable or input to predict the y variable or response. For example, in MIR spectroscopy the absorption at multiple wave numbers are used as the x variables. The benefits of multivariate calibration is that it allows for multiple

components to be analysed simultaneously, the multiple redundant measurements provided by adjacent variables can increase precision and it has facilitated a paradigm shift in measurement, opening up a host of technologies as useful measurement techniques.

There are a number of steps to building a calibration model (Fig. 21.4):

1. Definition of the problem statement.
2. Selection of a suitable measurement technology.
3. Acquisition of high quality calibration set comprised of spectral data and their corresponding concentration information.
4. Application of appropriate pre-processing to the spectra to counteract sources of interference or noise such as baseline drifts, scattering etc.
5. Construction of a calibration model.
6. Validation of the calibration model by testing its performance against previously unseen spectra.
7. Use and maintenance the calibration model.

Firstly, it is important to clearly define the problem statement. The nature of the system, the parameters to be measured and the range expected during operation should be specified. Practical issues such as the sample interval required for the given application, physical restrictions such as a need for sterility or exposure to sterilization conditions should be considered. Clearly understanding the operational requirements is necessary to select a suitable measurement technology.

The choice of the type of spectroscopy and the offline reference measurement method is the second step. They should be selected based on knowledge of the system and analyte to be quantified. The common forms of spectroscopy have been discussed in the previous section on measurement. It is also important to understand the precision and accuracy of both the spectroscopy instrument and the offline reference method because these will influence the precision and accuracy of the calibration model. The greater the noise associated with the spectra or the reference value, the greater the error of prediction of the calibration model that can be expected.

Once this has been completed, a high quality calibration data set must be collected. The data set consists of spectra and the corresponding offline reference values for the parameters to be determined. Every effort should be made to ensure that the calibration set is as precise and accurate as possible. If there are doubts over the quality of the spectral or offline data, then the data point should be excluded. The calibration set should span the concentration range specified in the problem statement and capture the level of variability expected during use. In the case of animal cell culture, many factors change over the course of the culture due to the consumption of nutrients and the production of biomass, product and by-products. These changes in the measurement environment should be reflected in the calibration set to account for any potential interference.

The data is then imported into the MVDA software to be examined and pre-processed to remove any outliers and reduce any sources of error or noise. Pre-processing is any mathematical manipulation of the data prior to model

construction. It can both positively and negatively impact the results. If not used carefully, it may remove the concentration information that is being sought. Common pre-processing techniques include: normalization, weighting, smoothing to reduce random noise and improve the signal-to-noise ratio (SNR), baseline corrections such as derivatives or scatter correction algorithms to remove systematic variations, scaling and spectral window selection. The reader is referred to the literature for further details on selection of pre-processing methods for guidance. In practice, the choice is often based on experience or trial and error.

The calibration model is then built using either principal component regression (PCR) or partial least squares regression (PLS). Both techniques reduce the dimension of the x space by generating a new set of axes or components for the data in decreasing order of relevance. PCR selects the components to explain the greatest level of variance in the x data without reference to the y data. The PLS algorithm selects components in the x space to explain the maximum variance in the y space. PLS is generally favoured as it ensures that the components used best explain the variance in the y variables even if there are greater irrelevant effects in the x variables. For example, a variation in temperature will affect an IR or Raman spectrum significantly but not the glucose concentration. Therefore, provided a suitable calibration data set has been used, a PLS model should be able to predict glucose concentration despite the changing temperature.

Models of this sort are not based on any underlying physical principle. They are built on correlations between the x data and y data. The correlated changes in the spectra may be directly related to the y variable but there may be no causal link. It is vital that the models are sufficiently validated to ensure that they will accurately predict the concentration for unseen data. The risk is that, given the large number of x variables in a spectrum, too many components may be included. This results in the noise in the calibration set being captured, giving low errors of calibration but poor predictive capabilities. In order to avoid this pitfall, a fully independent validation set of spectra and reference values is required. The number of components used should be chosen to maximize the predictive capability of the model when applied to the validation set. If the performance is unsatisfactory, the model can be redeveloped with alternative calibration samples, pre-processing or number of components.

Once the model performance is deemed satisfactory, it can be put to use. It is important to monitor the performance of these types of calibration models. Changes such as an alternative raw material, instrument or operating parameter may impact the quality of the predictions. In this case, the model may re-enter the construction phase and be supplemented.

There have been numerous examples of such calibration models published (Whelan et al. 2012a; Sivakesava et al. 2001a, b; Foley et al. 2012). They have been used to interpret and translate the signals from NIR, mid-IR and Raman spectroscopies as well as other instruments such as multiwavelength spectrofluorometers.

21.3.1.2 Batch Analysis

The QbD approach requires extensive batch analysis in order to identify and understand the relationships between the CPPs and CQAs. The traditional PCA and PLS techniques have been modified to allow for the analysis of batch data sets such as those generated during a bioreactor run. These modifications are known as multiway PCA (MPCA) or multiway PLS (MPLS). The general concept is that an MPCA or MPLS model is built to describe a process. The selection of MPCA or MPLS depends on the application.

The workflow is similar to that for developing a multivariate calibration model (Fig. 21.4). The problem statement is defined and this in turn informs the choice of data to be inputted to the model. Data for the calibration and validation sets are then either generated according to a DoE designed to address the particular question or extracted from a historical data base if appropriate. DoE data is generally required when seeking to extract causal relationships. Data sets from historical data bases are more appropriate if building a model to facilitate process monitoring or fault detection. At this point, additional variables may be generated in order to better reflect the process. For example, the cumulative base flow required to maintain the pH setpoint is generally more informative than the instantaneous base flow rate. When selecting which batches and which variables should be inputted to the model, it is important to use theoretical and process-related knowledge and to consider observability. Knowledge of the underlying process will inform which variables should be inputted and calculated to best represent the process. It also allows for a suitable frequency for the data to be chosen. The frequency need not be constant across the whole process. It can be reduced in periods that are deemed less critical in order to reduce the computational load. The process dynamics must, however, be taken into account so that important patterns are not missed. Observability means that the data set on which the model is constructed contains measurements or variables which capture the effect being investigated. For example, if the model is to be used for fault detection, the fault must be detectable in the data set.

The data must be examined to identify any issues such as faulty sensors, spikes in the data, missing data etc. It is then pre-processed, generally by scaling and weighting the variables. Here, it is important to use process knowledge to inform the decisions made. The analyst should never work independently of the process knowledge. The model is then constructed.

There are two levels of batch models: batch evolution models and batch level models. Batch evolution models are MPLS models with batch time or maturity as the y variable. Each batch is reduced to a line in the scores space (Fig. 21.5a). Batch level models may be MPCA or MPLS models. Each batch is further reduced to a point in the scores space (Fig. 21.5b). Batch evolution models may be used offline or in real-time. It is only possible to use batch level models offline as the batch data set must be complete in order to collapse it onto a single point.

Either MPCA or MPLS is used to reduce the dimensionality of the data set. The batches are then visualized in the scores space which plots the model components

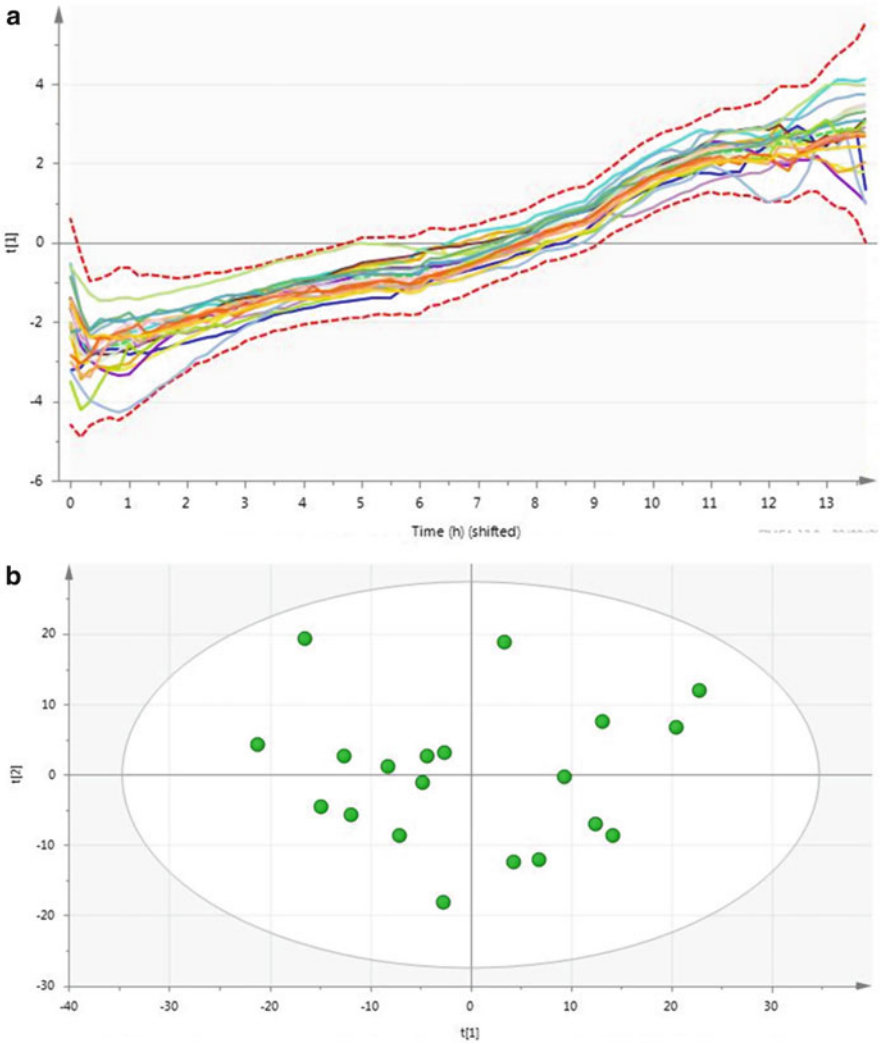

Fig. 21.5 Scores plot for (**a**) a batch evolution model and (**b**) a batch level model

against each other (Fig. 21.5). Batches close together in the scores space are statistically similar. The ellipse represents the 95 % confidence interval. The residuals for the batches should also be inspected. The residuals represent the variation in the batch that is not explained by the model. Batches with high residuals may be statistically different to the mean batch. It is possible to drill down to investigate which of the variables such as pH or temperature have contributed to either the variance explained by the model or the variance in the residuals.

As with all MVDA, it is important to validate the models developed with a validation set that spans the anticipated ranges. Once a satisfactory model has been

created, it is ready to be used. It will require maintenance and updates to capture any process changes.

There are numerous publications on the application of MVDA to cell culture processes. The range of applications are broad including: process characterization and design space mapping, development of scale up/scale down models and in tech transfer, batch consistency/root cause analysis, online process monitoring and fault detection and soft sensors/predictive monitoring. Amgen, in particular, has published numerous papers championing MVDA as a valuable tool in animal cell culture.

In 2008, Gunther et al. applied PLS techniques to the analysis of DoE data in order to identify similarities and dissimilarities between runs and to investigate which physical parameters contributed to these. The data set used was collected during a 92 run DoE conducted on an animal cell culture process. Online process data such as temperature, pH, DO and agitation along with the associated controller outputs and variables and offline measurements such as glucose, glutamine, lactate and ammonia concentrations amongst others were inputted for each batch. A PLS model was generated for each batch and these were then compared to all others by calculating a similarity factor. The similarity factor ranged from 0 to 1 with a value of 1 indicating identical batches. The PLS similarity factor was shown to reliably illustrate similarity or dissimilarity between batches. Analysis of the underlying PLS models then allowed the differences between the physical variables to be easily visualized. This work was extended in a further publication to broaden the analysis across multiple products and operating conditions (Gunther et al. 2009).

Gunther et al. (2009) also used online PLS modelling to predict a quality variable, product titre and for fault detection and diagnosis for a fed-batch cell culture process conducted at an Amgen pilot plant facility at a 2 L scale. Variables such as temperature, agitation, pH, DO, air, oxygen, carbon dioxide gassing rates, base, antifoam and feed flow rates were used as inputs to the MVDA. Fifteen batches were used in the calibration set with four being reserved for the validation set, one of which contained a fault. A single model was shown to be capable of accurately predicting protein titre over the time course of the batch. Interrogation of a number of the statistics associated with the models identified the abnormal batch and allowed the DO profiles to be identified as the deviation. Earlier in 2007, the same group had also demonstrated that PCA analysis could also be used for online fault detection and diagnosis (Gunther et al. 2007). In this paper, analysis of the variables contributing to abnormal behaviour in the PCA space were shown to successful identify the source of the fault, some of which had the potential to be addressed immediately and hence, avoid loss of the batch.

Kirdar et al., also of Amgen, used MVDA to facilitate the successful scale-up of a cell culture process from 2 to 15,000 L by identifying the root causes for differences in process performance across the scales (Kirdar et al. 2007, 2008). MVDA of the batches revealed two distinct clusters of data. Interrogation of the models suggested that raw materials and pCO2 levels were responsible for the differences between the groups and this was confirmed by experimental verification, demonstrating the power of MVDA.

Many other groups have reported on batch MVDA. Lopes and Menezes (2003) applied the techniques to predict final product concentration and to determine the time points in the process that most influenced the outcome. Ündey et al. (2004) implemented online MVDA for process monitoring and diagnosis of faults and the prediction product quality parameters. Others have taken it a step further and have used the multivariate models developed as part of a control strategy to ensure that the process follows a defined trajectory (Chiang et al. 2006). This application will be discussed further in the section on control.

21.3.2 Modelling

The value of modelling as a knowledge management tool can be clearly understood on examination of the application of MVDA to animal cell culture processes. However, this represents only one of a multitude of modelling approaches. At the simplest level, a model is a mathematical or statistical relationship that predicts certain outputs based on a series of inputs. The relationships may be purely empirical, that is data-driven, or based on fundamental scientific principles or a hybrid of both. The selection of the most appropriate modelling approach depends on a number of factors: the system to be modelled, the type of data available, the amount of data available and the ultimate goal of the model.

When developing the model, the first question that should be asked is what the intended application is. It is important to understand what knowledge is the model seeking to represent and to what use the knowledge captured within the model will be put. Common applications include process monitoring, process characterization, soft sensors, process optimization and as an element of a control strategy.

Once the application is understood, the system can be defined. In animal cell culture, the system can be thought of at the macro, meso or micro-scale (St Amand et al. 2012). The bioreactor along with its inputs and outputs constitutes the macro-scale. The individual cell can be considered as the meso-scale and the micro-scale is concerned with intracellular organelles such as ER, Golgi apparatus or ribosomes. All three scales are inter-related. For example, the nutrient concentrations in the bioreactor determine the state of the cell and hence, the activities within each of the organelles. The scale and hence, level of detail and complexity built into the model depends on the intended use.

At this stage, the data required should be specified based on the problem statement. Considerations such as what data are possible to collect, the quality and reliability of that data, the time and experimental runs necessary etc. should be taken into account. The data may consist of on-line parameters as well as at- and off-line sample results. Experimental investigation of the principles governing the system may be necessary in the case of mechanistic model development. Whatever data set is specified should be obtainable and contain information pertinent to the application, that is the phenomena being modelled must be observable within the data set. As the number of process analyzers and their sophistication increases, so

too does the amount and diversity of process data available, opening up further modelling opportunities.

Data-driven models do not make any mechanistic assumptions about the system and so can be a good option for complex systems. They do, however, require large amounts of high quality data for model development. Also, these types of models should not be used for extrapolation or predictive purposes as their validity in regions outside the ranges previously seen cannot be assured. Data-driven models are also known as "black box" models, reflecting the absence of the structure of the underlying physical mechanisms in the model.

Mechanistic models are based on the underlying processes and scientific principles. Having this mechanistic knowledge captured within the model allows it to be used for predictive purposes. These models can be developed on much smaller data sets. The challenge, however, is to represent the mechanisms of the system. This requires in depth understanding of the process and principles. Given the complexity of animal cell culture systems, this is often aspirational.

Hybrid models use both data-driven and mechanistic elements in order to balance their advantages and disadvantages. The mechanistic model components can be used to ensure that the mass balances are obeyed while the empirical model components can be used to describe the more poorly understood aspects of the system. One example of a hybrid model is where the equations governing cell growth are known and so modelled mechanistically but some of the inputs are not known. A neural network is trained to create the inputs for the mechanistic equations (Silva et al. 2000). Hybrid models will not be discussed in detail here.

21.3.2.1 Data-Driven Models

The most common form of data-driven model, bar the multivariate PCA and PLS models discussed above, are artificial neural networks (ANNs). ANNs are computing systems made up of a number of simple, highly interconnected processing elements called nodes. The structure of ANNs was inspired by biological neural networks in the brain. Standard computer processors solve problems in a sequential, deterministic manner. ANNs, similar to the brain, solves problems in a parallel, stochastic way.

ANNs are of value in modelling animal cell cultures as they are useful in situations where:

- The objective is to identify patterns
- The number of variables and volume of data is large
- The relationship between variables is poorly understood
- The relationship between variables is difficult to describe by conventional approaches, particularly if the relationship is dynamic or non-linear.

ANNs have been described in detail elsewhere (Boudreau and McMillan 2007). Briefly, the nodes are arranged in layers with an input and output layer with a number of hidden layers between. Each node processes its input via a simple

activation function, for example a sigmoid function. Each input is weighted. Suitable weights are determined by "training" the ANN with a training data set. Therefore, the model is determined by the ANN architecture, ie number of layers and nodes, the weights on the node connections which are adapted during training and the activation function within each node that converts the weighted inputs to an output.

ANNs have been applied in a number of ways to biological processes. They have been used as soft sensors to predict biomass concentration from indirect measurements such as oxygen uptake rate (OUR), carbon dioxide production rate (CPR) and base consumption rate (BCR) (Jenzsch et al. 2006). They have also been used to exploit data bases in order to identify the influence of process variables on performance and to model cell culture kinetics such as substrate consumption and by-product production rates (Shimizu et al. 1998).

21.3.2.2 Mechanistic Models

Mechanistic models seek to describe phenomena in terms of the underlying fundamental principles, such as mass balances or reaction pathways. One of the major benefits of developing a mechanistic model over a data-driven model is that it may be used for predictive purposes because of the knowledge that it contains. It could, for example, be used to explore the effect of different feeding regimes or process set-points in an animal cell culture process without the need to run many iterative experiments. In effect, the experiments could be run in the virtual environment. The challenge is to develop the model for such complex systems. The relationships between variables may be dynamic and non-linear and therefore, difficult to express mathematically. The multivariate nature of the relationships and challenges associated with some biological analytical techniques may result in the nature of the interactions being only vaguely understood and difficult to investigate. The advantages have ensured that, despite these challenges, mechanistic models are still pursued. Here the discussion will be limited to models based on engineering first principles rather than biological first principles.

First principle models can be delineated by degree of structure and segregation described (Table 21.3). Unstructured models treat the cell as a single unit while

Table 21.3 Categories of mechanistic models

	Unsegregated	Segregated
Unstructured	Simplistic representation	Heterogeneous cell population
	Homogeneous cell population comprised of single component cells	Individual cells are represented as simple, homogeneous single-component units
Structured	Homogeneous cell population	Heterogeneous cell population comprised of multi-component cells
	Individual cells are represented as multi-component units	

structured models separate the cell into a number of pools such as amino acids, proteins and lipids. Unsegregated models treat the cell population as a number of identical units, whereas segregated models differentiate subpopulations in the cell number, for example, based on the cell cycle.

The level of process understanding and complexity within process models can be categorized in increasing order from lowest to highest as unstructured and non-segregated to structured and segregated. While structured, segregated process models provide the greatest level of understanding and prediction capability, the large effort required for their development and use within biopharmaceutical industry may be too expensive due to the complex nature of most bioprocesses.

One of the most common mathematical models used for bioprocesses is based on the Monod kinetic model. It is an unstructured, non-segregated model. There are a number of examples of this type of model presented in the literature (Craven et al. 2013; Dalili et al. 1990; Liu et al. 2008; Tremblay et al. 1992). The specific growth and death rates are generally modelled using Monod type functions based on nutrient and metabolite concentrations. Unstructured models do not account for the intracellular processes and acknowledge only implicitly the change of cellular physiological state with the environment. Such models treat the cell as a single homogeneous unit, hence their biological significance is limited, and the mathematical equations involved are only phenomenological descriptions of the actual biological system. Pörtner and Schäfer (1996) conducted a survey on unstructured models for hybridoma cell growth and metabolite production which revealed that even though unstructured models contain limited process mechanistic information compared with structured models, they can still serve as an effective tool for process design as the model ensures that the engineering first principles such as mass and energy conservation are obeyed.

Structured models, on the other hand, are more complex because they attempt to incorporate biological knowledge by separating the biomaterial into compartments that are chemically and physically distinct. The compartments' interactions with each other and with the environment are described by stoichiometric equations that account for various metabolic pathways and/or kinetic rate expressions. For example, Batt and Kompala (1989) developed a structured kinetic model which distinguishes four compartments inside the cell and considers the major substrates (glucose and amino acids) and metabolites (lactate and ammonia). The development of such models can be time consuming and require a large knowledge base.

Not all cells in a mammalian cell culture are alike; they are a heterogeneous population. Process models may or may not take this heterogeneity into account. A non-segregated model views the population as consisting of identical "average cells" and uses a lumped variable such as total biomass per unit volume, to describe the entire population. Segregated models, in contrast, may take the cell cycle into consideration or distinguish different cell states (de Andrés-Toro et al. 1998; Uchiyama and Shioya 1999). Differentiating cells in the population with similar characteristics into groups is more representative of the true physiological state. Therefore, segregated models can account for the degree of heterogeneity of the cell culture with regards to cell age, size, growth rate and metabolic state. However,

segregated models are also more computationally difficult to handle (Bailey and Ollis 1986).

21.4 Control: Harnessing Process Knowledge

Biological systems have inherently large variations between batches. Controlling the bioreactor helps to reduce the variability and to protect product quality. However, bioprocesses are dynamically complex; therefore, their control is a challenging and delicate task. Process control attempts to influence the sophisticated metabolic reactions inside the cell by controlling the extracellular environment. QbD, risk-based manufacturing and PAT have become three cornerstones of the U.S. Food and Drug Administration's effort to modernize pharmaceutical and biopharmaceutical manufacturing. The application of multivariate, statistically designed experiments is valuable for identifying the CPPs that affect the CQAs of the bioprocess. However, the scarcity of on-line and at-line measurements for some of the major bioprocess CPPs such as, nutrient, metabolite and biomass concentrations, makes the task of controlling the CPPs and CQAs non-trivial. To improve the control of a bioprocess, the identified bioprocess CPPs must be continuously monitored on-line or at-line. In biopharmaceutical processes, the need for real-time process information is particularly high due to the complexity and unpredictability of the process. Traditionally, nutrient, metabolite and biomass concentrations are obtained through off-line laboratory methods. Such off-line analyses limit the possibility of controlling and optimising bioprocesses. However, PAT aims to close this gap through timely availability of such data, ultimately facilitating better control strategies and process optimisation. Automated process control is routinely used to some aspects of the bioreactor environment such as the temperature, pH and DO level. However, there are few reports of automated control of other potential CPPs or CQAs such as substrate concentrations or growth rate at the industrial production scale.

21.4.1 Automated Process Control

Automated process control encompasses a range of control strategies which seek to maintain specified process variables (PV) to a predefined value or set-point. Deviations from these defined set-points can dramatically diminish the growth and productivity of the bioprocess. The actual PV value is controlled by making changes to a manipulated variable. For example, in order to maintain a certain pH (the PV), the flow of base or carbon dioxide (the manipulated variables) are typically changed as required.

Control can be broken down into three stages: measurement, comparison and adjustment. Firstly, it is necessary to make some determination of the current state of the PV. This is accomplished through measurement. The PV may be measured directly or inferred through the measurement of a related parameter known as the

measured variable (MV). The current state of the system is then compared to the desired state in order to determine if it is necessary to take action to correct the PV. The controller computes the action required and adjusts the manipulated variables as required to remove any discrepancy between the actual and desired PV values.

There are a number of automated control loop configurations commonly used. Open loop and closed loop feedback control are the most relevant for animal cell culture. Open loop control is a controller which computes its action or adjustment based only on the current state of the system and its process model. There is no feedback in the loop so it cannot determine if its action has had the desired effect. Therefore, it cannot compensate for disturbances to the system or any errors it may make. An example of open loop control in animal cell culture processes would be substrate control via a fixed feed trajectory. Here, the feed supplied to the reactor at any given time is based on time elapsed and a priori knowledge of the culture requirements.

Closed loop feedback control measures the effect that its action has on the system and takes this into account when determining the action required (Fig. 21.6). The PV or related MV is measured and then compared to the set-point. Based on the error, the controller determines the action to be taken. The manipulated variable is then adjusted to reduce the error. The feedback allows for disturbance rejection and process variability. A standard pH control loop using base and carbon dioxide to maintain set-point control is one example. Given the level of variability typically observed in mammalian cell culture processes, the capability to

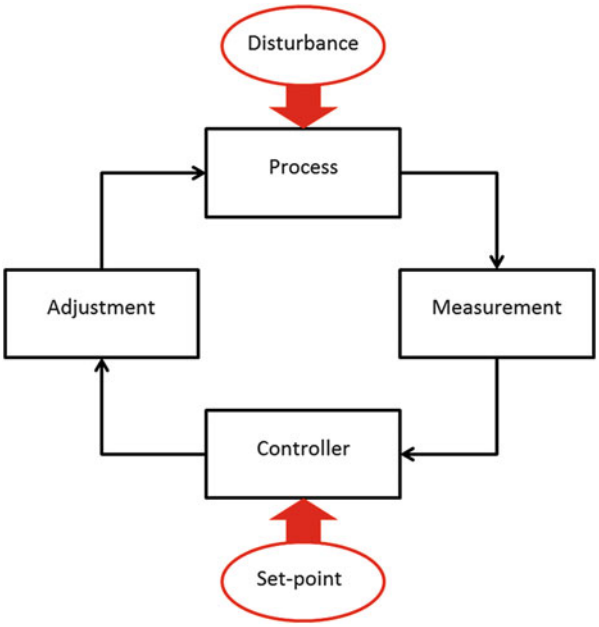

Fig. 21.6 Closed loop feedback control schematic

self-correct is extremely important if tight control of a parameter is to be realized. Currently, feedback control is only routinely applied to a small number of variables such as temperature, pressure, pH and DO in this field. However, as technology advances and the real-time measurement of a greater number of parameters become accessible, it is possible to control more CPPs to their optimum level and thus optimize the bioprocess.

There are many academic projects focusing on the application of automated open and closed loop nutrient concentration control strategies using predefined feeds and feeds determined using on-line or at-line sensors to measure the process variable (PV) respectively (Lu et al. 2013). On-line models have also been used to estimate the nutrient concentration with an at-line sensor used to periodically update the model. Lu et al. (2013) demonstrated two different automated cell culture control strategies. The first method was based upon on-line capacitance measurements where cultures were fed based on an on-line calculation involving growth and nutrient consumption rates. The second method was based upon automated glucose measurements obtained from the Nova Bioprofile FLEX (Nova Biomedical, UK) where cultures were fed to maintain a target glucose level by using an on-line feedback calculation.

Lee et al. (2003) used a low-glutamine fed-batch feedback control-loop process in attempting to control ammonia and lactate for a 293-HEK mammalian cell bioprocess for adenovirus production . The control algorithm consisted of a simple on-line calculation. Controlling glutamine levels at 0.1 mM, with no other modifications improved cell density and gave a tenfold improvement in virus titer. Li et al. (2005) controlled glucose at 0.3 mM and glutamine at 0.5 mM via an online closed loop feeding calculation, which related to ammonia and lactate levels decreasing by 74 % and 63 % respectively. Their cultures extended from 8 to 14 days, with a 1.7-fold increase in monoclonal antibody (MAb) titers.

The traditional bioreactor control loops are based on classical proportional, integral derivative (PID) feedback control strategies. The PID controller is by far the most dominant form of feedback control in use today with more than 90 % of the control loops in the entire manufacturing sector being based on PID controllers. PID control has been applied to a wide variety of processes with varying degrees of success and is thus the bread and butter of automatic control. It is the first control strategy that should be tried when feedback control is used. However, PID controllers are single input-single output (SISO) controllers and generally only perform best for processes characterised by linear, low-order dynamics. Overall, this linear, SISO PID controller framework is not optimally designed to control the inherently nonlinear and complex nature of bioprocesses with highly interacting process dynamics.

Commercial PID controllers generally have features to enhance controller performance such as gain scheduling. Gain scheduling is a PID enhancement that facilitates the control of a process with controller tuning parameters (gains and time constants) that vary according to the current value of the process variable. This gives the PID controller non-linearity. A gain scheduler runs in the PID controller

framework and monitors the process variable to determine when the process has entered a new operating range. It then updates the controller with a predetermined set of tuning parameters designed to optimize the closed-loop performance in that range. Gain scheduling is particularly appropriate for bioprocesses where the process dynamics change over the course of the process. Unfortunately, tuning a PID controller for different operating regions requires a lot of work. The control engineer implementing the gain schedule must first determine how the full span of the process variable should be partitioned into distinct operating ranges that adequately represent all the possible variations in the process's behaviour. The controller would then have to be tuned for each operating range. Various alternatives to the PID controller algorithm have been developed such as advanced and intelligent controllers that would be better suited to the nonlinear, complex, multivariate nature of bioprocesses (Boudreau and McMillan 2007).

21.4.2 Advanced Process Control

The limitations associated with the standard process control solutions have led to significant interest in advanced process control (APC) across numerous industries. The number of APC applications worldwide was estimated to be around 6,000 in 2005 and it is growing steadily (Canney 2005). As with new hardware technologies, the biopharmaceutical industry tends to move slowly and cautiously with regard to new approaches in control algorithms. Engineers are monitoring the evolution of techniques such as fuzzy logic controllers (FLC) and model predictive control (MPC) and considering their potential application to bioprocesses.

21.4.2.1 Model Predictive Control

MPC is currently the most widely used of all advanced control methodologies for industrial applications (Qin and Badgwell 2003). The essence of MPC is to optimise forecasts of process behaviour. The forecasting is accomplished with a process model, and, therefore, the model is an essential element of an MPC controller. A schematic of the MPC framework is presented in Fig. 21.7. The MPC can be classified as either linear MPC or nonlinear MPC depending on the model utilised within the MPC framework. Though manufacturing processes are inherently nonlinear, the vast majority of MPC applications to date are based on linear mathematical models, the most common being step and impulse response models (Özkan et al. 2000; Xie et al. 2011). This is because such models are easier to identify and less computationally intensive to solve in comparison with nonlinear models. However, there are cases where nonlinear effects are significant enough to justify the use of nonlinear MPC (NMPC) technology (Hauge et al. 2005). Nonlinear model predictive control (NMPC) is an algorithm of intense industrial and academic interest (Biegler 1998). Processes demonstrating highly nonlinear

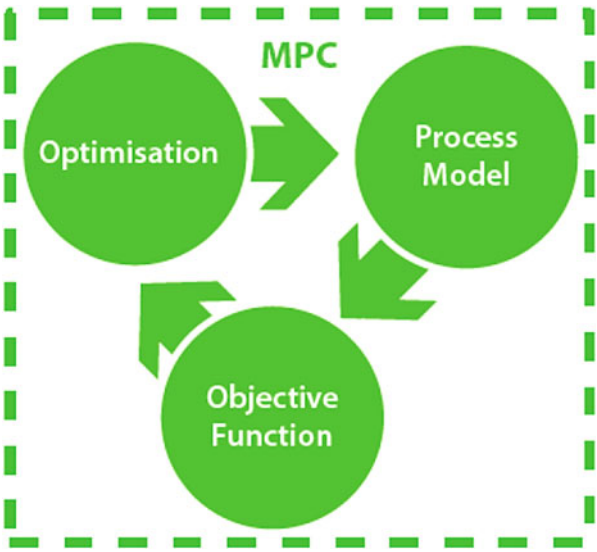

Fig. 21.7 Schematic of a model predictive controller

behaviour can be operated in regimes closer to the process optimum, where LMPC may fail (Morari 1983). Most of the non-linear MPC strategies used in industry have been based on the use of artificial neural networks (Bequette 2007).

Since MPC seeks to minimise the objective function over a prediction horizon, the short term effects of unknowns and erratic signals are minimised. In contrast, a PID controller only knows what is happening at any instant. Additionally, MPC can simultaneously manipulate multiple variables, whereas a PID controller can only handle one controller output and one controller input. For example, to optimise the growth rate of a mammalian cell bioprocess, it is necessary to control a number of interacting bioprocess parameters (culture pH, nutrient concentration, by-product concentration, culture temperature, osmolarity, DO, etc.). The ability to model process interactions often enables model predictive controllers to outperform multiple PID control loops, which require individual tuning.

MPC technology can now be found in a wide range of application areas, including chemicals, food processing, automotive, and aerospace applications. Bioprocess applications of MPC have appeared in a number of academic projects recently. Aehle et al. (2012) experimentally applied a MPC to indirectly control the oxygen mass consumed by mammalian cells in a bioreactor by manipulating the glutamine feed-rate. Ashoori et al. (2009) simulated the use of MPC based on a detailed model for penicillin production in a fed-batch bioreactor. The main control goal was to get a pure product with a high concentration, by regulating temperature and pH at certain levels.

One of the main challenges in mammalian cell culture relates to the cellular metabolism. By-products such as lactate and ammonia result from glucose and glutamine metabolism, respectively and are accumulated in cultures. Lactate build up can have negative effects on the pH and osmolarity of the culture medium and

thus the growth of the cells. Similarly high levels of ammonia in cell culture can lead to intracellular pH changes and inhibition of enzymatic reactions. Lowering lactate and ammonia production by mammalian cells can lead to increased process productivity, robustness and protein quality. Controlling the glucose and glutamine concentrations in the bioreactor at fixed low concentrations via the controlled addition of the nutrients to the bioreactor helps to avoid overflow metabolism (formation of high levels of by-products such as lactate and ammonia) and thus directly improves the culture.

To date, the most frequently employed fed-batch operation scheme uses concentrated solutions of key nutrients fed according to a predefined feeding strategy or based on offline integral viable cell density calculations or concentration measurements (deZengotita et al. 2000; Pörtner and Schäfer 1996; Zhou et al. 1997). Process control based on such open-loop feeding programs is well accepted in the biopharmaceutical industry due to its simplicity. However, such open loop modes of control are only effective if robustness is not significantly affected by uncontrollable input parameters and if the variability is not very large. In this case, closed loop control might be highly beneficial. With the development of suitable process models and the availability of reliable biosensors for real time monitoring, the application of an MPC strategy is a possibility. The MPC can calculate optimum, continuous feed profiles for the fed-batch bioprocess because it can predict future violations of constraints; has built in features for maximisation, minimisation and has a tuning adjustment to smooth out the optimisation in response to a noisy measurement signal. Noisy measurement signals are inherent when using on-line spectroscopic techniques such as IR and Raman. MPC also offers the simultaneous manipulation of multiple process inputs. There are huge benefits in being able to apply this strategy to multiple nutrients key in metabolism, protein expression and product quality.

Traditionally, in the biopharmaceutical industry, on-line sensors or automated at-line analytical techniques are not common place. As a result, due to the time lags associated with taking a sample from the bioreactor and awaiting analyses from the laboratory, closed loop feedback control is futile via classical PID controllers. However, based on the ability of the MPC controller to predict the trajectory of the nutrient consumption over a prediction horizon with the aid of an applicable process model, it is possible to implement closed loop feedback control for process parameters measured as infrequently as every 24 h. For example, an MPC can be used to successfully calculate a continuous optimum feed profile to control the glucose concentration in a bioreactor to a defined set-point with a measurement frequency of 12 h based on off-line analytical analyses (Craven et al. 2014).

Dissolved oxygen is an important factor because it is a regulator of metabolic activity. Several examples have demonstrated the successful use of MPC to control dissolved oxygen by manipulating multiple variables to do a better job than a PID controller in dealing with noise and dead time (response of a PV to the controller calculated manipulated variable). In one case, an MPC application for a microbial fermentation controlled DO in a bioreactor by manipulating air flow, agitation speed, and vessel pressure. The MPC controlled a single process variable by

manipulating three different controller outputs (Callanan 2004). In another case, an MPC application for a microbial fermentation controlled DO by manipulating air, oxygen, and nitrogen to the headspace and oxygen sparge to the broth and it controlled pH by manipulating carbon dioxide to the headspace and a sodium carbonate solution to the broth (Jerden et al. 2003). These microbial examples are directly applicable to mammalian cell cultures.

In summary, MPC can be beneficial for processes with (1) inherent measurement noise, (2) large dead times (response of a PV to a controller calculated manipulated variable), (3) multiple manipulated variables, (4) interactions, (5) constraints and (6) optimisation. The benefits from optimisation can provide the motivation to advance beyond classical single input/single output PID control to advanced multiple input/multiple output MPC control. Overall, batch manufacturers who use optimally tuned MPC are experiencing significant benefits in the form of improved product quality and consistency between batches (Bauer and Craig 2008).

21.4.2.2 Fuzzy Logic Control

Intelligent control is also an emerging area of interest in recent years for bioreactor control. Current developments in knowledge based, and associated learning systems, for real-time control offer opportunities for significant improvements in product quality assurance and fault tolerance. Intelligent controllers are adaptive controllers that automatically adapt to plant and environmental changes without prior knowledge of the changes. Such controllers use input/output data to formulate control strategies. Bioprocesses have traditionally been controlled by the judgement of the experts who are skilled operators and have much process experience. Such experience is normally described in terms of linguistic IF-THEN rules. Fuzzy logic control (FLC) is a popular intelligent control system based upon linguistic processes expressed in an interactive rule base and is often mentioned as an alternative to PID control (Passino et al. 1998). The main advantages claimed by fuzzy logic control over PID control are its nonlinearity, flexibility and ability to handle multiple inputs and outputs. In contrast to model predictive controllers, fuzzy logic control is known as a knowledge-based controller that does not require a mathematical model of the process at any stage of the controller design and implementation. In many cases, a mathematical model of the control process may not exist or maybe too expensive in terms of computer processing power and memory, and a system based on rules and human knowledge may be more effective. In this case, fuzzy logic control is a simple alternative to model-based advanced controllers. However, tuning of fuzzy logic controllers is not an easy task. Due to the many calibration possibilities, the tuning is a complex process which can take a relatively long time and require much expert experience in identifying the rules.

In summary, fuzzy control based on fuzzy set theory is a mathematical tool for dealing with qualitative information and linguistic expressions which can be applied to biotechnology (Galluzzo and Cosenza 2010). Nyttle and Chidambaram (1993) showed how a fuzzy logic controller could use glucose feed to force a

penicillin fermentation process to follow a predetermined product time varying concentration profile.

21.4.2.3 Multivariate Statistical Process Control

Statistical process control (SPC) is typically a technique for monitoring process performance. However, this technique is usually lacking closed loop feedback control on identified process deviations. Process deviations are identified by analysing batch control charts online and acting on identified excursions in an open loop fashion. When a process deviation is identified that could be critical to the process performance, an attempt is made by the process operator to identify the source of the deviation and to restore the process to its optimal trajectory.

Most manufacturers still rely on traditional univariate SPC which uses univariate statistical analysis such as mean, median, standard deviation etc. Traditionally, univariate SPC uses charts such as Shewhart, CUSUM and EWMA (Chiang et al. 2006; Lopes et al. 2002), which monitor a single process variable for the purpose of improving product purity and reducing batch to batch variability. An example of a shewhart control chart is shown in Fig. 21.8. Unfortunately, univariate statistical techniques often miss the underlying patterns in process data and do not perform well for multivariate systems such as bioprocesses. This is where Multivariate Statistical Process Control (MSPC) can give manufacturers and engineers an edge. MSPC has many advantages over traditional SPC. Overall, MSPC simplifies the job of process operators by showing all process variables, including relationships which cannot be detected with univariate statistics, on just one or two control charts. Therefore, the necessity of a control chart for every process variable is eliminated.

MSPC is not limited to academic or research purposes; MSPC has been applied across many industries during the past 10 years, with applications in the areas of polymer processing (Kourti 2005), semiconductors (Cherry and Qin 2005) and chemicals (Rotem et al. 2000). In comparison to these industries, the application

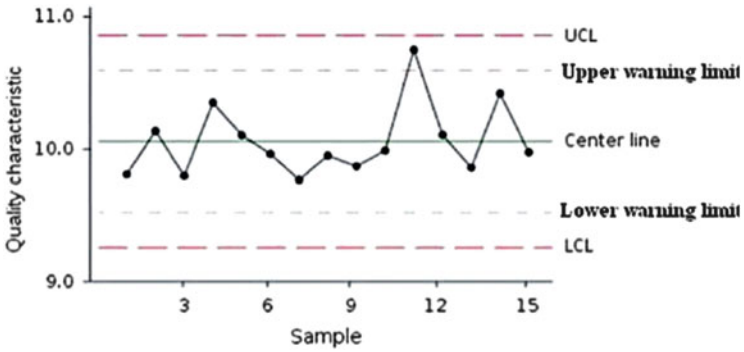

Fig. 21.8 Shewhart control chart (Shah et al. 2010)

of MSPC in the biopharmaceutical industry is in its infancy. However, due to the abundance of data available from bioprocesses, which has been augmented with the advent of online and at-line PAT, MSPC is a good candidate for achieving the goals of high quality product and low batch to batch variability. In bioprocesses, hundreds of variables can be recorded regularly for a single operating unit, resulting in a large data set that is hard to analyse via univariate methods. MSPC uses MVDA methods to reduce the dimension of the large raw data set. These methods are used to build a golden batch model of the process from historical data. MSPC applies this multivariate model online to real-time data from the distributed control system (DCS) or data historian to give the user a real-time indication of the current batch progress against the predefined model. Figure 21.5a demonstrates how on-line process monitoring can be conducted for a bioreactor process. It illustrates the golden batch trajectory (green line) with the associated control limits (red lines) that are typically $\pm 3\sigma$.

The MSPC allows for the operator to visualise in real-time excursions from the golden batch trajectory and detect which process variable(s) is contributing to the identified excursion from ideal batch process behaviour. Once the process variable(s) has been identified, its associated univariate chart(s) can be analysed to see if the process variable(s) is within the pre-specified limits or ranges. Following an identification of the root cause of the process deviation, the process and quality experts should be consulted to determine the appropriate course of action.

Many academic and industrial researchers have recognised the benefits of MSPC and applied the methodology to various bioprocesses. Lopes et al. (2002) applied MSPC to a pharmaceutical fermentation process and determined the most important process stages are inoculum production and API production fermentation. Also, Chiang et al. (2001) studied the performance of MSPC in an industrial fermentation process at the San Diego biotech facility of the Dow chemical company. Use of MSPC in conjunction with PAT instruments is gaining importance in process control (Machin et al. 2011). It focuses on describing the whole process and deviations to desired behaviour. In particular, batch trajectories (or batch evolution models) represent an easily comprehended, online, good-or-bad assessment of a batch in progress. It allows operators to identify deviations and counteract them immediately.

In recent years, work has been done by the MVDA software company, Umetrics, to combine the multivariate monitoring tools of MSPC and the prediction and control tools of advanced MPC. Harnessing the benefits of both these techniques, an MVDA model representing the ideal batch behaviour can be used as the process model within an MPC framework. If the process is predicted to deviate outside the specified control limits, adjustments can be implemented to correct the process before the control limits are reached. The benefits in terms of reduced batch-to-batch variability, increased product quality, greater process understanding can be enormous.

Overall, increased bioreactor monitoring and control have led to reduced bioprocess variability, productivity improvements and increased on-line monitoring

and troubleshooting capabilities. On-line monitoring and control of bioprocesses has become an expectation and no longer needs justification for most bioprocesses.

21.5 Optimization: Managing Process Performance

The ultimate benefit of applying PAT to animal cell cultures is the delivery of an optimized, robust process. The process insight gained through the application of measurement, analysis and control allows for the development of fundamental understanding of which process parameters are important in determining the process outputs. The enhanced capability to peer into the process has many benefits both during development and manufacture. These benefits have been highlighted previously but in summary, the most significant benefit is simply that the application of PAT to a process confers upon us the ability to manage the process performance.

Greater understanding and control makes it possible to more robustly deliver a high quality product. By embedding scientific understanding into the process, the scaling from the development laboratory to the production plant is more reliable. It is easier to ascertain the root causes for process deviations and process drifts and to put effective remedial actions in place. The sophistication of PAT applied to cell cultures is ever increasing and so, the value and impact it makes to these processes is growing. Although in its infancy in this sector, advanced measurement and closed loop control strategies are enabling the reduction of process and product variability by creating the opportunity to "tune" the process within the design space to cope with variation in inputs such as raw materials and inoculum.

The future will see advances in all aspects of PAT: measurement, analysis and control. As the technology advances and strategies evolve to deal with the current challenges, the rate of adoption will significantly increase until PAT enabled animal cell cultures are the norm. The outcome will be a higher quality product produced more robustly at lower cost and with a lower environmental footprint – a win for both the manufacturer and patient.

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Chapter 22
Biosafety Recommendations on the Handling of Animal Cell Cultures

Philippe Herman and Katia Pauwels

Abstract The first steps in tissue culture are dating back to the beginning of the nineteenth century when biosafety measures did not yet exist. Later on, animal cell culture became essential for scientific research, diagnosis and biotechnological activities. Along with this development, biosafety concerns have emerged pointing to the risks for human health and in a lesser extent for the environment associated to the handling of animal cell cultures. The management of these risks requires a thorough risk assessment of both the cell cultures and the type of manipulation prior the start of any activity. It involves a case-by-case evaluation of both the intrinsic properties of the cell culture genetically modified or not and the probability that it may inadvertently or intentionally become infected with pathogenic micro-organisms. The latter hazard is predominant when adventitious contaminants are pathogenic or have a better capacity to persist in unfavourable conditions. Consequently, most of the containment measures primarily aim at protecting cells from adventitious contamination. Cell cultures known to harbour an infectious etiologic agent should be manipulated in compliance with containment measures recommended for the etiologic agent itself. The manipulation of cell cultures from human or primate origin necessitates the use of a type II biosafety cabinet. The scope of this chapter is to highlight aspects relevant for the risk assessment and to summarize the main biosafety recommendations and the recent technological advances allowing a mitigation of the risk for the handling of animal cell cultures.

Keywords Biosafety • Risk assessment • Risk management • Contained use • Genetically modified organisms • Pathogenic organisms

Abbreviations

BSC	Biosafety cabinet
BSL	Biosafety level
GMO	Genetically modified organism
LAIs	Laboratory-acquired infections

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SPF Specified-pathogen-free
TSE Transmissible spongiform encephalopathies

22.1 Introduction

Biosafety is a concept that refers to the need to protect human health and the environment from the possible adverse effects of pathogenic and/or genetically modified organisms and micro-organisms used in basic research, research and development (R&D) and modern biotechnology. To this end, a case-by-case risk assessment is conducted which consists in the identification and characterisation of potential effects, which may be intended or unintended, together with an assessment of the likelihood and consequences should any effect occur. Depending on the risks identified, risk management measures can be proposed.

The use of cell cultures may fall within the scope of one or several regulatory provisions which consider an assessment of biological risks. For example in Europe, tissue culture work will in many cases involve the use of genetically modified cell lines as well, in which case a risk assessment should be made in accordance with the provisions of the Directive 2009/41/EC related to the contained use of genetically modified micro-organisms (European Commission 2009). Cell culturing activities aiming at manufacturing biopharmaceuticals are covered by the Regulation (EC) No 726/2004 and its amending acts laying down procedures for the authorisation and supervision of medicinal products for human and veterinary use (European Commission 2004a), whereas activities that involve the use of human cells and tissues for application to the human body falls within the scope of the Directive 2004/23/EC and its amending acts (European Commission 2004b). The manipulation of animal cell cultures also exposes the worker to potential biological risks which are considered under the provision of the European Directive 2000/54/EC (European Commission 2000). It should be pointed out that guidelines aiming at mitigating the biological risks for the laboratory workers, public health and the environment have been issued by some scientific advisory bodies or competent authorities (World Health Organization 2004; Centers for Disease Control and Prevention 2009a; Swiss Expert Committee for Biosafety 2011).

While biosafety recommendations (as outlined hereafter) are principally aimed at providing maximal protection of human health (including laboratory workers) and the environment, it is recognised that many of the precautionary measures will also directly benefit the quality of research activities involving animal cell cultures. Indeed, cross-contamination (Lucey et al. 2009; Capes-Davis et al. 2010; Stürzl et al. 2013; Jäger et al. 2013; Johnen et al. 2013; MacLeod et al. 2013) or inadvertent contamination with infectious micro-organisms (bacteria, fungi, yeasts, virus, and prion) are plaguing many researchers, often leading to unproductive data, misinterpretation of results and a considerable waste of time and energy (Mahy et al. 1991; Drexler and Uphoff 2000; Mirjalili et al. 2005; Cobo et al. 2007; Pinheiro de Oliveira et al. 2013). It should also be emphasized that even if Good

Manufacturing Process (GMP) aim at protecting the product, some of the GMP measures are compatible with biosafety measures and reveal to be complementary.

The objective of this chapter will be to address and review biorisk assessment and management considerations of diagnostic and research activities involving cell cultures.

22.2 Biological Risk Assessment

From an historical perspective, the assessment of biological risks has an empirical basis and has resulted from the awareness of the scientific community with regards the risks associated with the handling of pathogenic organisms, as demonstrated through many reported cases of laboratory-acquired infections, followed by the potential risks associated with experiments involving recombinant DNA. In the 1970s, some initiatives for implementing measures guaranteeing the safe use of recombinant DNA were linked to the safety measures which were at that time already successfully applied in microbiology for the containment of pathogenic organisms (National Institute of Health 1979). The conjunction of these two aspects constitutes two pillars of biosafety and has led to a classification system of organisms into risk classes or risk groups. So far, many risk assessments have been carried out among the scientific community on the use of pathogenic organisms (genetically modified or not), regardless of the scale or the purpose of the activity. The basis for this risk assessment methodology takes into account the most recent scientific, technical data and uses a scientifically sound approach.

The methodology of biological risk assessment of contained use activities involving pathogenic and/or genetically modified organisms (GMO) identifies and takes into account the probability of occurrence and the severity of a potential negative effect on public health (including the exposed workers) and/or the environment. As a result of this methodology, a well characterised risk will lead to the choice of appropriate preventive measures encompassing the adoption of an appropriate containment level, the use of safety equipment including personal protective equipment, work practices and waste management.

The risk assessment methodology is commonly used and its 4-step approach is described in Fig. 22.1. The first step (1) takes into account the characteristics of the organism(s) used and, in the case of genetic modification, the genetic material introduced and the resulting GMO. Based on information relative to their harmful characteristics, natural pathogenic micro-organisms can be categorised in several classes of risk or risk groups. This classification takes into account the severity of the disease that pathogenic organisms may cause to human or animal health, their ability to spread amongst the population and the availability of prophylaxis or efficient treatment (World Health Organization 2004). For zoopathogens, the classification system is mainly based on the definitions of the World Organisation of Animal Health (OIE), which categorises animal pathogens into four groups according to their risk to animal health, and since 2008, their risk to human health

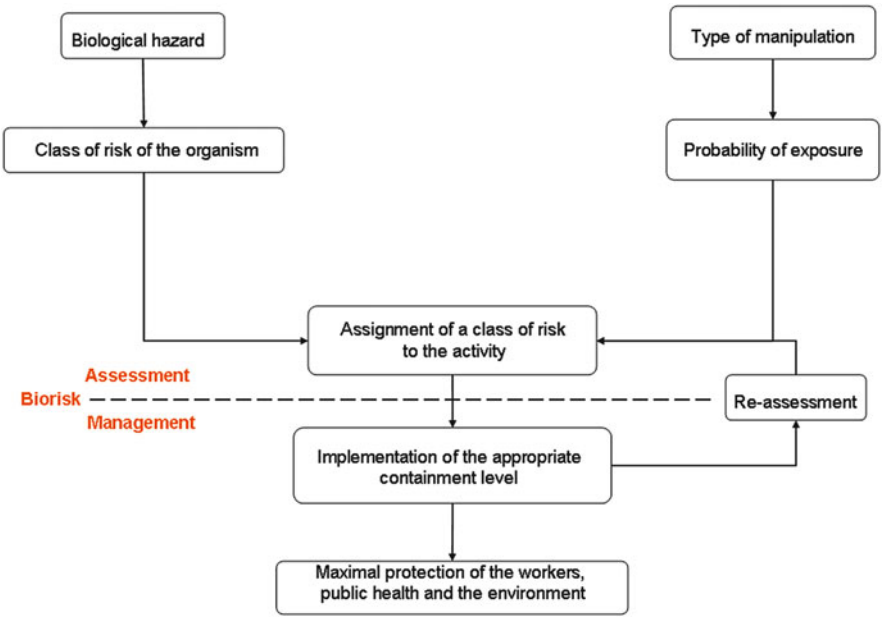

Fig. 22.1 Flow diagram summarizing the Biorisk assessment and management methodology

as well (World Organisation for Animal Health 2009). Micro-organisms that are unlikely to cause disease are classified into class of risk 1 while etiologic agents responsible for severe diseases with a high potential of transmissibility and for which no prophylaxis or treatment is available are assigned to class of risk 4. As such, pathogenic organisms will be categorised from class of risk 2 to class of risk 4. Some periodically revised reference lists issued by international and national authorities or advisory committees classify natural biological agents (not genetically modified) into risk groups or assign the biosafety level under which these should be manipulated (World Health Organization 2004; European Commission 2000; Swiss Agency for the Environment, Forests and Landscape 2005; Centers for Disease Control and Prevention 2009a; Belgian Biosafety Server 2010; Advisory Committee on Dangerous Pathogens 2013).

In the second step (2), the magnitude of the identified negative effects such as human diseases, including allergenic or toxic effects or the transfer of genetic material to other organisms are characterized. In a third step (3), an assessment is performed of the exposure of the laboratory worker, the population and/or the environment to the considered organism and the consequences of each negative effect should it occur. In the fourth step (4), a characterization of the risk is performed resulting in the assignment of the risk level associated with the contained use involving the use of the organism(s). On this basis, the containment measures and other protection measures (e.g. safe work practices, safety equipment and biological waste management) to be adopted are determined. There are four levels

of risk to which contained uses can be assigned, with level of risk increasing from 1 to 4. The final step consists in definitively classifying the contained use activity by conducting a re-assessment of the whole procedure before starting the research, diagnosis or production activity.

In this chapter, the risk assessment methodology applied to animal cell cultures is developed and illustrated by examples. It is important to mention that such a risk assessment is always performed on a case by case basis by the scientist (s) responsible for the activity and the biosafety officer (or biosafety professional) in compliance with local guidance and regulatory requirements.

22.2.1 Risk Assessment of Cell Cultures

The risk assessment applied to animal cell cultures relies on a thorough evaluation of both the intrinsic properties of the cell culture – including subsequent properties acquired as a result of genetic modification(s) – and the possibility that the cell culture may inadvertently be contaminated or deliberately infected with pathogenic micro-organisms. It also includes an exposure assessment which means that the type of manipulation carried out with the cell cultures is taken into account.

The assessment of cell cultures harbouring pathogens follows the same principles as the assessment of the pathogens itself. First, the main organism characteristics (a comprehensive description of the pathogen) is considered by taking into account the following parameters (not by order of importance): (1) the pathogenicity and, when available, the infectious dose (2) the mode of transmission, (3) the host range, (4) the epidemiology (assignment of appropriate risk group may depend on the geographic localisation), possible reservoir and vector(s), and the ability to zoonosis (5) the stability and the persistence of the organism in the environment (i.e. survival outside the host). In addition, information related to the physicochemical properties of the pathogenic organism is considered such as: (1) susceptibility to disinfectants, (2) physical inactivation and (3) drug susceptibility (e.g. sensitivity and known resistance to antibiotics or antiviral compounds). Finally, aspects related to the disease caused by the pathogen are also to be taken into consideration. This includes (1) the availability of an effective prophylaxis, (2) the availability of an efficient therapy and (3) any reported case of laboratory-acquired infection (s) (LAIs).

Although underestimated, many cases of LAIs related to the handling of cell cultures and/or containing virus suspension have been reported. Among them are the reported laboratory worker's exposure to (recombinant) vaccinia viruses amplified in cell culture resulting into infections (Jones et al. 1986; Openshaw et al. 1991; Mempel et al. 2003; Moussatché et al. 2003; Wlodaver et al. 2004; Lewis et al. 2006; Centers for Disease Control and Prevention 2009b). Recommendations to work safely with vaccinia virus have been reviewed recently together with an overview on the reported cases of LAIs involving this virus (Isaacs 2012).

The risk assessment of cell cultures that are genetically modified basically follows a comparative approach: the characteristics of the GMO are compared to those of the non-modified (wild-type) organism from which it is derived under corresponding situations of use. The distinctive feature of the risk assessment of genetically modified cell cultures which consists of the evaluation of the recipient cell, the vector, the donor organisms and the inserted genetic material (insert) is developed in Sect. 22.2.5.

22.2.2 Intrinsic Properties of Cell Cultures

Good knowledge and characterisation of the intrinsic properties of cells are key to successful and safe cell culturing. With respect to the biological risks and the risk assessment associated with the manipulation of animal cell cultures, three properties intrinsic to cell cultures should be considered : the species of origin, the cell type or type of tissue, organ from which the cell line is derived and the status of the culture (Fig. 22.2).

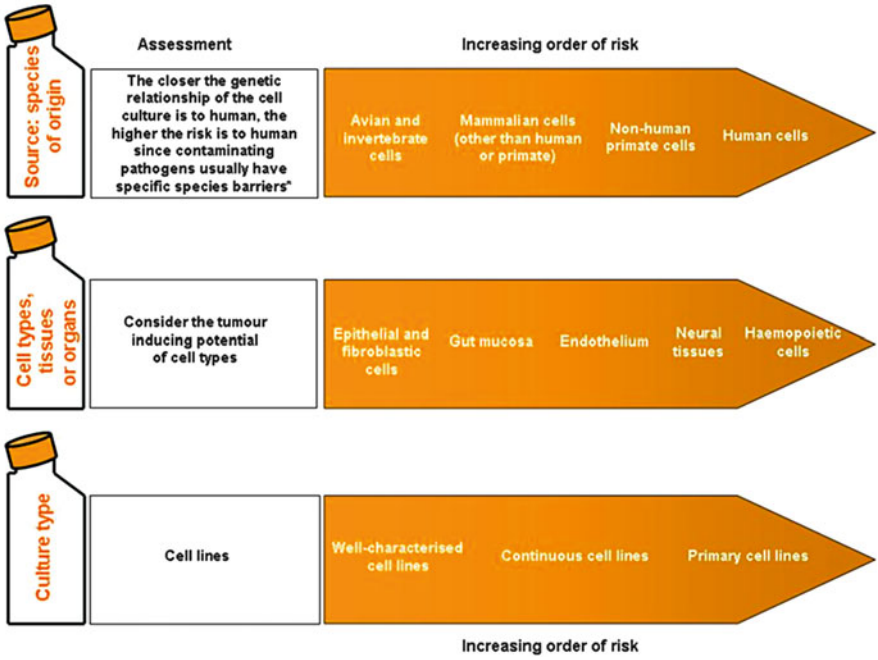

Fig. 22.2 The three intrinsic properties of animal cell cultures distributed in increasing order of risk. *Some contaminating organisms might cross the usual species barrier (e.g. H5N1 influenza, BSE, SARS, etc.)

With respect to the species of origin and based on the fact that pathogens usually have specific species barriers, it is considered that the closer the genetic relationship of the cell culture is to humans, the higher the risk is to humans. The incidence to harbour organisms that could cause harm to human health is therefore considered higher in human or primate cells compared to cells of non-human origin (Brown 1997). Accordingly, mammalian cells other than human or primate cells are considered to represent less risk, followed by avian and invertebrate cells. However, it should be kept in mind that some infectious agents are able to cross the species barrier and to persist in new host species, leading to zoonotic diseases. It is acknowledged since many years that more or less 70 % of the emerging infectious diseases are zoonotic (Chomel et al. 2007). Well documented cases of viruses that have crossed the species barrier from animal reservoirs to humans include hantavirus (murine reservoir), haemorrhagic fever viruses (Ebola, Marburg) (Peters et al. 1992), avian Influenza virus (Reperant et al. 2012) and Severe acute respiratory syndrome (SARS) associated coronavirus (Ksiazek et al. 2003; Herman et al. 2004). These examples show that incidences of cross-species transfer can occur and that occupational risks related to exposure to infected animal tissues or cell cultures should not be underestimated (Mahy and Brown 2000; Louz et al. 2005).

Cells may dramatically differ in their *in vivo* half-life depending on the cell type or type of tissue from which these are derived. For example, intestinal and certain leukocytes have a half-life of a few days, human erythrocytes have approximately a 55–60-day half-life, healthy liver cells have a life span of several months, whereas, in adults, there is a slow loss of brain cells with little replacement. Partly due to this fact, some cell lines can be more readily obtained than others. The establishment of cell lines is often obtained by a series of (generally uncontrolled) mutations that occur by culturing cells for a longer period. It is known that cells cultured for extensive periods of time display changing growth properties. A reduction of the doubling time, as a result of transformation, may give cells the ability to overgrow the rest of the population and to survive for a large (infinite) number of passages compared to primary cells with a finite life span. Therefore, the establishment of cell cultures of a certain cell type upon extensive passage relies on the positive selection for cells that have a growth advantage. These transformed cells can have an increased tumorigenic potential and may present more risks of becoming/being fully neoplastic upon accidental (Gugel and Sanders 1986) or deliberate introduction into the human body. Therefore, taking the tumorigenic potential into account, the following cell types may be ranked in increasing order of risk: epithelial and fibroblast cells, gut mucosa, endothelium, neural tissues, haematogenous (e.g. blood, lymphoid) cells and tissue.

A third inherent property to consider is the status of cell culture. Diagnostic and research activities involve the manipulation of primary cultures or cell lines as well as continuous cell lines derived from primary cultures. Primary cell cultures and cell strains are produced directly from organs or tissues and are often the most accurate *in vitro* tool for reproducing typical cellular responses observed *in vivo*. However, as they are characterised by a finite life span, the time available for

characterisation and detection of contaminating agents remains limited. Also, because typical cell characteristics are often lost during the passage of cells, primary cell cultures are repeatedly obtained from fresh tissue, resulting in increasing risks for potential contaminating pathogens.

A feature that distinguishes continuous cell lines from primary cell cultures is the ability to survive if not infinitely, at least for a great number of passages. These immortalised cells are obtained by isolating cells from tumours, by mutating primary cells with mutagens, by using viruses or recombinant DNA to generate indefinitely growing cells or by cell fusioning of primary cells with a continuous cell line. Due to their increased life span, the time left for thorough characterisation and detection of contaminating agents is considerably increased. Within this respect, well-characterised cell lines present the lowest risks compared to primary cultures or less characterised cell lines as the origin, the source and suitability are well-known and well-defined.

For cell lines obtained from external sources (e.g. different laboratory), cross-contamination of cell-lines and/or a lack of proof of identity is actually a widespread problem (Buehring et al. 2004; Capes-Davis et al. 2010). In order to have at least evidence of the species of origin of a cell line and to be able to conduct a thorough risk assessment, it may be necessary to fully characterise the used cell lines. For this purpose, a number of techniques are available such as cytogenetic analysis, DNA fingerprinting, PCR, flow cytometry and isoenzyme analysis. (Matsuo et al. 1999; Cabrera et al. 2006).

22.2.3 Intentional Infection of Cell Cultures

Many micro-organisms benefit from a cell's machinery to complete their life cycles and to disseminate. Hence the study of a pathogens' lifecycle or immunity escape mechanism requires the intentional *in vitro* infection of animal (or human) cells. The identification of potential hazards associated with infected cell cultures requires a consideration of the intrinsic cell properties and the inherent properties of the infecting pathogen. The latter implies an assessment of a number of pathogen specific criteria along with aspects such as the existence of effective treatment or prophylaxis. On the basis of these criteria the WHO defines a classification system that enables the categorisation of micro-organisms into four risk groups (World Health Organization 2004). A fundamental rule is that the biological risk of infected cell cultures will depend on the infecting pathogen(s) class of risk. For example, cell cultures deliberately infected with Hepatitis C virus (HCV) in order to produce virus particles are assigned to class of risk 3, as HCV is a class of risk 3 virus. Human cells infected with an airborne pathogen like species of the *Mycobacterium tuberculosis* complex are also assigned to class of risk 3 and are requiring the adoption of Biosafety Level 3 containment. However, as discussed below, the class of risk to which the infected cell cultures are assigned will not necessarily indicate the level of containment to be implemented as the latter will also be determined by

the nature of the work carried out with these cells. An example is the infection of bovine leukocytes with *Theileria parva*, a tick-transmitted, intracellular protozoan of veterinary importance and the causative agent of East Coast fever among domestic livestock. It is an animal pathogen of risk group 3, which is not pathogenic to humans. The sporozoite form (infective form) invades bovine lymphocytes where it develops into a non-infective form (shizonts) and induces host cell transformation and clonal expansion of the cell. These infected bovine leucocytes may be categorised under class of risk 2 for animals, while the biosafety level (BSL1 or 2) appropriate for handling is determined by the presence or absence of the infectious form of the parasites.

Cell culture can also be coupled with electron microscopy to identify viral diseases of unknown cause as shown in a recent study published by the Centers for Disease Control and Prevention (Goldsmith et al. 2013). In case of outbreaks the harvested tissues from dead or living infected patients are inoculated to a permissive cell line (generally on VERO E6 cells) and eventually subjected to electron microscopy for morphological analysis of the causal virus. Although alternatives methods such as high throughput DNA sequencing are available to identify a microorganism without a prior *in vitro* expansion, cell culture followed by electron microscopy remains the complementary approach of choice to molecular methods for the unbiased diagnosis of ill-defined infectious disease. Some of the diagnostic activities presented by the authors required the adoption of BSL3 measures to handle the cell cultures. It illustrates how activities involving the *in vitro* amplification of unknown virus may represent a risk for the laboratory personnel and requires the adoption of an appropriate containment level and work practices.

22.2.4 Adventitious Contamination of Cell Cultures

Adventitious contamination of cell cultures is a major drawback for any activity that involves cell culturing (Langdon 2004). Causative agents of cell contamination include bacteria, fungi, mycoplasms, parasites, viruses, prions and even other animal cells. Beside the fact that contamination of cell cultures may place experimental results in question or may lead to the loss of cell cultures, one of the main biosafety concerns when manipulating animal cell cultures for research, diagnosis or production purposes is the fact that they may provide a support for contaminating agents that cause harm to human health.

Generally, bacterial or fungal contamination can be readily detected because of their capacity to overgrow cell cultures. Typically, these organisms cause increased turbidity, pH shift of media (change in media colour), slower growth of the cells and cell destruction. Antibiotics may be used to prevent cell bacterial contamination, however, continuous use of antibiotics in cultures may lead to development of resistant organisms with slow growing properties, which are much more difficult to detect by direct visual observation. Compared to bacterial or fungal infections, mycoplasma contamination gives more problems in terms of incidence,

detectability, prevention and eradication. Mycoplasma, an intracellular bacterium, is one of the most common cell culture contaminants. It may go unnoticed for many passages and can change several cell properties such as growth, metabolism, morphology and genome structure (Paddenberg et al. 1996; McGarrity and Kotani 1985). It has also been reported to influence the yield of virus production in infected cells (Hargreaves and Leach 1970). Mycoplasmal contamination is also a biosafety concern, because some of the contaminating *Mycoplasma* spp. belong to risk group 2. Together with *M. arginini, M. orale*, *M. pirum* and *M. fermentans*, pathogenic organisms like *M. gallisepticum* (risk group 3 for animals), *M. hyorhinis* (risk group 2 for animals), *M. pneumoniae* and *M. hominis* (risk group 2 for humans) account for more than 96 % of mycoplasma contaminants in cell cultures. Primary sources of contamination with *M. orale*, *M. fermentans*, and *M. hominis* in the laboratory are infected people who handle cell cultures and suspensions of viruses. Sources of *M. argini* and *M. hyorhinis* are usually animal donors of tissues and biological constituents used for cell culture, e.g. calf serum and trypsin (Razin and Tully 1995; Pinheiro de Oliveira et al. 2013). It was already reported that the contamination of cell cultures by Mycoplasma occurs via aerosols (O'Connell et al. 1964).

Viral contamination merits particular attention because infected cells may pose a serious harm to human health, especially when infected cells are able to release infectious particles. Human cells may be infected by various viruses like hepatitis viruses, retroviruses, herpes viruses or papillomaviruses. Although cell cultures from non-human origin may pose less risk, it should be emphasised that many viruses have a broad host range and can cross species barriers. Since a number of non-human viruses are capable of infecting and/or replicating in human cells *in vitro*, their possibility to infect human cells *in vivo* if human exposure occurs should be carefully considered. Well-known viral contaminants of primate tissues or cells from non-human origin that can cause human disease are listed in Table 22.1. While contamination with some viruses may be associated with changes in cell morphology or behaviour – such as the formation of syncytia (HIV, herpes viruses), swelling of cells (adenoviruses) or haemagglutination or haemadsorption – viral contamination may be harder to detect when cytopathic effects remain absent. Viral contamination could also trigger adverse effects as a result of recombination events or phenotypic mixing between contaminating components and experimentally introduced agents, creating agents with new properties. For example, experimental results suggested that HTLV-I or HTLV-II undergo phenotypic mixing with HIV-1 in HTLV/HIV-1 co-infected cells, leading to an increase of the pathogenicity of HIV-1 by broadening the spectrum of its cellular tropism to CD4 negative cells (Lusso et al. 1990). Another example is the contamination of murine cell cultures by Lymphocytic choriomeningitis virus (LCMV). LCMV is an arenavirus that establishes a silent, chronic infection in mice but causes aseptic meningitis, encephalitis or meningoencephalitis to humans. The significance of LCMV contamination has been reinforced by the description of cases of laboratory-acquired LCMV infections arising from contaminated murine tumour cell lines (Mahy et al. 1991). Manipulation of LCMV infected material or material with an increased likelihood of LCMV contamination necessitates the

Table 22.1 Main viral contaminants of animal cell cultures or tissues that can cause human infectious diseases

Virus	CR[a]	References
In human tissues		
Hepatitis viruses : HBV, HCV, HDV, HEV, HGV	**3**[b]	Simmonds 2001
Human Retroviruses : HIV-1, HIV-2, HTLV-1, HTLV-2	**3**[b]	Popovic et al. (1984), Clavel et al. (1986), Poiesz et al. (1980), Kalyanaraman et al. (1982), Bhagavati et al. (1988), Hjelle et al.(1992)
Herpesviruses : EBV, CMV, HHV-6, HSV-1, HSV- 2	**2**	Whitley (2001)
Papovaviruses	**2**	Butel (1996)
In primate tissues		
Flaviviruses : Yellow Fever virus, Kyasanur forest Virus	**3**	Tomori (2004)
Filoviruses : Marburg, Ebola	**4**	Shou and Hansen (2000), Mahy et al. (1991), Mahy (1998), Peters et al. (1992)
Simian hemorrhagic virus	**2**[c]	Mahy (1998)
Rabies virus	**3**	Brown (1997)
Hepatitis A virus	**2**	Dienstag et al. (1976)
Poliovirus	**2**	
Herpesviruses (Herpes B Virus and others)	**2**	Hummeler et al. (1959), Davidson and Hummeler (1960), Weigler (1992)
Cercopithecine herpesvirus **Simian**	**3**	Estep et al. (2010)
SV40 (non-pathogenic for humans)	**2**	Vilchez and Butel (2004), Dang-Tan et al. (2004)
Simian Immunodeficiency virus (SIV)	**3**[b]	Hahn et al. (2000), Khabbaz et al. (1994)
Monkeypox	**3**	Likos et al. (2005)
Simian Foamy virus 1&3	**2**[c]	Delelis et al. (2004)
In rodent tissues		
Lymphocytic choriomeningitis virus (LCMV)	**2**	Hinman et al. (1975), van der Zeijst et al. (1983), Mahy et al. (1991)
Hantaan virus (hemorrhagic fever with renal syndrome)	**3**	Lloyd and Jones (1986), Mahy (1998)
Murine leukemia virus (MuLV)	**2**	Stang et al. (2009)
Monkeypox	**3**	Likos et al. (2005)

[a]*CR* Class of risk for human, from available lists from different sources (Swiss Agency for the Environment and Landscape 2005; Belgian Biosafety Server 2010; Advisory Committee on Dangerous Pathogens 2013)
[b]Not infectious via the airborne route
[c]Animal only

implementation of a BSL2. However, it should be kept in mind that the handling of the neurotropic LCMV itself should be performed in a BSL 3 laboratory.

Adventitious contamination with parasites may be an issue when handling primary cell or organ cultures originating from a donor organism that is known or suspected to be infected with a specific parasite. As the life cycle of most parasites comprises distinct developmental stages, transmission and survival of the parasite will strongly depend on the ability of the invasive stage to recognize and invade specific host cells. But even with cells developing the non-infectious form of parasites, possible harmful effects remain to be considered since natural modes of transmission could be bypassed during the manipulation of infected cells. It is recognised that most of the parasitic laboratory acquired infections are caused by needle stick injuries (Herwaldt 2001).

Finally, the use of bovine-derived products as tissue culture supplements may also lead to the contamination with unconventional agents that cause transmissible spongiform encephalopathies (TSE), the so-called prions (Solassol et al. 2003; Cronier et al. 2004; Vorberg et al. 2004). Contrary to the majority of the infectious agents, TSE agents are resistant to most of the physical and chemical methods commonly used for decontamination of infectious agents. It has been shown that neuroblastoma cell lines, primary cultured neurons and astrocytes can serve as hosts (Butler et al. 1988). Although many studies have suggested that the risk of propagation of TSE agents in tissue culture cells cultivated in the presence of bovine serum potentially contaminated with TSE was restricted to neurons or brain-derived cell cultures, it has been shown recently that non-neuronal cells can also support TSE infection, suggesting that any cell line expressing normal host prion protein could have the potential to support propagation of TSE agents (Vilette et al. 2001; Vorberg et al. 2004). While the understanding of the transmission of prions is still in progress (natural transmission seems mainly to take place via oral route in human and animals), investigators using cell cultures need to take into account different routes by which these agents may be transmitted experimentally. Mouse scrapie aerosol transmission has been successfully obtained in mice (Stitz and Aguzzi 2011; Haybaeck et al. 2011). In cervids, Chronic Wasting Disease (CWD) was already proposed as a natural airborne pathogen (Denkers et al. 2010). In the case of Creutzfeldt Jacob Disease (CJD), there is to date no proof of release of prion into aerosols.

Animal cell cultures can also harbour unknown pathogens or whose tropism has not been defined yet. Examples described in the literature include viruses such as Hepatitis G (Linnen et al. 1996), HHV8 (Moore et al. 1996), TT virus (Nishizawa et al. 1997) or human pneumovirus (van den Hoogen et al. 2001).

Cell cultures can be contaminated by different sources. Infected organisms or infected animal cells or tissues from which a cell line has been established are the primary source of contamination. An accidental contamination can also occur through the material used for cell culturing including glassware, storage bottles and pipettes due to incorrect maintenance or sterilisation. Before the use of disposable material the lip of the culture flask and the outside of the used pipette were important sources of contamination with mycoplasma (McGarrity 1976).

Nowadays, the use of disposable and sterile pipettes has significantly decreased the likelihood of adventitious contamination. A third source of contamination resides in culture media and its components such as serum, basic culture media and salt solutions and enzymes (trypsin, pronase and collagenase). For example, media and additives derived from bovine sources are often contaminated with bovine viral diarrhoea virus (BVDV) (Levings and Wessman 1991). As mentioned above the relative resistance of TSE agents may also be an issue when using bovine-derived products as tissue culture supplements. Finally, non-filtered air supply, clothing, personnel and floor can be a source of airborne contamination (Hay 1991).

22.2.5 Genetically Modified Animal Cell Cultures

Genetically modified (GM) animal cell cultures are employed for a number of different activities. For example, the expression of transgenes and production of proteins of interest whose function depends on methylation, sulfation, phosphorylation, lipid addition, or glycosylation may necessitate the capacity of higher eukaryotic cells to perform post-translational modifications. GM animal cell cultures may also be chosen for the replication of defective recombinant or even wild type viruses. The risk assessment of GM cells should follow the five-step methodology as outlined in Fig. 22.1, which means that an evaluation of each individual aspect in the process of genetic modification should be performed. This includes an evaluation of the recipient cell, the vector, the donor organism properties and an assessment of the characteristics of the inserted genetic material. A comprehensive risk assessment of genetically modified cells expressing transgenes should also take into account the risk associated with the transgene products. A gene product may be intrinsically harmful (e.g. toxic properties) or could induce hazardous properties via its expression in GM cells, dependent upon the genome integration site, promoter activity and expression of regulatory sequences governing expression. The risk assessment for transgenes is not straightforward and demands appropriate consideration. Comprehensive reviews have specifically addressed this topic (Bergmans et al. 2008; van den Akker et al. 2013). Genetic modification may confer an expanded life-span, immortalisation or increased capacity for tumour induction. However, it is unlikely that recombinant properties obtained by genetic modification may have an adverse effect upon release of the recombinant animal or human cells into the environment. Cells (genetically modified or not) have difficulties to survive in a hostile environment where control of temperature and osmolality is lacking or where cell-specific nutrients (e.g. glucose, vitamins, lipids) are not balanced or missing. Hence, the survival of such primary cells or cell lines outside of proper conditions is unlikely to occur. Apart from the fact that GM cell cultures may harbour pathogens and pose serious biological risks to human health (as discussed above), recombinant cells are more likely to cause harm when entering the body of animals or humans. However, the extent of the harmful effect remains hard to predict. It should be kept in mind that the lack of histocompatibility

between recombinant cells and the host organism remains a major obstacle for these cells to survive and to multiply as the natural immune response of the healthy (immunocompetent) host will recognise foreign cells and eventually destroy them. This is also one of the main reasons why the culturing of cells originating from the laboratory worker is not allowed for research and diagnostic activities (risk associated with autologous cells).

Particular attention should be paid to the use of packaging cell lines. These are established cell lines that are deliberately and stably transfected with "helper constructs" to ensure the replication and packaging of replication deficient viral vectors. For example, in case of retroviral packaging cell lines, the expression of "helper genes" allows high-level constitutive production of viral proteins (e.g. gag, pol and env proteins), which are missing in the genome of the replication deficient viral vector but are crucial for viral replication. One of the main biosafety issues related to the use of packaging cell lines is the fact that replication-competent viruses may be generated as a result of (homologous) recombination between the replication deficient viral vector and viral sequences present in the packaging cell. These events could result in the formation of viruses with novel yet unwanted properties such as the generation of replication-competent viruses. One of the strategies to engineer safer generations of packaging cell lines consists in minimising the likelihood of generating replication-competent viruses by separating viral functional elements into different expression plasmids, thereby increasing the number of recombination events necessary to generate replication-competent viruses (Dull et al. 1998) or by reducing or eliminating the sequence homology between the viral vector and the helper sequences. However, endogenous retrovirus genomes expressed in safer generations of retroviral packaging cell lines may still give rise to unwanted recombination events (Chong et al. 1998). This means that the possibility to generate replication-competent viruses cannot be ruled out.

Clearly, the risk group of the transfected packaging cell line will depend on the risk group of the viral vector itself. Consequently, risk assessment of packaging cell lines should be based on the biosafety of the produced viral vectors, including an evaluation of their infectivity, spectrum of host range, capacity of integration (insertional mutagenesis), stability and physiological role of the transgene(s) if expressed (Baldo et al. 2013).

22.2.6 Type of Manipulation

Aside from the identification and characterisation of hazards intrinsic to the cell culture, a thorough risk assessment must also consider the exposure pathways through which cell cultures may present a risk to human health or the environment. This necessitates an evaluation of the type of manipulation, because processes, methods and/or equipment involved may increase or decrease the likelihood of exposure and hence the resulting potential risks. For instance, while established cell lines inherently present low risks, large scale operations involving the culturing of

large volumes (from 10 to 1,000 l) are prone to contamination when inadequate containment measures are applied. This is exemplified by continuous processes such as cell cultivation in bioreactors where an appropriate design of seals, valves, pumps and transfer lines is required to guarantee long-term sterility of the operation to avoid inadvertent contamination. At the opposite, the handling of cell cultures belonging to risk group 2 may present less risk once they have been fixed by glutaraldehyde or formaldehyde/acetone for immunostaining and may therefore require less stringent containment measures

Manipulations that are common to research and diagnostic activities and warrant consideration with respect to the risk assessment of animal cell cultures are described hereunder:

- Procedures generating aerosols: pipetting, vortexing, centrifugation, opening of wet cups, etc.;
- Handling cells outside of a class II BSC: flow cytometric analysis and cell sorting constitute a special case of cell manipulation in which cells are handled outside of a BSC. The use of a fixative is in many cases not appropriate (e.g. viable cell sorting for subsequent further cell culturing) and the risk of aerosol formation can be particularly high, especially during sorting experiments and upon instrument failure such as a clogged sort nozzle. All scientists in the field of flow cytometry must be aware of the potential hazards associated with their discipline and only experienced and well-trained operators should perform potentially biohazardous cell sorting. General recommendations approved by the International Society of Analytical Cytology should help to set a basis for biosafety guidelines in flow cytometry laboratories (Schmid 2012). Some standard operating procedures and methods have also been described for ensuring the cell sorting under optimal biosafety conditions even under BSL3 conditions (Lennartz et al. 2005; Perfetto et al. 2011).
- Altering culture conditions: changing the availability of cell-specific nutrients, growth factors, signal molecules or adopting co-culture techniques may have significant effects on animal cell cultures as it may result in altered neoplasia (Stoker et al. 1990), altered expression of (proto)onco-genes or cell surface glycoproteins and release of endogenous viruses (Cunningham et al. 2004). As a consequence, changing culture conditions may lead to altered susceptibility of cultured cells to biologic agents such as viruses (Anders et al. 2003; Vincent et al. 2004).
- Manipulations involving use of needles or sharps: due to injuries cell material may be accidentally transferred directly to an operator's tissue and/or blood stream.
- *In vivo* experiments involving animals: major risks are self-inoculation (needle-stick injury) and exposure to aerosols.

Laboratory workers handling infected rodents or cell cultures originating from infected animals expose themselves at risk by directly exposing cuts, open wounds or mucus membranes with infected body fluids or by inhaling infectious aerosolized

particles of rodent urine, faeces or saliva. The risk can be minimised by utilising animals or cell cultures from sources that are regularly tested for the virus.

Finally, the purpose of cell culturing should be taken into consideration as many clinical approaches such as stem cell therapy, gene therapy, xeno- or allotransplantation involve cell culturing *ex vivo* for therapeutic purposes. This latter clearly justifies more careful consideration regarding safety, ethical, social and regulatory issues, which will not be addressed in this chapter (Food and Drug Administration; European Medicines Agency; ICH 1997).

22.3 Biological Risk Management

22.3.1 General Biosafety Recommendations

The assessment of biological risks related to animal cell cultures and the type of manipulation allows the determination of an adequate containment level in order to optimally protect human health and the environment. The implementation of an appropriate containment level includes a list of general and more specific work practices and containment measures.

Table 22.2 lists precautionary measures that should be applied whenever handling animal cell cultures. Much of these measures focus on reducing the risk of contamination with adventitious agents. It should be emphasised that the drawn-up of an appropriate set of standard operating procedures and adequate training of the staff is of crucial importance.

As a general rule, cell cultures known to harbour an infectious etiologic agent should be manipulated in compliance with containment measures recommended for the etiologic agent. When cell cultures are not known to harbour infectious agents, cells may be considered free of contaminating pathogens as long as a number of conditions are fulfilled. This implies the use of well-characterised cell lines or controlled cell sources for primary cells such as specified-pathogen-free (SPF) animals. If no well-characterised cell lines or SPF are available, tests for detection of likely contaminating agents should be negative. Second, whenever cell cultures are manipulated, media sources should be pathogen free and appropriate containment measures should be adopted to reduce potential contaminations during sampling or subsequent manipulation of cells (re-feeding and washing steps).

As the history of a cell culture may be poorly documented when a given cell culture is manipulated for the first time in the laboratory, it often remains unclear whether all appropriate measures have been implemented regardless of the fact that it may have been manipulated for years in another laboratory facility. In this case, cell cultures should be considered to be potentially infectious and should be manipulated in a class II BSC. If the presence of adventitious agents of a higher risk group is considered likely, the cell line should be handled under the appropriate

Table 22.2 Precautionary measures for handling animal cell cultures

To respect good microbiological practices, especially those that are aimed at avoiding accidental contamination.
To be in compliance with good cell culture practice (GCCP), especially in industrial settings for vaccine production.
To avoid opening of culture vessels or contact with culture fluid through a defective culture vessel, stopper or poor technique because of the ever present likelihood of contamination with airborne pathogens.
To treat each new culture that is manipulated for the first time in the laboratory facility as potentially infectious.
To clean up any culture fluid spills immediately with an appropriated and validated disinfection protocol.
To work with one cell line at a time and disinfect the work surfaces between two operations involving cell lines.
To aliquot growth medium and other substrates so that the same vessel is not used for more than one cell line.
To mitigate cross-contamination by avoiding pouring actions.
To proceed to the use of the biosafety cabinet (BSC) by adequately trained staff, t.i. turn on for a period before and after use, thoroughly disinfect BSC surfaces after each work session and do not clutter the BSC with unnecessary materials.
To restrict the use of antibiotics in growth media.
To quarantine new cell cultures to a dedicated BSC or separate laboratory until the culture has been shown negative in appropriate tests.
To carry out a quality control of cells demonstrating the absence of likely contaminating pathogens on a regular basis or whenever necessary.
To operate cell cultures from undefined sources as risk group/class of risk 2 organism. If the presence of adventitious agents of higher risk class is expected, the cell line should be handled under appropriate containment level until tests have proven safety.

Adapted from Pauwels et al. (2007)

containment level until tests have proven the absence of such organisms. Good documentation of the history of cell cultivation is mandatory.

The extent to which cell cultures should be controlled on the likelihood of contaminants strongly depends on the nature of activity. For example, guidelines have been issued aiming at minimising any potential risk for transmission of infectious agents with respect the use of animal cell cultures for industrial production of biopharmaceuticals (European Medicines Agency; Food and Drug Administration; ICH 1997; World Health Organization 1998). Hardly any guidance has been provided for the extent of detecting possible contaminants in case animal cell cultures are used for *in vitro* research or diagnostic activities, or for purposes other than therapeutics or production of biopharmaceuticals. The choice of the detection technique depends on the contaminating pathogen and often a combination of methods is recommended for important samples such as master cell banks.

The implementation of BSL2 measures is adequate for most of the work carried out with cell cultures from human or primate origin. BSL1 measures may be considered provided that all manipulations occur in a class II BSC and the cell culture is a well-characterised and certified cell line that presents no increased risk resulting from genetic modification or contaminating pathogen. The contained use

in a BSL1 of viral infected cells could be envisaged if no viral particles are detectable in the supernatant of the infected cells. However, it should be emphasized that procedures for viral clearance are virus-host specific and that variables such as vector titre and the infection protocol can influence the degree and rate of clearance (Bagutti et al. 2012). Since the implementation of good laboratory practices and the use of a BSC is usually the norm in most laboratories dealing with cell culturing, we think that BSL1 laboratories can relatively easily be upgraded to BSL2 facilities by implementing a restricted number of simple additional safety measures. It is important to notice that horizontal laminar air flows and clean benches minimise the risk of adventitious contamination of the cell cultures, however they offer no protection for the manipulator or the environment. Bearing in mind that biosafety measures intend to provide a maximal protection of human health and the environment, it is important to note that the sole use of a horizontal laminar "clean bench" should be prohibited.

Based on, but not limited to, key features of risk assessment and the type of manipulation performed as discussed in former paragraphs, we developed a flow diagram providing the cell culture users a schematic guidance for the assignment of an appropriate containment level when manipulating human or primate cells *in vitro* (Fig. 22.3). This flowchart is indicative and should be applied and/or reconsidered according to case specific conditions and risk assessments proper to the activities performed.

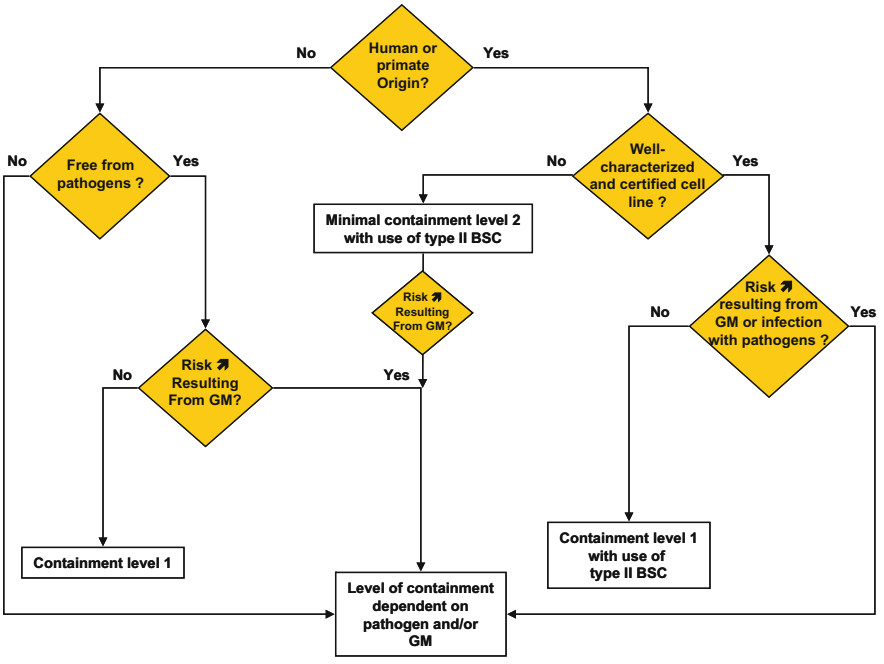

Fig. 22.3 Flow diagram helping for the assignment of the containment level to adopt as a function of the handled biological material

22.3.2 *Novel Approaches for Reducing Hazard and/or Exposure Associated with Handling of Cell Cultures*

Whenever possible approaches developed to *ab initio* reduce the risk associated with the handling of animal cell cultures should be favoured. Such approaches involve the use of dedicated instruments or safer biological material. The examples hereunder illustrate how the choice to apply one or several of these approaches is determined on a case-by-case basis, depending on the intrinsic characteristics of the biological material and the intended use.

During the last couple of years instruments enabling automated mechanical passaging and nutrient supply for *in vitro* cell expansion have been developed for primary cells, adherent and non–adherent mammalian cells (Kato et al. 2010; Thomas and Ratcliffe 2012). Some of these developments also provide a safer approach with respect to biosafety considerations by including a biosafety module in the design of such automated platforms.

For example a long-term cell culture device ensuring both the protection of the product and the operator has been designed for the culturing of embryonic stem cells in an antibiotic-free medium by means of an integrated automation platform using a class II BSC confining the microwells (liquid handling robot contained in a BSC) (Hussain et al. 2013)

An automation system has also been deployed for the production (140–1,000 ml) of HIV-1 pseudovirus for HIV vaccine trials in compliance with GCLP. This robust automated system for cultivation of 293 T/17 cells is contained in a class II BSC that guarantees the protection of the workers, environment and the product. This system can be implemented to produce other biological reagents under standardised large-scale conditions (Schultz et al. 2012).

Lowering the hazards associated with the cell cultures handled is another approach that was applied in the characterisation of pandemic influenza viruses under emergency situations. While such activities typically require BSL3 conditions, inactivation protocols applied to influenza virus culture allows for performing the virological and immunological assays under BSL2 conditions (Jonges et al. 2010).

Another example where the assignment of a less stringent containment level was enabled relates to the use of insect cells and GM bacteriophage Lambda capsids in cases where usually the maintenance of infectious stocks of highly pathogenic or emerging influenza viruses is involved (Domm et al. 2014). The bacteriophages are "decorated" by the viral glycoproteins of interest in an insect cell-derived system so as to use hemagglutinin displaying bacteriophages in hemagglutination-inhibition assay on pathogenic influenza viruses. The same approach was already applied for HIV-envelope protein (Mattiacio et al. 2011).

With regards to HIV-1 *in vitro* testing, a non-infectious cell-based assay to assess the HIV-1 susceptibility to protease inhibitors was also developed recently (Buzon et al. 2012). Here again, activities that normally require relatively high containment level can be performed in facilities with less expensive infrastructure. It also shows that careful consideration of the material handled can lead to cost-effective choice while maintaining the same objectives.

Concluding Remarks

Biosafety is an internationally recognized concept referring to the maximal protection of laboratory workers, public health and the environment from the possible adverse effects associated to the use of organisms and micro-organisms (genetically modified or not). Within this respect activities involving the use of animal cell cultures for fundamental research, R&D or *in vitro* diagnosis purposes pose biosafety considerations as well. Before starting any of such activities biosafety considerations should be addressed by performing a biological risk assessment allowing the identification and characterisation of any potential adverse effect together with an evaluation of the likelihood and consequences should any effect happen. Such an approach is made on a case-by-case basis, taking into account the type of manipulation and the type of cell culture handled. A risk assessment will result in biosafety recommendations in view of the implementation of an adequate containment level. Considering the very limited persistence capacity of animal and human cells outside non-optimised culture conditions and the fact that many cell lines have a long history of safe use, it is generally considered unlikely that cell cultures may inherently cause harm to humans or the environment. The main hazard associated with the handling of animal cell cultures resides in the presence of adventitious pathogenic micro-organisms, which are often difficult to detect and hence less controllable. In contrast to their host cells, adventitious organisms can persist in more hostile conditions and may present risks for human health or the environment in case they are pathogenic (Bean et al. 1982; Walther and Ewald 2004; Kallio et al. 2006; Kramer et al. 2006). For this reason, a risk assessment of cell cultures will frequently lead to a risk assessment of the potential adventitious contaminants, the organisms used for cell's immortalisation (viruses, viral sequences, etc.) and/or the micro-organisms intentionally used to experimentally infect them. Though the assignment of biosafety containment level requirements cannot be generalised and should be performed on a case-by-case basis, it is recognised that most of the containment measures primarily aim at protecting cells from adventitious contamination in order to mitigate potential risks for the laboratory worker. Except for authenticated cell lines proved to present no risk, the activities involving cell cultures from human or primate origin should generally be performed under containment level 2 involving the use of a class II Biosafety cabinet and Good Laboratory Practice.

Whilst the occurrence of adverse effects related to the handling of cell cultures cannot be excluded, a thorough risk assessment and the implementation of the appropriate containment level offer an optimal protection for the laboratory worker and by extension, public health and the environment. Continuous efforts have been made to mitigate the risk associated with the handling of cell culture in laboratory settings. These biorisk management

(continued)

measures (i.e. adoption of the appropriate containment level) can be coupled with the implementation of automation platforms allowing the reduction of cell culture cross-contamination and accidental contamination with infectious agents.

It is anticipated that future efforts contributing to the improvement of the quality of the data necessary for the risk assessment, the containment measures and the increased awareness of biosafety considerations within the scientific community will also directly benefit the quality of research activities involving animal cell cultures.

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Glossary

Authentication Is the process by which the true origin and identity of cell lines are determined and should form an essential part of any cell culture operation.

Biosafety In the context of this chapter, biosafety relates to the evaluation of the potential risks to human health and the environment associated with the use of genetically modified organisms (GMOs) or pathogenic organisms.

Biosafety Cabinet (Class II) Safety cabinet with a front aperture through which the operator can carry out manipulations inside the cabinet and which is constructed so that the laboratory worker is protected, the product and cross contamination is low. The escape of airborne particulate contamination generated within the cabinet is controlled by means of an appropriate filtered internal airflow and filtration of the exhaust air (HEPA filters).

Contained use Any activity in which (micro)-organisms are genetically modified or in which such organism (pathogenic or not) are cultured, stored, transported, destroyed, disposed or used in any other way, and for which specific containment measures are used to limit their contact with the general population and the environment.

Culture Type: Primary Cell Cultures, Diploid Cell Lines, Continuous Cell Lines

Primary cell cultures Are established directly from tissues of animals and are often the most appropriate *in vitro* tool for reproducing typical cellular responses observed *in vivo*. However, as typical cell characteristics are lost during passaging, these cultures must be obtained from fresh tissue that may contain or may become inadvertently contaminated with pathogens. Consequently, primary cell cultures may potentially present increased risks compared to continuous, established cell lines.

Diploid cell lines Are similar to primary cells, are considered non-tumourogenic and have a finite capacity for serial propagation. They are used for the preparation of viral vaccines and are from human or monkey origin.

Continuous cell lines Are immortalized cells that may survive almost infinite serial passages. These cells are obtained by either isolating cells from tumours (neoplastic origin), primary cells treated with mutagens, oncogenic viruses or recombinant DNA (oncogenes) or by cell fusioning of primary cells with a continuous cell line. As a consequence, the class of risk of these cell lines is often correlated to the class of risk of primary cells of whom they are derived. Due to the "immortality" of the continuous cell lines, their ability to induce tumours has also to be considered.

Transfection Gene transfer of DNA in eukaryotic cells using non-viral delivery methods.

Transduction Gene transfer of DNA in eukaryotic cells using viral vectors.

Infection Process occurring when a virus (wild type) that typically replicates and spreads into neighbouring cells mediates a gene transfer belonging to its own genome or extra genes it may carry.

Viral vector Is a protein particle derived from a replicative virus that contains genetic information in the form of RNA or DNA. A viral envelope may be present as well.

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Chapter 23
Biopharmaceutical Products from Animal Cell Culture

Darrin Kuystermans and Mohamed Al-Rubeai

Abstract Animal cell culture bioprocesses based on mammalian expression systems have given the pharmaceutical industry a means to produce complex glycosylated therapeutic proteins that is projected to be at least a US$500 billion dollar market by 2020. Medicinal products produced by mammalian cell cultures include hormones, enzymes, cytokines, bone morphogenic proteins, clotting factors, antibodies, and fusion protein therapeutics. Activase®, a recombinant thrombolytic enzyme, was the first approved mammalian cell culture drug to be produced from Chinese hamster ovary cell culture and marketed to the public. Over time, other mammalian derived products followed and have evolved from simple replicas of endogenous proteins to complex engineered bio-molecules. Among the existing mammalian expressed biological drugs discussed, that have been produced in the USA and EU till early 2014, monoclonal antibody therapeutics have become the top earning products being over 40 % of products produced. The development of chimeric, humanized and eventually fully human antibodies has also decreased immunogenic reactions in human patients to below 10 % for the majority of engineered monoclonals with some even reaching below 1 %. Enbrel®, the first Fc-fusion protein introduced onto the market has also led to engineered therapeutic proteins with a longer half-life and multiple functions as with the introduction of bispecific antibody therapeutics. The introduction of the first biosimilars, starting in 2007, can further lower the cost of access to mammalian produced biologics and has also meant a further increase in the overall mammalian cell culture capacity around the globe.

Keywords Biopharmaceuticals • Monoclonal antibodies • Fusion proteins • Mammalian cell culture • Biosimilars • EMA • FDA • Drug approvals

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23.1 Introduction

Over the recent years the small molecule pharmaceutical industry has slowed and shifted emphasis towards biopharmaceuticals. This shift is due to thinning pipelines and higher success rates of clinical trials for biopharmaceuticals compared to small molecules drug entities, but also pressure from the generics industry lowering the profit margins of blockbuster chemical compounds. The global biopharmaceutical market was estimated to be around US$199.7 billion in 2013 and was further projected to reach close to US$500 billion by 2020 growing at a compound annual growth rate (CAGR) of 13.5 % CAGR between 2010 and 2020 (Research and Markets 2013) compared to the 0.6 % growth rate of more small-molecules entities in the pharmaceutical market (Beck et al. 2008). The United States biopharmaceutical market alone is estimated to reach US$144 billion by 2016 due to an increase in new product launches but also due to an aging population, with a large majority reaching 65 years and above and the approval of new indications for existing drugs (Markets and Markets 2011). In 2012, 58 % of United States (US) and/or European approved and marketed biopharmaceuticals are produced via a mammalian cell culture (Ecker and Ransohoff 2014). The area of monoclonal antibody (mAb) constitutes the largest growing segment of the market with an estimated share of 25.6 % in 2013, US$51.1 billion of the global market and predicted to reach sales of US$70 billion by 2015 (Chon and Zarbis-Papastoitsis 2011). Generally, over the last two decades the amount of therapeutic recombinant glycoproteins on the market produced by animal cell culture has increased significantly from only 18 approved products in the 1990s to approaching close to 200 approved products by the end of 2015 at the current approval rates.

The first biopharmaceuticals consisted of replacement hormones and first generation vaccines, followed by monoclonal antibodies, recombinant proteins and second generation vaccines that are now maturing in the industry. Monoclonal antibodies (mAbs) have taken the leading segment in biopharmaceuticals, and are also the fastest growing segment (Reichert et al. 2005; Reichert 2014) predominantly produced via mammalian cell culture processes. Over thirty new biological entities (NBE) produced by mammalian cell culture came onto the US and European Union (EU) market between 2010 and the first quarter of 2014 which is a slight increase from the 3 years prior, before 2010. The market for glycosylated biopharmaceuticals has had a ramp up in manufacturing since the year 2000 after the first monoclonal antibody entered the market in 1986, known as Orthoclone®, opening the gateway for the first generation recombinant glycosylated protein approvals. This first generation consisted, initially, of recombinant tissue plasminogen activator (rtPA) and recombinant erythropoietin followed by a plethora of recombinant human proteins that entered the market with a majority showing great success over the past 25 years.

The top best-selling biopharmaceuticals in 2013 all were produced from mammalian cell culture with the top three drugs, Humira® (Adalimumab, a fully human mAb), Remicade® (Infliximab, a chimeric mAb) and Enbrel® (Etanercept, a

Fc-fusion based) all treating autoimmune diseases such as rheumatoid arthritis (RA). Cancer drugs are also a big hit with the sixth best-selling biopharmaceutical drug being a chimeric mAb initially used to treat non-Hodgkin lymphomas and follicular lymphoma which can also be used to treat RA. In fact, the sales of non-antibody based biopharmaceuticals produced in mammalian cell culture has slowed over the recent years due to decreases in the development pipeline for these kind of drugs (Ecker and Ransohoff 2014). This slowdown has had little effect on the overall growth of mammalian cell culture produced biopharmaceuticals with the once small segment in the overall pharmaceutical product pipeline becoming a major global segment for the pharmaceutical industry.

This chapter will give an overview of the biopharmaceutical products manufactured using animal/mammalian cell culture that have been approved by the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) to be introduced onto the market, and how these products have evolved from simple copies of endogenous proteins to the engineered proteins we see today. The arrival of biosimilars is discussed and along with current market trends, what might be the impact on biopharmaceutical cell culture capacity. The recombinant proteins manufactured via mammalian cell culture brought onto the EU and US market from 1989 till the first quarter of 2014 have also been tabulated for reference along with their respective platform host cell lines and indications.

23.2 Early Mammalian Based Biopharmaceuticals

Mammalian cells are well suited to carry-out post-translational modifications similar to the native proteins found in the human body as opposed to microbial and yeast systems at this moment in time. The superior ability to perform these modifications can become a necessity for retaining biological activity of complex proteins such as mAbs, thus the first therapeutic monoclonal antibody, a mouse IgG2a, was produced using the mouse ascites, in 1986, Muromonab which was marketed as OrthoClone OKT3®. The ascitic fluid was harvested from a peritoneal tumor in mice that was induced by injecting hybridoma cells into the peritoneum. This essentially makes the rodent a mini bioreactor for cell growth so that the hybridoma densities could increase as they secreted antibodies until a concentrated solution of mAb's (~1–10 mg/ml) could be harvested (McGuill and Rowan 1989). Apart from this in vivo method of mAb production from ascites causing pain and significant distress in mammals the economic implications for large scale production have directed pharmaceutical manufacturers to utilize in vitro cell culture processes.

Over the past decades several cell lines have become popular hosts of marketed recombinant protein products. The past and current cell lines utilized as a main platform for mammalian biopharmaceutical culture are murine myeloma lymphoblastoid type cells such as NS0 (Bebbington et al. 1992; Barnes et al. 2001) and Sp2/0-Ag14 (Shulman et al. 1978), Chinese Hamster Ovary

(CHO) (Cockett et al. 1990; Milbrandt et al. 1983), baby hamster kidney (BHK-21) (Carvalhal et al. 2001; Christie and Butler 1999; Geserick et al. 2000; Kirchhoff et al. 1996), and human embryonic kidney epithelial cells (HEK-293) (Baldi et al. 2005; Schlaeger and Christensen 1999). From these cell types, CHO cell lines were the host that dominated the first round of commercial mammalian cell culture produced drugs, with the first recombinant therapeutic protein produced from animal cell culture being a tissue plasminogen factor marketed in 1987, by Genentech, under the trade name Activase® (alteplase), FDA-approved for the treatment of myocardial infarctions (Walsh 2004; Cannon et al. 1998; Gillis et al. 1995; Kunadian and Gibson 2012). With CHO based biopharmaceuticals gaining regulatory approval, most manufacturers considered CHO an acceptable host system for intravenous drug production and naturally the popularity of CHO cells as hosts increased. Other factors such as the CHO being a robust cell line that is adaptable, with the ability to reach a high cell density suspension culture also reinforced the CHO cell line popularity amongst manufacturers as time progressed. As of early 2014, CHO based cell culture represents over 75 % of all mammalian expressed biopharmaceuticals produced (Fig. 23.1). Since a host of highly glycosylated protein could not be produced by microbial systems such as *Escherichia coli* (*E.coli*), tPA, EPO (erythropoietin), and factor VIII recombinant therapeutics (see Table 23.1) became attractive first time biopharmaceuticals produced in mammalian cell culture systems in the late 1980s and early 1990s.

The popularity of commercial suspension mammalian cell culture produced mAb based therapeutics and their derivatives started in the late 1980s, soon after the release of Orthoclone OKT3®. While the first round of biopharmaceuticals were mainly copies of recombinant anti-hemophilic factors (clotting factors), cytokines, and enzymes such as the thrombolytic agents (see Table 23.1), this

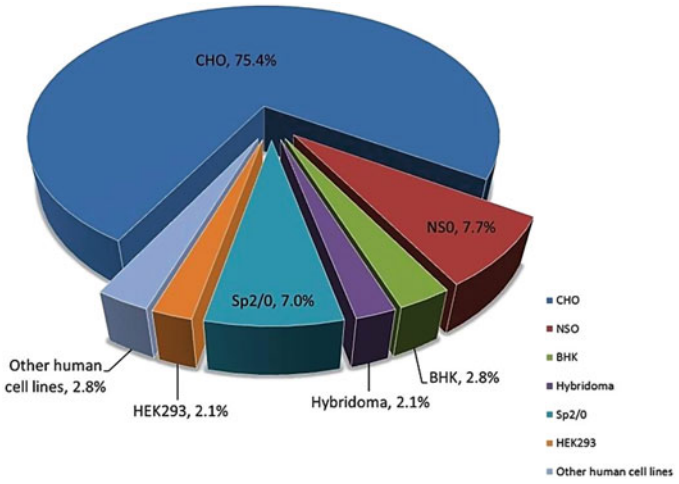

Fig. 23.1 Percentage of the type of mammalian cell lines used in commercial scale manufacturing of biopharmaceuticals from 1987 till April 2014

Table 23.1 The biopharmaceutical drug approvals in the EU and USA between 1989 and April 2014 which are produced by mammalian cell culture are summarized in table below. The table starts from the most recent approvals. Data was collected from several sources (European Medicines Agency 2014; United States Food and Drug Administration 2014). Those shaded in *green* are biosimilars and those shaded in *blue* indicates that the drug was already approved in another region the previous calendar year or earlier

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2014						
Entyvio™ Vedolizumab	Takeda Pharmaceutical Company Limited	Antibody that binds to integrin α4β7 blocking the α4β7 integrin resulting in gut-selective anti-inflammatory activity, for the treatment of ulcerative colitis and Crohn's disease.	Humanized Monoclonal Antibody	CHO	In Review	EU & USA
Cyramza™ (Ramucirumab)	Eli Lilly & Company	Binds to VEGFR2 blocking the binding of vascular endothelial growth factor (VEGF) to VEGFR2 indicated for the treatment of patients with advanced or metastatic, gastric or gastro-esophageal junction adenocarcinoma	Fully Human Monoclonal Antibody	NS0	23-Apr-14	USA
Sylvant™ Siltuximab	Janssen Biotech (Johnson & Johnson)	Antibody that specifically binds to and neutralizes human IL-6 with high affinity for the treament of adult patients with multicentric Castleman's disease (MCD)	Chimeric Monoclonal Antibody	CHO	23-Apr-14	USA
Alprolix™ (Coagulation Factor IX)	Biogen Idec.	Coagulation factor human IgG1 Fc fusion , which binds to the neonatal Fc receptor (FcRn) for treatment of hemophilia B	Clotting factor / Fc-Fusion Biologic	HEK293	28-Mar-14	USA
VIMIZIM™ (elosulfase alfa)	BioMarin Pharmaceutical Inc.	Recombinant N-acetylgalactosamine-6-sulfate sulfatase replacement therapy for treatment of Mucopolysaccharidosis type IVA (MPS IVA; Morquio A syndrome)	Enzyme	CHO	14-Feb-14	USA

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2013						
Obinutuzumab® (Gazyva GA101-CD20 mAb)	Genentech/Roche	Treatment of chronic lymphocytic leukemia (CLL) - Obinutuzumab targets CD20 and kills B cells	Humanized Monoclonal Antibody	CHO	01-Nov-13	USA
Novoeight®, Antihemophilic Factor VIII	Novo Nordisk	Recombinant factor VIII for the treatment and prophylaxis of bleeding in patients with haemophilia A (congenital factor VIII deficiency).	Clotting Factors	CHO	15-Oct-13	EU (13-Nov) & USA (15-Oct)
Ovaleap™ Follitropin	Teva Pharma B.V.	A biosimilar of follitropin alfa, recombinant follicle-stimulating hormone for use in conjunction with assisted reproductive technologies, such as in vitro fertilization by the induction of ovulation	Hormone	CHO	27-Sep-13	EU
Inflectra™ Infliximab	Hospira	A biosimilar to Remicade, against tumour necrosis factor alpha (TNF-α) used to treat Crohn's disease, ankylosing spondylitis, alcerative Colitis, psoriatic arthritis, plaque psoriasis and rheumatoid arthritis	Chimeric Monoclonal Antibody	Sp2/0	10-Sep-13	EU
Remsima™ Infliximab	Celltrion	A biosimilar to Remicade, against tumour necrosis factor alpha (TNF-α) used to treat Crohn's disease, ankylosing spondylitis, alcerative Colitis, psoriatic arthritis, plaque psoriasis and rheumatoid arthritis	Chimeric Monoclonal Antibody	Sp2/0	10-Sep-13	EU
Simponi® Aria™ Golimumab	Janssen Biotech (Johnson & Johnson)	Inhibits inflammatory response by suppressing tumor necrosis factor (TNF) as a treatment of moderately to severely active rheumatoid arthritis	Fully Human Monoclonal Antibody	CHO	18-Jul-13	USA
Rixubis®- (Coagulation Factor IX)	Baxter Interantional Inc.	Recombinant Coagulation Factor IX for treatment of hemophilia B, indicated for the control and prevention of bleeding episodes (prophylaxis)	Clotting Factors	CHO	27-Jun-13	USA
Kadcyla® Ado-Trastuzumab Emtansine, T-DM1	Genentech/Roche	Trastuzumab alone stops growth of cancer cells by binding to the HER2/neu receptor, whereas mertansine enters cells and destroys them by binding to tubulin	Humanized Monoclonal Antibody	CHO	22-Feb-13	EU (15-Nov) & USA (22-Feb)
Zaltrap™ Aflibercept	Regeneron/Sanofi Aventis	Fc fusion protein consisting IgG1 Fc fused with VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2 for the treatment of metastatic colorectal cancer as Zaltrap (aka Elyea)	Fc-Fusion Biologic	CHO	01-Feb-13	EU (already approved in the USA in 2012)

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2012						
Abthrax® Raxibacumab injection	GlaxoSmithKline (GSK)	Monoclonal antibody that neutralizes the toxins produced by the anthrax bacterium Bacillus anthracis intended for the prophylaxis and treatment of inhaled anthrax	Human Monoclonal Antibody	NS0	14-Dec-12	USA
Eylea™ Aflibercept	Regeneron/Bayer	Fusion protein with VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2 for the treatment of neovascular (wet) Age-related Macular Degeneration (AMD) as Eylea (aka Zaltrap)	Fc-Fusion Biologic	CHO	22-Nov-12	EU (already approved in the USA in 2011)
Adcetris™ Brentuximab vedotin	Millennium Pharmaceuticals/Takeda & Seattle genetics	Antibody that which targets the cell-membrane protein CD30 linked to cathepsin cleavable linker for the treament of Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (sALCL).	Chimeric Monclonal Antibody	CHO	25-Oct-12	EU (already approved in the USA in 2011)
Zaltrap™ ziv-aflibercept	Regeneron/Sanofi Aventis	Fusion protein consisting IgG1 Fc fused with VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2 for the treatment of metastatic colorectal cancer as Zaltrap (aka Elyea)	Fc-Fusion Biologic	CHO	03-Aug-12	USA
Perjeta® Pertuzumab	Genentech/ Roche	Indicated for the treatment of HER2-positive breast cancer, in combination with trastuzumab and docetaxel.	Humanized Monoclonal Antibody	CHO	04-Mar-12	EU (4-Mar) and USA (8-Jun)

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2011						
Eylea™ Aflibercept	Regeneron/Bayer	Fusion protein with VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2 for the treatment of neovascular (wet) Age-related Macular Degeneration (AMD) as Eylea (aka Zaltrap)	Fc-Fusion Biologic	CHO	18-Nov-11	USA
Adcetris™ Brentuximab vedotin	Millennium Pharmaceuticals/Takeda & Seattle genetics	Antibody that which targets the cell-membrane protein CD30 linked to cathepsin cleavable linker for the treament of Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (sALCL).	Chimeric Monclonal Antibody	CHO	19-Aug-11	USA
Xgeva® Denosumab	Amgen	Inhibits RANK ligand, which acts as the primary signal for bone removal for the treatment of bone loss due to cancer	Fully Human Monoclonal Antibody	CHO	13-Jul-11	EU (already approved in the USA in 2010)
Nulojix® Belatacept	Bristol-Myers Squibb (BMS)	Fc fragment of a human IgG1 linked to the extracellular domain of CTLA-4 blocking the process of T-cell activation for the prevention of acute rejection in adult kidney transplants	Fc-Fusion Biologic	CHO	15-Jun-11	EU (17-Jun) & USA (15-Jun)
Yervoy® Ipilimumab	Bristil-Myers Squibb (BMS)	Activates the immune system by targeting CTLA-4 for the treatment of late-stage melanoma	Fully Human Monoclonal Antibody	CHO	25-Mar-11	EU (13-Jul) & USA (25-Mar)
Benlysta® Belimumab	GlaxoSmithKline (GSK)	Inhibits B-cell activating factor, also known as B-lymphocyte stimulator for treatment of treatment of adults with active, autoantibody-positive systemic lupus erythematosus	Fully Human Monoclonal Antibody	NS0	09-Mar-11	EU (13-Jul) & USA (9-Mar)

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2010						
Xgeva® Denosumab	Amgen	Inhibits RANK ligand, which acts as the primary signal for bone removal for the treatment of bone loss due to cancer	Fully Human Monoclonal Antibody	CHO	18-Nov-10	USA
Prolia® Denosumab	Amgen	Inhibits RANK ligand, which acts as the primary signal for bone removal for the treatment of osteoporosis, bone metastases, multiple myeloma, and tumors of the bone	Fully Human Monoclonal Antibody	CHO	01-Jun-10	EU (26-Mar) & USA (1-Jun)
Lumizyme® Alglucosidase alfa	Genzyme Corporation	Lysosomal glycogen-specific enzyme indicated for patients 8 years and older with late (non-infantile) onset Pompe disease (replaces Myozyme®)	Enzyme	CHO	24-May-10	USA
Arzerra® Ofatumumab	GlaxoSmithKline (GSK)	CD20 monoclonal antibody for the treatment for chronic lymphocytic leukaemia in patients who have not responded to Campath (alemtuzumab) or fludarabine	Fully Human Monoclonal Antibody	NS0	19-Apr-10	EU (already approved in the USA in 2009)
VPRIV® Velaglucerase alfa	Shire Pharmaceuticals Ireland Ltd	VPRIV® is used as a long term treatment as an enzyme replacement in patients with Type I Gaucher disease.	Enzyme	Human Fibroblasts	26-Feb-10	EU (26-Aug) and USA (26-Feb)
Elonva® Corifollitropin alfa	Merck Sharp & Dohme Limited	A modified rhFSH in which the carboxy-terminal peptide of the beta subunit of hCG is fused to the FSH beta chain indicated for the controlled stimulation of the ovaries	Hormone	CHO	25-Jan-10	EU
Actemra ® Tocilizumab	Genentech/ Roche	Targets interleukin-6 receptor (IL-6R) for the treatment of rheumatoid arthritis (RA) and systemic juvenile idiopathic arthritis	Humanized Monoclonal Antibody	CHO	08-Jan-10	USA

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2009						
Eporatio® Epoetin theta	Ratiopharm GmbH (Biopoin® and Eporatio® are the same product under two brand names)	Recombinant erythropoietin that stimulates the production of red blood cells from the bone marrow and is used to treat anaemia, chronic renal failure, and in adults with non-myeloid cancer.	Cytokine	CHO	29-Oct-09	EU
Arzerra® Ofatumumab	GlaxoSmithKline (GSK)	CD20 monoclonal antibody for the treatment for chronic lymphocytic leukaemia in patients who have not responded to Campath (alemtuzumab) or fludarabine	Fully Human Monoclonal Antibody	NS0	26-Oct-09	USA
Arcalyst® Rilonacept	Regeneron Pharmceuticals Inc.	For treatment of two Cryopyrin-Associated Periodic Syndromes (CAPS) disorders: Familial Cold Auto-Inflammatory Syndrome (FCAS) and Muckle-Wells Syndrome (MWS).	Fc-Fusion Biologic	CHO	23-Oct-09	EU (already approved in the USA in 2008)
Biopoin® Epoetin theta	Teva GmbH (Biopoin® and Eporatio® are the same product under two brand names)	Recombinant erythropoietin that stimulates the production of red blood cells from the bone marrow and is used to treat anaemia, chronic renal failure, and in adults with non-myeloid cancer.	Cytokine	CHO	23-Oct-09	EU
Stelara™ Ustekinumab	Janssen Biotech (Johnson & Johnson)	Directed against interleukin 12 and interleukin 23, proteins that regulate the immune-mediated inflammatory disorders to decrease the inflammatory response	Fully Human Monoclonal Antibody	CHO	16-Jan-09	EU (16-Jan) & USA (25-Sep)
Ilaris® Canakinumab	Novartis Pharmaceuticals	Monoclonal antibody targeted at interleukin-1 beta for treatment of Cryopyrin Associated Periodic Syndrome (CAPS)	Fully Human Monoclonal Antibody	Sp2/0	17-Jun-09	EU (23-Oct) & USA (17-Jun)
Simponi® Golimumab	Janssen Biotech (Johnson & Johnson)	Inhibits inflammatory response by suppressing tumor necrosis factor (TNF) as a treatment of moderately to severely active rheumatoid arthritis	Fully Human Monoclonal Antibody	CHO	24-Apr-09	EU (1-Oct) & USA (24-Apr)
Removab® Catumaxomab	Neovii Biotech GmbH /TRION Pharma	Attaches to two antigens: EpCAM, on cancer cells and CD3 on T cells forming a bridge for T-cells to attack the cancer and treat malignant ascites, peritoneal fluid accumulation caused by a cancer	Bispecific Antibody	Hybrid Hybridoma Cells	20-Apr-09	EU
Opgenra® Eptotermin alfa	Olympus Biotech International Limited	Recombinant version of osteogenic protein 1, also known as bone morphogenic protein 7 (BMP-7) which used for treating adults with spondylolisthesis	Bone Morphogenic Protein	CHO	19-Feb-09	EU
RoActemra® Tocilizumab	Genentech/ Roche	Targets interleukin-6 receptor (IL-6R) for the treatment of rheumatoid arthritis (RA) and systemic juvenile idiopathic arthritis	Humanized Monoclonal Antibody	CHO	16-Jan-09	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2008						
Arcalyst® Rilonacept	Regeneron Pharmceuticals Inc.	For treatment of two Cryopyrin-Associated Periodic Syndromes (CAPS) disorders: Familial Cold Auto-Inflammatory Syndrome (FCAS) and Muckle-Wells Syndrome (MWS).	Fc-Fusion Biologic	CHO	27-Feb-08	USA
Xyntha® Factor VIII	Wyeth Pharmaceuticals/ Pfizer	Recombinant factor VIII (updated version of Refacto with no-animal origin raw materials used) for the treatment and prophylaxis of bleeding in patients with haemophilia A (congenital factor VIII deficiency).	Clotting Factors	CHO	21-Feb-08	USA
Recothrom® Thrombin	ZymoGenetics, Inc.	Recombinant thrombin used in the prevention of minor bleeding during surgery	Clotting Factors	CHO	17-Jan-08	USA
2007						
Silapo® Epoetin-Zeta	Stada Arzneimittel AG	A biosimilar medicine of Eprex/Erypo, a recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, chronic renal failure related to cancer chemotherapy.	Cytokine	CHO	18-Dec-07	EU
Retacrit® Epoetin-Zeta	Hospira UK Limited	A biosimilar medicine of Eprex/Erypo, a recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, chronic renal failure related to cancer chemotherapy.	Cytokine	CHO	18-Dec-07	EU
Vectibix® Panitumumab	Amgen	Monoclonal antibody specific to the epidermal growth factor receptor (EGFR)for the treatment of EGFR expressing metastatic colorectal cancer	Fully Human Monoclonal Antibody	CHO	03-Dec-07	EU
Abseamed® Epoetin alfa	Medice Arzneimittel Pütter GmbH & Co. KG	A biosimilar medicine of Eprex/Erypo, a recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, chronic renal failure related to cancer chemotherapy.	Cytokine	CHO	28-Aug-07	EU
Binocrit® Epoetin alfa	Sandoz GmbH	A biosimilar medicine of Eprex/Erypo, a recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, chronic renal failure related to cancer chemotherapy.	Cytokine	CHO	28-Aug-07	EU
Epoetin alfa Hexal®	Hexal AG	A biosimilar medicine of Eprex/Erypo, a recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, chronic renal failure related to cancer chemotherapy.	Cytokine	CHO	28-Aug-07	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2007						
Mircera® Methoxy polyethylene glycol-epoetin beta	Roche	Chemically linked erythropoietin with methoxy polyethylene glycol butanoic acid stimulating erythropoiesis and is used to treat anemia associated with chronic kidney failure.	Cytokine	CHO	20-Jul-07	EU (20-Jul) & USA (14-Nov)
Pergoveris® Follitropin alfa/Lutropin alfa	Merck Serono Europe Ltd.	Recombinant hormone combination indicated to stimulate the development of follicles in the ovaries of infertile adults with low FSH and LH.	Hormone	CHO	25-Jun-07	EU
Orencia® Abatacept	Bristol-Myers Squibb (BMS)	IgG1 Fc fusion to CTLA-4 binding with more avidity to CD80 (B7-1) than to CD86 (B7-2) for second-line treatment of rheumatoid arthritis in moderate to severe adult patients.	Fc-Fusion Biologic	CHO	21-May-07	EU
Soliris® Eculizumab	Alexion Pharmceuticals, Inc	Monoclonal antibody that is a terminal complement inhibitor and approved for the treatment of paroxysmal nocturnal hemoglobinuria (PNH)	Humanized Monoclonal Antibody	NS0	16-Mar-07	EU (20-Jun) & USA (16-Mar)
Elaprase® Idursulfase	Shire Pharmaceuticals	Recombinant lysosomal enzyme iduronate-2-sulfatase for the treatment of Hunter syndrome (mucopolysaccharidosis II; MPS II).	Enzyme	HT-1080	08-Jan-07	EU (already approved in the USA in 2006)
2006						
Vectibix® Panitumumab	Amgen	Monoclonal antibody specific to the epidermal growth factor receptor (EGFR)for the treatment of EGFR expressing metastatic colorectal cancer	Fully Human Monoclonal Antibody	CHO	27-Sep-06	USA
Elaprase® Idursulfase	Shire Pharmaceuticals	Recombinant lysosomal enzyme iduronate-2-sulfatase for the treatment of Hunter syndrome (mucopolysaccharidosis II; MPS II).	Enzyme	HT-1080	24-Jul-06	USA

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2006						
Tysabri® Natalizumab	Biogen Idec.	Monoclonal antibody against the cell adhesion molecule α4-integrin for treatment of multiple sclerosis	Humanized Monoclonal Antibody	NS0	27-Jun-06	EU
Myozyme® Alglucosidase alfa	Genzyme Corporation	Recombinant glucosidase for enzyme replacement therapy for the treatment of Pompe disease (Glycogen storage disease type II), a rare lysosomal storage disorder (LSD)	Enzyme	CHO	29-Mar-06	EU (29-Apr) & USA (28-Apr)
Naglazyme® galsulfase	BioMarin Pharmaceutical Inc.	Recombinant N-acetylgalactosamine 4-sulfatase increasing the catabolism of glycosaminoglycans (GAG)is indicated for patients with mucopolysaccharidosis VI (MPS VI)	Enzyme	CHO	24-Jan-06	EU
2005						
Orencia® Abatacept	Bristol-Myers Squibb (BMS)	IgG1 Fc fusion to CTLA-4 binding with more avidity to CD80 (B7-1) than to CD86 (B7-2) for second-line treatment of rheumatoid arthritis in moderate to severe adult patients.	Fc-Fusion Biologic	CHO	26-Dec-05	USA
Hylenex®, Cumulase® Hyaluronidase	Halozyme Therapeutics, Baxter Healthcare Inc.	Recombinant hyaluronidase for use as a "spreading agent" to enhance the delivery of local anesthesia, contrast agents, and for subcutaneous fluid replacement (hypodermoclysis)	Enzyme	CHO	05-Dec-05	USA
Xolair® Omalizumab	Genentech/ Novartis	Antibody that specifically binds to free human immunoglobulin E (IgE) used to reduce sensitivity to allergens for treatment of moderate-to-severe allergic asthma	Humanized Monoclonal Antibody	CHO	25-Oct-05	EU (already in the USA since 2003)
Naglazyme® galsulfase	BioMarin Pharmaceutical Inc.	Recombinant N-acetylgalactosamine 4-sulfatase increasing the catabolism of glycosaminoglycans (GAG)is indicated for patients with mucopolysaccharidosis VI (MPS VI)	Enzyme	CHO	31-May-05	USA
Avastin ® Bevacizumab	Genentech/ Roche	An angiogenesis inhibitor (inhibits VEGF-A)where the drug slows the growth of new blood vessels to treat colorectal, lung, breast, glioblastoma, kidney and ovarian cancers	Humanized Monoclonal Antibody	CHO	12-Jan-05	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2004						
Tysabri® Natalizumab	Biogen Idec.	Monoclonal antibody against the cell adhesion molecule α4-integrin for treatment of multiple sclerosis	Humanized Monoclonal Antibody	NS0	24-Nov-04	USA
Raptiva® Efalizumab	Genentech	Binds to the CD11a subunit of lymphocyte function-associated antigen 1 and acts as an immunosuppressant for treatment of autoimmune diseases, originally marketed to treat psoriasis	Humanized Monoclonal Antibody	CHO	09-Sep-04	USA
Neutrospec® Fanolesomab	Palatin Technologies, Ben Venue Labs, Mallinckrodt Inc.	Indication to aid in the diagnosis of appendicitis. It is labeled with a radioisotope, technetium-99m (99mTc). Withdrawn in Dec-2005	Murine Monoclonal Antibody	Hybridoma	30-Jun-04	USA
Luveris® LH	EMD Serono, Inc.	Luteinizing hormone (recombinant human LH) for the treatment of female infertility and it is indicated for use in combination with human follicle-stimulating hormone (Gonal-f®).	Hormone	CHO	24-May-04	USA
Advate® Antihemophilic Factor	Baxter Interantional Inc.	Recombinant factor VIII for the treatment and prophylaxis of bleeding in patients with haemophilia A (congenital factor VIII deficiency).	Clotting Factors	CHO	02-Mar-04	EU (already in the USA since 2003)
Avastin ® Bevacizumab	Genentech/ Roche	An angiogenesis inhibitor (inhibits VEGF-A)where the drug slows the growth of new blood vessels to treat colorectal, lung, breast, glioblastoma, kidney and ovarian cancers	Humanized Monoclonal Antibody	CHO	26-Feb-04	USA
Erbitux® Cetuximab	Bristol-Myers Squibb, Eli Lilly and Company, Merck KGaA	Mouse/human antibody for treatment of patients with epidermal growth factor receptor (EGFR)-expressing, metastatic colorectal cancer, head and neck cancer	Chimeric Monoclonal Antibody	Sp2/0	12-Feb-04	EU (29-Jun) & USA (12-Feb)
Zevalin® Ibritumomab tiuxetan	Spectrum Pharmaceuticals /Biogen Idec	Radioimmunotherapy treatment for relapsed or refractory, low grade or transformed B cell non-Hodgkin's lymphoma, a lymphoproliferative disorder	Murine Monoclonal Antibody	CHO	16-Jan-04	EU (already approved in the USA in 2002)

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2003						
Humira® Adalimumab	Abbott Laboratories (AbbVie)	Adalimumab binds to tumor necrosis factor-alpha (TNFα) and this TNFα inactivation has proven to be important in downregulating the inflammatory reactions	Fully Human Monoclonal Antibody	CHO	08-Sep-03	EU (already approved in the USA in 2002)
Advate® Antihemophilic Factor	Baxter Interantional Inc.	Recombinant factor VIII for the treatment and prophylaxis of bleeding in patients with haemophilia A (congenital factor VIII deficiency).	Clotting Factors	CHO	25-Jul-03	USA
Bexxar® Tositumomab-I131	GlaxoSmithKline (GSK)	Tositumomab binds to the CD20 antigen, which is predominantly expressed on mature B cells for treatment of non-Hodgkin's lymphoma (CD20 positive, follicular)	Murine Monoclonal Antibody	Hybridoma	27-Jun-03	USA
Xolair® Omalizumab	Genentech/ Novartis	Antibody that specifically binds to free human immunoglobulin E (IgE) used to reduce sensitivity to allergens for treatment of moderate-to-severe allergic asthma	Humanized Monoclonal Antibody	CHO	20-Jun-03	USA
Aldurazyme® Laronidase	Genzympe Corporation	Recombinant alpha-L-iduronidase used in enzyme replacement therapy for the treatment of Mucopolysaccharidosis I (MPS I)	Enzyme	CHO	30-Apr-03	USA
Fabrazyme® Agalsidase	Genzyme Corporation	Recombinant alpha-galactosidase A or alpha-GAL for enzyme replacement indicated for the treatment of Fabry disease	Enzyme	CHO	24-Apr-03	USA (already approved in the EU since 2001)
Amevive® Alefacept	Astellas Pharma Inc.	Inhibits the activation of CD4+ and CD8+ T cells by interfering with CD2 on the T cells and it is used for treatment of moderate-to-severe chronic plaque psoriasis	Fc-Fusion Biologic	CHO	30-Jan-03	USA

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2002						
Humira® Adalimumab	Abbott Laboratories (AbbVie)	Adalimumab binds to tumor necrosis factor-alpha (TNFα) and this TNFα inactivation has proven to be important in downregulating the inflammatory reactions	Fully Human Monoclonal Antibody	CHO	30-Dec-02	USA
Inductos® dibotermin alfa	Medtronic BioPharma B.V.	Acts on the bone structure since it is a recombinant version of bone morphogenetic protein 2 (BMP-2) stimulating bone formation to help heal fractures and aid in lower-back spine fusion surgery	Bone Morphogenic Protein	CHO	09-Sep-02	EU
Xigris® Drotrecogin Alpha	Eli Lilly & Company	Recombinant form of human activated protein C that has anti-thrombotic, anti-inflammatory, and profibrinolytic properties for the treatment of sepsis (withdrawn)	Clotting Factors	HEK293	22-Aug-02	EU
InFUSE™ Bone Graft/LT-CAGE™	Medtronic Sofamor Danek	Acts on the bone structure since it is a recombinant version of bone morphogenetic protein 2 (BMP-2) stimulating bone formation to help heal fractures and aid in bone graft surgery	Bone Morphogenic Protein	CHO	02-Jul-02	USA
Rebif® Interferon Beta-1a	Pfizer, EMD Serono	Recombinant interferon beta 1a for the treatment of relapsing forms of multiple sclerosis	Cytokine	CHO	07-Mar-02	USA (already luanched in the EU in 1998)
Zevalin® Ibritumomab tiuxetan	Spectrum Pharmaceuticals /Biogen Idec	Radioimmunotherapy treatment for relapsed or refractory, low grade or transformed B cell non-Hodgkin's lymphoma, a lymphoproliferative disorder	Murine Monoclonal Antibody	CHO	19-Feb-02	USA

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2001						
Xigris® Drotrecogin Alpha	Eli Lilly & Company	Recombinant form of human activated protein C that has anti-thrombotic, anti-inflammatory, and profibrinolytic properties for the treatment of sepsis (withdrawn)	Clotting Factors	HEK293	21-Nov-01	USA
Aranesp® Darbepoetin alfa	Amgen	Recombinant erythropoetin to simulate erythropoiesis (increases red blood cell levels) and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy	Cytokine	CHO	17-Sep-01	EU (8-Jun) & USA (17-Sep)
Fabrazyme® Agalsidase	Genzyme Corporation	Recombinant alpha-galactosidase A or alpha-GAL for enzyme replacement indicated for the treatment of Fabry disease	Enzyme	CHO	03-Aug-01	EU
Replagal® agalsidase beta	Shire Human Genetic Therapies AB	Recombinant alpha-galactosidase A or alpha-GAL for enzyme replacement indicated for the treatment of Fabry disease	Enzyme	CHO	03-Aug-01	EU
Nespo® Darbepoetin alfa	Dompé Biotec S.p.A.	Recombinant erythropoetin to simulate erythropoiesis (increases red blood cell levels) and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy	Cytokine	CHO	08-Jun-01	EU
Campath®, Mabcampath® Alemtuzumab	Genzyme Corporation	Binds to CD52, on the surface of mature lymphocytestreatment of chronic lymphocytic leukemia (CLL), cutaneous T-cell lymphoma (CTCL) and T-cell lymphoma	Humanized Monoclonal Antibody	CHO	07-May-01	EU (6-Jul) & USA (7-May)
Metalyse® Tenecteplase	Boehringer Ingelheim	Recombinant fibrin-specific plasminogen activator indicated for use in the reduction of mortality associated with acute myocardial infarction (AMI).	Enzyme	CHO	23-Feb-01	EU
Ovitrelle® Choriogonadotropin Alpha	Merck Serono Europe Limited	Ovidrel (recombinant Chorionic Gonadotropin (Hcg) used as part of a treatment program for certain fertility problems in women and generally in combination with another hormone (FSH).	Hormone	CHO	02-Feb-01	EU (already launched in the year 2000 in the USA)

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
2000						
Ovidrel® Choriogonadotropin Alpha	EMD Serono, Inc.	Ovidrel (recombinant Chorionic Gonadotropin (Hcg) used as part of a treatment program for certain fertility problems in women and generally in combination with another hormone (FSH).	Hormone	CHO	20-Sep-00	USA
Herceptin® Trastuzumab	Genentech/ Roche	A treatment that mainly interferes with the HER2/neu receptor to treat certain breast cancers.	Humanized Monoclonal Antibody	CHO	28-Aug-00	EU (already approved in the USA in 1998)
Helixate FS® / NexGen® Factor VIII octocog alfa	CSL Behring	A recombinant antihemophilic factor that is indicated for the control and prevention of bleeding episodes in patients with hemophilia A	Clotting Factors	BHK	04-Aug-00	EU
Kogenate Bayer® Factor VIII, octocog alfa	Bayer Healthcare	A recombinant antihemophilic factor that is indicated for the control and prevention of bleeding episodes in patients with hemophilia A	Clotting Factors	BHK	04-Aug-00	EU
TNKase® Tenecteplase	Genentech/Roche	Recombinant fibrin-specific plasminogen activator indicated for use in the reduction of mortality associated with acute myocardial infarction (AMI).	Enzyme	CHO	02-Jun-00	USA
Mylotarg® Gemtuzumab ozogamicin	Wyeth Pharmaceuticals	An antibody-drug conjugate targeting CD33 for the treatment of acute myelogenous leukemia (withdrawn 2010)	Humanized Monoclonal Antibody	NS0	17-May-00	USA
'Thyrogen® (thyrotropin alfa)	Genzyme Europe B.V.	Recombinant form of human thyroid-stimulating hormone (TSH) which binds to TSH receptors used as a diagnostic tool for patients that may have thyriod cancer	Hormone	CHO	09-Mar-00	EU (already luanched since 1998 in the USA)
ReFacto® Antihemophilic Factor	Wyeth Pharmaceuticals/ Pfizer	Recombinant factor VIII for the treatment and prophylaxis of bleeding in patients with haemophilia A (congenital factor VIII deficiency).	Clotting Factors	CHO	05-Mar-00	USA (already launched 1999 in EU)
Enbrel® Etanercept	Pfizer	Tumor necrosis factor receptor2-immune globlulin G1 Fc fusion protein for treatment of rheumatoid arthritis, psoriasis, and ankylosing spondylitis.	Fc-Fusion Biologic	CHO	03-Feb-00	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
1999						
Remicade® Infliximab	Janssen Biotech (Johnson & Johnson)	Chimeric monoclonal antibody against tumour necrosis factor alpha (TNF-α) used to treat autoimmune diseases such as Crohn's disease and rheumatoid arthritis	Chimeric Monoclonal Antibody	Sp2/0	13-Aug-99	EU
Synagis® Palivizumab	MedImmune Inc.	Monoclonal antibody (IgG) directed against an epitope in the A antigenic site of the F protein of respiratory syncytial virus (RSV) for treatment of RSV infections	Humanized Monoclonal Antibody	NS0	13-Aug-99	EU
ReFacto® Antihemophilic Factor	Wyeth Pharmaceuticals/ Pfizer	Recombinant factor VIII for the treatment and prophylaxis of bleeding in patients with haemophilia A (congenital factor VIII deficiency).	Clotting Factors	CHO	13-Apr-99	EU
NovoSeven® Factor VIIa	Novo Nordisk	Treating or preventing bleeding episodes in certain patients with bleeding problems such as hemophilia A or B, acquired hemophilia, or congenital FVII deficiency.	Clotting Factors	CHO	25-Mar-99	USA (already launched 1996 in EU)
Zenapax® Daclizumab	Roche	First humanized antibody that binds to CD25, the alpha subunit of the IL-2 receptor of T cells for treatment of organ transplant rejection and investigated for treatment of multiple sclerosis.	Humanized Monoclonal Antibody	Sp2/0	26-Feb-99	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
1998						
Thyrogen® (thyrotropin alfa)	Genzyme Corporation	Recombinant form of human thyroid-stimulating hormone (TSH) which binds to TSH receptors used as a diagnostic tool for patients that may have thyriod cancer	Hormone	CHO	30-Nov-98	USA
Enbrel® Etanercept	Amgen	Tumor necrosis factor receptor2-immune globlulin G1 Fc fusion protein for treatment of rheumatoid arthritis, psoriasis, and ankylosing spondylitis.	Fc-Fusion Biologic	CHO	02-Nov-98	USA
Herceptin® Trastuzumab	Genentech/ Roche	A treatment that mainly interferes with the HER2/neu receptor to treat certain breast cancers.	Humanized Monoclonal Antibody	CHO	25-Sep-98	USA
Humaspect® Votumumab	KS Biomedix Limited	Diagnostic radiopharmaceutical for detection of carcnima of the colon or rectum	Human Monoclonal Antibody	Human Lymphoblastoid Cell Line	25-Sep-98	EU
Remicade® Infliximab	Janssen Biotech (Johnson & Johnson)	Chimeric monoclonal antibody against tumour necrosis factor alpha (TNF-α) used to treat autoimmune diseases such as Crohn's disease and rheumatoid arthritis	Chimeric Monoclonal Antibody	Sp2/0	24-Aug-98	USA
Synagis® Palivizumab	MedImmune Inc.	Monoclonal antibody (IgG) directed against an epitope in the A antigenic site of the F protein of respiratory syncytial virus (RSV) for treatment of RSV infections	Humanized Monoclonal Antibody	NS0	19-Jun-98	USA
Mabthera® (Rituxan®) Rituximab	Genentech (Roche)/Biogen Idec	Monoclonal antibody against the protein CD20 found primarily on B cells allowing for the treament of diseases with excessive or dysfunctional B cells	Chimeric Monoclonal Antibody	CHO	02-Jun-98	EU (already in USA since 1997)
Simulect® Basiliximab	Novartis Pharmaceuticals	Mouse-human monoclonal antibody to the α chain (CD25) of the IL-2 receptor to prevent organ transplantation rejection	Chimeric Monoclonal Antibody	Sp2/0	12-May-98	USA
Rebif® Interferon Beta-1a	Merck Serono Europe Ltd.	Recombinant interferon beta 1a for the treatment of relapsing forms of multiple sclerosis	Cytokine	CHO	04-May-98	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
1997						
Zenapax® Daclizumab	Roche	First humanized antibody that binds to CD25, the alpha subunit of the IL-2 receptor of T cells for treatment of organ transplant rejection and investigated for treatment of multiple sclerosis.	Humanized Monoclonal Antibody	Sp2/0	10-Dec-97	USA
Rituxan® Rituximab	Genentech (Roche)/Biogen Idec	Monoclonal antibody against the protein CD20 found primarily on B cells allowing for the treament of diseases with excessive or dysfunctional B cells	Chimeric Monoclonal Antibody	CHO	26-Nov-97	USA
Cerezyme® Imiglucerase	Genzyme Europe B.V.	Recombinant analogue of human β-glucocerebrosidase used in the treatment of Gaucher's disease, in which a fatty substance (lipid) accumulates in cells and certain organs	Enzyme	CHO	17-Nov-97	EU
Follistim ® Follitropin-Beta	Merck & Co	Recombinant follicle-stimulating hormone for the treatment of infertility by stimulating ovaries to produce one or more eggs during each treatment.	Hormone	CHO	29-Sep-97	USA
Gonal-F® Follitropin-alfa	EMD Serono, Inc.	Recombinant follicle-stimulating hormone for use in conjunction with assisted reproductive technologies, such as in vitro fertilization by the induction of ovulation	Hormone	CHO	29-Sep-97	USA
NeoRecormon® Epoetin-Beta	Roche	Recombinant erythropoietin. It stimulates erythropoiesis and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy.	Cytokine	CHO	16-Jul-97	EU (already launched in the USA in 1996)
Avonex® Interferon Beta-1a	Biogen Idec, Inc.	Recombinant interferon beta 1a is a drug in the interferon family used to treat multiple sclerosis (MS) by balancing the expression of pro- and anti-inflammatory agents in the brain.	Cytokine	CHO	13-Mar-97	EU
BeneFIX® Factor IX	Wyeth Pharmaceuticals/ Pfizer	For the control and prevention of hemorrhagic episodes in patients with hemophilia B (congenital factor IX deficiency and Christmas disease), including control and prevention of bleeding in surgical settings	Clotting Factors	CHO	11-Feb-97	EU (27-Aug) & USA (11-Feb)

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
1996						
Cathflo®, Activase®, Actilyse® (Alteplase)	Genentech, Boerhringer Ingelheim	Recombinant tissue plasminogen activator that is responsible for clot breakdown an indicated for treating heart attacks and acute massive pulmonary embolism such as in strokes.	Enzyme	CHO	18-Jun-96	USA
NeoRecormon® Epoetin-Beta	Roche	Recombinant erythropoietin. It stimulates erythropoiesis and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy.	Cytokine	CHO	14-Jun-96	USA
Avonex® Interferon Beta-1a	Biogen Idec, Inc.	Recombinant interferon beta 1a is a drug in the interferon family used to treat multiple sclerosis (MS) by balancing the expression of pro- and anti-inflammatory agents in the brain.	Cytokine	CHO	17-May-96	USA
Puregon® Follitropin beta	N.V. Organon	Recombinant follicle-stimulating hormone for the treatment of infertility by stimulating ovaries to produce one or more eggs during each treatment.	Hormone	CHO	03-May-96	EU
NovoSeven® Factor VIIa	Novo Nordisk	Treating or preventing bleeding episodes in certain patients with bleeding problems such as hemophilia A or B, acquired hemophilia, or congenital FVII deficiency.	Clotting Factors	CHO	23-Feb-96	EU
1995						
Gonal-F® Follitropin-alfa	Merck Serono Europe Ltd	Recombinant follicle-stimulating hormone for use in conjunction with assisted reproductive technologies, such as in vitro fertilization by the induction of ovulation	Hormone	CHO	20-Oct-95	EU

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
1994						
Reopro® Abciximab	Janssen Biotech and Eli Lilly and Company	Antibody fragment and a platelet aggregation inhibitor mainly used during and after coronary artery procedures like angioplasty to prevent thrombus (blood clots)	Chimeric Fab Antibody	Sp2/0	14-Nov-94	EU (country specific) & USA
Cerezyme® Imiglucerase	Genzyme Corporation	Recombinant analogue of human β-glucocerebrosidase used in the treatment of Gaucher's disease, in which a fatty substance (lipid) accumulates in cells and certain organs	Enzyme	CHO	06-Jun-94	USA
1993						
Bioclate™ Factor VIII	Aventis Behring	A recombinant antihemophilic factor that is indicated for the control and prevention of bleeding episodes in patients with hemophilia A	Clotting Factors	CHO	31-Dec-93	USA
Pulmozyme® human deoxyribonuclease I (rhDNase)	Genentech/ Roche	Recombinant human deoxyribonuclease I (rhDNase) hydrolyzes the DNA present in sputum/mucus of cystic fibrosis patients and reduces viscosity in the lungs, promoting improved clearance of secretions.	Enzyme	CHO	30-Dec-93	USA
Kogenate FS® Factor VIII, octocog alfa	Bayer Healthcare	A recombinant antihemophilic factor that is indicated for the control and prevention of bleeding episodes in patients with hemophilia A	Clotting Factors	BHK	28-Feb-93	USA
Helixate FS® Factor VIII octocog alfa	CSL Behring	A recombinant antihemophilic factor that is indicated for the control and prevention of bleeding episodes in patients with hemophilia A	Clotting Factors	BHK	28-Feb-93	USA

Table. 23.1 (continued)

Product	Company	Target/Initial Therapeutic Indication	Product Category	Mammalian Cell Line	First Approved	Region
		1992				
Recombinate™ Antihemophilic Factor VIII	Baxter Interantional Inc.	A recombinant antihemophilic factor that is indicated for the control and prevention of bleeding episodes in patients with hemophilia A	Clotting Factors	CHO	21-Dec-92	USA
		1989				
Procrit® , Epogen® (Epoetin-Alfa)	Amgen, Janssen Biotech (Johnson & Johnson)	Recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy.	Cytokine	CHO	01-Jun-89	USA (already approved in Europe)
		1988				
Eprex® , Erypo® (Epoetin-Alfa)	Janssen Biotech (Johnson & Johnson)	Recombinant erythropoietin that stimulates erythropoiesis and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy.	Cytokine	CHO	04-Aug-88	Europe
		1987				
Activase® Alteplase	Genentech	Recombinant tissue plasminogen activator that is responsible for clot breakdown an indicated for treating acute mycardial infarctions.	Enzyme	CHO	13-Nov-87	USA
		1986				
Orthoclone OKT3® Muromonab	Ortho Biotech (Janssen Biotech)	Immunosuppressant drug given to reduce acute rejection in patients with organ transplants (First monoclonal antibody drug to be approved for clinal use in humans)	Monoclonal Antibody	Mouse Ascites	19-Jun-86	USA

changed in 1994, when the first approved and successfully marketed antibody drug produced via mammalian cell cultured was manufactured by Centocor (now known as Janssen Biotech from Johnson & Johnson) with the trade name ReoPro® (Abciximab) (Tam et al. 1998). Abciximab is a fragment antigen-binding (Fab) fragment of the chimeric human murine monoclonal antibody 7E3 which was designed to overcome the obstacles of murine based antibodies for human therapeutics since murine antibodies can have glycosylation patterns that are highly immunogenic to humans (Butler 2005; Jenkins et al. 1996) as can be seen with Orthoclone OKT3 studies where 50 % patients have experienced a potentially lethal human anti-mouse antibody (HAMA) response (Niaudet et al. 1993; Richards et al. 1999). While Orthoclone OKT3® can be considered a first generation biopharmaceutical, along with protein drugs that are simply engineered copies of native endogenous proteins. Abciximab is also one of the first efforts of designing a second generation mAb biopharmaceutical produced from mammalian cells. Second generation biopharmaceuticals have been engineered to improve their performance by a combination or single alteration of the following; reengineering the amino acid sequence or glycoproteins, the addition of chemical conjugates or the creation of fused protein structures that improve drug function such as stability and targeting. Abciximab was specifically designed to reduce immunogenicity, (Tam et al. 1998). To reduce possible complement-activating and immunogenicity reactions from Abciximab, the Fc fragment is removed from the complete antibody so that the fragment antigen binding structure is only left (Knight et al. 1995).

Years prior to Abciximab, Centocor had almost reached the brink of bankruptcy due to approval denial for an IgM antibody drug expressed from a Sp2/0 cell culture in an industrial scale perfusion process. The drug, known as nebacumab (Centoxin®), was already approved in The Netherlands, Britain, Germany and France in 1991, where it was indicated as a treatment for Gram-negative sepsis but soon after the FDA rejected approval in the USA due to new clinical trial data that eventually led to the discontinuation of Centoxin® from the market. The lessons learned and the bioprocesses developed from Centoxin® allowed Centocor in partnership with Eli Lilly to develop the perfusion process and gain marketing approval of ReoPro®(Marks 2012), 8 years after the first antibody based drug was introduced into the market.

After ReoPro® showed success, a flood of chimeric and humanized monoclonal antibody drugs that have the ability to trigger effector functions in humans, longer circulatory half-life, and decreased immunogenicity compared to murine antibodies came to the market (see Table 23.1). These engineered antibodies appeared first in 1997 starting with the chimeric molecule Rituximab under the trade names Rituxan® and Mabthera®. Rituximab was conceived and developed by IDEC Pharmaceutical Corporation, San Diego, CA (now known as Biogen Idec) under the development name IDEC-C2B8 (Maloney et al. 1997). The drug was brought to market in collaboration with Genentech, Inc., South San Francisco, CA and F. Hoffman-LaRoche (Nutley, NJ) as the first mAb approved for the treatment of cancer, specifically the treatment non-Hodgkin's lymphoma. Rituximab was also the first mAb approved for the treatment of cancer, specifically the treatment

non-Hodgkin's lymphoma (Grillo-Lopez 2000). To generate rituximab, the variable regions of a murine anti-human CD20 that are found on the surface of malignant and normal B cells were fused to the human IgG and kappa-constant regions (Silverman and Weisman 2003). Rituximab is designed to promote antibody-dependent cellular cytotoxicity (ADCC) with human effector cells and mediate complement-dependent cell lysis. The U.S. patent for rituximab was issued in 1998 and will expire in 2015. Apart from Rituximab Roche also had the first humanized MAb approved for marketing in 1997, a few months after rituximab, known as daclizumab (Zenapax®) and used in the treatment of organ transplant rejection similar to Orthoclone OKT3®. Humanization of an antibody, usually involves reengineering of antibodies, where the complimentary determining regions from the rodent antibody V-regions are combined with framework regions from human V-regions in an attempt to decrease immunogenicity even further than chimeric antibodies. Soon after Zenapax®, in 1998, Novartis got marketing approval for Simulect® (basiliximab) and Johnson & Johnson got approval for Remicade® (Infliximab), both chimeric mAbs (see Table 23.1). In the case of the humanized mAbs that followed Zenapax®, it was followed a year later with Synagis® (Astra Zeneca) and Herceptin® (Roche) in 1998. Currently, a plethora of chimeric and humanized antibodies have been approved (reviewed in Table 23.1), but with the advent of technology to produce fully human antibodies Abbot was able to create the first fully human mAb drug, marketed as Humira® (adalimumab) in 2002. The fully human antibody is another variant of engineered mAbs harnessed as a therapeutic drug that can provide reduced immunogenicity and a longer half-life compared to the use of murine antibodies.

The early mammalian cell culture based production processes had very low yields of recombinant protein product, sometimes a 100 times less when compared to today's processes. This was due to both upstream and downstream processes lacking optimization. Firstly the bioreactor cultures gave low titers of <50 mg/l for mammalian cells, a far cry from today's average of 2,000–5,000 mg/L for fed batch process and beyond that titer for perfusion processes where up to 25 g/l has been reported (Kelley 2009; Chon and Zarbis-Papastoitsis 2011). On top of that the purification steps were sometimes giving yields below 20 % and these aspects meant that the manufacturer had to increase the scale of the process in order to achieve enough products to serve the market with reasonable economics. To meet market demand with low yielding processes, large 10,000–15,000 L bioreactor capacity facilities were build, usually designed for mono-product operations making it difficult to transfer different products across facilities (Werner 2013). Over time the optimizations of cell productivity due to improved cell line development and selection methods, optimization of bioreactor designs and configurations (Kuystermans and Al-Rubeai 2011), and improved downstream process technology and design has facilitated increases in efficiency of bioprocesses (Low et al. 2007; Shukla et al. 2007) affording them to operate at a smaller scale to satisfy market demand. These smaller scales can operate with disposable systems in conjunction with high yielding engineered cell lines to allow for flexible multi-product operating facilities. In addition and concurrently, market demand for complex

glycosylated recombinant protein drugs has increased at such a pace that large scale facilities with multi-product capabilities are still feasible options in order to supply today's market for certain high demand products. Although, there is still a considerable amount of further development expected in the areas of bioprocess optimizations, much has already been done over the last two decades and contributed to reduced production costs (Shukla and Thömmes 2010). With reported research of fed batch CHO culture reaching titers of more than 10 g/l (Huang et al. 2010), continued development of mammalian cell culture processes for high value drug production will reduce the cost to market even further and enable wider access to biopharmaceuticals around the world.

23.3 Monoclonal Antibodies as Drugs

From the data shown in Table 23.1, it is apparent that monoclonal antibody drugs have become important driver in the biopharmaceutical market that is now dominated by biomolecular manufactured drugs as the fastest growing source of innovation and revenue with a total of more than 140 mammalian cell culture derived drugs approved between 1986 and 2014 in the EU and USA. As antibody drugs evolved from murine to chimeric, humanized, and finally fully human antibodies, the concerns regarding immunogenicity, weak efficacy, and short serum half-life, has been reduced significantly. For example Orthoclone OKT3 had a serum half-life of 0.3–0.75 days and immunogenicity chance of 50 % compared to the fully human mAb drug approved in 2010 with a serum half-life of 26 days and the chance of immunogenicity being >1.6 % marketed under the trade name Ilaris® (Canakinumab) from Novartis Pharmaceuticals (Wilde and Goa 1996; Yoon et al. 2010). Apart from Orthoclone OKT3, two other murine mAb produced therapeutics have been approved for marketing, named Zevalin® (Ibritumomab tiuxetan) and Bexxar® (Tositumomab-I131) as they showed a lower immunogenicity, with below 8 % of patients only having HAMA responses, whereas normally it is observed that with murine therapeutic antibodies HAMA responses can range within the 50–100 % range for the majority (Hwang and Foote 2005). Zevalin® was approved in 2002 as a CD20 targeting IGg1 conjugate drug for radio-immunotherapy therapy for difficult to manage low grade or transformed B cell non-Hodgkin's lymphoma, a type of lymphoproliferative disorder. The murine antibody is conjugated to the radioactive isotope yttrium 90 via the chelate tiuxetan and has a half-life of 1.25 days. Bexxar® is also targeting the B cell marker CD20 to treat non-Hodgkin's lymphoma with an Iodine 131 conjugate for radio-immunotherapy treatments, although this time; the isotope is linked directly to the antibody instead of through a chelate. Again, Bexxar® like Zevalin® has a short half-life, being only 2.7 days, something that is desirable to avoid excess exposure to the antibody and conjugate (Leveque et al. 2005).

As indicated earlier, the introduction of chimeric full antibodies as drugs started with rituximab (Rituxan®) from Genentech in 1997, used to treat non- Hodgkin's

lymphoma, this antibody is only immunogenic in 1.1 % of patients with no secondary conditions (Yoon et al. 2010). The serum half-life of Rituximab is 22 days (Genentech 1997) due to the increased stability of a human Fc region. Chimeric antibodies have generally shown varied immunogenicity with chimeric antibodies such as basiliximab and infliximab both demonstrating immunogenicity in patients of up to 44 % and 37 % respectively (Leveque et al. 2005), rituximab, demonstrates much lower immunogenicity. With humanized and fully human mAb drugs, there has been a further decrease in the average immunogenicity within patients, as the majority has shown immunogenicity's below 10 % (Yoon et al. 2010). By engineering antibodies with a reduced murine amino acid derived sequence makeup it is possible to reduce the immunogenicity. Humanized and fully human antibodies can still have immunogenicity risks since the variable regions can be murine derived such as the complementarity determining regions (CDR)-sequence that can contain these murine regions. Apart from the presence of murine amino acid sequences that can contribute to increased immunogenicity of mAb therapeutics, there can be several other intrinsic and even extrinsic factors that may increase immunogenicity for mAb therapeutics. It is known that the carbohydrate side-chains attached via glycosylation has a major impact on immunogenicity of an antibody and plays a major intrinsic role as well as other post translational events that may modify the antibody sequence such as oxidation, non-enzymatic glycosylation, and deamination of the amino side chains (Arnold et al. 2007; Sheeley et al. 1997) . It has also been found that antibodies that target insoluble factors, such as cell surface markers, may pose a risk of increased immunogenicity to the patient. Another intrinsic factor is the presence of CD4+ T helper epitopes that can lead to an immune response depending on the amino acid sequence (Harding et al. 2010) . Apart from a patients immunological status and the effects of co-medication (Harding et al. 2010; Hendrickson et al. 2006), extrinsic factors may arise due to the composition of the antibody drugs manufacturers formulate. Some formulations may be able to cause increased immunogenicity issues due to the presence of adjuvant-like contaminants and aggregates (Shire 2009; Rosenberg 2006).

Adalimumab, the first fully human antibody, was selected via phage display of the human variable heavy and light chain sequences, but it is also possible to produce fully human antibodies from an engineered mouse via a process known as XenoMouse technology. With XenoMouse technology, the immunoglobulin genes within the transgenic mouse are of human origin (Lonberg et al. 1994; Green 1999) making the possibility of natural in vivo affinity maturation of the sequences which may contribute to a further reduction in immunogenicity. The first therapeutic mAb to be approved for marketing that utilized the XenoMouse technology was panitumumab (Vectibix®), in 2006 (Jakobovits et al. 2007). Panitumumab has a very low immunogenicity of 3–4 %, due to the antibody development strategy employed, thus fully human derived antibodies can contain no murine sequences, unlike humanized antibodies, but immune responses can still occur. Thus the development of fully human antibodies are not a guarantee of non-immunogenicity, but it is possible that with further development steps

immunogenicity of engineered mAbs can be reduced or even eliminated by a combination of CDR-sequence engineering, optimized cell culture bioprocess development strategies, and formulation engineering to help fine tune the intrinsic and extrinsic factors that can reduce immunogenicity.

What has also been observed is that serum half-life can vary greatly with humanized and fully human antibody drugs compared to natural antibodies such as IgG which has a mean half-life of 25–32 days (Maarschalk-Ellerbroek et al. 2011). These engineered therapeutic antibodies have a serum half-life that varies greatly from a low of 7.5 days to a range similar to natural antibodies (Yoon et al. 2010). Varying serum half-life can also be the result of variations in post translational processing of these recombinant antibodies with the use of non-human originating cell lines including the culture conditions during manufacturing as we know that glycans also influence immunogenicity and efficacy (Ghaderi et al. 2012).

The serum half-life of mAb's is usually high compared to other recombinant proteins due the neonatal Fc receptor of IgG (FcRn). The FcRn is a MHC Class I like molecule that binds to the CH2-CH3 hinge region of IgG which starts a process that ultimately protects IgG from degradation thereby promoting the extended half-life of this class of antibody in the serum (Simister and Mostov 1989; Kuo et al. 2010). In further detail, IgG is bound to the Fc receptor of a cell within an acidic endosome that is destined to be internalized via pinocytosis, the IgG can be recycled to the cell surface and released back into a neutral pH environment preventing the faith of lysosomal degradation that unbound proteins face when taken in by the endosome. This recycling can extend the serum half- life of IgG (Rodewald 1976), although, further studies are required since studies have shown that an increase in binding affinity of an engineered IgG molecule to the FcRn is not proportional to half-life (Roopenian and Akilesh 2007). One study demonstrated this with variants of Mab drug, Herceptin™, from Genentech with 3 and 12-fold higher binding affinities for the FcRn that still had similar half-life compared to Herceptin at the end (Petkova et al. 2006). Currently, more than 20 glyco-engineered mAbs, with enhanced ADCC, are being evaluated in clinical studies. Two of these mAbs have already been approved, mogamulizumab (Poteligeo®) on March 30th 2012 for marketing in Japan, an antibody developed exclusively by Kyowa Hakko Kirin, and obinutuzumab (Gazyva®), approved on November 1st 2013 in the USA (see Table 23.1), confirming the success of this approach. Although mogamulizumab has not been approved in Europe or the USA as of this writing, it is under review for treatment peripheral T-cell lymphoma while clinical studies have shown that the engineered obinutuzumab has a half-life of 28 days (Reichert 2011). The glyco-engineered Fc region of obinutuzumab has a bisected, complex, non-fucosylated oligosaccharides attached to asparagine 297, that enhances the binding affinity to FcγRIII an Fc receptor (Mossner et al. 2010). The glycol-engineering of obinutuzumab has significantly improved the efficacy over earlier therapeutic molecules such as rituximab and earlier developed mAb in B-cell malignancies.

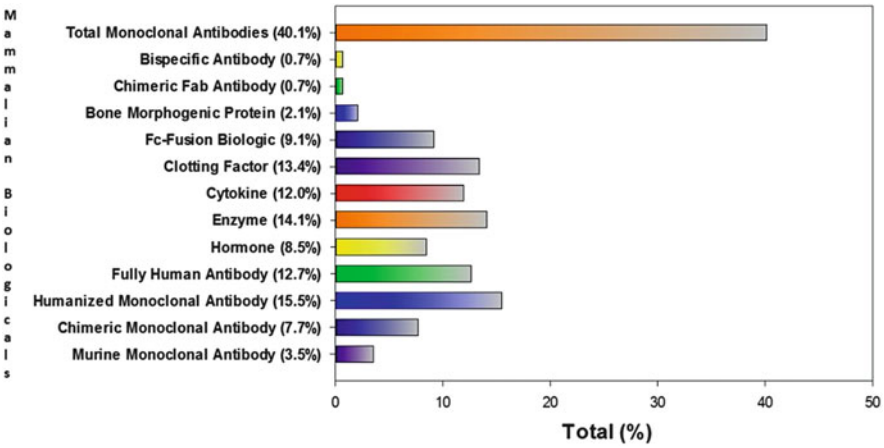

Fig. 23.2 A representation of the variety of mammalian cell culture products approved in the EU and USA, from the year 1987 till April 2014, revealing that monoclonal antibodies take 40.1 % of the approved mammalian biologicals on the market. Humanized and fully human antibodies make up the majority of the monoclonal antibodies approved

The majority of mammalian expressed biologics approved from 1987 up until April 2014, are monoclonal antibodies, with 40.1 % of the market (Fig. 23.2). In the month of January 2014, a total of 7 mAb therapeutics were undergoing their first regulatory review with the first submission of marketing applications being for; vedolizumab, siltuximab, ramucirumab, secukinumab, dinutuximab, nivolumab, and pembrolizumab. As of April 23th 2014, the FDA approved the chimeric antibody drug Sylvant™ (siltuximab) and the fully human antibody Cyramza™ (ramucirumab) for marketing (see Table 23.1). The median circulating half-life of siltuximab has been shown to be 17.8 days and siltuximab treatment was well tolerated and non-immunogenic according to in-house studies (Puchalski et al. 2010) and an external clinical lab (Kurzrock et al. 2013; van Rhee et al. 2010). Ramucirumab has a low immunogenicity with only 7.4 % of patients developing anti-ramucirumab antibodies in clinical trials when administered every 2 weeks. As screening and antibody engineering technologies improve, along with mammalian cell culture processes and cell line development techniques it is expected that the majority of mAb drugs will be non-immunogenic or have a very low chance of immunogenicity as well as a similar half-life to natural antibodies when required by their medicinal indication.

Apart from the typical antibody constructs used a therapeutic agents in 2009, the first bispecific antibody, under the trade name Removab® (catumaxomab), was approved in the EU for the treatment of cancer, malignant ascites, and peritoneal fluid accumulation caused by a cancer (European Medicine Agency 2014). The antibody structure consists of a mouse κ-light chain, a rat λ-light chain, a mouse IgG2a-heavy chain and a rat IgG2b-heavy chain that has two antigen binding sites where one mouse derived Fab region of the antibody binds an epithelial cell

adhesion molecule (EpCAM) and the second rat derived Fab region binds to CD3 (Walsh 2010). The hybrid antibody is manufactured via a rat-mouse hybrid-hybridoma cell culture process and the bispecific antibody functions by bringing together CD3-expressing T-cells, EpCAM-expressing tumor cells, and immune effector cells such as natural killer (NK) cells, macrophages, or dendritic cells that would bring about the destruction of the tumor cells through multiple immune system mechanisms.

The future outlook of mAb drugs is bright, with the start of this decade having close to 300 mAb's in various stages of clinical development. Of these mAb's approximately 150 new monoclonal antibodies are in development for the area of oncology treatments and close to 70 mAb's are in clinical development for treatment of inflammatory and autoimmune diseases with the rest are for indications that include metabolic disorders, cardiovascular disorders, CNS disorders, infectious diseases, and transplant rejection (Norman 2011). Currently, mAbs are the strongest growing segment of the pharmaceutical market and is expected to further grow at a fast pace along with sub categories such as fusion protein drugs that use antibody components to carry-out their function.

23.4 Fusion Protein Drugs

Since the Fc region of antibody binds to the FcRn to confer longer circulatory half-life there has been great success with the use of this natural molecular process to engineer proteins that can take advantage of this. In 1998, Enbrel® (etanercept) was the first CHO cell culture produced Fc fusion biologic, to gain marketing approval by the FDA. Enbrel®, a recombinant human soluble tumor necrosis factor (TNF) receptor able to bind and inactivate soluble and cell bound TNF and lymphotoxin competing with the cellular TNF receptors for the treatment of rheumatoid arthritis. Enbrel® has been one of the most successful biopharmaceuticals on the market with global sales reaching $8.4 billion in 2013 just behind Humira® of $10.7 billion, the two of these being the most successful drugs the biopharmaceutical industry has ever developed. Enbrel® consists of an intracellular portion of the human p75 TNFR linked to the Fc portion of IgG1 to form a dimeric protein. The benefit of the Fc fusion bestows the etanercept molecule with an extended median half-life of 4.8 days, together with a high binding affinity this contributes to Enbrel® overall effectiveness as an arthritis drug compared to others on the market (Mohler et al. 1993) at the time of its approval. A CHO cell line is used as the host for expression of the 150 kDa dimeric etanercept molecule. Enbrel® is part of a class of biologics that work by inhibiting the binding of TNF such as adalimumab (Humira®), golimumab (Simponi®, Simponi ARIA®), and infliximab (Remicade®), this allows these biologics to suppress the cascade of reactions that lead to an inflammatory response within the body that can actually destroy joint tissue as is characteristic with rheumatoid arthritis.

After market approval and release of Enbrel®, several other Fc fusion molecules were approved in the EU and USA that required a mammalian cell culture process in order to produce their complex fusion molecules. In 2003, a second mammalian cell expressed fusion product was approved by the name of Amevive® (alefacept) (Krueger and Callis 2003; Krueger and Ellis 2003) which utilized the Fc portion for apoptosis induction apart from boosting half-life. This 91.4 kDa protein has a Fc region of IgG1 linked to human leukocyte function antigen 3 (LFA-3) that can bind, with high affinity, to CD2, a functionally important and widely distributed T lymphocyte surface glycoprotein. Upon human LFA-3/IgG1 fusion protein administration the LFa-3 binds to CD2 inhibiting T-cell activation and proliferation. The Fc portion extends the circulatory half-life to 11.25 days (Kimchi-Sarfaty et al. 2013) in addition to interacting with the FcγRIII receptor on the surface of NK cells which results in NK induced apoptosis of T-lymphocytes (Majeau et al. 1994). This overall effect suppresses the immune system and can be used in the treatment of psoriasis, a skin condition that causes skin redness and irritation.

The CHO cell line has been the favorite for Fc fused recombinant proteins produced as biopharmaceuticals since all except one of the Fc-biologics from mammalian cell culture have at the time of this writing been produced in CHO cells, sticking to the formula of not changing what already works well. Fc fusions have also benefited from extended half-life, similar to Enbrel®. For example, Orencia® (Abatacept), Arcalyst® (Rilonacept), Nulojix® (Belatacept), Eylea®/Zaltrap® (Aflibercept), and Alprolix™ (Coagulation Factor IX) have all had an increased half-life due to an Fc fusion. For example, Orencia® has a half-life of 13.1 days. The therapeutic is a second-line treatment of rheumatoid arthritis in moderate to severe adult patients but works via a different biological mechanism than Enbrel®, acting as the first in a new class of agents that acts as a co-stimulation modulator able to inhibit full T-cell activation (Quan et al. 2008). This fusion protein is engineered as an IgG1 Fc fusion to CTLA-4 binding that acts by binding to CD80 and CD86 on antigen-presenting cells which will inhibit interaction with CD28 on T cells suppressing T-cell activation. Orencia® has been shown, in vitro, to decrease T-cell proliferation and inhibit the production of tumor necrosis factor alpha, interferon-gamma and interleukin-2 (Vital and Emery 2006). The extended half-life provided by the benefit of the glycosylated FC fusion structure for Orencia® means that it will only need to be taken once a month at maintenance dosage by the patient compared to once weekly with Enbrel®. Arcalyst® is an orphan designated drug also known as an interleukin-1 (IL- 1) trap since the fusion protein inhibits IL-1 which in turn reduces inflammatory responses due to unbalanced IL-1 stimulation. As with other Fc fusions the half-life is considerably extended to 8.6 days (Hoffman et al. 2008). Nulojix® is another IgG1 Fc fusion to CTLA-4 immunosuppressive agent but this time approved for the prevention of kidney transplant rejection therapy. Belatacept only differs from abatacept by two amino acids and although half- life is considered reasonable, it has been shown to be a little shorter than abatacept, at 8–10 days.

Two of the Fc-fusion biologics are not solely immunosuppressive and these are Elyea® and Alprolix™. The drug Eylea® is an anti-angiogenic used in the

treatment of neovascular age-related macular degeneration, an eye disease due to blood vessels leaking fluid into the macula. Zaltrap® (named ziv-aflibercept for distinction) is the same drug but approved as an anti-cancer agent for treatment of metastatic colorectal cancer. Aflibercept is a vascular endothelial growth factor (VEGF) trap that consists of an Fc region fused with the VEGF-binding portions from the extracellular domains of human VEGF receptors 1 and 2. The VEGF trap has a highly variable half-life of 1.7–7.4 days depending on the dosage. Alprolix™, which was approved for marketing on the 28th of March 2014, is the only Fc-fusion protein produced in HEK293 cells that has been approved by the FDA and also the first fusion drug for the treatment of hemophilia B (Food and Drug Administration 2014). The Fc fusion increased the half-life of the drug to 3.6 days, a considerable increase over the other coagulation factor IX drugs, Rixubis® and BeneFIX®. Rixubis® was introduced in 2013 with a half-life more than three times less than Alprolix®, at 26 h, and BeneFIX® was approved in 1997 with a maximum half-life of 24 h (Pfizer 1997; Baxter 2014). The coagulation factor IX drugs activate the coagulation pathway to ultimately convert prothrombin to thrombin which converts fibrinogen to fibrin so that a clot can be formed for the treatment of bleeding episodes.

More innovative fusion drugs are also being introduced onto the US market, such as the fertility drug Elonva® (corifollitropin alfa), a fusion drug Merck is currently seeking FDA approval for and has already been approved in the EU in 2010 (Table 23.1). Corifollitropin alfa is a modified recombinant human follicle stimulating hormone (rhFSH) in which the carboxy-terminal peptide of the beta subunit of human chorionic gonadotropin (hCG) is fused to the FSH beta chain. This drug is the first long-acting hybrid molecule that has a prolonged half-life and a slower absorption to serum peak concentrations meaning sustained follicle stimulating activity for the controlled stimulation of the ovaries. The benefits of corifollitropin alfa compared to other follicle stimulating drugs on the market today is that it remains effective for 7 days whereas other approved recombinant human FSH drugs require daily injections (Bouloux et al. 2001; Duijkers et al. 2002; Fares et al. 1992).

23.5 Drug Approvals and Regulation

The actual approval process for a biopharmaceutical product to be able to come on the market has evolved over the years and since the EMA and the FDA have different regulatory systems for the review and approval of new drugs there has been some harmonization between the two agencies over the years. The FDA implements regulations based on legislative law in the USA and the majority of activities related to biopharmaceutical regulation and the marketing approval process is done via two FDA divisions known as the Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER). Briefly, in the USA, a potential new drug has to undergo pre-clinical trials

and the data submitted as an investigational new drug (IND) application to either CDER or CBER and when a biopharmaceutical seeks marketing approval for a new biological entity (NBE) they would either submit an application to CDER as a new drug application (NDA) or to CBER as a biologics license application (BLA) (Walsh 2003). The EMA was originally known as the European Agency for the Evaluation of Medicinal Products from 1995 to 2004, and the agency was setup by funding from the individual member states in western Europe, the European Union (EU) and the pharmaceutical sector. Headquartered in London, the EMA was born after years of negotiations among EU governments and since individual member states have their own national medicine regulatory bodies the EMA was setup to work closely with the agencies in the 28 European Union Member States as well as the European Economic Area (EEA) countries such as Norway, Iceland and Liechtenstein (European Medicine Agency 2014). Since by law, a pharmaceutical company can only market a medicine once it has received a marketing authorization, the EMA allows pharmaceutical companies to submit a single marketing-authorization application to the EMA for approval for all the member states and EEA countries as part of a centralized system. Like the FDA, the EMA requires pre-clinical and clinical trial data before marketing authorization application can take place and thus a clinical trial authorization (CTA) application has to be filed with the relevant governing body of the country the trial is being conducted.

Over the past few years, the EMA and FDA have taken several steps to harmonize and align regulatory practices for the approval and marketing of drugs (Trotta et al. 2011). These efforts have brought the differences in the time required for approval of the same drug between the two agencies closer together as can be seen in Table 23.1. One important collaboration established between the two agencies in 2003 are arrangements to allow the exchange of confidential information between the EU and the FDA as part of their regulatory and scientific processes (European Medicine Agency 2014). Further collaboration has brought about the development of common procedures for good-manufacturing-practice (GMP) and good-clinical-practice (GCP) inspections including applications for orphan drug designations.

23.6 Biosimilars or Follow on Biologics Approvals

As competition drives the price of the generic products down in the pharmaceutical sector, the impact of biosimilars would be expected to have a similar affect. Once a patent expires generic drugs can be legally produced, although loop holes can exist in areas or countries where the patent is not enforceable or the patent can be proven invalid. While small-molecule formulated pharmaceuticals can have exact copies made that can pass the regulatory framework, biopharmaceutical manufactured products such as, recombinant proteins can have a high degree of molecular complexity that includes the post translational modification which are all affected by the manufacturing process. The term biosimilar, or follow-on biologic, was

introduced by the regulatory authorities in the EU and USA to imply that the newly introduced product would be similar to the original biologic, but might not be an identical molecular copy of the parent biologic. The FDA tends to use the term follow-on biologics while in the EU biosimilar is used by the EMA (European Medicine Agency 2014; Food and Drug Administration 2014). In order to prove a biological entity is a biosimilar to the regulatory authorities, data has to be compiled through clinical, animal and analytical studies where the results must indicate that the biological entity reproduces the same clinical results as the parent drug. The time and costs associated with mammalian biopharmaceutical development and manufacturing of biosimilars will be a far greater investment for a pharmaceutical compared to what is required for small-molecule generics as it is estimated that the development time for a biosimilar recombinant proteins could range from 5 to 8 years compared to the 1–2 years for a generic small molecule (Lanthier et al. 2008; Grabowski et al. 2006). This is due to the complexity of large glycosylated molecule and the process development required. For example, the host cell will tend to go through the process of cell line selection and development to create a suitable host for the target bioprocess to produce the biosimilar, which can take months to years depending on the biological product. This is in addition to the development of the commercial scale manufacturing process that requires strict quality controls and process monitoring of all upstream and downstream processes till final formulation and product testing.

In 2006, the first biosimilar was approved in both the EU and the USA under the trade name Omnitrope™. This biosimilar was an *E. coli* expressed 22.1 kDa recombinant growth hormone (hGH) identical to the native protein consisting of a 191 amino acid single chain polypeptide manufactured by Sandoz for the treatment of growth hormone deficiencies (European Medicine Agency 2014). This approval was due to the regulatory framework that was established since 2005 by the EMA and established legislation in the USA (Woodcock et al. 2007; Schneider and Kalinke 2008). The EU has further build on their regulatory framework that by 2010 draft guidelines were established for Mab biosimilars leading to a final version of the guidelines completed by the EMA's Committee for Medicinal Products for Human Use (CHMP) that included IgG1 Fc-fusion protein biosimilars in the scope of MAb biosimilars (Schneider et al. 2012). The regulatory framework in the EU allowed the EMA to approve several biosimilars that are recombinant biopharmaceutical proteins produced in mammalian cell culture (see Table 23.1) beginning with a biosimilar for recombinant erythropoietin (Epoetin alfa). The first five Epoetin alfa biosimilars were approved in 2007 each marketed by Hexal AG, Sandoz GmbH, Medice Arzneimittel Pütter GmbH & Co. KG, Hospira UK Limited, and Stada Arzneimittel AG under the trade names; Epoetin alfa Hexal®, Binocrit®, Abseamed®, Retacrit®, and Silapo® respectively. This was a milestone accomplishment for a getting biosimilars of a glycosylated protein onto the market and helped set the stage for mAbs. In June 2013, Celltrion and Hospira received permission from the EMA's CHMP to market their biosimilars to Johnson & Johnson's Remicade® (infliximab) under the trade names Remsima® and Inflectra® respectively (see Table 23.1 for further details).

The impact of mammalian cell produced biologics is becoming the major contributor to pharmaceutical industry growth pipelines and the existence of a regulatory framework for biosimilars has meant further increases in mammalian cell culture capacity. BioProcess Technology Consultants, Inc. have given an interesting analysis of the global mammalian cell culture capacity with currently, the existence of one contract manufacturing organization (CMO) (Lonza), one excess capacity company acting as both product manufacturer and CMO (Boehringer Ingelheim), and 10 product companies with an installed capacity greater than 100,000 L each. These companies are; Roche, Johnson & Johnson, Amgen, Pfizer, Sanofi-Aventis, Novartis, Eli Lilly, Biogen Idec, Bristol-Myers Squibb, and Celltrion. Samsung Biologics being an additional CMO to join the list by 2017 (Ecker and Ransohoff 2014). Celltrion, Samsung BioLogics, and Innovent Biologics are examples of companies outside of the USA and Europe increasing capacity due to growing interests in mammalian cell culture biopharmaceutical manufacturing and the biosimilar market. It is predicted that by 2017 the worldwide capacity for mammalian cell culture manufacturing will be close to 4,400,000 L (this includes a perfusion factor of 5× to adjust for productivity differences between fed-batch and perfusion facilities) due to the further expansion of capacity from now till then (Ecker and Ransohoff 2014). This growth in worldwide capacity is an approximate 57 % increase in capacity since 2010.

Conclusion

As the pharmaceutical market has demonstrated over the last decade, the mammalian cell derived biologicals market has continued to thrive and drive major growth in the pharmaceutical industry. The ability to provide post translational modifications and the continued need for monoclonal antibody therapies and the rise of Fc-fusion protein therapies have given mammalian expression systems a dominant advantage over other expression systems for the next generation of engineered biopharmaceuticals. With the introduction of disposable technologies, the improvement of cell culture processes, cell line selection and development strategies giving higher titers and specific productivities, including the establishment of EU and US regulatory pathways to bring biosimilars to the market, an infusion of growth has occurred at a rate currently faster than any other pharmaceutical sector. This growth is exemplified by the increase in global manufacturing capacity, including a substantial increase in the construction of Asian GMP mammalian culture facilities over the last few years. The introduction of new players in the biopharmaceutical industry, alongside the arrival of biosimilars promising lowered healthcare expenses of animal cell derived biopharmaceuticals, allows us to make the prediction that the next decade of mammalian bioprocesses and their biological products will continue to grow at a remarkable pace in innovation and discovery providing an increased affordability of these biological medicines.

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