

Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives

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Abstract The expansion of the biologics pipeline depends on the identification of candidate proteins for clinical trials. Speed is one of the critical issues, and the rapid production of high quality, research-grade material for preclinical studies by transient gene expression (TGE) is addressing this factor in an impressive way: following DNA transfection, the production phase for TGE is usually 2–10 days. Recombinant proteins (r-proteins) produced by TGE can therefore enter the drug development and screening process in a very short time—weeks. With “classical” approaches to protein expression from mammalian cells, it takes months to establish a productive host cell line. This article summarizes efforts in industry and academia to use TGE to produce tens to hundreds of milligrams of r-proteins for either fundamental research or pre-clinical studies.

Keywords Mammalian cells · Non-viral gene delivery · Recombinant protein · Transient gene expression

Introduction

The production of recombinant proteins (r-proteins) has been an important technology over the last twenty years with the main focus being on biopharmaceutical products with medical applications. Most r-proteins in this category are complex molecules that need to be post-translationally modified for full bioactivity. As such modifications are only accurately performed by mammalian cells, they have become the major host for complex r-protein expression (Wurm 2004). Pharmaceutical companies routinely establish recombinant cell lines that constitutively produce r-proteins for extended cultivation times. This stable gene expression is achieved through the insertion of the recombinant gene(s) into the host genome. However, identification and characterization of recombinant cell lines is a costly and time-consuming process. Faster approaches to r-protein production are sometimes preferred when one must rapidly evaluate many candidate proteins or when several variants of a single protein need to be studied for their potential as biologics. Transient gene expression (TGE) is well-suited to fill this need. In contrast to stable gene expression, TGE involves short-term r-protein production typically for up to 10 days post-transfection in the absence of genetic selection of the plasmid DNA. Secreted, membrane, and intracellular proteins have been successfully

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expressed by TGE (Meissner et al. 2001; Geisse and Henke 2005; Shi et al. 2005), and high-throughput approaches for TGE in human embryonic kidney (HEK 293) cells have recently been described for the production of G-protein coupled receptors (Davies et al. 2005). This review emphasizes the main advantages and drawbacks of large-scale TGE performed with suspension cultures of mammalian cells (the term “large scale” is implied for work with cultures greater than 1 l in working volume) and describes challenges for further improvement of the technology.

Expression vectors

Different expression vectors, both non-viral and viral, have been designed to transfer foreign genes into mammalian cells. The choice of the vector depends on the application, the host cell, the time limitation, the level of desired product, and safety. Only a brief overview of this subject is provided here. Additional information may be found in recent reviews (Makrides 1999; Van Craenenbroeck et al. 2000).

Non-viral expression vectors

Essential elements of typical TGE vectors include (1) a constitutive or inducible promoter; (2) a transcription terminator; and (3) a prokaryotic cassette with a replication origin and a selection marker for vector production in bacteria. Most of the commonly used strong constitutive promoters are from viral genomes including the human cytomegalovirus (HCMV) immediate early promoter, the simian virus 40 (SV40) early promoter, and Rous sarcoma virus long-terminal repeat promoter. Cellular promoters such as that of the human elongation factor-1- α (EF-1 α) gene have been shown to be similar in strength to the HCMV promoter (Gill et al. 2001). Although most cDNAs can be expressed at high levels from a strong constitutive promoter, insertion of an intron between the promoter and the 5'-end of the cDNA has been shown to increase the r-protein expression level (Buchman and Berg 1988). Mammalian cells expressing the Epstein-

Barr virus (EBV) nuclear antigen 1 (EBNA1) or the SV40 large-T antigen, support the episomal amplification of vectors carrying the EBV or the SV40 origin of replication, respectively. This strategy improves r-protein production levels by increasing the plasmid copy number in the transfected cell population (Van Craenenbroeck et al. 2000; Young et al. 1988).

TGE requires a significant amount of plasmid DNA. Typical protocols use about 1–1.25 μ g plasmid DNA per ml culture. DNA is thus a major cost-factor for large-scale TGE. Two of the most important aspects of the plasmid purification are the recovery of a high percentage of supercoiled DNA and the elimination of contaminants such as bacterial RNA and endotoxin (Stadler et al. 2004). Although it has been reported that these contaminants do not significantly affect the efficiency of transfection with the calcium phosphate (CaPi) method (Wright et al. 2003), the removal of lipopolysaccharide (LPS) is critical in downstream processing of the r-protein (Wakelin et al. 2006).

Viral expression vectors

Semliki Forest virus (SFV) is the most widely used viral expression vector for TGE. Large-scale production of a recombinant G-protein coupled receptor has been performed with SFV-infected mammalian cells cultured in suspension in bioreactors or spinner flasks (Blasey et al. 2000; Hassaine et al. 2006). Baculovirus vectors have also been used to produce r-proteins in both insect and mammalian cells (Boyce and Bucher 1996; Hofmann et al. 1995; Kost et al. 2000). The disadvantage of using insect cells is that they are unable to produce fully active recombinant mammalian glycoproteins due to the absence of sialylation. Thus, efforts have been made to engineer baculovirus expression systems that allow production of humanized recombinant glycoproteins in insect cells (Jarvis 2003; Seo et al. 2001). For the expression of r-proteins in mammalian cells, baculovirus vectors have numerous advantages including: (1) the absence of viral replication, (2) the lack of cytotoxicity, (3) the technical simplicity of vector production, and (4) the large DNA insert capacity (up to 38 kb)

allowing the delivery of multiple genes from a single vector (Kost et al. 2000). Finally, vaccinia virus vectors have been used to produce r-proteins such as nerve growth factor (Edwards et al. 1988), Factor VIII (Pavirani et al. 1987), and human pro-thrombin (Falkner et al. 1992). The infectious nature of the virus is a major concern, and it requires containment in a Biosafety Level 2 laboratory. To reduce safety risks, less infectious strains such as the modified vaccinia virus Ankara (MVA) have been used (Sutter and Moss 1992).

Non-viral gene delivery vehicles

A multitude of physical and chemical methods have been developed for the transfer of DNA into cultivated mammalian cells, but only a few of these are applicable to large-scale TGE. In general, the chemical agents used for transfection can be classified into inorganic compounds, cationic polymers, and cationic lipids. Among the methods employing inorganic compounds, CaPi-DNA coprecipitation is still the most widely used (Jordan et al. 1996). The most efficient cationic polymers are polyethylenimines (PEIs), most notably either branched or linear 25 kDa PEI (Boussif et al. 1995; Thomas and Klibanov 2003). Even though cationic lipids are very effective transfection agents, they have not been frequently used for large-scale TGE due to the comparatively high cost. All of these chemical agents face the same barriers regarding the delivery of functional DNA into the nucleus of cells (Lechardeur et al. 2005). Physical gene transfer methods such as microinjection, electroporation, and ballistics bypass these cellular barriers and deliver naked plasmid DNA directly to the nucleus. However, these methods have not been successfully adapted to large-scale TGE.

CaPi remains one of the most efficient DNA delivery agents, as it allows transfer of plasmid DNA to approximately 80–90% HEK 293 cells under some conditions (Meissner et al. 2001). Although it is inexpensive and does not interfere with protein recovery, there are several drawbacks to its use including the time-sensitive

formation of the coprecipitate, making transfection of large volumes technically challenging. The requirement for serum in the transfection medium to limit the “growth” or aggregation of the coprecipitate into large inefficient complexes is another critical constraint. Furthermore, CaPi is not an efficient delivery method for chinese hamster ovary (CHO) cells without an osmotic shock (Batard et al. 2001). In a variation of the traditional CaPi method, Lindell et al. (2004) demonstrated that addition of DNA and CaCl₂ directly to a suspension culture of HEK 293 cells (“Calfection”) resulted in the production of about 3 mg/l antibody in a 30 l culture.

Currently, the most widely used DNA delivery agent for large-scale TGE appears to be PEI. Transfections with linear 25 kDa PEI have been the most productive to date with reported IgG yields up to 80 mg/l in HEK 293 cells (Wuhlfard and Wurm, unpublished results) and 22 mg/l in CHO cells (Muller 2005) (see Table 1). PEI is moderately cytotoxic and non-biodegradable. Importantly, the effects of residual PEI on the downstream processing of r-proteins and on r-protein quality have not been systematically addressed.

Large-scale TGE

Cell lines

Several cell lines have been used for transient expression of r-proteins, but only a few of them present the advantages of (1) high transfectability with common methods (2) ease of adaptation to suspension cultivation in serum-free conditions, and (3) cost-effective scalability. HEK 293 is the most commonly used cell line for large-scale transient transfection. It was created nearly 30 years ago by transformation of primary human embryonic kidney cells with sheared fragments of adenoviral DNA (Ad5) (Graham et al. 1977), and they constitutively express the adenovirus E1A and E1B genes. In order to support large-scale adenovirus production in bioreactor cultures, this cell line was adapted to suspension growth, and the resulting cell line was named 293N3S

Table 1 Transient expression of recombinant proteins at scale of more than 1 l final volume

Product	Cells	Transfection agent	Serum	Scale (litre)	Culture system	Maximum titer (mg/L)	Maximum yield (mg)	Reference
TNF-Rp55	HEK293E	PEI	no	2	Erlenmeyer	0.55	n.d.	Schläger and Christensen (1999)
huIgG	HEK293E	CaPi	yes	110	10 l STR	7.7	500	Girard et al. (2002)
SEAP (a)	HEK293E	PEI	yes	3.5	Helical ribbon	20	60	Durocher et al. (2002)
Tie-2 ED	293-SFE	PEI	no	14	STR	n.d.	33	Pham et al. (2003)
NP-1	293-SFE	PEI	no	10	STR	n.d.	35	Pham et al. (2003)
various	HKB11	DMRIE-C	no	10	Wave™	40 (b)	400	Cho et al. (2003)
hu-IgG	HEK293E	Calfection	yes	30	110 l STR	2.5	75	Lindell et al. (2004)
hu-IgG	CHO (c)	PEI	no	13	20 l STR	6	n.d.	Derouazi et al. (2004)
SEAP	HEK293E	PEI	yes	1	1 l STR	25	n.d.	Pham et al. (2005)
SEAP	HEK 293F	PEI	no	1	1 l STR	10	n.d.	Pham et al. (2005)
>30	HEK293E	PEI	no	10	Wave™	12	n.d.	Geisse and Henke (2005)
various	HEK293E	PEI	no	2	Shake bottle	27	145	Baldi et al. (2005)
hu-IgG	CHO (c)	PEI	no	80	110 l STR	22	n.d.	Muller (2005)
various IgGs	CHO-K1s	LF (d)	no	1	Spinner	20	n.d.	Rosser et al. (2005)
hu-IgG	HEK293E	PEI	no	4	Biotainer™	4.25	17	L. Baldi (e)
B2-CD1d	HEK293E	PEI	no	2	Shake bottle	n.a.	24	L. Baldi (e)
hu-IgG	CHO (c)	PEI	no	10	Biotainer™	17.5	n.a.	M. Stettler (e)
hu-IgG	CHO (c)	PEI	no	10	Wave™	9	n.a.	M. Stettler (e)
hu-IgG	HEK293E	PEI	no	10	Shake bottle	80	n.a.	S. Wuhlfard (e)

(a) and 13 other proteins; (b) only 8 over 50 proteins express at titers >10 mg/l; (c) CHO DG44 cells; (d) LF, Lipofectamine; (e) unpublished results

Abbreviations: n.d., not described; n.a., not available; DMRIE-C, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide/cholesterol; STR, stirred-tank bioreactor; HEK293E, HEK 293 EBNA; 293-SFE, HEK 293 serum-free EBNA cells; HEK 293F, HEK 293 Freestyle (commercially available from Invitrogen).

(Graham 1987). Yet another cell line (293S) was generated through gradual adaptation to serum-free medium (Garnier et al. 1994). A subclone of this cell line (293SF-3F6) has been further selected for its capacity to grow at high density (8×10^6 cells/ml) in serum-free suspension and is frequently used for the production of adenoviral vectors (Côté et al. 1998). In the attempt to specifically improve TGE through promoting episomal replication of the exogenous plasmid DNA, two other HEK 293 derivatives have been generated: HEK 293-T and HEK 293-EBNA that constitutively express from stably transfected gene the SV40 large T antigen and the EBV EBNA1 protein, respectively (see section on non-viral expression vectors). Another cell line

(HKB11) was derived from HEK 293 cells through the fusion with a human B cell lymphoma (Cho et al. 2002). This cell line was successfully used in a 10 l Wave bioreactor system for TGE (see below). Recently a few groups described successful TGE in CHO cells previously adapted to serum-free suspension growth (Derouazi et al. 2004; Rosser et al. 2005). The two strains of CHO most commonly used for TGE are K1 and the dihydrofolate reductase mutant DG44 (Urlaub and Chasin 1980). These cells have been widely used for decades in industrial laboratories. They have a very high productivity potential and there is a well-developed understanding of their behaviour in bioprocesses. In particular, when TGE is used as screening tool for drug development, it is

highly desirable to rapidly obtain CHO-derived material for preclinical tests, as this will be the host cell for stable cell line development. Although there is no evidence that HEK 293-derived r-proteins differ in any way from CHO-derived material, the pharmaceutical industry prefers to use CHO cell lines for production because of their established track record. A few successful reports of TGE in CHO at large scale (larger than 1 l) are summarized in Table 1.

Production media

Due to intensive research into media formulations for the manufacture of r-proteins, serum-free cultures are now the standard for growth of mammalian cells in suspension (Barnes and Sato 1980). The need to minimize animal-derived components in production processes, both for biosafety reasons and to facilitate downstream processing, has created an interest in using protein hydrolysates (peptones) as a protein supplement to replace serum. Hence a number of chemically defined media have been developed which do not contain animal-derived material. These media generally support high cell densities and facilitate the expression and purification of r-proteins. Most often these media have been developed specifically for serum-free cultivation of HEK 293 or CHO cells. Initial attempts to perform transient transfection in these serum-free media failed, mainly because of the presence of polymers that hinder transfection complex stability and uptake. Transfection in these media has then been made possible through accurate optimisation of the gene delivery protocols (Derouazi et al. 2004; Muller 2005). Supplementation of standard protein-free media with peptones yielded a significant increase in TGE productivity in HEK 293-EBNA cells (Pham et al. 2003; Pham et al. 2005).

Large-scale processes

There are only a few published reports on TGE at scales larger than 1 l, and even less for 10 l and more. Most of these publications have originated from academic laboratories. Large-scale processes employ mostly standard stirred-tank

bioreactors (STR), but a recently developed bioreactor (the WaveTM), with disposable plastic bags, has proven to be particularly well-suited for TGE (Geisse and Henke 2005). Disposable bioreactors are raising interest mainly due to their low initial investment cost and their ease of operation, maintenance, and validation. In addition, TGE in non-instrumented round- or square-shaped bottles of nominal volumes of 1–20 l is being explored. In head-to-head comparisons these containers have outperformed instrumented bioreactors, making them an attractive, inexpensive alternative for large-scale TGE (Wuhlfard, Hildinger, Stettler and Wurm, manuscript in preparation).

Expression level

Table 1 summarizes the publications reporting TGE at scales of 1 l or more, together with a few unpublished results from our laboratory. However, several leading biopharmaceutical companies are routinely employing TGE at scales up to 100 l for their drug discovery programs.

Volumetric productivity with TGE depends to a great extent on the intrinsic structural features of the r-protein, thus it is difficult to compare the efficacy of various TGE protocols (Bentley et al. 1998). In general, with efficient non-viral gene delivery agents like PEI, titers range from 1 to 80 mg/l with either CHO or HEK 293 cells (Table 1). IgG yields in the range of 2–80 mg/l have been achieved in instrumented bioreactors ranging in size from 3 to 150 l using either batch or fed-batch approaches (Meissner et al. 2001; Girard et al. 2002).

Conclusions and future perspectives

The technology to generate research-quality r-proteins by TGE has been under development for more than 10 years in both academic and industrial laboratories. During this period, the major improvements in TGE have come via the formulation of serum-free media that support high-density growth of cells in suspension and efficient serum-free transfection methods. Despite these advances, the specific productivity of

TGE remains typically far below 10 pg/cell · day, whereas that of some recombinant cell lines has been reported to be over 50 pg/cell · day (Wurm 2004). It is therefore likely that the upper limit for the specific productivity by TGE has not yet been achieved. One of the major challenges for the future is to close the gap between transient and stable r-protein productivity. Another gap exists with respect to cell number in batch processes between transient and stable expression. Whereas most transient protocols result in cell densities below 3×10^6 cells/ml, optimized high-yield stable processes achieve $10\text{--}15 \times 10^6$ cells/ml over 10–14 days (Wurm 2004). While the exact nature of the improvements are not known in detail for proprietary reasons, it is likely that they involve (i) the development of both serum-free and protein-free media to support high-density cultivation of cells in suspension, (ii) improved feeding strategies (for fed-batch cultures), and (iii) the genetic modification of the host cells. This results in volumetric yields of up to 5 g/l in the best cases. Targets for cell engineering may include metabolic and apoptotic pathways (Fussenegger and Bailey 1998). For example, strategies involving the over-expression of exogenous anti-apoptotic genes have led to higher cell viability and improved r-protein yield (Mastrangelo et al. 2000; Tey et al. 2000; Kim and Lee 2002). Similar strategies are also likely to benefit TGE. As an example, it has been shown that improved transient r-protein production results from the inhibition of the cell cycle (Tait et al. 2004; Galbraith et al. 2006). To establish more efficient host cells for TGE through genetic manipulation, the over-expression of exogenous genes as mentioned above and the downregulation of host genes through siRNA technology (Hacker et al. 2004) should be attempted.

A very promising route and probably the best one for providing high volumetric yields is the increase in biomass before, during and/or after transfection. Other improvements to TGE are likely to come from the transfection process itself. All of the major chemical transfection agents used for TGE including CaPi, PEI, and liposomes have major drawbacks as described above. In addition, they only deliver a small percentage of the transfected DNA (<10%) to

cells (Batard et al. 2001; Lechardeur et al. 2005). More efficient DNA transfer would allow the amount of plasmid DNA, a major cost factor in addition to a major source of variability for TGE, to be reduced. Besides increased transfection efficiency, the ideal transfection reagent will be non-cytotoxic and will not interfere with r-protein purification or quality. The development of novel transfection agents such as dendrimers (Lee et al. 2005), chemically-modified PEI (Thomas et al. 2005), and chitosan (Dang and Leong 2006) continues and may lead to more efficient delivery methods. Finally, it may be possible to adapt physical DNA delivery methods such as electroporation to large-scale TGE (Li et al. 2002).

Another major challenge remains the establishment of TGE as a bioprocess for the manufacture of r-proteins. Currently, there are no FDA-approved r-proteins generated by TGE, probably because it appears to be a “revolutionary” approach. In our mind, there is no reason to prevent eventual approval of a TGE-based manufacturing process. For certain low-dose products such as erythropoietin (EPO) or granulocyte colony-stimulating factor (G-CSF), current TGE yields are quite sufficient to establish economically feasible processes. R-proteins used for individualized medicine could also be manufactured by TGE. This is of course not the case when considering high-dose antibody products. For these it will be necessary to increase the specific productivity, the volumetric productivity and, last but not least, the maximum operating volume for TGE. The largest volume reported for TGE is 100 l (Girard et al. 2002), and volumes from 1–100 l are probably sufficient for the production of milligram to gram quantities of r-proteins for research or for pre-clinical studies. Larger TGE processes are undoubtedly possible, but this parameter has not been adequately explored. Besides the issue of the feasibility of scaling-up the production process, there is also concern that the product heterogeneity within a batch and from batch to batch may restrict the manufacturing potential of TGE. However, not enough detailed studies on product heterogeneity in TGE production runs have been published to make a conclusive statement on this point.

Summary

The future challenges for the biotechnology industry require a wide-angle, integrative view spanning the cell's metabolism (from genomics to proteomics and metabolomics), innovative bio-process engineering strategies for high-density, large-scale cell cultivation, efficient gene delivery methods, and more efficient and economical downstream recovery and analytical methods. Through such an integrated approach involving both biology and engineering, we believe that in the upcoming years specific productivity of transiently transfected mammalian cells will increase to 20–50 pg/cell · day. Combined with increased process robustness, reproducibility, scalability and speed, a substantial improvement in protein yield will make TGE an option for therapeutic protein manufacturing and open a whole new chapter in industrial biotechnology.

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