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Better and faster: improvements and optimization for mammalian recombinant protein production

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Abstract

Thanks to numerous technological advances, the production of recombinant proteins in mammalian cell lines has become an increasingly routine task that is no longer viewed as a heroic enterprise. While production in prokaryotic or lower eukaryotic systems may be more rapid and economical, the advantages of producing large amounts of protein that closely resembles the native form is often advantageous and may be essential for the realization of functionally active material for biological studies or biopharmaceuticals. The correct folding, processing and post-translational modifications conferred by expression in a mammalian cell is relevant to all classes of proteins, including cytoplasmic, secreted or integral membrane proteins. Therefore considerable efforts have focused on the development of growth media, cell lines, transformation methods and selection techniques that enable the production of grams of functional protein in weeks, rather than months. This review will focus on a plethora of methods that are broadly applicable to the high yield production of any class of protein (cytoplasmic, secreted or integral membrane) from mammalian cells.

Cell type

The workhorse of mammalian protein expression in an a pharmaceutical company is Chinese Hamster Ovary cells (CHO), due to their relative ease of use and long history of regulatory acceptance for the production of biopharmaceuticals [1]. The top selling biologic in 2012 was Humira, a monoclonal antibody made in CHO cells, directed against tumor necrosis factor alpha for the treatment of rheumatoid arthritis, with sales of close to 9 billion dollars [2]. In development and laboratory settings, Human Embryonic Kidney 293 (HEK) cells are commonly used and, other novel cell lines may provide even more desirable properties for protein production. These include the human retina-derived Per.C6 and amniocyte-derived Cap-T lines, which are capable of very high-density growth (~5–15*10^7 cells/ml) that supports concomitant increases in protein yield and decreased media costs (reviewed in [3, 4]). Cell types may also be chosen, or engineered, to alter the extent of post-translational modifications such as glycosylation, lipidation, sulphation etc., which can

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modulate the activity of the target protein (e.g., 10–20 fold in the case of an anti-CD20 antibody) [5, 6]. Unfortunately these post-translational modifications, while essential for function, may be inversely correlated with the success of structural studies, as increased heterogeneity can adversely affect crystal packing [7]; but numerous options are available for the mitigation of these challenges. Several investigators have attempted to limit or homogenize glycosylation by the use of processing-deficient strains, such as the N-acetylglucosaminyl transferase I (GnTI) deficient HEK293S GnTI(−) line in the production of the hormone glucagon. Alternatively, inhibitors of glycosyl processing enzymes, such as kifunensine (that targets mannosidase 1) or swainsonine (that targets mannosidase 2), can be added to the growth media, which result in modifications that are more amenable to enzymatic removal of sugar moieties post-production. [8, 9].

Cell growth conditions

Advances in serum free media formulation allow for high-density cell growth (>1*10^6 cells/ml) in the absence of serum, which simplifies downstream purification and eliminates animal-based components, which alleviates some regulatory hurdles. Media has been optimized for protein production using design of experiment (DOE) approaches or metabolic analysis to derive optimized media, with the goal of increasing the protein yield per cell or volumetric yield (e.g., optimized CHO cell media supporting the ten-fold greater production of Tumor Necrosis factor fusion protein over yields from unoptimized basal media [10–12]). Additives can be used to supplement growth media such as histone deactylase inhibitors (e.g. valproic acid or sodium butyrate) to de-condense chromatin and increase the transcriptional activity of integrated genes with a concomitant enhancement in protein yield (e.g., four-fold increase in yield for an antibody produced in HEK293E cells after valproic acid addition). Proprietary feed solutions (HyClone Cell Boost, Thermo Scientific Inc) have been shown to increase yields and growth times by supplementing essential components that have become depleted in conditioned media; for example, doubling of the lifetime of a batch culture of CHO cells producing tissue plasminogen activator [13, 14]. Growth factors can also be added to the media. For example, the LONG R^3 IGF-I engineered peptide appears to show culture enhancing properties, such as doubling cell viability over a 12 day experiment for some cells, including CHO, HEK293 and PER.C6, when compared to more routinely used insulin additives [15–17]. Growth factors and cell cycle regulators may also be introduced by co-transfection of expression plasmids (such as acidic fibroblast growth factor as demonstrated in Backliwal et al, [18]), rather than by addition of purified proteins, with a resulting savings in cost.

Cell growth and protein yields may also be made more robust by the use of carefully controlled growth environments (such as bioreactors) or by providing microenviroments via the use of micro or macrocarriers (Cytodex or Cytopore from GE Lifesciences or Fibra-Cel from New Brunswick) which shield cells from harmful shear forces and can increase yields 2–5 fold [19–21]. Hypothermic shifts (37°C to 33°C) have also been found to be advantageous for increasing cell specific productivity (e.g., at least two-fold for a variety of test proteins) and in particular for CHO cells [20, 22]. Growth vessels for cell culture have been improved by the introduction of convenient disposables for high-density growth in suspension such as the Tubespin bioreactor and the Optimum Growth™ flask (Thompson

Instrument Company), which allows high-density growth with a smaller footprint than fernbach flasks [23]. For scale up (i.e., lab scale), many variants of 'wave' reactors (GE Lifesciences) are available including newer versions that rock in two rather than one direction, giving rise to more efficient mixing and mass transfer and potentially high cell viabilities (XRS Bioreactors from Pall Corporation); other platforms include hollow fiber bioreactors (FiberCell Systems Inc) which provides very high cell densities of cells in low volumes (up to $1*10^8$/ml), various air lift bioreactors (Cellexus Inc), and even those with merry-go-round type waterwheels inside (PBS Biotech Inc); many of these technologies are relatively novel (especially in the disposable format), so head-to-head comparisons of utility are currently unavailable.

Transforming the cells and expression vectors

Large-scale transient transfection, which is useful for very rapid (days) protein production, is now an effective option, with more than 50 reported successful attempts to produce useful proteins, and various optimizations have lead to simpler operational procedure at even the hundred liter scale, giving rise to gram levels per liter of production [23–26]. In a highly optimized large-scale transient transfection protocol, grams per liter of recombinant antibodies have been generated by engineering the expression vector to utilize a human CMV promoter, an artificial intron and woodchuck post-transcriptional regulatory element (WPRE) [18]. This result highlights, not surprisingly, that the choice of promoter and other vector elements are critical factors in determining protein expression yields. This study also utilized high cell density transient transfection mediated by the economical transfection reagent, polyethylenimine (PEI), along with a series of plasmids expressing growth factors and cell cycle regulators. The importance of different 5′UTR and polyadenylation sequences in expression vectors has been investigated in other studies and they have been found to be of utility in particular cases. [27–29].

Design of experiment has also been utilized to optimize various parameters for large-scale transient gene expression and has shown that DNA concentration may be held at a relatively low level whist achieving good protein expression yields. This work is interesting as the production of large amounts of high quality (i.e. endotoxin free) DNA could be a severely limiting factor [30]. Relatively inexpensive, yet effective transfection reagents, in particular PEI, allow for efficient transfection at both small (sub milliliter) and large-scale (hundreds of liters) [24, 31, 32].

Piggybac transposons offer a method of integrating many copies of the transgene into the genome of the cell, leading to high levels of stable protein production (i.e., months) from clonally sorted or polyclonal populations. [33, 34], The highly active piggybac transposase specifically targets transposition into active areas of the mammalian genome (not silenced by chromatin structure) resulting in greater levels of protein expression. PiggyBac systems have been used to produce some highly interesting and active complexes (such as human gamma secretase) and also come in a single plasmid version that confers the ability to exploit inducible expression without extra modification of the host cell type (e.g., in contrast to the T-rex system [35–37].

Multiple systems are available for gene delivery and protein expression based on viral vectors. The optimized “Daedalus” lentiviral protein production system [38] has demonstrated the effectiveness of using lentivirus to rapidly (within a few weeks) generate stable cell lines for the production of mammalian proteins at up to the 100mg/L scale. This system employs a unique and minimized Ubiquitous Chromatin Opening Element (UCOE) of only 0.7kb to help maintain stable protein expression over several months. This optimized UCOE fragment supports the long-term culture of transduced cell lines in shake flasks or bioreactors for maximizing protein yield. Inducible systems are also available that may benefit from the absence of negative selection due to expression of toxic gene products during viral amplification, which could reduce titer [39].

Other popular systems are the BacMam system, in which insect cell viruses are generated in insect cells and subsequently used at high multiplicities of infection (MOI) to transduce mammalian cells [40]. Dukkipati *et al*. have used BacMam viruses to express GPCR-protein ligand complexes for structural studies in mammalian cells; the virus was engineered to encode a promoter (CMV) that is only active in mammalian cells, and not the insect cell. Another viral system derived from Semliki forest virus has been utilized for protein production after optimization to reduce cell cytopathic effects leading to more stable expression over time [41], with yields as high as 2mg/L/day.

Selection methods for high yields

Stable cell lines have a long history of being generated by integrate-and-select approaches, which while effective, are slowly being superseded by more rapid and facile technologies (i.e., a couple of weeks rather than ~6 months) [7]. Several selection (and amplification) techniques have exploited the ability of the expression plasmid to rescue the growth of an auxotropic strain in media deficient for a crucial compound, with the severity of auxotrophy being augmented (or produced) by supplementation of the growth media with inhibitory compounds that block key biosynthetic pathways. A notable example is dihydrofolate reducatase (DHFR)-enabled gene amplification, which can be used in DHFR-deficient cells in conjunction with inhibition of residual DHFR enzyme by the drug methotrexate, leading to amplification of the gene of interest that is supplied in an expression vector physically linked to the rescuing DHFR gene. Selection and amplification via this system has enabled the production of gram levels of protein (often antibodies) per liter in CHO cells, and improvements are being developed to increase the rapidity of amplification. Strategies to increase the speed of gene amplification include weakening the selection marker (such as via fusion of the murine ornithine decarboxylase PEST protease targeting sequence) and the use of DNA sequences that lead to spontaneous gene amplification in transformed cells (such as the cassette of the mammalian replication initiation region (IR) matrix attachment region (MAR)). [42–45]. In highly analogous systems, selection by the introduction of the glutamate synthase gene into cells that are deficient in this vital enzyme, or rendered so by the inhibition using methionine sulfoximine, is frequently used for protein production (maximal antibody yields of 5g/L, with a 20–25 weeks timeline from cloning to large-scale fermentation) and is offered commercially by Lonza, Inc [46]. Another system, called OSCAR™, uses a cell line that is deficient for purine synthesis. Cells are transfected with a rescue vector that supplies the gene of interest and a partially disabled hypoxanthine

phosporibosyltransferase gene thus enabling selection for cell survival and amplification of the gene of interest in a single step [47]. Upon transformation and selection, a polyclonal pool of cells is established and further selection to a pure clonal line may be desirable to tease out highest producing cell lines. This often involves limited dilution to raise a single cell population. To aid in these efforts, flow cytometry has been successfully employed to enrich for high-level expressors via selection of cells that are fluorescently marked, either by co-expression of a fluorescent protein, or by a fluorescent protein fusion [48–50]. An intriguing selection method is Laser Enabled Analysis and Processing (LEAP), which involves imaging live adherent cells in bright field and fluorescent mode, with the degree of fluorescence being a marker of target protein expression [51]. The cells with zero or low target protein expression are destroyed via a laser pulse, leaving all other cells untouched. This process can be repeated at time intervals leaving a high expressing population of cells that have remained unmolested by the rigors of flow cytometric processing.

Discussion

It is an exciting time for the production of increasingly complex proteins and assemblies in greater yields and with ever decreasing timescales. Enormous attention continues to focus on the generation of mammalian expression systems to support academic and biopharma efforts. Overall, there are many effective paths for generating proteins and optimizing yields; perhaps lacking at this point are objective comparative studies that accurately evaluate the benefits (and weaknesses) of the many system with respect to one another. Despite this shortcoming, it is clear that the production of eukaryotic proteins via mammalian cell culture is becoming a more routine, rapid and successful route to realizing material for structural, functional and therapeutic use.

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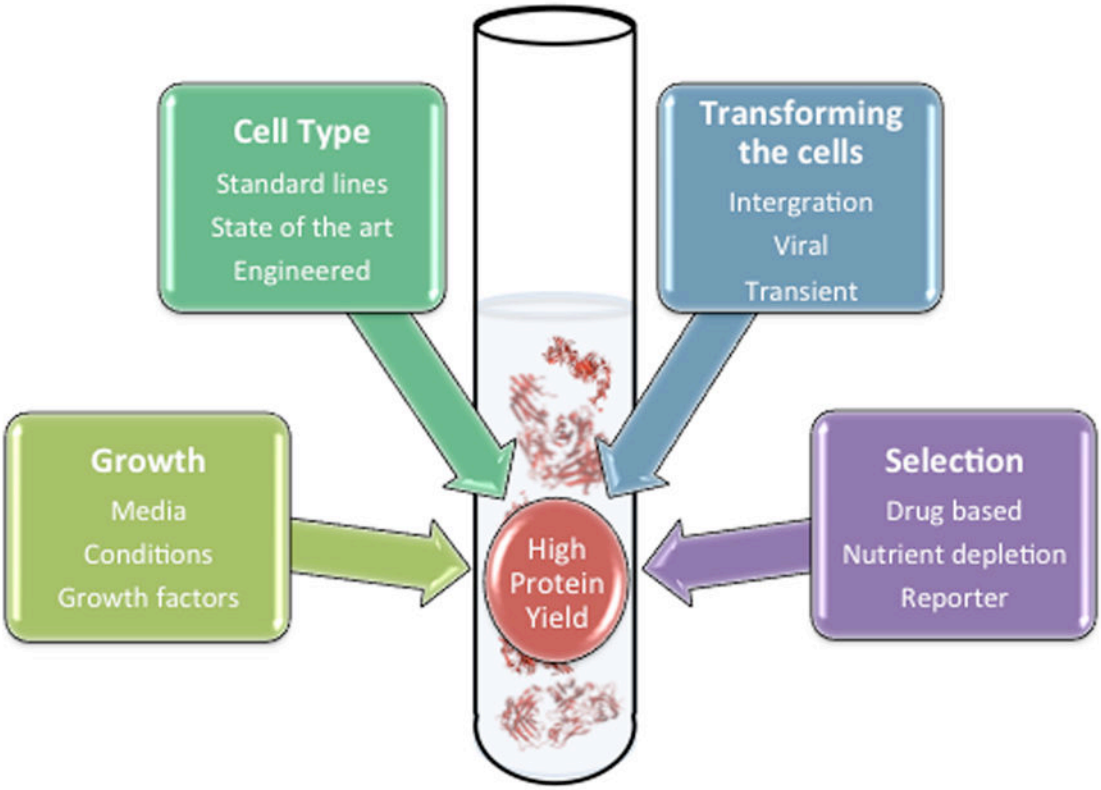

Figure 1.
Options for generating and selecting cell lines to produce high yields of functional proteins.