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A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications

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ABSTRACT

Transient gene expression in mammalian cells is a valuable alternative to stable cell lines for the rapid production of large amounts of recombinant proteins. While the establishment of stable cell lines takes 2–6 months, milligram amounts of protein can be obtained within a week following transfection. The polycation polyethylenimine (PEI) is one of the most utilized reagents for small- to large-scale transfections as it is simple to use and, when combined with optimized expression vectors and cell lines, provides high transfection efficiency and titers. As with most transfection reagents, PEI-mediated transfection involves the formation of nanoparticles (polyplexes) which are obtained by its mixing with plasmid DNA. A short incubation period that allows polyplexes to reach their optimal size is performed prior to their addition to the culture. As the quality of polyplexes directly impacts transfection efficiency and productivity, their formation complicates scalability and automation of the process, especially when performed in large-scale bioreactors or small-scale high-throughput formats. To avoid variations in transfection efficiency and productivity that arise from polyplexes formation step, we have optimized the conditions for their creation directly in the culture by the consecutive addition of DNA and PEI. This simplified approach is directly transferable from suspension cultures grown in 6-well plates to shaker flasks and 5-L WAVE bioreactors. As it minimizes the number of steps and does not require an incubation period for polyplex formation, it is also suitable for automation using static cultures in 96-well plates. This “direct” transfection method thus provides a robust platform for both high-throughput expression and large-scale production of recombinant proteins.

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1. Introduction

The rising of protein–protein interaction studies as well as the constantly increasing demand of proteins in drug discovery programs have brought the need for high-throughput protein expression and automation to the foreground [1–5]. The need for recombinant proteins with post-translational modifications has fostered the development of mammalian cell-based expression systems rather than prokaryotes and lower eukaryotes [6]. Transient gene expression in mammalian cells is widely used for the fast production of recombinant proteins, mainly for pre-clinical *in vitro* and *in vivo* applications. Over the last 10 years, major improvements of the technique leading to better yields have made transient gene expression a competitive alternative to the estab-

lishment of stable cell lines as it shortens the time required to obtain the target protein in sufficient amount from several months to a few weeks only [7–12]. The capacity to quickly express recombinant proteins in mammalian cells by transfection is thus of great benefit not only for large-scale applications but also for high-throughput expression for functional screening and crystallization purposes [3,11–15].

The human embryonic kidney 293 cell line (HEK293) and its derivatives, such as the HEK293-EBNA1 that stably expresses the Epstein-Barr virus Nuclear Antigen 1 (293E) or the simian virus 40 Large-T antigen (293T), are the most commonly used cell lines for transient gene expression [10–12]. Indeed, HEK293 cells allow proper folding and relevant post-translational modifications of the proteins. They are also easy to grow in serum-free suspension culture and highly transfectable with most gene transfer agents [11]. Polyethylenimine (PEI) is a widely used transfection agent for small to large-scale protein expression as it is highly efficient and cost-effective [10–12]. PEI is a cationic polymer which binds to and condensates DNA into small cationic nanoparticles, thus

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facilitating DNA cell uptake, most likely via their interaction with cell-surface heparan sulfate proteoglycans [16,17]. Linear and branched 25 kDa PEIs are the most widely used polymers, the linear PEI being more efficient and probably less toxic for the transfection of HEK293 cells grown in suspension cultures [7,18,19]. The current transfection procedure consists in the formation of PEI–DNA complexes (polyplexes) prior to their addition to the cell culture (indirect method). Typically, PEI and DNA are mixed together and then incubated at room temperature for 10–15 min to allow complexation before their addition to the cells. This incubation time allows polyplexes to grow up to the most suitable size for a maximum transfection efficiency, which significantly decreases when incubation time exceeds ~30 min [20]. The preparation procedure (order of addition of DNA and PEI, DNA concentration, addition and mixing speed) also influences the size and polydispersity of the polyplexes [21–23]. However, polyplexes formation becomes difficult to control when working with large-scale productions, as issues such as mixing parameters and time required to transfer the polyplexes into the bioreactor become significant. This DNA–PEI mixing step and the following incubation time are also complicating automation when using high-throughput robotic systems.

An alternative technique to the indirect method is the consecutive addition of DNA then PEI directly to the cell culture, allowing the formation of polyplexes *in situ* (direct method) [19]. The present study aims to evaluate the direct transfection method as an alternative technique for high-throughput, medium- and large-scale protein production purposes. It has been optimized with 293-6E cells (a HEK293 cell line expressing a truncated version of EBNA1) cultured in 96-well plates, 6-well and 6-deep well agitated plates, 125-mL shaker flasks and 5L WAVE bioreactor, transfected with plasmids encoding green fluorescent protein (GFP), human secreted alkaline phosphatase (SEAP) or a chimeric IgG1/4 monoclonal antibody using linear or branched 25 kDa PEI. The direct transfection process is performed in serum-free medium with cells grown in suspension (except for the 96-well plate format) and does not require a medium exchange at any time. We show here that our optimized direct method provides yields comparable to the indirect method, greatly simplifies process scale-up and is amenable to automation, thus providing a robust and simple platform for protein expression at any scales.

2. Materials and methods

2.1. Cell lines

The human embryonic kidney 293 cell line stably expressing a truncated Epstein-Barr virus Nuclear Antigen-1 (293-6E) was grown in suspension in a serum-free F17 medium (Invitrogen, Carlsbad, CA) supplemented with 0.1% Pluronic-F68, 25 µg/mL geneticin G418 and 4 mM glutamine [24]. Cultures were maintained between 0.1 and 2.0×10^6 cells/mL in 125 or 250 mL Erlen-

meyer flasks shaken at 120 rpm in a humidified incubator at 37 °C with 5% CO₂ (standard conditions).

2.2. Plasmids

Secreted human placental alkaline phosphatase (SEAP) and green fluorescent protein (GFP) were used as reporter genes in this study. The pTT vectors encoding SEAP or GFP were essentially as previously described [7]. The chimeric monoclonal antibody against the colorectal carcinoma TAG72 antigen consists of the variable heavy and light-chain domains of the murine monoclonal IgG1 antibody B72.3 coupled to human gamma 4 and kappa constant regions [25,26]. The heavy and light chains (HC and LC) were each cloned between the EcoRI and BamHI sites of the pTT5 vector [27,28]. Plasmids were amplified in *Escherichia coli* (DH5α) grown overnight in CircleGrow medium (MP Biomedicals, Solon, OH) supplemented with 50 µg/mL ampicillin and purified using MAXIprep or QIAprep spin Miniprep columns (Qiagen, Mississauga, ON). Endotoxins in selected plasmid preps were determined by the LAL method using an Endosafe®-PTS™ spectrophotometer and FDA-licensed cartridges (Charles River Laboratories, Wilmington, MA).

2.3. Cell counts

Cell density and viability were determined using either a hemacytometer (Hausser Scientific, Horshaw, PA) after erythrosine B staining, or the Cedex Innovatis' automated cell counter Cedex Analyzer (Roche, Laval, Qc) based on the trypan blue exclusion method.

2.4. Transfection

Linear 25 kDa polyethylenimine (LPEI) was obtained from Polysciences (Warrington, PA), and branched 25 kDa PEI (BPEI) from Aldrich (Milwaukee, WI). Stock solutions (1 mg/mL) were prepared in ultrapure water, sterilized by filtration (0.2 µm), aliquoted and stored at –20 °C. Cells were diluted 2 days before transfection in fresh medium at $0.40\text{--}0.45 \times 10^6$ cells/mL. No centrifugation step was necessary to eliminate spent medium as the latter has no impact on transfection efficiency or productivity (data not shown). Cells were transfected with viability greater than 98% at densities between 1.5 and 2.0×10^6 cells/mL, except for the 96-well plates for which cell density was reduced to 0.2×10^6 cells/mL the day of transfection, as cells were grown in static instead of suspension culture. DNA and PEI were separately diluted in complete serum-free F17 medium in sterile tubes. Transfection reagents volume was generally 10% of the final culture volume. The final DNA concentration was 1 µg per mL of culture, unless indicated otherwise. **Direct method:** the DNA:PEI ratio was 1:3 (w:w). Plasmid DNA was directly added to the cells and the suspension was allowed to incubate under agitation for 5 min at 37 °C before the addition

Table 1
Direct transfection parameters from 96-well plates to 5L WAVE batches.

	96-well plate	6-well plate	6 deep-well ThinCert™ plate	125 mL shake flask	10 L WAVE Cellbag™
Working volume	200 µL	2 mL	6 mL	25 mL	~5 L
Seeding cell concentration ($\times 10^6$ cell/mL)	0.2	1.5–2.0	1.5–2.0	1.5–2.0	1.5–2.0
Cell suspension initial volume	100 µL	1.8 mL	5.0–5.4 mL	22.5 mL	4.8 L
Final DNA concentration	1 µg/mL	1 µg/mL	1 µg/mL	1 µg/mL	0.75 µg/mL
DNA:LPEI ratio w:w	1:3	1:3	1:3	1:3	1:3
DNA solution volume	20 µL	100 µL	500–300 µL	1.25 mL	100 mL
PEI solution volume	80 µL	100 µL	500–300 µL	1.25 mL	100 mL
Agitation speed	No (adherent)	120 rpm	140 rpm	120 rpm	25 rpm, 8°; 28 rpm, 9.5° 24 hpt

of PEI. **Indirect method:** DNA:PEI ratio was 1:2.5 (w:w). PEI was added dropwise to DNA. The mixture was vortexed for 4 s and incubated 15 min at room temperature. Polyplexes were then added to the cells.

In all cases, cells were fed 24 h post-transfection with TN1 peptide to a final concentration of 0.5% (w/v) to enhance productivity [29]. Details specific to each scale and experiment are described in Table 1.

2.4.1. Transfection in 5 L WAVE Bioreactor

Scalability to 5 L bioreactors was assessed with the chimeric B72.3 antibody. The culture medium (3.5 L) was transferred and acclimatized for one hour at 37 °C in a 10L WAVE Cellbag™ (GE Healthcare, Piscataway, NJ) inflated at 0.1 bar and aerated at 100 ccm with 95% air + 5% CO₂. The rocking speed and angle were 18 rpm and 8° to minimize foaming. Cells grown in shaker flasks were transferred in the WAVE bag (final volume of 4.8 L) to obtain an initial density of 0.40–0.45 × 10⁶ cells/mL. The rocking speed and angle were then set at 25 rpm, 8° for the growth phase. Two days later, cells were co-transfected with the LC and HC plasmids at a 6:4 ratio (w:w). Plasmids were added to the culture at a final concentration of 0.75 µg/mL with a DNA:LPEI (w:w) ratio of 1:3 (direct method) or 1:2.5 (indirect method). Direct transfection was achieved by adding 100 mL of DNA followed 5 min later by 100 mL of LPEI. The transfer line was rinsed with 50 mL of medium after each addition. For indirect transfection, 200 mL of polyplexes were prepared and incubated for 15 min before being added to the cells, followed by a rinse of the transfer line with 50 mL of medium. Twenty-four hours post-transfection, the cultures were fed with TN1 (0.5% w/v final). Then the rocking speed was increased to 28 rpm and the angle to 9.5°. Culture samples were collected every day for 7 days post-transfection.

2.5. Products analyses

2.5.1. GFP

GFP was analyzed 48 h post-transfection (hpt) by flow cytometry using a BD LSRII cytometer (BD Biosciences, Mississauga, ON). For that purpose, 200 µL were taken out from the cultures in 6-well plates (500 µL from cultures of 5 mL and more). Only viable cells were analyzed for the expression of GFP. Remaining cultures were kept in the incubator for subsequent SEAP analysis.

2.5.2. SEAP

Unless otherwise indicated, SEAP was assessed at day 5 post-transfection (dpt). SEAP transfected cells were centrifuged and the supernatant was diluted in water (typically 1/1000). For the 96-well plate scale, the culture medium was sampled without centrifugation step and diluted in water (1/1000). Fifty microliter of diluted supernatant was transferred to a 96-well plate and mixed with an equal volume of SEAP assay solution containing 20 mM paranitrophenyl phosphate (pNPP), 1 mM MgCl₂, and 1 M diethanolamine. Absorbance was read at 405 nm at 1 min intervals for 5 min.

2.5.3. Chimeric B72.3 antibody

Concentration of the chimeric B72.3 antibody from direct and indirect methods in WAVE bioreactor were determined by protein-A HPLC using a 800 µL POROS® 20 micron Protein A ID Cartridge (Applied BioSystems, Foster City, CA) according to the manufacturer's recommendations. The antibody present in the culture medium was also visualized following reducing and non-reducing SDS-PAGE stained by Coomassie.

3. Results

3.1. Optimization of transfection parameters for the direct method

We first determined the optimal parameters for the direct transfection of 293-6E cells grown in agitated 6-well plates in serum-free F17 medium. The pTT vector expressing the human secreted placental alkaline phosphatase (SEAP) was used as it allows rapid and quantitative measurement of the product in the culture medium 5 days post-transfection using a simple and robust colorimetric assay [7,30]. The optimal DNA concentration and DNA:PEI ratio were first determined. Various concentrations of plasmid DNA were added to the culture followed by 5 min incubation under agitation to allow for a uniform mixing. Then various concentrations of PEI were added to the culture and the plates returned to the incubator for 5 days. Each set of experiment was independently repeated three times. SEAP activity in the culture medium was then measured using pNPP as a substrate and expressed as relative value (in %). Fig. 1 (panels A and B) shows that the optimal plasmid DNA concentration lies between 0.5 and 1 µg per mL of culture with 2.5–3.0 µg/mL LPEI, a slightly higher SEAP productivity being obtained at DNA and LPEI concentrations of 1 and 3 µg/mL, respectively. Maximal absolute SEAP titers were of 62, 67 and 75 mg/L for DNA:LPEI concentrations of 0.5:2.5, 0.75:2.5 and 1:3 µg/mL, respectively (averages of 3 independent experiments). For the branched 25 kDa PEI (BPEI), maximum SEAP productivity was observed with 1.5 µg/mL of DNA with 2.5–3.0 µg/mL BPEI. Maximal absolute SEAP titer was of 65 mg/L at a ratio of 1.5:3. As previously observed using the indirect method, LPEI was more efficient than BPEI at transfecting HEK293 cells [7,18] and increasing the concentrations of plasmid and PEI (Fig. 1B) did not increase productivities [31]. A DNA:LPEI ratio of 1:3 with 1 µg/mL of plasmid DNA was thus chosen for the subsequent experiments.

Since in the direct method plasmid DNA is directly added to the culture, a minimum incubation time may be needed to allow its adequate mixing prior to addition of PEI in order to obtain optimal transfection efficiency. In addition, the presence of nucleases released by the cells may reduce productivity by degrading the plasmid DNA. Thus the impact of incubation time with plasmid DNA prior to LPEI addition was evaluated. As shown in Fig. 2A, an incubation time of 5–15 min with the DNA prior to PEI addition yielded a maximum of 65–70% of GFP-expressing cells, as measured by flow cytometry 2 days post-transfection. Similarly, the same incubation time allowed to reach maximal SEAP expression as measured 5 days post-transfection (Fig. 2B). Longer incubation time yielded only slightly lower titers, suggesting that plasmid DNA is not significantly degraded by cell-derived nucleases that may accumulate in the conditioned medium.

3.2. Comparison of direct and indirect transfection methods

3.2.1. Comparison in ThinCert™ plates and 125 mL shake flasks

The direct transfection approach was then compared to the commonly used indirect method (i.e. formation of polyplexes prior to their addition to the culture) using the optimal parameters determined for both methods. For the direct transfection, DNA and PEI concentration used were 1 and 3 µg/mL with a DNA pre-incubation of 5 min prior to PEI addition. For the indirect transfection, previously determined optimal parameters (not shown) were 1 µg/mL of DNA, and 2.5 µg/mL of PEI (DNA:PEI ratio of 1:2.5). Transfections conditions were directly scaled up from 2 mL cultures in 6-well plates to 6 mL cultures in ThinCert™ 6-deep well plates and 25 mL cultures in 125 mL shaker flasks. SEAP expression was measured 5 days post-transfection for ThinCert™ plates

Fig. 1. Effect of DNA and PEI concentrations on SEAP production following direct transfection. Cells were grown in agitated 6-well plates at 1.5×10^6 cells/mL in 1.8 mL of complete culture medium. Plasmid DNA and PEI solutions were each diluted in 100 μ L of culture medium at various concentrations. Following DNA addition, the plates were returned to agitation in the incubator for 5 min, then PEI was added to the cultures. Tryptone N1 was added 24 hpt at 0.5% (w:v) and SEAP measured in the culture medium 5 days post-transfection (dpt) as indicated in Section 2. Error bars are standard deviation from three independent experiments.

(Fig. 3A) or followed daily over 8 days post-transfection for the shaker flasks (Fig. 3B). With both the direct and indirect methods, SEAP accumulation in the culture medium was very similar. For the ThinCert™ plates, slightly higher SEAP activity was obtained (70 vs 61 mg/L) when DNA and PEI solution volumes represented 16% vs 10% (1 vs 0.6 mL) of the final culture volume, respectively, and both conditions yielded higher titers compared to the direct method (58 mg/L). Surprisingly, transfection efficiencies (% GFP-positive cells) were higher in the indirect (53%) vs direct method (39–43%). For the shaker flask experiments, a steady SEAP increase was observed for up to day 4 post-transfection and then reached a plateau at day 5. Maximum titers found at day 5 post-transfection were of 75 and 88 mg/L for the direct and indirect transfection, respectively.

3.2.2. Comparison in a WAVE bioreactor

To demonstrate scalability of the process, a comparison of the direct and indirect methods was also done at the 5L-scale using a WAVE bioreactor with the chimeric B72.3 monoclonal antibody. Expression of the monoclonal antibody was achieved by co-transfection of two plasmids, one encoding the light chain (LC) and the other one the heavy chain (HC). The optimal LC:HC ratio for achieving highest Mab expression was assessed in 6-well plates and found to be 3:2 using 0.75 μ g/mL DNA and 2.25 μ g/mL PEI (DNA:-PEI ratio of 1:3, not shown). This optimal LC:HC ratio is slightly different to the 2:3 ratio previously found for the same antibody transiently expressed in CHO cells [32]. These optimal conditions were used to transfect two 5 L cell cultures in 10 L Cellbag™ using the direct and indirect methods (see Section 2 for culture and

Fig. 2. Impact of cells incubation time with DNA before LPEI addition on transfection efficiency. Cells (1.5×10^6 /mL) were plated in 6-well plates (1.8 mL per well). DNA and PEI solutions were prepared to get a DNA:PEI ratio of 1:3 in the culture. 100 μ L of plasmid DNA solution was added to the wells then 100 μ L of LPEI solution was added at various time points after DNA addition. Plates were returned to agitation in the incubator after each transfection, at 120 rpm under standard conditions. GFP was measured by flow cytometry 48 hpt (panel A) while SEAP activity was determined 5 dpt (panel B). Each point represents the average SEAP values from transfections done in triplicate $\pm$ SD.

transfection procedure). The cultures were sampled daily for up to 7 days post-transfection and antibody titers measured by protein-A HPLC using an in-house purified and quantified chimeric B72.3 standard (Fig. 4A). Supernatants from 5 days post-transfection were also analyzed by reducing and non-reducing Coomassie-stained SDS-PAGE (Fig. 4B). Maximum antibody titers were reached on day 6 and corresponded to 116 and 103 mg/L for the cultures transfected with the direct and the indirect methods, respectively. This shows that the direct method is a viable and simple alternative to the traditional polyplexes transfection approach for large-scale transfections.

3.3. High-throughput transfection and impact of culture mode

3.3.1. Miniprep plasmid DNA production

We tested here the feasibility and reproducibility of the direct transfection process in 96-well plates. We paid special attention to make the protocol easily amenable to automation. The first parameter that we addressed was suitability and reproducibility of miniprep plasmid DNA for efficient transfection of HEK293 cells. One critical aspect was to show that plasmid yield is constant not only between minipreps done in parallel, but also between minipreps done at different times. To that purpose, 24 *E. coli* colonies transformed with the pTT/SEAP plasmid were picked to inoculate 2 mL of culture medium per well in a 24-deep well plate. The cultures were then grown overnight at 37 °C. The following day, 24 pTT/SEAP plasmid minipreps were performed using a commercial kit (Qiagen). A second set of 24 minipreps was performed again as a completely independent experiment, for a total of 48 minipreps. The plasmid DNA was eluted from the columns using 200 μ L of TE buffer and quantified by absorbance at 260 and 280 nm using a NanodropTM spectrophotometer. The first minipreps series yielded an average of 26.6 ± 1.0 μ g while the second series gave 28.1 ± 1.6 μ g (Table 2). This suggests that the minipreps

Fig. 3. Comparison of SEAP production in 6-deep well ThinCertTM plates or 125-mL shaker flasks using the direct or indirect methods. (A) Cells in 6-deep well ThinCertTM (Greiner Bio-One) plates were transfected as follows: *direct method* – 5.4 or 5.0 mL of cultures at densities of $1.5\text{--}2.0 \times 10^6$ cells/mL were added to each well. Then 300 or 500 μ L of plasmid DNA (95% SEAP plasmid and 5% GFP plasmid) were added to the wells containing 5.4 or 5.0 mL of culture, respectively. After 5 min of incubation at 37 °C, 300 or 500 μ L of LPEI solution were added, for a final volume of 6 mL/well in all cases. GFP (black bars) was monitored by flow cytometry 48 hpt while SEAP activity (gray bars) was measured 5 dpt; *indirect method* – 5.4 mL of cultures at densities of $1.5\text{--}2.0 \times 10^6$ cells/mL were added to each well. 600 μ L of polyplexes were then added for a final volume of 6 mL/well. (B) 125 mL Erlenmeyer shaker flasks containing 22.5 mL of culture at densities between 1.5 and 2.0×10^6 cells/mL were transfected using 25 μ g of plasmid DNA (95% SEAP plasmid and 5% GFP plasmid) in 1.25 mL of medium followed 5 min later by the addition of 75 μ g of LPEI in 1.25 mL of medium (direct method; open squares) or 2.5 mL of polyplexes made of 25 μ g of DNA and 62.5 μ g of LPEI (indirect method; open triangles). GFP was monitored by flow cytometry 48 hpt while SEAP activity was measured daily until 8 dpt. Data are mean values of 3 independent experiments $\pm$ SD.

columns were all saturated as their expected capacity are 20 μ g of DNA according to the manufacturer. The plasmid quality was very good as indicated by the average $A_{260/280}$ ratio of 1.89. This suggests that plasmid DNA concentration determination prior to transfection is not necessary as long as column saturation is reached, thus greatly simplifying automation of the process.

3.3.2. Transfection in 96-well plates with miniprep DNA

The transfection conditions in 96-well plates were optimized in a final culture volume of 200 μ L. The optimal DNA:LPEI ratio and concentration was found to be 1:3 and 200 ng of plasmid per well, respectively (data not shown). The impact of cell number per well on volumetric yield was also tested. Volumetric SEAP yield increased steadily from 2×10^4 to 4×10^4 cells/well (e.g. 1×10^5 to 2×10^5 cells/mL) and then slightly declined at 5×10^4 cells/well (Fig. 5A). Subsequently, 72 wells of a 96-well plate were transfected with 24 pTT-SEAP plasmid minipreps in triplicates using the direct method (Fig. 5B). The average SEAP titer measured 5 days post-transfection was 17.2 ± 2.7 mg/L. This SEAP productivity observed in Fig. 5A and B was about four-times lower than that obtained with transfection in agitated 6-well plates. To verify that

Fig. 4. Chimeric B72.3 monoclonal antibody productions in 5 L WAVE bioreactors, following direct or indirect transfections. 4.8 L of culture in a 10 L Cellbag™ were transfected with the direct or indirect method as described in Section 2. The cultures were sampled daily for up to 8 days post-transfection. (A) Antibody titers were determined by protein-A HPLC against a purified chimeric B72.3 standard. (B) 20 μ L of culture medium sampled at day 5 was analyzed by SDS-PAGE under reducing or non-reducing condition. D: direct transfection; I: indirect transfection.

Table 2
Yield and purity of miniprep plasmid DNA.

Plasmid miniprep	1st series (average $\pm$ SD)	2nd series (average $\pm$ SD)	Average of 48 minipreps ($\pm$ SD)
Concentration (μ g/mL)	133.2 $\pm$ 5.0	140.5 $\pm$ 8.4	136.8 $\pm$ 7.8
Yield (μ g)	26.6 $\pm$ 1.0	28.1 $\pm$ 1.6	27.4 $\pm$ 1.6
A ₂₆₀ /A ₂₈₀	1.93 $\pm$ 0.01	1.86 $\pm$ 0.02	1.89 $\pm$ 0.04

this lower yield was not due to contaminants present in the minipreps DNA, we compared productivity of suspension cells in 6-well plates using miniprep and maxiprep plasmid DNA (Fig. 5C). The average SEAP productivity and transfection efficiency obtained with minipreps DNA was of 112.1 ± 2.3 mg/L and $78.9 \pm 0.6\%$ vs 123.3 ± 11.7 mg/L and $82.0 \pm 4.6\%$ for maxipreps. This demonstrates that the use of miniprep DNA provides volumetric yields only slightly lower to those obtained with maxiprep DNA. This might be due to the significantly higher level of endotoxins measured in those DNA preparations (see Fig. 5 legend for details).

3.3.3. Transfection of adherent vs suspension cultures

The impact of the culture mode (adherent vs suspension) on SEAP productivity was evaluated using the direct transfection

method (Fig. 6). Cells transfected in non-agitated 6-well plates secreted 25.4 ± 4.4 mg/L of SEAP while agitated cultures produced 117.1 ± 16.5 mg/L (average of two independent experiments done in triplicates). This clearly shows that suspension-growing cells allow reaching titers that are 4–5 times higher compared to adherent cells. This difference likely result from the higher optimal cell density at time of transfection of suspension vs adherent cells ($1.5\text{--}2.0 \times 10^6$ cells/mL vs 0.36×10^6 cells/mL, respectively). The lower SEAP yield measured in the 96-well plate format (17.2 mg/L, panel B) compared to the non-agitated 6-well plate (25.4 mg/L, panel D) may also be explained by the lower optimal cell density found for the former culture format.

4. Discussion

With the continual increase in demand for high-throughput and high-level protein expression, robust and simple processes providing predictable protein yields are needed. The robustness, versatility and relative simplicity of PEI-mediated transient expression systems concomitant with the use of performing cell lines and episomal expression vectors [10–12] have made protein expression in mammalian cells a valuable alternative to bacterial systems. For a given cell line, the efficiency of PEI-mediated transfection depends on many parameters such as the culture medium in which the transfection occurs, the mode of cell culture (i.e. adherent vs suspension), the cell density at time of transfection, the DNA concentration in the culture medium and the DNA:PEI ratio. The formation of polyplexes by mixing together plasmid DNA and a polycation is a dynamic process involving particle formation, growth and aggregation. As the size and polydispersity of polyplexes are important parameters for efficient gene transfer [20,33], it is a significant source of variation in transfection efficiencies, especially when up-scaling or down-scaling culture volumes [34]. The preparation of polyplexes, which consists of mixing DNA with a cationic polymer such as PEI, is typically done via pipetting and as such, is restricted to relatively small volumes. When performing large-scale (multi-liters) transfections, mixing of DNA with PEI is often done by manual swirling in glass bottles, a process that is difficult to control and which imposes a limitation on the volume that can be handled. In addition, high-throughput PEI-mediated transfection in 96-well plates is challenging as it involves very small volumes that are not easily amenable to efficient and reproducible mixing. To overcome these challenges we have optimized parameters for the direct transfection approach, which consists of the addition of plasmid DNA and PEI directly to the culture. We have shown that this approach provides recombinant protein titers similar to the usual indirect method based on the addition of pre-made polyplexes to the culture. As the direct transfection method implies less pipetting and mixing steps, it is also well suitable for automation in 96-well plates. Even though transfection of adherent cells generates 4- to 5-fold less product compared to transfection of suspension cells, a likely result of the 4- to 5-fold lower optimal transfection density of adherent vs suspension cultures, transfection in 96-well plate allows the high throughput parallel evaluation of multiple variants for expression screening.

The direct transfection approach has also been described recently by Backliwal et al. [35]. In their study, the authors performed the direct transfection using a high cell density suspension (20×10^6 cells/mL) that was obtained following centrifugation of a seed train. The cell suspension was then diluted with fresh medium to 1×10^6 cells/mL four hours post-transfection. While this approach was shown to provide twice as much titers compared to their direct method, it is difficult to perform at large-scale as it does involve a centrifugation step.

Fig. 5. Transfection in 96-well plates. (A) the optimal cell number per well was first evaluated. Twenty microliter of plasmid DNA were first added to the wells. Then, 100 μ L of cells at various densities were plated and mixed with DNA by one up and down pipetting. After 5 min at room temperature, 80 μ L of LPEI was added to each well. The large volume of PEI solution was increased to 40% of the final culture volume (200 μ L/well) to enhance mixing of DNA and PEI with the cells. After transfection, cells were returned to the incubator under standard conditions, without agitation. (B) SEAP titers from 72 transfections performed in 96-well plates using 24 pTT/SEAP plasmid minipreps. Twenty microliter of plasmid DNA were first added to the wells, then 100 μ L of cells at 0.4×10^6 cells/mL were added followed by 80 μ L of LPEI as described above. (C) SEAP titers 5 dpt (gray bars) and % GFP-positive cells 2 dpt (black bars) obtained from transfection suspension cultures in 6-well plates using three independent minipreps (mini) or maxipreps (maxi) DNA. (D) to evaluate the relative quality of miniprep vs maxiprep DNA, 6 μ L of the three minipreps (corresponding to about 850 ng) shown in panel C was loaded side-by-side with 1 μ g each of the three maxiprep on an agarose gel and stained with ethidium bromide. The endotoxin levels as determined by the LAL method was very high in the miniprep DNA (>75000 EU/mg) compared to maxiprep DNA (average of 2500 EU/mg).

Fig. 6. Transfection of adherent vs suspension cultures. Productivity of adherent vs suspension cells was compared in 6-well plates. Cells (1.8 mL) were respectively plated at 4×10^5 or $1.5\text{--}2 \times 10^6$ cells/mL for the adherent or suspension cultures. Plasmid DNA ($2 \mu\text{g}$) in $100 \mu\text{L}$ of culture medium was then added, followed by the addition of $100 \mu\text{L}$ of LPEI ($6 \mu\text{g}$). SEAP activity was measure 5 dpt. Data show the average of 2 independent experiments done in triplicates $\pm$ SD.

We have shown here that the direct transfection method can be applied to 6-well or 6-deep well plates, 125 mL shaker flasks and to large-scale multi-liter transfection in bioreactors using exactly the same conditions. As ThinCert™ 6 deep-well plates can be stacked easily and can accommodate up to 8 mL of culture/well, we found them convenient for producing significant quantities of proteins in a high-throughput manner. The method can even be adapted to 24 deep-well plates since transfection of 293-EBNA1 cells grown in these plates was shown to be efficient [36,37]. Moreover, we have shown that plasmid DNA prepared using silica-based commercial miniprep kits is fully compatible with PEI-mediated transfection, giving expression levels only slightly lower to those obtained with anion-exchange purified plasmids. The quantity of plasmid DNA generated with miniprep kits ($\sim 25 \mu\text{g}$) is sufficient to transfect up to 25 mL of cell culture, which is enough for small-scale affinity purification and crystallization trials [13]. For that purpose, the addition of the glycosyltransferase inhibitor kifunensine can be used to generate glycoproteins with high mannose-type glycosylation that are more susceptible to endoglycosidases treatment and to crystallization [38,39]. Preliminary results have shown that this transfection method can also be applied to suspension CHO cells grown in serum-free medium (data not shown).

Still today, many high-throughput transient expression platforms use adherent 293 cells [13,38,40]. We have shown here that optimal transfection conditions differ significantly between adherent and suspension cultures, and that suspension cultures provide significantly higher volumetric yields vs adherent ones. Also, cell density measurement at time of transfection, a critical parameter for optimal gene transfer and expression, can only be qualitatively estimated when growing adherent cultures. As for scaling-up production with adherent cells, it requires costly culture flasks such as cell factories (e.g. CellStack™ system) which involve extensive manipulations and complicate cell harvesting for intracellular or membrane-associated products. A single $\sim 6000 \text{ cm}^2$ cell factory (10 layers of $\sim 600 \text{ cm}^2$) that typically uses 2 L of culture medium provides up to 1×10^9 cells at time of transfection, compared to up to 4×10^9 cells with suspension cultures. As 2 L of suspension cells can be easily grown in two 3 L Erlenmeyer shaker flasks, the

costs for the culture vessels are reduced by a factor of ~ 4 . Combined with the significantly increased volumetric productivity obtained with suspension cells, these factors strongly illustrate the benefits of suspension over static cultures for recombinant protein production by transfection.

In conclusion, the direct transfection of 293 cells in serum-free suspension cultures offers a simple, robust and scaleable process for small-scale high-throughput and large-scale protein expression with volumetric yields comparable to the commonly used indirect method. As the process can use miniprep- or maxiprep-quality DNA without impacting transfection efficiency or productivity and does not require a medium exchange at any time, it greatly facilitates automation as well as large-scale operations.

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