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REVIEW

Production of recombinant protein therapeutics in cultivated mammalian cells

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Cultivated mammalian cells have become the dominant system for the production of recombinant proteins for clinical applications because of their capacity for proper protein folding, assembly and post-translational modification. Thus, the quality and efficacy of a protein can be superior when expressed in mammalian cells versus other hosts such as bacteria, plants and yeast. Recently, the productivity of mammalian cells cultivated in bioreactors has reached the gram per liter range in a number of cases, a more than 100-fold yield improvement over titers seen for similar processes in the mid-1980s. This increase in volumetric productivity has resulted mainly from improvements in media composition and process control. Opportunities still exist for improving mammalian cell systems through further advancements in production systems as well as through vector and host cell engineering.

In 1986 human tissue plasminogen activator (tPA, Activase; Genentech, S. San Francisco, CA, USA) became the first therapeutic protein from recombinant mammalian cells to obtain market approval. Today about 60–70% of all recombinant protein pharmaceuticals are produced in mammalian cells. In addition, estimates speak of several hundred clinical candidate proteins currently in company pipelines. Like tPA, many of these proteins are expressed in immortalized Chinese hamster ovary (CHO) cells, but other cell lines, such as those derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retinal cells have gained regulatory approval for recombinant protein production. Although the majority of today's mammalian cell culture processes for biopharmaceutical production are based on cells that are cultivated in suspension, the diversity of actual manufacturing approaches is considerable.

Production scheme

The development of a manufacturing process for a recombinant protein in mammalian cells usually follows a well-established scheme (**Fig. 1**). Initially, the recombinant gene with the necessary transcriptional regulatory elements is transferred to the cells. In addition, a second gene is transferred that confers to recipient cells a selective advantage. In the presence of the selection agent, which is applied a few days after gene transfer, only those cells that express the selector gene survive. The most popular genes for selection are dihydrofolate reductase (DHFR), an enzyme involved in nucleotide metabolism, and glutamine synthetase (GS). In both cases, selection occurs in the absence of the appropriate metabolite (hypoxantine and thymidine, in the case of DHFR, glutamine in the case of GS), preventing growth of nontransformed cells. In general, for efficient expression of the recombinant protein, it is not important whether the biopharmaceutical-encoding gene and selector genes are on the same plasmid or not.

Following selection, survivors are transferred as single cells to a second cultivation vessel, and the cultures are expanded to produce clonal populations. Eventually, individual clones are evaluated for recombinant protein expression, with the highest producers being retained for further cultivation and analysis. From these candidates, one cell line with the appropriate growth and productivity characteristics is chosen for production of the recombinant protein. A cultivation process is then established that is determined by the production needs. So far, all mammalian recombinant therapeutics are naturally secreted proteins or have been developed from gene constructs that mediate protein secretion.

Improving productivity

Although the basic concepts have not changed since the mid-1980s, the productivity of recombinant cell lines has increased dramatically in the past two decades. Comparing hypothetical data from a typical 'best case' stirred-tank production process in 1986 to an actual industrial production process performed in 2004 provides some insight into the basis for the improved productivity (**Fig. 2**). In 1986, cells typically reached a maximal density of about 2×10^6 cells/ml with a batch process production phase of about 7 days and a specific productivity slightly below 10 pg/cell/day. The product titer reached about 50 mg/l. In the process performed in 2004, the culture was started at a low cell density of about 100,000 cells/ml and rapidly grew to a density of more than 10×10^6 cells/ml. A high level of cell viability was maintained for almost 3 weeks, and the accumulated yield reached about 4.7 g/l with a specific productivity up to ~90 pg/cell/day (data was calculated from **Fig. 2**).

Although this may be among the highest specific productivities reported from industrial sources, it is similar to that of processes reported much earlier. For example, in 1991 specific productivities

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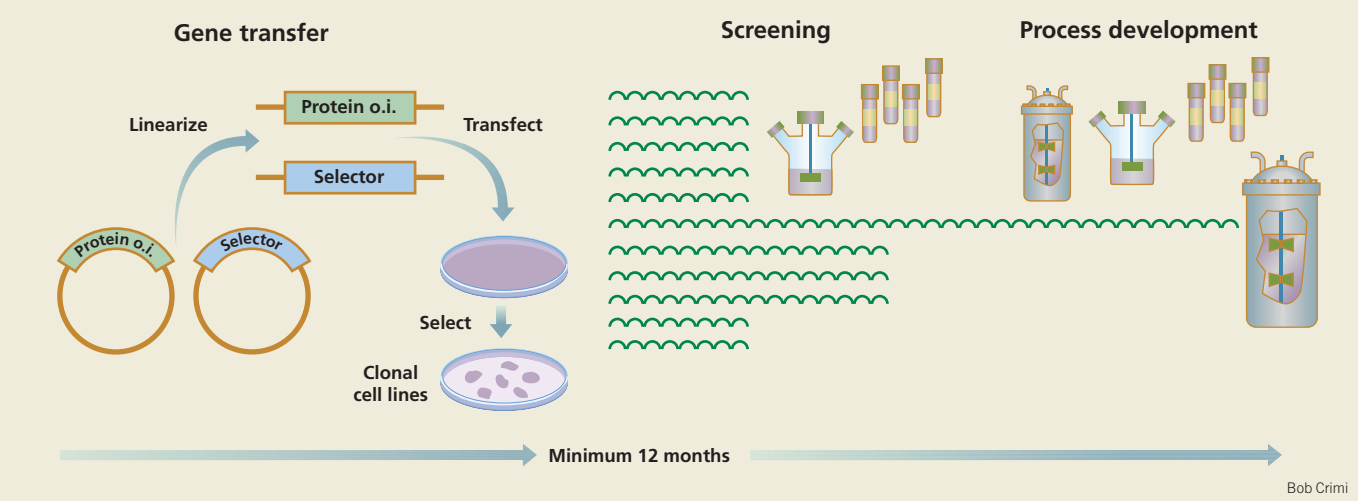

Figure 1 Cell line generation and development for cell culture processes for the generation of recombinant proteins of interest (o.i.). The wavy lines indicate subcultivations of individual cell lines that are in a screening program to obtain the final producer. Vials indicate banks of cells frozen in liquid nitrogen. Spinner flasks represent scale-down systems for process optimization, and bioreactors represent large-scale production processes. (Artwork after an original design by Danièle Fraboulet, Lyon, France.)

for the synthesis of a recombinant antibody in a myeloma cell line ranged from 20 pg/cell/day to 80 pg/cell/day[1]. **Figure 2a** (shaded areas) shows, however, that the accumulated viable cell number was 17-fold higher for the 2004 process compared with the typical process performed in 1986.

The high yields obtained in today's processes are the result of years of research that has led to a better understanding of gene expression, metabolism, growth and apoptosis delay in mammalian cells. Overall, efforts have led to improvements in vectors, host cell engineering, medium development, screening methods and process engineering and development. The concepts discussed here are based on published sources that may not precisely reflect production processes that are currently used by manufacturers of therapeutic proteins. The companies with record recombinant protein yields in large-scale processes neither present methods used for breakthrough achievements nor do they provide a comprehensive disclosure of manufacturing aspects from vector construction to production process.

DNA delivery and integration

Expression vectors for recombinant cell line generation generally use a strong viral or cellular promoter/enhancer to drive the expression of the recombinant gene[2,3]. In most cases, the gene of interest is isolated as a cDNA without introns. It is known, however, that efficient cytoplasmic transport and translation of the mRNA in eukaryotic cells depends on splicing[4]. Most expression vectors now include at least one intron sequence, which is usually located between the promoter and the cDNA coding sequence. Modifying the coding region itself may also improve the level of gene expression. For example, a mammalian gene that uses rare tRNA codons, and thus may not be expressed at a high level, can be converted to a high-expressing gene by changing the tRNA codons to more abundant ones[5].

In 1973, Graham and van der Eb showed that exposing cells to nanoparticles of DNA and calcium phosphate makes possible DNA transfer into cultivated mammalian cells[6]. Nonviral gene transfer remains the preferred approach to generate stable cell lines for manufacturing purposes. Calcium phosphate transfection, electroporation, lipofection and biolistic- and polymer-mediated gene transfer are routinely used and are all reasonably efficient and reliable[7]. It is impossible to assess whether any one of these methods is superior to the others because comprehensive studies are lacking.

The recombinant gene and the selection gene can be present on the same vector or on separate vectors. When present on the same vector they can be expressed from a polycistronic mRNA[8]. To increase the chance for obtaining high-level producer cell lines, the selective gene can be driven from a weak promoter. Although this approach usually reduces the efficiency of stable transfection, the cells that survive selection yield more recombinant product.

Linearization of plasmids before transfection improves the efficiency of stable transfection, but supercoiled plasmid DNA molecules will also be converted into linear molecules within the nucleus after 1–2 hours because of the activity of endo- and exonucleases[9]. Nuclear ligases assure the covalent joining of individual plasmid DNA molecules[10], and recombination enzymes catalyze the random integration of these concatamers, as well as single plasmid molecules, into the host genome by nonhomologous recombination[9]. The ligation of plasmid DNA before integration accounts for the frequent cointegration of multiple plasmid molecules at a single site. All manufacturing processes discussed here are based on cell lines with transgenes integrated into the host chromosome.

The site of integration has a major effect on the transcription rate of the recombinant gene (a phenomenon known as the position effect). Integration into inactive heterochromatin results in little or no transgene expression, whereas integration into active euchromatin frequently allows transgene expression. However, integration into euchromatin may not be sufficient to ensure long-term expression of the recombinant gene. Transgene expression in mammalian cells is rapidly inactivated (silenced) in many cases, probably because of the influence of neighboring condensed chromatin. Gene silencing correlates with histone hypoacetylation, methylation of lysine 9 of histone H3, and an increase in CpG methylation in the promoter region of the transgene[11,12].

Several strategies have been developed to overcome the negative position effects of random integration. Protective *cis*-regulatory elements include insulators, boundary elements, scaffold/matrix

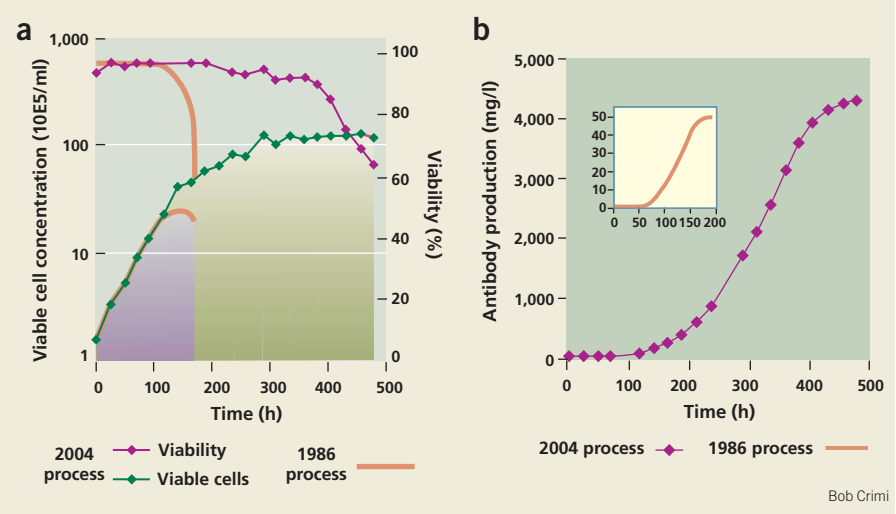

Figure 2 Comparison of cell culture processes from 1986 and 2004. (**a**,**b**) The viable cell mass and viability (**a**) and the productivity (**b**) of both cell culture processes are shown. The examples are derived from processes in suspension cultures of CHO cells. The data from 2004 was generated from a process for the expression of a human recombinant antibody at the 10-liter scale. The hypothetical process from 1986 is based on unpublished results. For clarity the product accumulation data for the hypothetical 1986 process is also represented in the insert in **b**. Data courtesy of Lonza Biologics, Slough, UK.

attachment regions[13], ubiquitous chromatin opening elements[14] and conserved antirepressor elements[15]. Flanking transgenes with these elements reduces the effects of heterochromatin and allows stable expression of the transgene[16,17]. Another way to inhibit silencing is to block deacetylation of histones using butyrate[18,19]. Targeting transgene integration to transcriptionally active regions of the genome is another possible strategy to avoid position effects, and presentations at conferences in recent years have hinted at the use of gene targeting in industry.

However, homologous recombination between transfected plasmid DNA and the genome rarely occurs[20,21]. One way to enhance the probability of targeted integration is to use enzymes, such as bacteriophage P1 Cre recombinase, lambda phage integrase or yeast Flp recombinase to exchange DNA between the genome and a transfected plasmid. These enzymes catalyze the exchange at high frequency if the donor and recipient DNAs are bordered by specific attachment regions[22,23]. The identification of a highly active site of transcription in gene targeting is crucial. If an active site is not found, the receptor site for recombination is inserted randomly and hundreds of clones are screened for those that have integrated in so-called good sites.

Host cell engineering and medium optimization

Commercial media for cell culture of excellent quality are now available from a few leading suppliers. However, the leading recombinant protein manufacturers probably invest considerable work in optimization of their own media formulations. Usually, several different formulations are necessary for a single manufacturing process, each one of them designed for a specific phase. Media for rapid growth involving subcultivation every 3–5 days have formulations different from those of production media. The production process in a batch (6–8 days) or extended batch (10–21 days) process is much longer than a typical subcultivation period. Medium development is of utmost importance and has to be done on an individual basis, for each process and cell line.

In the past, fetal bovine serum, added at a concentration of 1–20%, was essential for the propagation of mammalian cells. Today, most large-scale cell culture processes are executed in serum-free media. Modern media compositions support excellent cell culture performance, in the absence of serum-provided peptides, growth factors and an undetermined collection of proteins, lipids, carbohydrates and small molecules. Some reasons for excluding serum are its undefined character, the risk of transmitting adventitious agents (for example bovine viruses and prions) and its cost.

Like media, host cells have been improved. It is likely that all super-high producers, like the one represented in **Figure 2**, are cell lines genetically enhanced to make them resistant to influences reducing viability and/or to carry growth-promoting transgenes. Reports, mainly provided by academic laboratories, provide ample examples of how the growth, survival and productivity of recombinant host lines can be improved by genetic engineering. Proto-oncogenes, cell cycle control genes (cyclins), growth factor genes (e.g., insulin-like growth factor) and antiapoptotic genes have been inserted into cell lines for the generation of superior production hosts[24]. Unfortunately, industrial laboratories have not published results in this area, probably because of proprietary concerns.

In the example shown in **Figure 2**, the rapid growth from about 100,000 cells/ml to more then 10×10^6 cells/ml in one week indicates an unusually healthy cell line whose performance exceeds anything reported to date in the literature. Interestingly, during this one-week period very little product was made. Eventually, the logarithmic growth profile reached a plateau, probably because of declining supply of nutrients and the accumulation of cellular waste products, such as lactate and ammonia. One of the consequences of the changing environment in culture is the induction of apoptotic death and a rapid drop in cell viability[25]. In the case shown, the consequence of apoptotic signals appeared to be delayed for almost two weeks, and the cells remained in a stable, highly productive phase. However, this only works in combination with well-balanced feeding of concentrates of carbohydrates, amino acids, lipids, nucleic acid precursors and rare metal ions.

The improvement in post-translational protein modification and processing is another promising development. For example, Umana *et al.*[26] showed that the efficacy of antibodies can be improved by enhancing the potency of their natural immune effector functions. Stable overexpression of *N*-acetylglucosaminyltransferase-III, an enzyme not naturally expressed in CHO and NS0 cells, in recombinant antibody-producing cells generates IgGs with high levels of bisected, nonfucosylated oligosaccharides in the Fc region. These modifications of the glycoform result in 5–10-fold increases in antibody-dependent cellular cytotoxicity[26].

Selection, gene amplification and cell line screening

A variety of systems for selecting transfected cells exist, including resistance to antibiotics such as neomycin, hygromycin and puromycin. However, selection for the presence of the transfected DHFR gene in CHO cells deficient in DHFR activity is still the most

frequently used approach. An important consideration in choosing a selection agent is the degree of selectivity or stringency. A more stringent agent will select for cells that express the selection gene and in many cases the recombinant gene at higher levels. The higher stringency level can also reduce the work required for screening of candidate cell lines.

With the DHFR system, expression of a recombinant protein can be augmented by exposing cells to methotrexate (MTX), a drug that blocks the activity of DHFR[27,28]. After 2–3 weeks of exposure to MTX the majority of cells die, but nearly any level of MTX can be overcome by a small number of cells that overproduce DHFR. Upon MTX treatment, ramped upwards in concentration, the surviving cells frequently contain several hundred to a few thousand copies[29] of the integrated plasmid embedded in chromosomes that are frequently elongated[27]. Most 'amplified' cells produce more recombinant protein than the unamplified cells, but the improvement of specific productivity (up to 10- to 20-fold) varies among individual clones[30].

Although gene amplification is not always necessary because of a sufficiently high expression level in primary cell clones, it can also be done with GS-transfected NS0 cells. GS catalyzes the production of glutamine from glutamate and ammonia, the latter being an undesirable waste product of cells. Thus the GS system offers the twofold advantage of reducing the level of ammonia in the cell culture medium and providing an unstable amino acid (glutamine) to cells. A specific and irreversible inhibition of GS can be mediated by methionine sulphoximine (MSX). At a concentration of 10–100 μM MSX, resistant clones can be identified in selected NS0 cell populations that have amplified the transgene complex containing the GS gene and the desired gene(s) of interest[31,32].

The protein expression levels of different cell clones, including those derived from gene amplification, cover a wide range that may exceed two orders of magnitude[33,34]. As a consequence, the identification of high-producer cell lines is a tedious and labor-intensive exercise that requires the screening of hundreds of cell lines. Several methods for the isolation of clones exist, the most popular one being cloning by limiting dilution using multiwell plates. In addition, glass or steel cloning rings can be used to separate the colony physically from other cells. Cells can be enzymatically detached and then removed from the cloning ring with a micropipette. Alternatively, cotton swabs gently scraped over a colony can also be used to isolate clones. Cells attached to the cotton can subsequently be released by moving the cotton swab into medium. However, in the industrial setting, these more traditional methods are being replaced by cell sorting and by robotics that reduce the workload for screening of candidate cell lines.

For high-volume products, like monoclonal antibodies, cell lines with a specific productivity less than 20 pg/cell/day are probably discarded early on. Screening for highly productive cell lines requires the execution of multiple transfection experiments because the number of individual integration events in transfected populations is low. A single transfection plate rarely delivers more than 500 or 1,000 stable cell lines. Thus, screening is not done at the same scale as other high-throughput systems. The screening of production candidates also needs to take cell growth into account. Very high specific-productivities are usually seen in slowly growing cell lines, but it is the combination of fast growth and high specific-productivity that makes a cell line suitable for scale-up and manufacturing. How growth and productivity in immortalized cell lines are interacting with each other has been studied in some instances[35,36]. Because there is little literature on this matter, it is difficult to state in a general way how the apparent inverse relationship of productivity and growth in cells is being addressed by process-development scientists in companies.

An important consideration is the stability of clonal cell lines over extended periods of time. The inherent trend of immortalized cell lines towards genetic[37] and therefore also physiological heterogeneity requires that hosts for production be analyzed carefully before considering them as stable. This work can take several months of study, as a recent paper on the production stability of clonal cell lines of the GS-NS0 system indicates[38]. The maintenance of MTX as a selective drug in culture may in some cell lines maintain the phenotypic appearance of a 'stable' cell line with respect to productivity; however, genetic studies have shown that the presence of the drug promotes cytogenetic heterogeneity, an undesirable feature, especially with respect to the regulatory process of approval of the host line. Thus, it might be advantageous to omit selective drugs once promising clonal cell lines have been identified[39,40].

Cell culture formats

Two main formats have been employed for the production of recombinant proteins in mammalians cells: cultures of adherent cells and suspension cultures. The latter is by far the most common.

Adherent cell culture. Erythropoeitin (Epogen; Amgen, Thousand Oaks, CA, USA) was the first recombinant protein from mammalian cells to achieve blockbuster status with sales of over $1 billion annually. Epogen is produced with a simple technology. CHO cells are seeded into roller bottles that are filled to 10–30% of capacity with medium and slowly rotated, allowing cells to adhere. The rotation assures a regular wetting of the cells and oxygen is supplied by the ample 'head space' in the bottle. After a period of growth and maintenance of the culture at confluency for a few days, the product is harvested from the decanted supernatant. The process can be scaled-up easily because the number of roller bottles handled in parallel determines scale. Product concentrations in the 50–200 mg/l range are possible, providing protein in the kilogram range annually. It is unlikely that such a process would deliver gram/liter product concentrations because the ratio of cells/volume is much lower than in an optimized stirred-tank reactor process. Today's Epogen process is essentially a robot-based manufacturing procedure whereby all the critical handling steps, including the seeding of cells, filling of bottles with media and harvesting of cell culture fluids are executed within air-filtered environments without human interaction.

Adherent cells have also been cultivated on polymer spheres termed microcarriers that are maintained in suspension in stirred-tank bioreactors. They allow for easy scale-up in bioreactors. CHO cells on microcarriers are being used for the production of follicle stimulating hormone[41] and of virus-vaccines[42].

Suspension culture. CHO cells now dominate the domain of mass production of recombinant protein products because of their capacity for single-cell suspension growth. Other cell lines grown well in suspension are mouse myeloma–derived NS0[32] cells, BHK[43], HEK-293[44] and human retina–derived PER-C6[34] cells. With the exception of blood-derived cells (NS0), most established cell lines maintain their anchorage-dependent character unless special efforts are undertaken to adapt them to suspension growth. The transition of adherent cells to suspension cultivation was difficult in the 1980s. Today, commercially available media formulations make this transition much easier. However, it still requires effort to screen several media formulations that support the transition[45,46]. In my laboratory, we usually allow a cell line to grow for a minimum of six passages in suspension medium, and we require that the cell line achieve at least 2×10^6 cells/ml within 3–4 days of subcultivation. Only after this time period do we consider the cell line to have a potential for scalability.

In a 'simple' batch or extended batch production process, the scale-up to very large volumes can occur by the dilution of the content of a bioreactor into 5–20 volumes of fresh medium held prewarmed in a larger reactor. The entire process from the thawing of banked cells to the production vessel consists of three separate phases—seed train, inoculum train and production phase. The seed train is usually performed at a small scale to provide fresh cells for scale-up during the period chosen for the production. The inoculum train starts with a small volume of cell suspension from the seed train and its volume is expanded so that a sufficient cell number will be generated for the final production phase. Process conditions optimized for a given cell line can rarely be considered generic, because mammalian cell lines have a highly individual character highlighted by different glucose consumption rates, lactate production rates and sensitivity towards stress signals.

Although the timing of the termination (that is, harvest) of a culture is driven mainly by plant capacity and productivity kinetics, another important factor is the quality of the derived product. The continuously changing composition of the culture medium during the production phase can affect the quality of earlier synthesized product through degradative activities mediated by cell-released enzymes. Also, a diminishing supply of nutrients as energy providers or as building blocks for the synthesized product can change its molecular composition. Reproducible processes will, however, produce populations of protein molecules within a definable range of molecular variation.

Process optimization occurs in 'scale-down' systems. The most popular and widely used scale-down reactor has a volume of 1–2 liters. But reactors can be replaced by noninstrumented systems. Spinner flasks have volumes from 200–5,000 ml when filled to 30–50% capacity. Cell cultures in spinner flasks frequently show signs of oxygen limitation, especially at high-cell densities. Shaken multiwell plates have also been used as scale-down systems, but they suffer from evaporation and hence their usefulness is limited, especially for extended production cultures. A shaken 50-ml conical centrifuge tube with a ventilated cap recently has been developed and shows promise as a scale-down system, with reactor-like growth and productivity performance[47].

Extended batch cultures and perfusion processes

Most high-yielding processes today are extended batch cultures whereby medium components are added in small batches or semi-continuously. The development of these extended batch processes requires a good understanding of the cell line and the product, and is usually only applied to processes that supply material for phase 3 clinical trials and for the market.

An entirely different philosophy for manufacturing is represented by continuously perfused production processes. Perfused cultures can achieve even higher cell densities than batch or extended batch cultures and can be maintained for many weeks and months, with product harvests occurring repeatedly throughout that period. Several reactor volumes of fresh medium can be fed into the culture per day, while the same volume is being withdrawn from the reactor. The antihemophilic factor VIII (Kogenate, Bayer, Berkeley, CA, USA), of which the market requires about 150 g per year, is reliably being manufactured using perfusion technology with suspension-cultivated BHK cells. Factor VIII may be the largest protein (2,332 amino acids) ever produced in bioreactors. It is harvested continuously from ongoing perfusion cultures. This very sophisticated, highly controlled process runs for up to 6 months and assures that the fragile protein is of reproducibly high quality[43].

Conclusions and future developments

Mammalian cells cultivated in bioreactors have surpassed microbial systems for the production of clinical products, both in number of product entities and in recoverable final product titer. The need to obtain complex, modified proteins has driven science and technology to better understand the biology of mammalian cell lines under production conditions. The principle benefit of this understanding is the ability to obtain higher cell numbers, even under simple batch culture conditions. The trend for higher cell density in reactors will continue and will be the driving force for even higher productivity. It should be noted that with a density of 10×10^6 cells/ml and a typical diameter of 10–15 μm of cells, the volume of cells in a reactor corresponds only to about 2–3% of the total volume (packed cell volume). In contrast, microbial cultures can achieve a packed cell volume of 30% or more. Thus, there is ample opportunity to increase cell mass in mammalian cell cultures. Higher cell masses will require only minor adjustments in process reactor engineering and control (e.g., the use of pure oxygen instead of air). However, they will require a more stringent control over the medium composition at any time in the reactor.

On the molecular and cellular side of things, numerous opportunities for further improvements exist. Gene transfer and site-specific integration will eventually become routine. Information derived from genomic studies in man, mouse and hamster will help to guide this work. The growth and productivity of cells can be further improved through host cell engineering, in particular by controlling growth and productivity with regulated promoters[25,48,49]. Inducible promoters can also be used for some protein products that confer toxicity when expressed from a constitutive promoter[49].

Finally, transient gene expression, currently being explored for the production of proteins for research purposes and for preclinical studies, is on the verge of becoming a production system in its own right. Although expression levels from transiently transfected cells are presently only in the tens-of-milligrams/liter range, the short time span of a few days between DNA delivery and protein harvest makes this approach very appealing. Many companies (Genentech (S. San Francisco, CA, USA), Roche (Basel, Switzerland), Novartis (Basel, Switzerland) and Bayer (Berkeley, CA, USA), among them) are now using scale-up approaches for nonviral DNA delivery to cells in bioreactors to transiently produce hundreds of milligrams if not grams of recombinant protein in a matter of days[44].

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The authors decline to provide information about competing financial interests.

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