RESEARCH ARTICLE

High cell density transient transfection of CHO cells for TGF- β 1 expressionAbdalla A. Elshereef^{1,2} | André Jochums¹ | Antonina Lavrentieva¹ |
Lena Stuckenberg¹ | Thomas Scheper¹ | Dörte Solle¹¹Institute of Technical Chemistry, Gottfried Wilhelm Leibniz University of Hannover, Hannover, Germany²Chemistry of Natural and Microbial Products Department, Pharmaceutical and Drug Industries Research Division, National Research Centre, Giza, Egypt

Correspondence

Dr. Abdalla Elshereef and Dr. Dörte Solle,
Institute of Technical Chemistry, Gottfried Wilhelm Leibniz University of Hannover,
Callinstr. 5, 30167 Hannover, Germany.
Email: aa.elshereef@nrc.sci.eg;
solle@iftc.uni-hannover.de

Funding information

Deutscher Akademischer Austausch Dienst
Kairo, Grant/Award Number: 57076387

Abstract

High cell densities for transient transfection with polyethyleneimine (PEI) can be used for rapid and maximal production of recombinant proteins. High cell densities can be obtained by different cultivation systems, such as batch or perfusion systems. Herein, densities up to 18 million cells/mL were obtained by centrifugation for transfection evaluation. PEI transfection efficiency was easily determined by transfected enhanced green fluorescence protein (EGFP) reporter plasmid DNA (pDNA). A linear correlation between fluorescence intensity and transfection efficiency was improved. The transfection efficiency of PEI was highly dependent on the transfection conditions and directly related to the level of recombinant protein. Several factors were required to optimize the transient transfection process; these factors included the media type (which is compatible with low or high cell density transfection), the preculture CHO-K1 suspension cell density, and the pDNA to PEI level. Based on design of experiment (DoE) analyses, the optimal transfection conditions for 10×10^6 cells/mL in the CHOMACS CD medium achieved 73% transfection efficiency and a cell viability of over 80%. These results were confirmed for the production of transforming growth factor-beta 1 (TGF- β 1) in a shake flask. The purified TGF- β 1 protein concentration from 60 mL supernatant was 27 μ g/mL, and the protein was biologically active.

KEYWORDS

CHO cells, EGFP transfection efficiency, PEI transient transfection, TGF- β 1 purification and bioactivity

1 | INTRODUCTION

The production of recombinant proteins is an important issue for biotechnology research, bioprocess development, and industry. Due to advances in molecular biology, construction of a plasmid with the desired gene and expression of the desired protein after transfection into an appropriate cell host

are now fairly easy processes. Cell line establishment through a selection process following stable transfection requires an extended period of time, and thus, transient transfection remains the first choice method for rapid recombinant protein production [1–4]. Although the protein production from transient transfection is still lower than that via stable transfection, this difference can be overcome by a repeated transient transfection strategy. This strategy extends the production time and increases the protein production [5]. Another promising approach to increase protein production is the high cell density transfection strategy [6,7]. More recently, the ExpiCHO expression system, utilizing media

Abbreviations: DoE, design of experiment; hpt, hours post transfection; pDNA, plasmid DNA; PEI, polyethyleneimine; TE%, transfection efficiency.

for a high CHO cell density, has become commercially available [8]. Scalability of PEI-mediated transient transfection of CHO culture for protein production in industry has been developed up to 500 L scale [9].

One of the most frequently used cationic polymers for transient transfection is the linear 25 kDa polymer polyethyleneimine (PEI). PEI is cost-effective compared to other transfection reagents, such as Lipofectamine® 2000, TransIT-PRO® ExGen 500, and Effectene [10,11]. The PEI transfection method is based on the formation of nanoparticles (polyplexes) between the negatively charged plasmid DNA (pDNA) and cationic polymers [12]. These polyplexes can enter cells carrying the genetic information. The formation of PEI/pDNA polyplexes with desirable features (small size and positively charged) for efficient transfection strongly depends on the preparation method and the medium volume and composition. The formation of PEI/pDNA polyplexes occurs either through the *in situ* or the conventional method. By *in situ* transfection, the polyplexes are formed spontaneously in the culture, while in the conventional method, the polyplexes are formed before being added to the culture, and the concentrations of pDNA and PEI required in each method are different [13,14]. High concentrations of pDNA and PEI due to a strong interaction between them lead to partially formed polyplex particles and undesirable aggregation [15]. The medium compositions also affect transfection efficiency by changing the polyplex formation behaviour [16]. Some commercially available media have anionic components that inhibit PEI-mediated transfection, such as dextran or heparin sulphate, ferric ammonium citrate, and certain hydrolysates or other unknown components [17–19]. The anionic component reduces the overall positive charges of the polyplexes, dissociates pDNA from the polyplex and consequently prevents the transfection process. For effective gene delivery, neutralization (balance) of the charge outside and inside the cell is required to maintain low cytotoxicity and high protein expression [20]. Excess cationic charge density (the unbound free PEI chain) disturbs the integrity of the cell membrane's and polyplex formation, potentially leading to cytotoxicity [21].

Concerning the media compatibility issue, one study reported that Pro-CHO5 medium mediates PEI transfection, while another study contradicted this finding [6,22], which may be due to the differences between the applied transfection conditions. This result indicates the importance of the media and viability in transfection efficiency before starting optimization analyses [23]. For these analyses, basic factors, including the cell density and pDNA/PEI concentration or ratio, play a relevant role during optimization [4]. Hence, optimization of already identified factors is required; the quadratic model “response surface modelling” (RSM) is flexible and develops the relationship between factors and response. One commonly used RSM design applied in this study is a central composite circumscribed (CCC) design [24,25].

PRACTICAL APPLICATION

The protocol assessed in this study can quantify important parameters that strongly influence the polyethyleneimine (PEI)-mediated transient transfection efficiency (TE). These parameters including the cell density effect before and at transfection time. This experimental approach is capable of optimizing the PEI and plasmid DNA (pDNA) levels required for high TE. TGF- β 1 is a well-known immunosuppressor and proangiogenic factor used for cancer treatment and was assessed as a target protein in this study. The high cell density transfection protocol paves the way for bioprocess volume scale-up and for further development of recombinant protein production.

A wide range of recombinant proteins, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and others have been transiently produced by PEI transfection of CHO and HEK-293 cells [22]. In the present work, the product of interest is transforming growth factor-beta1 (TGF- β 1).

For direct protein purification of secreted protein in the transfected culture supernatant, short peptide affinity tags are useful in protein downstream research. The Twin-Strep-tag® is particularly popular for obtaining recombinant proteins with a high purity and functionality by using physiological conditions within a rapid one-step protocol [26]. The proliferative effect of TGF- β depends partially on the cell type; it can promote the growth of several mesenchymal cells fibroblasts in soft agar in combination with either TGF- α or EGF. However, this protein inhibits the monolayer growth of epithelial cells, stem cells, and lymphocytes through the extension of the cell cycle time [27,28].

In this study, high cell density transfection was the main target for improving recombinant protein production. Due to the complexity of the media effect, we chose one medium to study the effects of different parameters, such as (1) cell density at transfection time, (2) preculture cell density, and (3) pDNA and PEI concentrations. This study provides new insight into the effects of the media as well as the different transfection efficiencies under different conditions.

2 | MATERIALS AND METHODS

2.1 | Plasmids

For transfection efficiency evaluation, the plasmid pEGFP-N1 (Clontech Laboratories Inc., USA) encoding enhanced green

FIGURE 1 Structure of the TGF- β 1 fusion protein. The fluorescent tag consists of three tryptophan residues, which are linked to a cleavable enterokinase site through a peptide GS-linker. For the purification process, the TGF- β 1 segment is linked to the Twin-Strep-tag. The amino acid (aa) sequence consists of 39 aa for the fluorescent tag, 112 aa for mature TGF- β 1 (monomer form) and 28 aa for the Twin-Strep-tag

fluorescence protein (EGFP), which remains inside cells, was used.

The plasmid TGF- β 1 was designed by IBA (IBA, Germany) to express a fusion TGF- β 1 protein. The fusion protein consists of a tryptophan tag, the TGF- β 1 coding sequence and a Twin-Strep-tag. The plasmid map is displayed in Figure 1. The tryptophan tag (W2) sequence was designed according to GenBank accession no: JN120907. The plasmids were amplified in transformed TOP10 *Escherichia coli* cells and purified by Giga Qiagen column tips using the Qiagen Giga plasmid kit (Qiagen, Germany).

2.2 | Media and cell cultivation

CHO-K1 cells were maintained in CHOMACS CD serum-free medium (Miltenyi Biotec, Germany) supplemented with 8 mM L-glutamine (Biochrom, Germany). The cells were cultivated at 37°C and 5% CO₂ in 90% humidified incubators and shaken at 160 rpm (ELMI DOS-20L, USA) in shake flasks or at 250 rpm (KS 260 basic, IKA, Germany) in a tube spin bioreactor 50 (TPP, Trasadingen, Switzerland). The initial cell density for seeding was 0.5×10^6 cells/mL, and viability was >99%. The cell cultures were subcultured

frequently every 3–4 days to maintain a high cell density and viability. For high cell density transfection screening, CD CHO (Thermo Fisher Scientific, Germany) and ProCHO-5 (Lonza, Sartorius AG, Germany) media were used along with CHOMACS CD medium. The media were used for the low cell density transfection screening are indicated in the results section.

2.3 | Cell analysis

Total cell number was estimated by an automatic cell counter (Innovates Cedex cell analyser, Roche Diagnostics GmbH, Germany), according to the manufacturer's protocol. Viable cells were counted based on the trypan blue exclusion method [29]. The hydrophilic trypan blue stain diffuses through the cell membrane of the dead cells, which will be then coloured and can be easily counted. The difference between total and dead cells will give the concentration of the viable cells. Cell viability was calculated as a percentage of surviving cells compared to the total cell count.

2.4 | PEI transfection reagent

Linear PEI (MW 25 kDa; Polysciences GmbH, USA) in a stock solution of 1 mg/mL was used for transfection. This solution was prepared according to the Cold Spring Harbour protocol [30].

2.5 | Transfection protocol

Preculture (one day prior to transfection) was prepared with CHO-K1 cells seeded at $1.2\text{--}1.5 \times 10^6$ cells/mL (unless otherwise noted) in CHOMACS CD medium plus 8 mM L-glutamine. After 24 h (duplication time), the required cell amounts were collected by centrifugation at 200× g for 5 min. The cells were resuspended in fresh medium. After 1 h of incubation, the corresponding amounts of pDNA followed by PEI were added (in situ transfection). At 5 h post transfection (hpt), the transfected culture was diluted with fresh medium. The transfection parameters and procedures are illustrated in Table 1.

2.6 | Transfection efficiency evaluation

Following the transfection protocol described above, the cells were washed twice with PBS solution. Next, a total 10 000 cells were examined with a flow cytometer (BD Accuri™ C6, BD Biosciences, USA) equipped with a 488 nm solid-state laser. Cell fluorescence intensity was detected in the FL1-H channel with a bandpass filter (BP) (wavelength: 533 nm; bandwidth: 30 nm), and the number of EGFP-positive viable cells was counted. The percentage of EGFP-positive cells was determined using BD Accuri C6 Software and compared to

TABLE 1 Various transfection conditions

Experiment ID	Media volume	Viable cells	pDNA [μ g]	PEI [μ g]	Medium addition at 5 hpt	Culture vessel
Low cell density transfection screen	5 mL	10×10^6	EGFP 6 and 8	30	—	Tube spin
High cell density transfection screen	1 mL	10×10^6	EGFP 6 and 8	30	4 mL	Tube spin
Cell density	1 mL	$4\text{--}18 \times 10^6$	EGFP 24	45	1 mL	Tube spin
Preculture cell density ($1.2\text{--}5.3 \times 10^6$ cells/mL)	1 mL	10×10^6	EGFP 24	45	1 mL	Tube spin
DoE transfection conditions	1 mL	10×10^6	EGFP, PEI Figure 5	1 mL	Tube spin	
TGF- β 1 production	12.5 mL	10×10^6 /mL	TGF- β 1 17 μ g/mL	38 μ g/mL	12.5 mL	Shake flask

that of the non-transfected control. This value is expressed as the transfection efficiency at the indicated hpt.

2.7 | Software

GraphPad Prism version 7 software (GraphPad Software Inc., USA) was used to perform the analysis of variance (ANOVA) and P-value calculations.

The design of experiment (DoE) approach was used to determine the optimal transfection parameters. A series of experiments using a CCC design [24] were created by the MODDE software programme (MODDE 9, Umetrics, Sweden). These experiments were conducted under a fixed cell density and a range of PEI and pDNA concentration as showed in Supporting Information Table S1,S2.

2.8 | TGF- β 1 analysis

The recombinant fusion TGF- β 1 protein was purified directly from 60 mL of transfected culture supernatant through Twin-Strep-tag® technology [26]. Purified TGF- β 1 protein in the elution buffer fractions E1, E2, and E3 was quantified by Nanodrop spectrophotometry (Thermo Fisher Scientific, Inc., USA) using absorbance at 280 nm. TGF- β 1 bands were then separated by 15% SDS-PAGE under reduced condition and transferred onto polyvinylidene difluoride (PVDF) membranes. After the membrane was blocked with 5% skim milk, it was incubated with primary mouse anti-human-TGF- β 1 (Dianova GmbH, Germany) for one hour and then washed three times for 5 min in PBST solution (500 mL PBS buffer+0.5% Tween 20). The membrane was incubated with secondary goat anti-mouse IgG AP-conjugate (BD Biosciences Pharmingen, USA). The band colour was developed by using an AP conjugate substrate kit (Bio-Rad Laboratories, Inc., USA). The primary and secondary antibodies used in this study were diluted into 3 and 0.2 μ g/mL of (50:50) block solution: PBST solution, respectively. The mature TGF- β 1 concentration of the purified protein from the E2 fraction was quantified by indirect ELISAs using secondary goat

anti-mouse IgG HR-conjugate in a 96-well plate reader at 450 nm absorbance in a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Germany).

2.9 | Evaluation of TGF- β 1 bioactivity (cell-based assay)

A549 (adenocarcinoma human alveolar basal epithelial cells, ACC107, DSZM) cells were maintained in high glucose DMEM (Sigma Aldrich, now Merck, Germany) supplemented with 10% foetal calf serum (FCS) (Sigma Aldrich, now Merck, Germany) and 1% penicillin/streptomycin (Biochrom/Merck, Germany). Cultures were incubated at $37 \pm 1^\circ\text{C}$ in a humidified atmosphere of $5 \pm 1\%$ v/v CO_2 in the air. The cell number in the trypsinized cell suspension was determined using a haemocytometer (Neubauer 0.1 mm, Germany). Detached cells were collected and washed twice with PBS by centrifugation at $300 \times g$ for 5 min. The supernatant was discarded, and the harvested cells were counted and seeded in 96-well plates (Sarstedt AG & Co. KG, Germany) in fresh DMEM with 10% FCS. The cell suspension was diluted to give a final concentration of 4×10^4 cell/mL in 200 μ L pipetted into each well of 96-well plate (i.e., 8000 cells/well). After a 24 h adhesion time, the incubation medium was removed, and DMEM supplemented with 1% FCS and various TGF- β 1 concentrations ranging from 44 to 176 ng/mL and controls (0 ng/mL) were added. Each concentration was tested in four independent wells. Commercially available TGF- β 1 (Peprotech GmbH, Germany) served as a positive control.

After 5 days of incubation, the TGF- β 1-containing media and negative controls were removed, and an indirect estimation of cell proliferation was performed using CellTiter-Blue® Cell Viability Assays (Promega, Germany). The dye used for this assay is the blue, non-fluorescent resazurin (7-hydroxy-3h-phenoxazine-3-one-10-oxide). In the mitochondria of living cells, this dye is reduced to the pink fluorescent resorufin. Therefore, a measure of the

metabolic activity of the living cells is based on the degree of fluorescence intensity. Fluorescence was measured with a microplate fluorometer (544Ex/590Em) (Fluoroskan Ascent, Thermo Electron Corporation, USA).

3 | RESULTS AND DISCUSSION

3.1 | Screening of high cell density transfection medium

The cells were grown one day (preculture) in CHOMACS CD medium and directly resuspended in different media as indicated in Figure 2A and B prior to transfection. The CHO-K1 transfection was performed with different ratios of EGFP pDNA to PEI w/w (Table 1). Transfection efficiency was measured at different time points with a flow cytometer and viability assays with Cedex as indicated in Figure 2A and B. Two transfection screening methods, low cell density transfection (Figure 2A) and high cell density transfection (Figure 2B), were used. Here, 10 million cells were transfected with 6 and 8 μg of pDNA and 30 μg of PEI in medium volumes of 1 and 5 mL. Therefore, the two transfection methods resulted in different transfection conditions. For the high cell density transfection method, volume was increased to 5 mL at 5 hpt (Table 1).

For low cell density transfection (two million cells/mL at transfection time) (Figure 2A), some media, such as ProCHO-5, CD CHO, and CHOMACS CD media, supported high viability, while Opti-MEM, DEMEM/F12, and Ex-Cell CD CHO media showed a significant reduction in cell viability. This experiment showed that transfection in ProCHO-5, CD CHO, and Opti-MEM media resulted in relatively high transfection efficiency compared to that in CHOMACS CD, DEMEM/F12, and Ex-Cell CD CHO media. Thus, ProCHO-5 and CD CHO were the best transfection media for CHO-K1 cells. Only CHOMACS CD showed high cell viability, but this medium is not suitable for transfection with the low cell density transfection method because no fluorescent cells were detected.

Since CHOMACS CD medium showed high cell density growth up to 18×10^6 cells/mL [31], and the transfection efficiency still has not been investigated, the high cell density transfection protocol should be studied to confirm the transfection efficiency in the most promising media, as shown in Figure 2B. With the high cell density transfection method (ten million cells/mL at transfection time), relatively similar transfection efficiencies were obtained among CHOMACS CD, ProCHO-5, and CD CHO media, although the previously mentioned low cell density transfection conditions showed differences in the transfection efficiencies. When CHOMACS CD medium was used with a volume of 1 mL (Table 1), high cell density enhanced the transfection efficiency and reduced

the differences compared to ProCHO-5 and CD CHO and vice versa. In addition, to decrease the transfection rate of ProCHO and CD CHO under high density transfection conditions, CHOMACS CD medium was selected for further experiments. Very high cell density is an economical method for recombinant protein production [32].

The results also showed that with the addition of fresh CHOMACS CD medium up to 5 mL at 5 hpt, the unknown PEI inhibitory components of CHOMACS CD medium had a negligible effect on transfection efficiency. Consistent with this finding, most transfection events occurred within the first hour after transfection, when 80% of the pDNA/PEI polyplex is taken up by the cells [16,33]. Therefore, volumetric upscaling of the high cell density transfected culture through medium addition at 5 hpt decreased its cell density to extend the protein production time as showed in Supporting Information Figure S2. This high density transfection method was employed in the following experiments.

3.2 | The effect of cell density on transfection efficiency in CHOMACS CD medium

To investigate transfection efficiency of high cell density transfection in CHOMACS CD medium, we conducted four different experiments using different cell concentrations from 4×10^6 cells/mL to 18×10^6 cells/mL. The pDNA and PEI concentrations at the transfection time point were adjusted to 24 μg pDNA and 45 μg PEI per mL according to the preliminary experiments of the DoE study. Here, the different cell densities, as indicated in Figure 3, were transfected by the same amounts of pDNA and PEI (Table 1), and the cultures were diluted to 1:2 (v/v) fresh medium at 5 hpt. As shown in Figure 3, the highest transfection efficiency was observed at 10×10^6 cells/mL, but there was no significant difference in the transfection efficiencies obtained for higher cell densities. Although there is no significance difference between data of 18 million cells/mL and 10 million cells/mL, the later was preferred for comparing experiments. The former would prefer for conducting transfection in large scale high density perfusion bioreactor. The use of Alternating Tangential Flow (ATF) system hollow fiber module 50 KDa pore to CHO cells has been used in a concentrated fed-batch process providing high cell concentrations and high levels of recombinant protein over that produced by perfusion system [34].

The maximum cell density transfected here was 18×10^6 cells/mL, and the literature reports 5×10^6 cells/mL for CHO cells and 20×10^6 cells/mL for HEK-293 cells [14,22]. Here, the same amount of pDNA and PEI did not appear to be an issue for transfection efficiency at a low cell density of 4 million cells per mL or a high cell density of 18 million cells per mL. One possible explanation is that the applied pDNA and PEI concentrations in this experiment result in the formation of sufficient transfectable

FIGURE 2 Media screen for transfection. EGFP transfection efficiency (TE%) and cell viability measured at different time points. (A) Low cell density. Control represents non-transfected cells. (B) High cell density transfection media screen; error bar indicates the standard deviation of $n = 2$

pDNA/PEI polyplex particles for the whole tested cell densities range. Therefore, the transfection efficiency is based on the biophysical properties of polyplex particle formation in a specific medium regardless of the cell density. The cells would not interfere with the polyplex formation within the tested range. Polyplex particle formation with a small size, a highly positive charge and a high PEI/pDNA ratio enhanced entrance of pDNA into the cell nuclei [35]. In contrast, an increase in cell density should result in a change in PEI and pDNA concentrations for efficient transfection

because the extent of particle uptake decreased with an increase in cell density [16,33]. These data indicated that low amounts of pDNA and PEI per cell can be used to achieve the same transfection efficiency. Decreasing the amount of pDNA required for transfection will help reduce the production costs [36]. The cell viability of all transfected cultures was over 80%, except the cell density of cultures with 4 million cells per mL. This difference in culture viability was very significant. The toxic effect of free PEI (unbound to the pDNA complex) may be responsible for reducing cell

FIGURE 3 Various cell densities at fixed transfection time points. Different cell densities were applied at the time of transfection 1-Day of pre-culture. Transfection efficiency and cell viability were determined at 48 hpt. Statistical analysis of cell viability was performed by one-way analysis of variance (ANOVA), $n = 3$. Differences between viability at the lowest cell density of 4×10^6 and other cell densities up to 18×10^6 cells/mL show very significant P -values; ** $P < 0.01$

FIGURE 4 Effects of preculture cell density on transfection. Transfection efficiency and viability depend on different preculture cell densities and a constant transfected cell density. Comparisons of transfection efficiency and cell viability were performed using one-way ANOVA; $n = 3$. Summary of P -values; * $P < 0.05$, ** $P < 0.01$ were considered significant and very significant, respectively

viability [37]. Therefore, transfection with a low cell density is not preferable.

3.3 | Preculture cell density prior to transfection

To investigate the effect of preculture cell density before transfection, we conducted four different experiments using fixed cell densities for transfection of 10×10^6 cells/mL (based on previous findings) originating from different preculture cell densities as shown in Figure 4. All cultures were transfected by the same amounts of pDNA and PEI (Table 1).

High transfection efficiency was observed with high preculture cell densities, as shown in Figure 4. These conditions resulted in a twofold higher transfection efficiency (TE%) than that with a preculture density of 1.2×10^6 cells/mL, with a very significant difference. The cells originating from a preculture cell density in the range of

FIGURE 5 Identification of the optimal transfection area by the DoE method. Different amounts of pDNA interacting with PEI affect transfection efficiency. The design spaces areas A and B represent the interaction of high and low levels of pDNA and PEI, respectively, and their effect on transfection efficiency (TE%). The centre point is represented by the average of three experiments to validate the DoE design

2.6 – 5.3×10^6 cells/mL showed an increased susceptibility to PEI-mediated transfection. This result could be because the growth potential of cells strongly depends on preculture densities, as reported for hybridoma cells [38]. Monitoring of cell physiological state by intracellular nucleotide pools has been employed to improve transient gene expression (TGE) [39].

To determine the optimal correlation between high transfection efficiency and viability induced by pDNA and PEI concentrations, we conducted a DoE analysis and achieved a transfection efficiency of over 60% with a viability of over 80%, which are good indicators for the physiological culture state of TGE. A total of 33 combinations of pDNA and PEI, as shown in Figure 5, were carried out with a fixed density of cells (10×10^6 cells/mL) in a 1 mL transfection volume diluted with fresh medium at 1:2 v/v at 5 hpt. The plot of Figure 5 shows that the two transfection conditions called areas A and B were evaluated.

Unexpectedly, as shown in Figure 5, areas A and B reveal clearly that there are no major increases in transfection efficiency from the transfection condition applied in the previous experiment. The previously applied transfection condition of 24 µg pDNA to 45 µg PEI reached a transfection efficiency of 66%, while the maximum transfection efficiency achieved here was 73% using a pDNA amount of 17 µg and a PEI amount of 37 µg. This represents the optimal combination where the pDNA/PEI polyplex can overcome the inhibitory effect of CHOMACS CD medium. The power to increase transfection efficiency over 73% seems to be limited to the

Table 2: The whole transient transfection process

Cultivation time [h]	Viability %	Cell density * $[10^6 \text{ cells/mL}]$
Pre-culture 0-24 h	99.6-99.6	1.3-3.6
Hours post transfection 0-72 hpt	99.6-89.3	10.2-14.3

Table 3: Estimation of purified TGF- β 1 protein concentration by Nanodrop 27 $\mu\text{g/mL}$

Elution fraction	The volume of elution fractions [mL]	The concentration of purified protein $[\mu\text{g/mL}]$	The total amount of purified protein $[\mu\text{g}]$ from 60 mL
E1	1.2 mL	510	612
E2	5.2 mL	170	884
E3	1 mL	140	140
Σ	7.4 mL		1636

Evaluation of TGF- β 1 by western blotting

FIGURE 6 Transfection procedure for TGF- β 1 production. Table 2 represents the transfection process, which was started by preparing precultured cells with a low cell density inoculum (1.3×10^6 cells/mL). After 24 hours, cells at a high density were collected, resuspended in fresh medium and transfected by the appropriate amounts of TGF- β 1-pDNA and PEI. The transfected culture was left for 72 hpt before harvesting. Table 3 represents the calculation of total purified protein followed by the detection of TGF- β 1 with western blot analysis; Lane 1, a protein size marker is shown. Lane 2, commercial TGF- β 1 (CHO derived) and Lane 3, the purified TGF- β 1 from transfected culture supernatant. Tag that used here are Twin-Strep-tag 30 aa. (3 kDa) for protein purification and tryptophan tag 30 aa.(4.4 kDa) for online protein expression monitoring

CHOMACS CD medium. Target TGF- β 1 protein production was maintained under that condition by replacing the EGFP-pDNA without further optimization.

3.4 | Transient TGF- β 1 expression in shake flasks

The TGF- β 1 production steps are illustrated in Figure 6, starting from preculture preparation for 24 h followed by cell collection at a high cell density in fresh CHOMACS CD medium. Then, the cells were transfected by the optimal concentrations of TGF- β 1 pDNA and PEI followed by a 1:2 dilution v/v at 5 hpt (Table 1) and harvesting at 72 hpt.

As shown in Figure 6 (Table 2), all transfected cultures exhibited a high viability of $89\% \pm 1.9$, which is a good indication of successful transfection according to that achieved previously with EGFP transfection. The medium was harvested, and TGF- β 1 (latent and mature) was purified via Strep-Tactin affinity chromatography and collected in elution fractions E1, E2, and E3. The concentration determined by a Nanodrop spectrophotometer at a wavelength of A280 was 27 $\mu\text{g/mL}$ (i.e., 27 mg/L), as shown in Figure 6 (Table 3). The maximum concentration of TGF- β 1 reported in the literature was approximately 26.6 mg/L of total protein, which is comparable to these results [40]. The western blot results in Figure 6 revealed that in the commercial TGF- β 1 lane, the upper band was located at approximately 75 kDa, which can be assigned to the latent peptide. In the purified TGF- β 1 lane, the upper band was the latent TGF- β 1 fused tag protein. This band was located at 100 kDa compared to the commercial one at approximately 75 kDa. The latent TGF- β 1 can be visualized on a

non-reducing western blot at 75, 80, 84, 90, or 100 kDa [41–43]. The differences between the bands of the commercial and purified TGF- β 1 could relate to presence of tags, difference in glycosylation patterns or aggregation of monomers and dimers. The other bands of approximately 25 and 14 kDa were assigned to the active TGF- β 1-dimer and monomer, respectively (mature form). Both commercial and purified TGF- β 1 samples were denatured using β -mercaptoethanol, the former revealed monomer (14 kDa) and dimer subunit 25 kDa while the later revealed monomer subunit. Based on these results, the commercial and purified TGF- β 1 proteins revealed similarities in the band size of the mature TGF- β 1 monomer. These data are consistent with findings showing that the mature TGF- β 1 can be visualized as a dimer form of 25 kDa and a monomer form of 12–15 kDa [40,44].

To determine whether the purified TGF- β 1 is biologically active and to compare its activity with commercial TGF- β 1, we prepared various concentrations of purified TGF- β 1 based on ELISAs in DMEM as indicated in Figure 7. The equivalent concentrations of commercial TGF- β 1 were tested in parallel as a positive control. For the negative control, the same experiment was performed in the absence of TGF- β 1 addition. The bioactivity test was performed on A549 cells by measuring the inhibitory effect of TGF- β 1 on cell proliferation through cell viability %.

The results in Figure 7 show the inhibitory effects of different TGF- β 1 concentrations on A549 cell proliferation compared with that of the negative control (0 ng/mL TGF- β 1). The purified TGF- β 1 showed a comparable effect on cell proliferation to commercial TGF- β 1. A549 cells treated with concentrations of 88 ng/mL and 176 ng/mL

FIGURE 7 Bioactivity of TGF- β 1 secreted from transfected CHO-K1 cells. Percent changes in A549 cell viability depending on TGF- β 1 concentration. Data were analysed using two-tailed paired t-tests compared to the control ($N = 3$), and $*P < 0.05$, $**P < 0.01$ in t-tests were considered significant and very significant, respectively

showed significantly lower cell viability than those treated with 44 ng/mL. This result indicates that TGF- β 1 produced in CHO-K1 cells at a high density transfected with the PEI method has comparable (and higher) bioactivity to the commercially available TGF- β 1.

4 | CONCLUDING REMARKS

Polyethylenimine (PEI) transient transfection is of great interest for the production of recombinant proteins in various cell lines. The optimization of this gene delivery tool is important to achieve maximal production of recombinant protein either used for fundamental research or industry applications. A multitude of optimization approaches are available with a wide range working on cell line engineering, media type, plasmid and transfection reagent concentrations. Our optimized protocol mentioned preculture (cells cultured for one day) cell density as a novel important factor on the transient transfection step. Also, it classified the culture media into that supports high or low cell density transfection. In the literature, the maximum cell density used with PEI transient transfection was 20 million HEK-293 cells per mL. This optimized protocol concentrated the CHO cells for transfection up to 18 million cells per mL without affecting the transfection efficiency level $60 \pm 5\%$ with an advantage to reduce the amount of pDNA needed per cell transfection by a factor of 4.5 compared to 4 million cells per mL. Protein production can be increased by different strategies such as extending the production time, transfecting high cell density culture as well as transfecting large culture volumes. Scalability of PEI-mediated transient transfection of CHO culture in the industry

has been developed, the scale up of our own process up to 30 L disposable bioreactor will be studied. Remain the need to prepare preculture to achieve the optimal transfection efficiency and protein yield one of the main limitations of transient transfection method. This makes transient gene expression at large scale culture volume (liters) very challenging due to the need to centrifuge large culture volumes. Currently, the Alternating Tangential Flow (ATF) providing a promising solution with high density transfection protocol to overcome the medium replacement and concentrate the preculture cells.

Interestingly, the applicability of our optimized EGFP transient transfection protocol to produce bioactive recombinant TGF- β 1 titer similar to that produced in literature was achieved; this reflects the advantage of this protocol to produce other recombinant proteins at the optimal level just by exchange EGFP-N1 plasmid with the desired one. Size of plasmid seems to be not a critical factor while further study will be studied.

ACKNOWLEDGMENT

The author would like to thank the Deutscher Akademischer Austausch Dienst 57076387 (DAAD) for financial support. Thanks are also extended to the Egyptian Knowledge Bank (EKB), group of nature research for the language editing of the manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ORCID

Abdalla A. Elshereef
<https://orcid.org/0000-0002-7713-8134>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Elshereef AA, Jochums A, Lavrentieva A, Stuckenberg L, Scheper T, Solle D. High cell density transient transfection of CHO cells for TGF- β 1 expression. *Eng Life Sci.* 2019;19:730–740. <https://doi.org/10.1002/elsc.201800174>