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Mammalian cell protein expression for biopharmaceutical production

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ABSTRACT

Mammalian cell expression has become the dominant recombinant protein production system for clinical applications because of its capacity for post-translational modification and human protein-like molecular structure assembly. While expression and production have been fully developed and Chinese hamster ovary cells are used for the majority of products both on the market and in clinical development, significant progresses in developing and engineering new cell lines, introducing novel genetic mechanisms in expression, gene silencing, and gene targeting, have been reported in the last several years. With the latest analytical methods development, more attention is being devoted towards product quality including glycol profiling, which leads to better understanding the impact of culture condition during production. Additionally, transient gene expression technology platform plays more important role in biopharmaceutical early development stages. This review focused on the latest advancements in the field, especially in active areas such as expression systems, glycosylation impact factors, and transient gene expression.

1. Introduction

Biopharmaceutical products are clinical reagents, vaccines, and drugs produced using modern biotechnology for in vivo diagnostic, preventive, and therapeutic uses. The first approved recombinant protein, insulin, was produced in the early 1980s. Since its successful entry onto the market, the U. S. Food and Drug Administration (FDA) has approved more than 100 new recombinant protein therapeutics, including several notable monoclonal antibodies (Mabs) and more than 300 non-recombinant biopharmaceuticals, such as vaccines and blood products (www.fda.gov; Rader, 2010).

Recombinant DNA and hybridoma technologies are used to engineer biological systems to produce 1) recombinant forms of natural proteins (including human growth hormones, cytokines, and insulin), 2) derivatives of natural proteins and living systems (including protein muteins, viral-like-particle vaccines, cancer cell vaccines, immunotoxins, and IgG fusion proteins), 3) viral vectors, plasmid vectors, and small interfering RNAs that carry genes or genetic information for vaccination or gene-therapy, and 4) in-vivo diagnostic and therapeutic monoclonal antibodies. The biopharmaceutical industry has been rapidly developing with 10–20% annual increases in revenue worldwide (Global, 2008; Huang, 2005; Junker, 2007; Mammalian, http://www.researchandmarkets.com, 2005; Matasci et al., 2009). Of the 27 latest (Jan. 2008–June 2011) FDA approved biopharmaceutical entities (Table 1, www.fda.gov), 18 are recombinant proteins manufactured using cells, organisms, or animals. The other 9 are vaccines and therapeutics manufactured from natural product sources such as human plasma, tissue, cancer cells, attenuated virus, and bacteria. Among the 18 recombinant products, 12 are produced using mammalian expression systems, 3 are produced by *Escherichia coli* (*E. coli*), and the remaining 3 are produced by baculovirus, yeast, and transgenic goats. This indicates that mammalian expression systems are the dominant choice for biopharmaceutical manufacturing. Furthermore, with respect to the economic impact of recently marketed top selling biopharmaceutical products (Table 2), two thirds of the revenue has come from products manufactured using mammalian systems, while only one third has come from microorganisms, such as *E. coli* and yeast. This clearly shows the preeminence of mammalian expression systems in the contemporary biopharmaceutical production enterprise.

Biopharmaceutical products are made using micro-organisms (Shiloach and Rinas, 2010; Zhang and An, 2010), animal cell lines

Abbreviations: 6-TG, 6-thioguanine; ACE, Artificial Chromosome Expression; ADCC, Antibody-dependent Cellular Cytotoxicity; BGH, Bovine Growth Hormone; BHK, Baby Hamster Kidney; CHO, Chinese Hamster Ovary; CLL, Chronic Lymphocytic Leukemia; CMV, Cytomegalovirus; CTE, Constitutive Transport Element; DHFR, Dihydrofolate Reductase; DO, Dissolved Oxygen; EBV, Epstein-Barr Virus; EBVNA 1, Epstein-Barr Virus Nuclear Antigen 1; *E. coli*, *Escherichia coli*; EPO, Erythropoietin; FDA, United States Food and Drug Administration; FGF, Fibroblast Growth Factor; FUT8KO, FUT8 Knockout; Gal, Galactose; GS, Glutamine Synthetase; HEK, Human Embryonic Kidney; HGPRT, Hypoxanthine-guanine Phosphoribosyltransferase; HPRT, Hypoxanthine-guanine Phosphoribosyltransferase; HIV, Human Immuno Deficiency Virus; hTPO, Human Thrombopoietin; IgG, Immunoglobulin G; IFN, Interferon; LDH-A, Lactate Dehydrogenase-A; LV, Lentiviral Vector; Mab, Monoclonal Antibody; MTX, Methotrexate; NANA, *N*-acetylneuraminic Acid; NCI, National Cancer Institute; NIH, National Institutes of Health; NHL, Non-Hodgkin's Lymphoma; PB, Piggybac; PEI, Polyethylenimine; rhIL-15, Recombinant Human Interleukin-15; RNAi, RNA Interference; SEAP, Secreted Alkaline Phosphatase; STR, Stirred-Tank; TGE, Transient Gene Expression; tPA, Tissue Plasminogen Activator; TNFR, Tumor Necrosis Factor Receptor; UCOE, Ubiquitous Chromatin Opening Elements; VPA, Valproic acid; WPRE, Woodchuck Hepatitis Virus Post-transcriptional Regulation Element.

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Table 1
New biopharmaceutical products approved by FDA.

Product	Year approved	Description	Manufacturer	Expression system	Indication
Fibroblasts, Autologous	2011	A formulation of autologous fibroblast skin-derived cells	Fibrocell science	Patient fibroblasts	Appearance of nasolabial fold wrinkles
Belatacept (CTL4-Ig Fusion)	2011	CTLA4-Ig Mutant	BMS	Mammalian	Prevention of acute rejection in adult kidney transplant patients
Yervoy (Ipilimumab)	2011	Anti- CTLA-4 MAb	BMS	Mammalian	Metastatic melanoma
Benlysta (Belimumab)	2011	Anti BLyS MAb	HGS	Mammalian	Systemic lupus erythematosus
Prolia (Denosumab)	2010	Anti RANK Ligand MAb	Amgen	Mammalian	Osteoporosis
Pegloticase (Krytexxa)	2010	Pegylated urate oxidase	Savient	*E. coli*	Chronic refractory gout
Victoza (Liraglutide)	2010	GLP-1 Analog	Novo Nodisk,	Yeast	Diabetes
Pancreaze (Pancrelipase)	2010	Pancreatic enzyme	J & J	Tissue Extraction	Exocrine Panceatic insufficiency
Xeomin (Incobotulinumtoxin A)	2010	Botulinum toxin A	Merz	Bacteria	Cervical dystonia, blepharospasm
Vpriv (Velaglucerase)	2010	Human glucocerebrosidae	Shire	Mammalian	Gaucher Disease
Menveo (Meningitis Vaccine)	2010	Meningococcal Conjugate Vacccine (3)	Novartis	Bacteria (Corynebacterium)	Prevention of invasive meningococcal disease
Provenge (Prostate Cancer Cellular Vaccine)	2010	Prostatic Acid Phosphastase (PAP)—GM-CSF	Dendreon	Cancer cell	Metastatic prostate cancer
Xiaflex (Collagenase)	2010	Clostridial Collagenase for Injection	Auxilium	Bacteria (Clostridium histolyticum)	Dupuytren's Disease
Lumizyme (Alglucosidase alfa)	2010	Glucosidase alfa	Genzyme	Mammalian (CHO)	Pompe Disease
Prevnar 13	2010	Pneumococcal 13-Valent Conjugate Vaccine	Wyeth	Bacteria (Corynebacterium)	Pneumoniae
Actemra (Tocilizamab)	2010	Anti IL-6 Recetor MAb	Genentech	Mammalian	Systemic juvenile idiopathic arthritis
Cervarix MEDI 501	2009	Human Papilloma Virus (HVP) Vaccine Type 16 and 18 VLP	GSK	Baculovirus	Cervical cancer
Stelama (Ustekinumab)	2009	Anti IL-12 and IL-23 MAb	Centocor/J&J	Mammalian	Plaque Psoriasis
Arzerra (Ofatumumab)	2009	Anti CD20 MAb	Genmab	Mammalian	CLL (Chronic Lymphocytic Leukemia)
ATryn (rhATIII)	2009	Antithrombin III	GTC (Genzyme)	r Transgenic Goat	Blood clots
Simponi (Golimumab)	2009	TNF alfa human MAb	Centocor/J&J	Mammalian	Immune dysfunction-related arthritis
Ilaris (Canakinumab)	2009	Anti IL-1 beta MAb	Novartis	Mammalian	Cryopyrin-associated periodic syndromes
Crtmzia (Certolizumab Pegol)	2008	TNF alfa Inhibitor (r humanized Fab covalently bond to ethylene glycol)	UCB	*E. coli*	Crohn's disease
Nplate (Romiplostim)	2008	Thrombopoietin Peptibody, Fc Fusion	Amgen	*E. coli*	Chronic Immune Thrombocytopenic Purpura (ITP)
Arcalyst (rilonacept)	2008	Interleukin-1 trap	Regeneron	Mammalian (CHO)	CAPS and FCAS
Rotarix (Rotavirus Vaccine)	2008	Live attenued oral vaccine	GSK	Virus	Rotavirus infestion
Cinryze (C1 Inhibitor)	2008	C1 inhibitor derived from human plasma	Lev	Human plasma	Angioedema attacks in with hereditary angioedema

(Matasci et al., 2009; Wurm, 2004; Zhang, 2010), plants (Franconi et al., 2010; Pogue et al., 2010; Ruffoni et al., 2011) or animals (Redwan, 2009). Plants have been proposed as an attractive alternative for pharmaceutical protein production to current mammalian or microbial cell-based systems. Eukaryotic protein processing coupled with reduced production costs and low risk for mammalian pathogen contamination and other impurities have led many to predict that agricultural systems may offer the next wave for pharmaceutical product production (Pogue et al., 2010). However, for this to become reality, the quality of products produced at a relevant scale must equal or exceed the predetermined

Table 2
Top selling biopharmaceuticals in 2007.[a]

Product	Revernue US $ (M)	Date approved	Manufacturer	Expression system	Indication
EPOs	10,794	1989	Amgen, Roche, J & J	Mammalian	Anemia
Insulins	10,132	1982	Eli Lilly, Novo Nodisk, Sanofi-Aventis	*E. coli*	Diabetes
IFNs	7455	1993	Schering-Plough, Roche, Biogen-Idec Bayer-Schering, Merck-Serono	*E. coli* and Yeast	Viral infection and cancer
Enbrel	5275	1998	Amgen, Wyeth	Mammalian	Rheumatoid arthritis
Remicade	4948	1998	J & J, Schering-Plough	Mammalian	Rheumatoid arthritis
Rituxan	4600	1997	IDEC, Genentech, Roche	Mammalian	NHL and CLL
Neupogen/Neulasta	4277	1998/2002	Amgen	*E. coli*	Myelosuppressive in chmotherapy
Clotting Factors	4168	1997	Novo Nordisk, Wyeth, Bayer, Baxter	Mammalian	Hemophilia episodes
Herceptin	4046	1998	Genentech and Roche (Trastuzumab)	Mammalian	Breast cancer
Lovenox	3605	2007	Sanofi-Aventis	N/A	Coronary artery disease
Avastin	3424	2004	Genentech and Roche (Bevacizumab)	Mammalian	Cancer
Humira	3000	2002	Abbott Laboratories (Adalimumab)	Mammalian	Rheumatoid arthritis
Growth Hormones	2545	1985	Pfizer, Novo Nordisk, Eli Lilly, Serono, Roche/Genentech	*E. coli*	Growth hormone deficiancy
Prevnar/Prevenar	2439	2002	Wyeth	Baterial	Prevention of invasive pneumococcal disease
Gardasil	1481	2006	Merck	Yeast	Prevention of vulvar and vaginal cancer
Erbitux	1336	2004	Merck-Serono, BMS	Mammalian	Cancer
Lucentis	1219	2006	Genentech, Novartis (Ranibizumab)	*E. coli*	Macular degeneration
Synagis	1200	1998	MedImmune	Mammalian	Respiratory syncytial virus infection
Cerezyme	1144	1994	Genzyme	Mammalian	Gaucher Disease

[a] Data are from the reference: Darby (2008).

release criteria of identity, purity, potency and safety as required by pharmaceutical regulatory agencies (Pogue et al., 2010). Microbial systems, especially *E. coli*, have the advantage of low cost in establishing a production strain, quick production cycle, easy in-process control, and high productivity compared to mammalian expression systems. However, there are various limitations for prokaryote systems like *E. coli*. Expression of a large complex protein containing multiple subunits, cofactors, disulphide bonds, and posttranslational modifications is a quite challenging (Mahmoud, 2007), since the posttranslational metabolic machinery is only available in mammalian cells (Butler, 2005; Zhang, 2010). Many recombinant proteins, for instant, Tissue Plasminogen Activator (tPA) and Erythropoietin (EPO), need to have posttranslational modification such as glycosylation for its biological function (Sasaki et al., 1987). Likewise, glycosylation of Mab is important for optimal biological function and pharmacokinetics (Beck et al., 2008; Jefferis, 2005).

3 More than 50% of therapeutic proteins approved and on the market are produced using mammalian cells, mainly due to the ability of mammalian cells to synthesize proteins that are similar to those naturally occurring in humans with respect to molecular structures and biochemical properties. Recently, the productivity of mammalian cells cultivated in bioreactors has reached 10–15 g/L in Mab and Fc-fusion protein production (Huang et al., 2010), which was mainly resulted from improvements of cell line development through effective selection methods, media optimization, and process control. There have been many outstanding reviews (Birch and Racher, 2006; Geisse and Fux, 2009; Wurm, 2004; Zhang, 2010) that summarized development of mammalian cell protein expression for biopharmaceutical production. Additionally, Zhang provided a detailed description of mammalian expression system with information regarding cell lines, medium and process development (Zhang, 2010). Monoclonal antibody expression systems typically include commonly used glutamine synthetase (GS) and dihydrofolate reductase (DHFR) selective markers to amplify transfected cells as summarized (Birch and Racher, 2006). Gene amplification and vector engineering to achieve rapid and high-level therapeutic protein production were reviewed by Cacciatore (Cacciatore et al., 2010) with emphasis on the DHFR selection system. Protein glycosylation was reviewed by Hossler (Hossler et al., 2009). Technological progresses in Mab production systems were described by Rodrigues et al. (2010). This review will focus on the latest advancements in the field, especially in active areas such as expression systems, glycosylation impact factors, and transient gene expression.

4 2. Expression system

Most marketed biopharmaceutical products have been produced in Chinese hamster ovary (CHO) cells, murine myeloma lymphoblstoid-like (NS0 and Sp2/0-Ag14) cells, Human Embryonic Kidney 293 (HEK 293) cells, and baby hamster kidney cells (BHK-21) (Birch and Racher, 2006; Meyer et al., 2008; Walsh, 2006; Zhang, 2010). Various expression systems have been investigated to enhance the production in mammalian cells. Vast improvements have been made in the last two decades in vector design and construction, codon optimization, gene amplification approaches, host cells, transfection methods, and screening tools (Aldrrich et al., 2003; Birch and Racher, 2006; Jalah et al., 2007; Wurm, 2004; Zhang, 2010). Of the many available systems, two have been commonly used: CHO cell lines for recombinant proteins and Mabs and murine myeloma (NS0 and Sp2/0) cell lines for Mabs (Zhang, 2010). Improvement of mammalian expression may be achieved through proper vector design including using strong promoter, proper signal peptide, selected introns, product gene codon optimization (Jalah et al., 2007) and use of transcription control regions (Deer and Allison, 2004). Common approach used in generating cell lines for the production of therapeutic proteins relies on gene amplification induced by a selective marker such as dihydrofolate reductase (DHFR) (Solomon et al., 2003) or glutamine synthetase (GS) (Bebbington et al., 1992; Birch and Racher, 2006). To achieve high levels of gene expression, vectors usually have strong promoters such as cytomegalovirus (CMV) promoter to drive high level messenger RNA transcription (Deer and Allison, 2004). Codon optimization for the target cell type, GC/AT ratio balancing, and signal sequence optimization have been shown to accelerate mRNA processing and improve secretion (Jalah et al., 2007). Besides, gene-targeting technology, chromatin opening elements and attachment regions have been incorporated into vector optimization to improve final product production (Cacciatore et al., 2010).

2.1. PER.C6 cell line

The retina-derived Per.C6 cell line, a human cell line with human glycosylation and other post-translational modification machinery, has been quite attractive to the biopharmaceutical industry for producing therapeutic proteins and Mabs. This cell line requires no gene amplification or selection marker. High producing stable clones can be developed within a few months. A low copy number is sufficient to retain stable and efficient protein expression.

The cell line has demonstrated its capacity of producing >2 g/L of recombinant protein in fed-batch culture (Zhang, 2010). A very high production titer of 25 g/L associated with a very high cell density (>150 million cells/mL) has been achieved using the eXtrem-Density (XD) continuous process. In this process, both cells and product are retained in a stirred-tank bioreactor using a suspension culture of PER.C6 (Schirmer et al., 2010). Recently PER.C6 was used to express multiple antibodies in one cell (Kruif et al., 2010). The cells were transfected with a combination of plasmids containing genes encoding three different antibodies with identical light chains. Triple positive clones were identified. Stable clones were selected through dilution cloning and stability testing. Volumetric IgG productions up to 387 mg/L IgG and specific productions up to 24 pg/cell/day were reached, which are compatible to a single IgG and IgM batch production as reported (Yallop et al., 2008).

2.2. UCOE Expression System

5 Gene amplification methodologies are frequently employed for the generation of large quantities of recombinant proteins in mammalian cells. Current expression systems rely on screening a large number of clones. Due to substantial variation and unpredictable stability of expression amongst transfected cells however, extensive clone screening is required to identify suitable high producers (Nair et al., 2011; Pilbrough et al., 2009). Although these systems usually guarantee very high yields, they are also very time-consuming. In addition, due to the large genomic re-arrangements that frequently occur with amplification, the resulting high-producing clones can be unstable. The instability of a cell line may involve silencing of the exogenous gene resulting from modifications such as methylation of CpG DNA sequences (Zhang et al., 2010), histone deacetylation and chromatin condensation (Kim et al., 2011).

The use of un-methylated CpG island fragments from housekeeping genes referred to as ubiquitous chromatin opening elements (UCOE) in plasmid vectors was established for increased stability of transgene expression (Benton et al., 2002; Cacciatore et al., 2010; Nair et al., 2011; Zhang, 2010). UCOE vectors contain non-tissue specific chromatin-opening-elements that permit rapid expression of a protein in an integration independent manner. Efficient expression can be derived from a single copy of an integrated gene site resulting in a higher percentage of cells expressing the marker gene in the selected pool in comparison to standard non-UCOE containing vectors (Benton et al., 2002). UCOE technology is potentially a useful tool for rapid protein production. It was initially reported that in combination with a serum-free and suspension adapted parent cell line, rapid production of over 300 mg of a recombinant antibody proteins in less than 1 month from transfection

pools in shake flasks can be achieved (Benton et al., 2002). Recently, when UCOE was incorporated in the expression vectors, many more transfectants with higher expression levels were found (Ye et al., 2010). By using a transfection pool of various clones from a single transfection to produce large quantities of therapeutic protein, UCOE improved the yield 6-fold by increasing the portion of high producers in the mixed population. Further optimization through UCOE-promoter combinations may result in expression higher than that from the CMV promoter (Nair et al., 2011).

2.3. Gene targeting

Random integration linking genomic amplification is widely used to generate desired cell lines for stable high-level expression of recombinant proteins. Expression level is unpredictable due to the randomized location of integration. A site-specific recombinase-recognition sequence, Flp/FRT, for gene targeting has been studied (Huang et al., 2007; Raymond and Soriano, 2007; Zhou et al., 2007, 2010). Several proteins, tissue plasminogen activator (tPA), secreted alkaline phosphatase (SEAP), and erythropoietin (EPO) were tested with the gene targeting procedure and some of them showed constant high expression (Zhou et al., 2007, 2010). A stable cell line generated by site-specific integration was able to reach productivities at 17.1 p/c/d (Zhou et al., 2007). Using a similar approach, anti-CD20 antibody was produced at 200 mg/L (Huang et al., 2007). Another recombination system known as the ΦC31 system has an advantage of two integration sites (Cacciatore et al., 2010) wherein integration is irreversible. One successful example was that luciferase expression was found to be 60-fold higher using this recombination system as compared to random transfection (Thyagarajan and Calos, 2005).

To improve the transfection process, the use of engineered chromosomes has been considered. An artificial chromosome expression (ACE) System has been used for the targeted transfection of cells containing mammalian-based artificial chromosomes with multiple recombination acceptor sites. This ACE System allows for the specific transfection of single or multiple gene copies and eliminates the need for random integration into native host chromosomes. The utility of using artificial engineered mammalian chromosomes, specifically the ACE System, has been demonstrated in several case studies covering the generation of CHO cell lines expressing monoclonal antibodies (Kennard, 2011).

Transposable elements such as piggyback (PB) and sleeping beauty have been shown to support the integration of recombinant genes into cultivated mammalian cells (Ding et al., 2005; Wu et al., 2006). Recombinant CHO cell lines expressing a tumor necrosis factor receptor-Fc fusion protein were generated based on transgene integration mediated by the PiggyBac transposon (Matasci et al., 2011) to show that pools of transposed cells produced up to fourfold more recombinant protein than did the pools generated by standard transfection. Those cell lines showed stable expression for up to 3 months in the absence of selection (Matasci et al., 2011).

2.4. Other progress

High-efficiency expression regulated through lambda phage P_L has been commonly used in the *E. coli* expression system. The promoter activity of P_L is fully repressed at low temperature by a thermolabile repressor product of the λcI1857 gene, and can be activated by heat induction (Remaut et al., 1981). Similar temperature-sensitive promoters were not reported with a mammalian expression system until recent publication by Thaisuchat et al. (2011). A novel, endogenous, and highly active gene promoter obtained from CHO cells shows conditionally inducible gene expression at reduced temperature (Thaisuchat et al., 2011). Upon a shift to 33°C, a two to three-fold increase of basal productivity was achieved. The promoter region S100a6 (calcyclin) and its flanking regions were identified from a genomic CHO-K1 lambda-phage library. It showed higher-than-SV40 promoter activity with potential further increased by duplication of a core promoter sequence (222 bp) (Thaisuchat et al., 2011). This property is particularly advantageous for processes with reduced expression during initial cell growth followed by a boost in expression during the production phase at low temperature (Thaisuchat et al., 2011).

A simplified process to amplify the DHFR expression system was reported as optimized through the coupling of codon adaptation with gene amplification (Kotsopoulou et al., 2010). As a result, expression saturation can be achieved rapidly, in as low as 5 nM MTX, with minimal effort and without compromise in final yields (Kotsopoulou et al., 2010).

Lentiviral vectors (LVs) derived from human immunodeficiency virus type 1 (HIV-1) have been widely used in applications for gene therapy because of their efficient transduction (Wiznerowicz and Trono, 2005). LV-mediated gene transfer provided an efficient alternative to plasmid transfection. Recently, an efficient method for the rapid generation of high-producing recombinant CHO cell lines was reported (Oberbek et al., 2011). Tumor necrosis factor receptor (TNFR) Fc fusion protein was expressed at a 50–250 mg/L level in a 4-day culture.

RNA interference (RNAi) technology has become a novel tool for silencing gene expression in cells (Maliyekkel et al., 2006; Tiscornia et al., 2004; Wu, 2009). DHFR was targeted for silencing, resulting in higher producing clones with more stable expression in the absence of MTX (Hong and Wu, 2007). To reduce lactate formation, lactate dehydrogenase-A (LDH-A), an enzyme catalyzing the conversion of glucose-derved pyruvate to lactate, was down-regulate by an expression vector of small interfering RNAS (siRNA) in CHO cells producing human thrombopoietin (hTPO). LDH-A activities were decreased by 75–89% compared with that of the control CHO cells (Kim and Lee, 2007). The effect of siRNA is more significant than that of other methods such as homologous recombination and antisense mRNA (Kim and Lee, 2007). Potentially, the approaches can be applied as to silence apoptosis-associated gene expression, protein glycosylation-associated gene expression, cellular metabolism gene that lactate dehydrogenase involved in, and other genes used for gene amplification. However, all of these belong to single targeting approach and depends strongly on the identification of the critical target gene to down regulate. Only then can silencing be used to stably influence the cellular functions through down-regulation of the target protein expression in mammalian cells (Wu, 2009). Future RNAi approaches can be extended to silence multiple targets involved in different cellular pathways to change the global gene regulation in cells, as well as the targets related to microRNA molecules for cellular self-regulation (Wu, 2009).

DHFR-deficient CHO cells have been the most commonly and successfully used host cells in the biopharmaceutical industry for the years. There has been one recent observation reported that different DHFR-deficient CHO cells (CHO-DG44 and CHO-DuxB11) show poor growth in fed-batch cultures even in HT supplemented medium, whereas antibody-producing cells derived from these hosts achieved least 2–3 fold higher peak cell densities. It may be associated with a direct consequence of DHFR deficiency (Florin et al., 2011).

Early monitoring of product quality should be an essential part of production cell line development. Many factors, particularly the choice of the host cell line had a significant effect on the overall product quality. Results of expressing CNTO736, a glucagon like peptide-1-MIMETIBODY showed that product expressed in mouse myeloma host cell lines had a lesser degree of proteolytic degradation and variability in O-linked glycosylation as compared to that expressed in CHO host cell lines. The choice of a specific CHOK1SV derived clone also had an effect on the product quality. In general, molecules that exhibited minimal N-terminal clipping had increased level of O-linked glycosylation in the linker region, giving credence to the hypothesis

that O-linked glycosylation acts to protect against proteolytic degradation. Moreover, products with reduced potential for N-terminal clipping had longer in vivo serum half-life (Dorai et al., 2009a).

2.5. A case study for construction design and optimization

Optimization of protein expression can be a comprehensive project that involves the selection of an expression system, choice of an expression cell line, and design of construction including promoter, lead signal sequence, codon optimization, and untranslated regions. One example of expressing cytokine interleukin-15 can be dissected for illustrating construction design and optimization of expression. Designing process from this case is applicable to other expression systems.

Recombinant human interleukin-15(rhIL-15) has remarkable biological function in promoting NK- and T-cell activation and proliferation as well as enhancing anti-tumor immunity of CD8+ T cells in pre-clinical models (Klebanoff et al., 2004; 'Mac' Cheever, 2008; Teague et al., 2006). A phase I clinical trial to evaluate the safety, dosing, and anti-tumor efficacy of IL-15 in patients has begun at the NIH. Though rhIL-15 expression in mammalian cells was attempted, the material for clinical trials was made from *E. coli* expression systems. Mammalian expression of rhIL-15 is highly desirable, since it may represent the natural form of the cytokine and possess longer half life in circulation system and/or less immunogenic. Efficient transient expression vectors for IL-15 were developed by combining RNA/codon optimization and modification of the IL-15 native long signal peptide. These changes resulted in elevated cytoplasmic levels of the optimized mRNA and more than 100 fold improved production of secreted human IL-15 protein (Jalah et al., 2007). While the results were still limited to laboratory use and not feasible for clinical manufacturing, the principle of construction design including coding optimization by increasing GC content, removal of potential splicing sites and leader sequence optimization, is applicable to all systems with mammalian expression.

The basic components of those vectors used are CMV promoter, bovine growth hormone (BGH) polyadenylation site, and kanamycin resistance gene (Jalah et al., 2007). A wild-type human IL-15 open reading frame and leader native long signal peptide were inserted into downstream of the CMV promoter (Table 3). By inserting of 173 bp constitutive transport element (CTE) in between the IL-15 coding sequence and the BGH signal, expression was doubled (Table 3). Further codon optimization including removing instability signals sequences (such as AUUUA and variant), removing potential splicing sites, and an increasing of GC content from 35% (wild type) to 57% (optimum) increased expression 11-fold as compared to the non-optimized construct. Next, secretory signals on IL-15 were examined and a construct with tPA leader showed a 75-fold increased expression of IL-15. The linker between the tPA promoter and the IL-15 coding sequence was optimized and a sequence with GAR 3 amino acids showed the highest expression (Table 3). Even with the optimized construct, however, it was still not feasible to proceed as a production cell line to deliver clinical material for human trials.

Low expression of rhIL-15 in mammalian cells is at least partially due to instability of the expressed product in cells. IL-15 expressed in HEK 293 cells was degraded immediately (Bergamaschi et al., 2008). IL-15 co-expression of IL-15 and IL-15 receptor alpha in the same cell resulted in significantly increased expression levels with increased stability and secretion of both molecules as a complex (Bergamaschi et al., 2009). IL-15 fusion with its receptor alpha showed increased biological activity (Mortier et al., 2006).

Table 3
Recombinant human interleukin-15 expression in mammalian cells.

Construct key point	Structure Promoter—Sig—Coding Sequence—Terminator	Fold Increase	Expression (ng/mL)
Wild type IL-15	CMV—Wile type IL-15—BGHpA	1	10
CTE	CMV—Wild type IL-15—CTE**—BGHpA	2	20
Opt	CMV—LSP—Opt IL-15—BGHpA	11	110
Native Sig (SSP)	CMV—SSP—Opt IL-15—	1	3
Native Sig (LSP)	CMV—LSP—Opt IL-15-	18	50
tPA 2 sig	CMV—tPAsig-GARA-Opt IL-15-	75	250
tPA 6 sig	CMV—tPAsig-GAR—Opt IL-15-	116	300
tPA 7 sig	CMV—tPAsig-G—Opt IL-15-	25	70
tPA 8 sig	CMV—tPAsig—Opt IL-15-	10	20

Data are from reference: Jalah et al. (2007).

2.6. Cell line engineering

There are about 200 recombinant biopharmaceutical products currently on the market and several hundreds are in clinical development (Rader, 2010). More than half of them are glycosylated proteins. Development of an expression system allowing the efficient manufacturing of quality glycoprotein is highly desirable.

The sialylation of glycoproteins for therapeutic use is important in maintaining a long residence time in circulation. The degree of sialylation is variable depending on product, host cell line, and culture conditions. The limiting steps of sialylation include the biosynthesis of sialic acid, the availability of nucleotide-sugars, and the CMP-sialic acid transporter and sialyl-transferase (Durocher and Butler, 2009). Overexpression of sialyl-transferase in CHO cells provided moderate improvement (Bork et al., 2009; Wong et al., 2006). Besides of sialylation, expression of IgG in CHO cells normally leads to a conserved fucosylated, biantennary glycan structure. Binding of the non-fucosylated IgG to human FcγRIII was improved 50-fold (Shields et al., 2002) as compared to the fucosylated IgG. Nonfucosylated anti-CD20 showed markedly higher (over 100-fold based on EC50) ex vivo B-cell depletion activity than its fucosylated counterpart in the presence of plasma IgG (Lida et al., 2006). Hence, non-fucosylated IgG1 exhibits strong therapeutic potential through dramatically enhanced ADCC at low doses in humans in vivo (Lida et al., 2006). In order to produce afucosylated antibodies using transient transfection, a FUT8 knockout (FUT8KO) cell line was generated in a CHO host cell line (Wong et al., 2010). Transfection of the cell using the cationic liposome, DMRIE-C, resulted in human IgG production titers comparable to the wild-type. The cell line may also be co-transfected with the exostosin-1 (EXT1) gene to increase heparin sulfate content in order to achieve similar expression levels (40–50 mg/L) as the wild-type (Wong et al., 2010).

Recombinant EPO production and sialylation in CHO cells were enhanced through transient expression of the *Bombyx mori* 30Kc19, which may represent a novel approach to improve the production and sialylation of recombinant glycoproteins in CHO cells (Wang et al., 2011). 30Kc19 protein was found to inhibit nuclear fragmentation and apoptotic body formation in Sf9 cells (Rhee et al., 2009). When a stable cell line containing 30Kc19 was established, its expression significantly improved EPO production and sialylation by 102.6% and 87.1% respectively. The enhanced productivity from 30Kc19 expression is believed to occur because the 30Kc19 protein suppresses the loss of mitochondrial membrane potential and consequently improves the generation of intracellular ATP. In addition, the positive effect of 30Kc19 expression on sialylation is believed to be due to its ability to maintain sialyltransferase activity (Wang et al., 2011).

Culture conditions such as nutrient starvation, oxygen limitation, toxic by-product accumulation, and high osmolality may lead to cell apoptosis, which has negative impact on the productivity of a recombinant protein in mammalian cells (Krampe and Al-Rubeai, 2010). Co-transfection and over-express of an anti-apoptosis gene in to a production cell line may extend cell viability and increase expression

yields (Chiang and Sisk, 2005; Dorai et al., 2010; Majors et al., 2008; Nivitchanyong et al., 2007; Rossi et al., 2011). Anti-apoptotic genes Aven and E1B-19K enhanced baby hamster kidney cell line to produce recombinant factor VIII in 12-L perfusion bioreactor studies (Nivitchanyong et al., 2007). A set of Bcl family members, Bcl-XL, Bcl-2Δ, combining with E1B-19K, were over-expressed in CHO cells, resulting in 80% increased productivity of a Mab as compared with an optimum clone from the control cell line (Dorai et al., 2010), as well as lactate consumed and culture longevity extended (Dorai et al., 2009b). A new cell line was engineered for extended cell survival, i.e., myeloma Sp2/0 transfected with Bcl2-EEE, the constitutetively active phosphomimetic mutant of Bcl-2 (Rossi et al., 2011). The clone referred as SpESFX-10 exhibited robust growth and resisted apoptosis induced by sodium butyrate or glutamine starvation (Rossi et al., 2011). The advantage of SpESFX-10 as a host for generating Mab-production cell lines was demonstrated by its increased transfection efficiency, culture longevity, as well as terminal Mab titer increased from 500 to 1300 mg/L at 3 L fed-batch bioreactor (Rossi et al., 2011).

3. Process development

Optimization of culture medium and feeding strategies are the key factors that contributed to increase production from below 100 mg/L to 13 g/L (Huang et al., 2010; Wurm, 2004). Various methods have been investigated to increase production yield including engineering host cells (Seth et al., 2006) and expression vectors (Aldrrich et al., 2003), optimization of culture media (Altamirano et al., 2004; Huang et al., 2010) with additives (Allen et al., 2008; Bai et al., 2011) various feeding strategies, and improvement of process control such as temperature, pH, and osmolality (Gagnon et al., 2011; Rodriguez et al., 2005; Trummer et al., 2006; Yoon et al., 2005).

To minimize lactate's negative impact, a simple method for controlling of lactate accumulation in suspension cultures of CHO cells was developed based on the culture's pH (Gagnon et al., 2011). When glucose dropped to a low level (generally below 1 mM), cells begin to take up lactic acid from the culture medium resulting in a rise in pH. Based on pH control, a nutrient feeding method was developed to deliver a concentrated glucose solution. It was demonstrated that the high-end pH-controlled delivery of glucose can dramatically reduce or eliminate the accumulation of lactate during the growth phase of a fed-batch CHO cell culture. The method was scaled from the bench scale to a large 2500 L scale (Gagnon et al., 2011). Furthermore, this method has proven applicable to the majority of CHO cell lines producing monoclonal antibodies and other therapeutic proteins with results that showed doubling of the final titers for eight cell lines (Gagnon et al., 2011).

A highly productive chemically-defined fed-batch process was recently developed to maximize Mab and Fc-fusion protein production titers to 10–13 g/L. Cell density reached 20 million/mL and cell viabilities were maintained above 80% on day 18 without the use of antiapoptotic genes or temperature shift. The process was scaled-up to 100 L (Huang et al., 2010). In this study, a plant hydrolysate was used to improve productivity from 44 p/c/d to 54 p/c/d. Volumetric productivity was over 500 mg/L/day, which is one of the highest reported for mammalian cell culture using defined medium (Huang et al., 2010).

Complex cell culture media components used for industrial mammalian cell culture can be analyzed by Raman spectroscopy for identification, characterization and quality assessment (Li et al., 2010). It can be potentially useful in large scale production to insure lot-to-lot consistency in biopharmaceutical production.

To reduce time from seed build-up to inoculums ready for production, a large 100 mL cryobage banking procedure was developed (Heidemann et al., 2010). No significant difference in recovery rate and productivity was observed (Heidemann et al., 2010).

3.1. Media development

The inhibitory threshold values of ammonia, lactate, osmolality, and carbon dioxide for cell growth and protein quality were examined in a CHO cell culture in 5000 L bioreactors (Xing et al., 2008). Lactate at 60 mM inhibited cell growth by 25% but increased specific protein production by 10% (Lao and Toth, 1997). In mammalian cell culture, lactic acid secretion during cell growth and production of protein therapeutics have been problematic due to resulting increases in lactate concentration and osmolality, both of which can adversely affect cell growth and productivity (Yoon et al., 2006).

Synthetic nonionic surfactant Pluronic F-68 (PF-68) is widely used to protect mammalian cells from sparging- and agitation-related injuries in a stirred-tank bioreactor (Clincke et al., 2011; Murhammer and Goochee, 1990). Under mild (low or without agitation) conditions, addition of PF-68 at 0–0.1% level may increase CHO cell growth and recombinant protein production (Clincke et al., 2011). Increased production titer may be partially due to the contributed by reduced the adherence of the product on the CHO cells (Clincke et al., 2011; Tharmalingam et al., 2008) in a decrease of the cell membrane hydrophobicity after PF-68 adsorption (Murhammer and Goochee, 1990). For glycosylation of recombinant glycoprotein, no significant differences were observed between the presence and absence of PF-68 (Clincke et al., 2011; Kochanowski et al., 2008).

Iron plays a critical role in supporting healthy cell growth. Iron deficiency causes poor cell growth and eventually cell death. Use of 0.25 mM iron with 0.5 mM sodium citrate was found effectively improved Mab production titer by 30–40% (Bai et al., 2011). Selenite was studied for its additional function as an iron carrier for CHO cell growth and Mab production (Zhang et al., 2006). Cell density as high as 10 million viable cells/mL and ~3 g/L product titer were achieved in 14-day fed-batch cultures in shake flasks, followed by successful scale-up to stirred bioreactors (Zhang et al., 2006).

3.2. Process control to improve product quality

Mis-incorporation of serine for asparagine during production of a recombinant Mab using CHO cells has been reported (Khetan et al., 2010; Wen et al., 2009). It was discovered as a result of modern analytical technologies including intact mass measurement, peptide mapping, and tandem mass spectroscopy sequencing (Wen et al., 2009). Mis-incorporation occurred during fed-batch processes under asparagine starvation. Maintenance of asparagine at low levels through controlled supplementation of asparagine-containing feed eliminated the occurrence of misincorporation (Khetan et al., 2010). This strategy was implemented in a clinical manufacturing process and scaled-up to 2000 L (Khetan et al., 2010). Unintended amino acid sequence changes in recombinant Mab expressed in CHO cells was also observed and reported by Guo et al. (2010). Since methotrexate (MTX) is often used to generate high producing cell lines, the genomic mutation rates of the hypoxanthine–guanine phosphoribosyltransferase (HGPRT or HPRT) gene using a 6-thioguanine (6-TG) assay under various concentrations of MTX selection in CHO cells. Results showed that 6-TG resistance increased as the MTX concentration increased during stable cell line development. Guo's data showed that two replacements of serine, one at position 167 by arginine (S167R) in the light chain and the other at position 63 by asparagine (S63N) in the heavy chain were due to a genomic nucleotide sequence change and translational mis-incorporation. This mis-translation is codon specific since S63N mistranslation is not detectable when the S63 AGC codon is changed to a TCC or TCT codon. Guo's results demonstrate that both a genomic nucleotide change and translational misincorporation can lead to low levels of sequence variants and mistranslation of serine to asparagine. More importantly, the misincorporation can be eliminated by substituting the TCC or TCT codon for the S63 AGC codon without impacting antibody productivity.

Biopharmaceutical product aggregation is one of complicated problems that require considerable amount of effort in both process and analytical development. Environmental conditions of production process such as temperature, protein concentration, pH, oxygen, shear force, and ionic strength may affect the amount of aggregate observed. The presence of certain ligands, including specific ions, may enhance aggregation. Stresses to the protein such as freezing, exposure to air, or interaction with metal surfaces may result in undesired post-translational molecule modification even unfolding, which then leads to the formation of aggregates (Vazquez-Rey and Lang, 2011).

During cell culture, protein aggregation is common phenomenon in Mab, Fc-fusion, and some cytokine production due to of the high concentration of protein accumulated. Selection of an optimum cell line and optimize cell culture conditions are key to minimize aggregation. High temperature and pH close to the product PI will favor the aggregation (Franco et al., 1999). Modifying culture medium in pH and addition of sodium chloride to medium to achieve certain osmolarity value (350 mOsm/kg) resulted in significant decrease of monoclonal antibody aggregates in the production cycle (Franco et al., 1999). The application of treatments with solvents and detergents to inactivate virus as an alternative to low pH inactivation will reduce the level of aggregations in the product (Vazquez-Rey and Lang, 2011). Strategies for aggregate removal and minimization during Mab manufacturing processes were well summarized by Vazquez-Rey. During downstream manufacturing, chromatography is typically the step that most contributes to aggregate removal. A new class of dextran-grafted agarose based ion exchange resin has gained popularity for process scale bioseparations (Suda et al., 2009; Vazquez-Rey and Lang, 2011). Besides of using size exclusion chromatography (SEC) to reduce the levels of aggregates in the final solution (Wang et al., 2006), alternatively, Lu et al. (2009) summarized recent advancement in application of hydrophobic interation chromatography for antibody aggregates and impurities such as host cell proteins removal in industrial purification process (Lu et al., 2009).

4. Glycosylation impact factors

11

Glycosylation is a crucial protein quality attribute that can affect efficacy and pharmacokinetics. Many factors including bioreactor/technology platform, cell type, medium, nutrients, and culture conditions have impacted glycosylation of therapeutic proteins and antibodies expressed in mammalian cells (Gawlitzek et al., 1995; Hossler et al., 2009). Overexpression of appropriate glycosyltransferases can enhance glycan quality (Andersen and Krummen, 2002), for instance, overexpression of a galactosyltransferase and a sialyltransferase in CHO cells led to corresponding increases in the galactose (Gal) and sialic acid content of expressed recombinant therapeutic proteins (Andersen and Krummen, 2002; Weikert et al., 1999). The effect of different culture conditions (temperature, pH, metabolic profile, and dissolved oxygen), bioreactor, processes, medium and nutrients on the structures of N-linked glycans attached to an antibody or a therapeutic protein has been widely investigated. However, due to complexity of glycan structure and glycosylation process, there is no consensus on culture system which may have a desirable glycol-profile for a product. For each individual expression system, cell line, and product, a systematic investigation would be the solution to understand each factor's impact on the final product (Hossler et al., 2009). Latest development of glycan profiling assays (Primack et al., 2011), particular high-throughput screening assay to quantify major glycan species in the crude mammalian cell culture samples for monoclonal antibodies, should facilitate antibody glycan profiling during cell culture expression, clone selection, and cell culture process optimization. The relative levels of high mannose (HM), fucosylated and galactosylated glycan species in the Fc domain can be determined for hundreds of crude cell culture samples in a few hours. (Primack et al., 2011).

4.1. Bioreactor and cell line

Detailed comparison studies were carried out by Nahrgang et al. (1999) to address the impact of bioreactors, and cell type on glycosylation of product. Adherent cells were cultivated in roller bottles and in suspension in a stirred tank (STR). No major differences in glycosylation were observed. SP2/0 galactosylated the IgG to a larger extent if cultivated in a STR than in a hollow fiber reactor (Nahrgang et al., 1999) with a shifted of G0/G1/G2 from 65%/31%/4% in hollowfiber bioreactor to 24%/56%/20% in STR. Cultivation of CHO was generally carried out in serum-free medium whereas SP2/0 often requires serum for growth. SP2/0 contained minor amounts of sialylated product (Nahrgang et al., 1999). The products from CHO contain less G2 and more G0 in CHO MDJ8S than two other cell lines, i.e., HEK 293 and Sp2/0 (Nahrgang et al., 1999). Among the two CHO cells (CHO MDJ8S and CHO MDS) tested, there was little differences in glycol profile (Kunkel et al., 1998).

4.2. Growth rate

12

Hahn and Goochee observed that the glycoprotein transferring, secreted by confluent and subconfluent cultures, contains different proportions of biantennary oligosaccharides. The biantennary glycoprotein was more biologically active. Confluent cells produce more active transferrin than the subconfluent cultures. Hahn and Goochee (1992) concluded that oligosaccharide synthesis is growth-dependent. Thus in standard batch culture where the growth rate varies throughout the fermentation, the glycosylation pattern will vary (Hahn and Goochee, 1992). Protein synthesis rates and the resulting protein glycosylation were investigated. Lowering the protein synthesis rate with cycloheximide improved the glycosylation site occupancy of recombinant protein produced by C127 murine cells (Shelikoff et al., 1994). However, studies on tPA synthesis in CHO cells suggested that the protein synthesis rate has little effect on protein glycosylation (Bulleid et al., 1992). Overall sialylation was increased in perfusion cultures compared to fed-batch (Kunkel et al., 2000). The slower growing cells in the perfusion mode facilitated a more fully glycosylated protein compared to the fed-batch mode where cells grew faster (Kunkel et al., 2000).

4.3. Medium and nutrients

Cell culture medium determines cell growth environment and physical conditions that have crucial impact on cell growth, productivity as well as product quality including glycosylation. Nutrient supplements include sugar feeding, nucleotide feeding, oxygen sparging, amino acid additions, and serum components.

4.3.1. Serum

Bovine serum has been used in mammalian cell culture as a nutrient supplement as well as to protect cells from pH fluctuations or shear forces for several decades. Serum contains growth factors which improve cell growth, and lipids which improve shear resistance. However, serum also contains waste products and proteases, which can be detrimental to the cell and glycoprotein products (Harcum, 2006). A monoclonal IgG1 produced by mouse hybridoma in serum-free media had higher levels of terminal N-acetylneuraminic acid (NANA) and Gal compared to cultures with serum, whereas terminal Gal was higher from CHO cells cultured in media with serum (Hossler et al., 2009; Patel et al., 1992).

4.3.2. Glucose

Glucose-limited chemostats were analyzed in order to demonstrate whether glycosylation was dependent on media components (Gawlitzek et al., 2000; Hayter et al., 1993). Hayter et al. (1993) examined the glycosylation pattern of Interferon (IFN)-gamma produced by

CHO cells at a constant dilution rate and with two different glucose concentrations. They were able to demonstrate that fully glycosylated IFN-gamma occurred more readily when glucose was not limited. It was concluded that this effect was due to the physiological state of the cells. Galactosylation could be slightly improved by addition of glucose to the culture medium (Nahrgang et al., 1999). The use of different media for production also resulted in minor variations in the ratio between galactosylated structures (Nahrgang et al., 1999). Galactose feeding can help facilitate a more fully galactosylated N-glycan profile (Hossler et al., 2009). Studies of CHO fed-batch cultures producing IFN-gamma revealed that glutamine and glucose levels below o.1 mM and 0.7 mM, respectively, led to decreased sialylation profiles and an increase in hybrid and high mannose type glycans (Wong et al., 2005). In contrast, continuous cultures of BHK-21 cells expressing a human IgG-IL-2 fusion protein under low glucose and glutamine concentrations, showed no difference in the oligosaccharide profile compared to a nonnutrient limited culture (Cruz et al., 2000).

13 *4.3.3. Ammonium*

The amount of Gal and NANA residues on TNFR-IgG correlated in a dose-dependent manner with the ammonium concentration under which the N-linked oligosaccharides were synthesized. As ammonium increased from 1 to 15 mM, a concomitant 40% decrease was observed in terminal galactosylation and sialylation of the molecule. Ammonium seems to alter the carbohydrate biosynthesis of TNFR-IgG by a pH-mediated effect on glycosyltransferase activity (Gawlitzek et al., 2000). The major effect of ammounia on glycosylation is a decrease in terminal sialylation. It was reported that even a low level of ammonia (2 mM) could affect the sialylation of O-glycans (Andersen and Goochee, 1994).

4.3.4. Others

Lipid supplements and carriers (dolichol) have been shown to improve N-glycan site occupancy of IFN-gamma (Castro et al., 1995; Jenkins et al., 1994). Manganese was added to the cell culture medium resulted in increased glycosylation both in O- and N-linked glycan (Crowell et al., 2007).

4.4. Culture condition

Control of the dissolved oxygen (DO) level is important to maintain optimal metabolism and growth of producer cells in bioprocesses. The effect of dissolved oxygen on the glycosylation of a recombinant protein from CHO cells was observed by a changing glycoform profile. By controlling DO set-points between a range of 1 to 100% air saturation, the terminal galactosylation of an IgG was changed significantly with a gradual decrease in the digalactosylated glycans (G2) from 30% at the higher oxygen level to around 12% under low oxygen conditions (Kunkel et al., 1998). The pH of the medium was shown to have some effect on the distribution of glycoforms of IgG secreted by a murine hybridoma (Muthing et al., 2003; Rothman et al., 1989). As pH increased from 6.9 to 7.4, the G2 content was increased from 16% to 32% (Muthing et al., 2003). During the cell death phase, the antibody produced from CHO cells showed a decrease in G2 and an increase in G0 (Kaneko et al., 2010).

5. Recombinant protein production by transient gene expression

14 Transient gene expression (TGE) has been actively pursued over the past decade. The approach offers the advantages of short development time (product production within several weeks), leading to much lower development costs when compared to stable cell line development (Table 4). Furthermore, the quality of the products obtained from TGE is suitable for preclinical assessment, thus speeding the "Proof of Principal" stage in which large biopharmaceutical companies screen multiple drug candidates prior to advancing them into the formal development pipeline. Technically, all the strategies used to optimize expression in a stable cell line development can be used and evaluated in TGE to assess their potential prior to committing significant resources to create a stable cell line. Due to quick turnover and low cost, TGE is used as the first step to screen expression strategy in terms of construction design and molecular candidates (Zhang et al., 2009).

Table 4
Transient vs stable expression in mammalian cells.

Transient expression	Stable expression
Expression of transfected genes are usually within 16–96 h, but expression does not propagate through generations	Heterologous DNA is integrated into the host genome and is maintained throughout many generations.
Transfection requires a large quantity of plasmid DNA (≥1 mg DNA/L cell culture).	Cell line development requires an extensive period (6–12 months).
Expression level usually is relatively low (1–100 mg/L).	After amplification and high producer selection, expression level is relatively high (50–5000 mg/L).
Expressed product is good for pre-clinical assessment, but not for clinical human use.	Stable cell lines can be used for large scale manufacturing leading to clinical trials and commercialization.
It takes only short period to express a product for "Proof of Principal" demonstration.	High cost
Low cost	

Transfection of DNA into a mammalian cell involves deliberately forcing nucleic acids into cells using high-voltage electric shock "electroporation" (Potter et al., 1984), or using chemical mediators such as calcium-phosphate or lipofection (Sambrook and Russell, 2001). Calcium phosphate is a well-established, inexpensive, high-efficiency DNA delivery vehicle. Unfortunately, it does not work well with CHO cells (Batard et al., 2001) and the time-sensitive nature of the transfection protocol makes implementation at large scale a challenge (Baldi et al., 2007; Majors et al., 2008). These established methods and protocols using lipofectamine demonstrated high transfection efficiency in introducing plasmid DNA into CHO and other cells (Matin-Montanez et al., 2010). However, the cost of lipofection transfection reagents is usually high. Therefore, it is not economically feasible to make gram-scale quantities of product using these reagents during pilot scale manufacturing. Likewise, electroporation devices are excellent to transfer DNA into milliliters of cell culture, making them efficient only for small-scale operation. Thus, transfection reagents and electroporation are limited to laboratory use, and are not practical for large scale production.

15 Polyethylenimine (PEI) was discovered to have had high gene transfer activity in many cell lines with an acceptable degree of cytotoxicity (Boussif, 1995; Ehrhardt et al., 2006; Godbey et al., 1999). More importantly, PEI enabled the cost-effective and practical transfer of DNA into many different cell lines with transfection rates in the range of 40–90% (Ehrhardt et al., 2006). For a 10 L transfection protocol, 293Fectin cost $4000; XtremeGENE cost $5000; whereas PEI cost was less than a dollar (Morrow, 2008). Given that their performances are roughly equivalent, PEI is preferably selected for large-scale manufacturing use (Morrow, 2008). PEI effectively condenses DNA into positively-charged particles that bind to anionic cell surface residues and enter into cells via endocytosis. Once inside the cell, protonation of the amines results in an influx of counter-ions and a lowering of the transmembrane osmotic potential. Osmotic swelling results and the vesicle bursts releasing the polymer–DNA complex (polyplex) into the cytoplasm. If the polyplex unpacks, the DNA is then free to diffuse into the nucleus (Akinc et al., 2004; Rudolph et al., 2000). Since the gene transfer vehicle was effective for large scale transfection of mammalian cells grown in suspension

culture, use of PEI as a transfection reagent was one of the major breakthroughs for rapid production of gram quantities of Mabs and recombinant proteins. Furthermore, the polymer/DNA ratio was carefully optimized and a simple robust transient expression system utilizing the PEI was developed with CHO (Derouazi et al., 2004; Tait et al., 2004), HEK293 and other cells (Ehrhardt et al., 2006). Subsequently, PEI has been widely used for transient gene expression, and has become a standard transfection reagent for recombinant protein production in many different cell types (Boussif, 1995; Cho et al., 2001, 2003; Godbey et al., 1999), and at production scales up to 100 L–150 L (Derouazi et al., 2004; Muller, 2005).

In addition to the low cost and possibility of producing gram quantities of final product, the PEI-based TGE technology platform has been successful for expression with high titers of antibodies and recombinant proteins (Table 5). Repeated reports of significantly increased expression levels have appeared. For recombinant proteins, 50–100 mg/L expression levels have been obtained. This enables gram-scale recovery from a small number of 20 L bioreactor runs, facilitating early biopharmaceutical assessment of promising therapeutics in animals and enabling determination of dosing ranges needed for toxicology and efficacy testing.

16 HEK293E and CHO cell lines are predominantly used in TGE technology for production at relative large-scales with expression levels up to 1 g/L for Mabs. The HKB-11 cell line, a hybrid of kidney cell and B-cell lines, was also used to express various recombinant proteins including IL-2SA (an interleukin 2 mutein) in a 10 L Wave bioreactors. Titers reached 40 mg/L, indicating the potential scalability of the cell line for production. In addition, many other cell lines, such as baby hamster kidney cell BHK-21, transformed African green monkey kidney fibroblast cell (cos-7) and mouse myeloma cell Sp2/0, have been used for transient expression (H.Y. Kim et al., 2011).

HEK293E cells expressing Epstein-Barr virus (EBV) nuclear antigen 1 (EBVNA1) allow episomal amplification of plasmids carrying the viral EBV origins of replication. Thus, they are expected to increase recombinant expression levels by permitting more plasmid copies to persist in the transfected cells throughout the production phase (Van Craenenbroeck et al., 2000). A detailed history of HEK 293E cells was summarized by Baldi (Baldi et al., 2007). HEK293E was used for a Mab production with yields over 1 g/L with TGE, which is a milestone breakthrough reported by Wurm's group (Backliwal et al., 2008a, 2008b, Table 5). An expression strategy incorporating multi-pathway modulation in HEK 293E cells was used to yield high production titers. Incorporation of a custom intron along with the Woodchuck Hepatitis Virus Post-transcriptional Regulation Element (WPRE) and promoter optimization increased expression from an initial value of 4 mg/L to optimized expression of 40 mg/L. After systematic comparison studies with human cell cycle regulatory proteins, growth factors, addition of histone deacetylase inhibitors and inhibitors of DNA methyltransferase, and an optimized combination of 37.5% of heavy chain, 10% of light chain, 10% of hp18, 10% of hp21 and 5% of fibroblast growth factor gene (FGF), transiently transfected HEK293E cells yielded over 1 g/L expression level. With this system, several non-IgG and Fc-fusion proteins were expressed with titers from 250 to 940 mg/L, the highest transient expression levels published to date. This strategy of co-expressing cell cycle, anti-apoptosis, and growth factor genes is extremely valuable for designing future optimized expression systems for either recombinant proteins or monoclonal antibodies.

17 The remarkable progress in TGE makes this approach attractive. There is growing interest in the possible use of this technology beyond the preclinical phase. This use will depend on convincing regulatory agencies that the products obtained from TGE have sufficient quality for first-in-human clinical trials. The HEK 293 cell line has been a host for the licensed glycoprotein, Xigris (activated Drotrecogin alfa, a recombinant form of human activated protein C), developed by Eli Lilly (www.fda.gov, Eichacker et al., 2006). The strategy of infecting cells to produce biopharmaceutical products has been commonly used in vaccine manufacturing. For example, an influenza viral-like particle vaccine is produced using baculovirus to infect sf9 cells and the product is in human clinical trials (www.novavax.com). From a regulatory perspective, a key question that must be answered is whether the lot–lot consistency and safety of the products produced by cell lines containing virus component nuclear antigens can be demonstrated. From safety point of view, and similar to stable cell lines, qualification of host cell bank and plasmid DNA, validation of viral clearance for a process using TGE are also critical expectations. In addition, product characterization through glycosylation pattern determination, specific antibody/antigen binding assays, PCR detection of residual plasmid DNA, and other modern analytical assays, such as high resolution mass-spectrum analysis, would be required to guarantee the quality and consistency of the products.

From a production process point of view, large quantities of plasmid DNA are required for transfection, which increases the overall manufacturing operation cost to make a large quantity of plasmid. Plasmid preparation requires *E. coli* fermentation, followed by cell lysis and DNA purification. Many high yield (500–2600 mg/L) fed-batch plasmid fermentation processes (Carnes et al., 2011; Singer et al., 2009; Williams et al., 2009) were reported. However, many of those studies were based on proprietary fed-batch media and heat inducible vector origin. One recent publication reported a simple large scale plasmid DNA preparation for TGE usage (Cheng et al., 2011). Fed-batch growth of *E. coli* was carried out in a 5 L bioreactor

Table 5
Recombinant protein produced with transient gene expression technology platform.

Cell	Expression	Product	Expression and process description	Reference
HEK 293E	81 mg/L	Mab	Enhancement by co-transfection of WPRE by 5 fold	Kim et al. (2009)
HEK 293E	1000 mg/L	Mab	1). Human CMV + Intron + WPRE (40 mg/L) 2). Co-transfection with 37.5% HC, 10%LC, 10% hp18 10% hp21, 5% FGF resulting in 1 g/L expression level 3). Process scaled-up to 2 L	Backliwal et al. (2008a)
HKB-11	40 mg/L	Various	Produced in 10 L Wave Bioreactor.	Cho et al. (2001, 2003)
HEK 293E	50 mg/L	r-protein	Human placental secreted alkaline phosphatase (SEAP) used as reporter	Durocher et al. (2002)
CHO K1SV	80 mg/L	Mab	1). DMSO and LiAc increased expression 2). Fed-batch process maintained 14 days with 3 post-transfections at 2–4 days intervals 3). Product showed similar glycosylation patterns from batch to batch 4). Process scaled-up to 20 L in Wave bioreactor	Ye et al. (2009)
CHO DG44	22 mg/L	Mab	Process scaled-up to 110 L bioreactor with 80 L working volume	Muller (2005)
CHO	300 mg/L (5 mL) 250 mg/L (500 mL)	Mab	1). Reduced plasmid DNA by 50% 2). Transfection at a high density 3). Eliminating the culture dilution step after transfection	Rajendra et al. (2011)
CHO DG44	90 mg/L	Mab	Valproic acid (VPA) increased mRNA and protein levels	Wulhfard et al. (2010)

by maintaining the glucose concentration below 1 g/L after the feeding has begun. Plasmid yields of 490 and 580 mg/L were achieved with *E. coli* Top 10 cells bearing two different plasmids respectively (Cheng et al., 2011). Furthermore, cell paste was lysed under alkaline condition as a standard method (Sambrook and Russell, 2001) and one step of alcohol precipitation was used to purify the plasmids for TGE. While the process is simple and the plasmid DNA produced showed excellent quality for TGE, reproducibility of the overall process particularly the downstream process needs to be demonstrated. Nevertheless, it is still debatable that the plasmid with high endotoxin level produced using described method resulted in highest expression when transfect CHO cells (Cheng et al., 2011).

18 In addition to PEI, transfection medium is another critical component in a successful large-scale transfection. A list of media used in TGE was compiled in a review (Geisse et al., 2005). In many cases, one optimized mediun for cell growth and another optimum medium for transfection were used; however, both may not be necessarily compatible. Development of or adaptation of the host cells to a single medium that is optimal for both cell growth and transfection is highly desirable since this would eliminate process steps needed for media exchange before and after transfection. Alternatively, transfection performed at high cell density (20×10^6 cells/mL) may be possible to use most commonly used media and not be restricted to a specific medium (Backliwal et al., 2008a, 2008b). Most of protocols for transient transfection of suspension-adapted HEK-293 cells still require a priori complex formation of PEI with plasmid DNA for optimal yields and limit the choice of transfection and production media. However at high cell density, and higher concentrations of PEI and DNA (100 and 50 mg/mL, respectively), in situ complex formation outperforms a priori complex formation (Backliwal et al., 2008a, 2008b).

Medium additives may play important roles as well. Valproic acid (VPA) enhances recombinant mRNA and protein levels in transiently transfected CHO DG44 cells (Wulhfard et al., 2010). The steady-state levels of the IgG light and heavy chain mRNAs were nearly 10 times higher than in the untreated control transfection even though the level of transfected plasmid DNA was the same in the presence or absence of VPA (Wulhfard et al., 2010). Besides the improvements achieved through medium components and additives, expression levels were increased more than 3-fold at low temperature of 31 °C compared with37 °C (Wulhfard et al., 2008). Additionally, the procedure was further simplified via elimination of a dilution step after transfection (Rajendra et al., 2011).

The productivity of biopharmaceutical manufacturing also can be improved through transiently transfecting a stable production cell line with apoptosis inhibitory gene (Majors et al., 2008; Wang et al., 2011). As mentioned in previous section (Cell Line Engineering of the EXPRESSION SYSTEM), recombinant EPO production and sialylation in CHO were enhanced through transient expression of the *B. mori* 30Kc19 gene, which may represent a novel approach to improve the production and sialylation of recombinant glycoproteins in CHO cells (Wang et al., 2011). In an alternative method, using a cell line (e.g. CHO DG44) stably transfected with an anti-apoptosis gene from the Bcl-2 gene family such as Bcl-xL increased a fusion protein transient expression by 70–270%, indicating anti-apoptosis engineering is one of many possible strategies that affect the physiological state of cells with significant benefits to TGE yields (Majors et al., 2008; Stettler et al., 2007). To improve specific antibody productivity in CHO cells, post-translational modifications in the endoplasmic reticulum during antibody production was enhanced through transient overexpression of protein disulfide isomerase (Mohan et al., 2007), resulting in a 27–37% increase in productivity. Furthermore, using an inducible transient expression to co-overexpress endoplasmic reticulum oxidoreductase with disulfide isomerase, productivity was enhanced by 55% (Mohan and Lee, 2010). In contrast, under stable inducible co-overexpression conditions, the productivity was not affected.

Having listed the progresses in developing TGE for protein production, there are still some questions regarding large quantity production due partly to relatively low expression level (Morrow, 2008). An alternative strategy using stable transfection pool technology was reported to produce gram quantities of Mabs for preclinical development (Ye et al., 2010). Expression levels for Mabs can be reached in a range of 100–1000 mg/L at the 200 L scale (Ye et al., 2010). Besides, there are concerns regarding DNA-cationic complexes or lipoplexes in terms of structures and teterogeneous, while a lot of fundamental investigation is aimed at acquiring a better understanding of their molecules (Morrow, 2008). Fortunately, a number of analytical techniques are being applied in basic science laboratories to the lipoplex research, including dynamic light scattering, analytical ultracentrifugation, gel electrophoresis, circular dichroism, and fluorescence spectroscopy. These may result in generating a protocol with reproducibility and consistency during manufacturing (Morrow, 2008). Recent progresses in many aspects of TGE technology development: cell line engineering (Majors et al., 2008; Wang et al., 2011), plasmid preparation (Carnes et al., 2011; Cheng et al., 2011; Singer et al., 2009; Williams et al., 2009), media and additives development (Wulhfard et al., 2010), procedural simplification (Rajendra et al., 2011), and process automation (Zhao et al., 2011) will likely contribute to realization of large scale biopharmaceutical manufacturing using TGE platforms for preclinical and early clinical development in the near future.

6. Conclusion

In last two decades, mammalian cell protein expression has become the dominant recombinant protein production system for clinical applications, producing more than half of the biopharmaceutical products on the market and several hundreds of candidates in clinical development. Significant progress in developing and engineering new cell lines, introducing novel genetic mechanisms in expression, gene silencing, and gene targeting have been achieved. Understanding of the glycosylation has become one of focuses and transient gene expression technology platform plays more important role in biopharmaceutical manufacturing. This review has summarized the latest advancements in the field of mammalian expression of recombinant proteins for biopharmaceutical development, especially in active areas such as expression systems, glycosylation impact factors, and transient gene expression.

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