Effect of addition of ‘carrier’ DNA during transient protein expression in suspension CHO culture

Ketaki Pradhan · Mugdha Gadgil

Received: 12 September 2011 / Accepted: 1 February 2012 / Published online: 14 March 2012
© Springer Science+Business Media B.V. 2012

Abstract Transient protein expression using polyethyleneimine as a transfection agent is useful for the rapid production of small amounts of recombinant proteins. It is known that an increase in extracellular DNA concentration during transfection can lead to a nonlinear increase in intracellular DNA concentration. We present an approach that hypothesizes that this nonlinearity can be used to decrease the amount of plasmid required for productive transfections. Through addition of non coding ‘carrier’ DNA to increase total DNA concentration during transfection, we report a statistically significant increase in protein (IgG) expression per unit plasmid used for transfection. This approach could be useful to increase protein yields for large scale transfections under conditions where plasmid availability is limited.

Keywords Transient protein expression · Carrier DNA · Salmon sperm DNA · pH excursion · Medium equilibration with CO₂ · Cell age · DNA: polyethyleneimine particle size

Background

Expression of recombinant proteins (r-proteins) in mammalian cells is predominantly achieved by

establishing a cell line in which the recombinant gene is integrated into the host genome and stably expressed over time. The cell line development process involves the isolation, characterization, and expansion of a highly productive clonal cell line from a pool of transfected cells. This labor intensive process for producing a r-protein can take between 6 and 18 months. It may frequently be necessary to rapidly produce small quantities of proteins, e.g. for pre-clinical evaluation of drug candidates. Such rapid expression of milligram to gram quantities of a recombinant protein can be achieved using transient protein expression (Baldi et al. 2005; Geisse et al. 2009; Pham et al. 2006). The two major hosts that have been used for transient protein production are Human Embryonic Kidney cells (HEK 293) and Chinese Hamster Ovary cells (CHO). Transient protein expression involves transfecting the host cell with plasmid encoding the recombinant protein. The amount of plasmid DNA delivered to cells and the transfection efficiency, i.e. fraction of cells which have received the DNA, influence the culture productivity. Both these factors (transfection efficiency and DNA delivered) are affected by the concentration of plasmid DNA in the culture (Glover et al. 2010). It has also been shown that the amount of DNA delivered to the nucleus is a nonlinear function of the extracellular DNA concentration, i.e. a twofold increase in external DNA concentration results in a greater than twofold increase in intra-nuclear DNA delivered (Glover et al. 2010; Tachibana et al. 2002). This suggests a strategy where the plasmid costs could be reduced by effecting an increase in the extracellular DNA

K. Pradhan · M. Gadgil (✉)
Chemical Engineering and Process Development,
CSIR - National Chemical Laboratory, Pune 411008,
India
e-mail: mc.gadgil@ncl.res.in

concentration by addition of ‘non-coding’ DNA such as salmon sperm DNA. This would be expected to result in increased protein expression even when the same amount of plasmid is used for transfection. Such an increase in DNA concentration by use of carrier DNA could be useful in situations when availability of plasmid DNA encoding the gene of interest is limited, which is possible at large scales. As an example of the plasmid DNA requirements at large scales, transfection of 100 L culture will need approximately 100 mg plasmid when transfected with 1 µg/ml plasmid DNA. Susa et al. (2008) showed that addition of carrier DNA enhanced transfection efficiency and reporter protein expression at low coding DNA concentrations (≤ 20 ng/well of 96 well plate) but had no effect on reporter expression at 40 ng/well using Fugene transfection reagent. However, to our knowledge, this has not been tested with secreted proteins and with PEI based DNA delivery.

Factors such as the medium composition and culture protocol, and the cell ‘age’ or number of passages after thawing, are also expected to affect transfection outcomes and hence productivity. Prior to evaluating the effect of carrier DNA, we tested the effect of these factors on culture productivity. DNA delivery agents such as calcium phosphate, liposomes and cationic polymers like polyethyleneimine (PEI) have been used to transfect 1 ml to 100 l suspension cultures of mammalian cells to transiently express recombinant proteins (Derouazi et al. 2004; Girard et al. 2002; Liu et al. 2008; Schlaeger and Christensen 1999; Meissner et al. 2001; Muller et al. 2007; Schlaeger et al. 2003). Polyethyleneimine is one of the widely used transfection agents used due to its ability to transfect a broad range of cell lines and low toxicity along with its cost effectiveness over commercially available reagents which could be an important consideration for scale up (Boussif et al. 1995). PEI effectively transfers DNA to the nucleus because of its natural buffering capacity which helps in endosomal escape (Boussif et al. 1995; Godbey et al. 2000). Both linear (Derouazi et al. 2004) and branched PEI (Tait et al. 2004) have been reported for transient expression. In this work we have restricted our analysis to the use of branched PEI as a delivery agent.

Formation of DNA:PEI complex depends on the electrostatic interaction between cationic charge of PEI and anionic charge of plasmid DNA. The particle size and zeta potential of the DNA:delivery agent complex can govern gene delivery to the cell (Kawaura et al. 1998; Ogris et al. 1998; Takeuchi et al. 1996) as they can

determine the binding, uptake and delivery of the complexes to the cell. DNA:PEI complexes are known to aggregate over time (Sharma et al. 2005), and this aggregation behavior is likely to differ in various medium compositions. Here we have tested the effect of two medium compositions on the particle size distribution and shown that the use of CHO-S-SFM II results in reduced aggregation.

It is known that transfection efficiencies and productivity can have significant day-to-day variability even though the same transfection parameters are rigorously applied at all times (Grosjean et al. 2002). We hypothesized that culture pH could play a part in this variability. Mammalian cell culture media are usually buffered using bicarbonate and require equilibration with appropriate CO₂ levels to attain optimal pH. Cells are frequently inoculated into medium which has not been pre equilibrated with appropriate levels of CO₂. This leads to a short-term pH excursion until CO₂ equilibration resulting in pH control to appropriate level is achieved. Another cause of the variability could be differences in cell cycle distribution (Männistö et al. 2005) which can affect DNA uptake. A third factor affecting protein expression could be the age of the culture (Bollin et al. 2011). Transfection efficiency and protein expression also depend on the cell density at the time of transfection (Backliwal et al. 2008; Sun et al. 2008). The plasmid DNA concentration has an ‘interaction’ with cell density. For instance, Bollin et al. (2011), observed an increase in protein expression with increasing DNA concentrations during transfections using linear PEI at high cell densities.

In this study, we have tested the effect of medium choice on DNA:PEI complex size, and of transient change in medium pH, cell age and cell density on transfection efficiency and protein expression to optimize operating conditions for transfection. For the best combination of these factors, we evaluated the use of carrier DNA on transient IgG expression and show that it results in higher protein expression per unit plasmid DNA used for transfection.

Materials and methods

Cell culture

CHO-S cells were obtained from Invitrogen Corporation, CA, USA and cultured in CHO-S-SFM II

medium (Invitrogen Corporation, CA, USA) without any added antibiotics or antifungal agents and maintained in shake flasks at 37 °C, 10% CO₂ and 110 rpm (shaker orbit diameter = 1.9 cm). Viable cell counts were performed using the trypan blue dye exclusion test. Cells were routinely maintained by seeding at a density of 0.2×10^6 cells/ml, and passaging after 2 days to maintain a similar cell 'state' for transfections. The number of cell doublings was calculated at every passage to maintain record of cell age in terms of number of generations.

DNA and transfection reagent

IgG heavy chain plasmid and IgG light chain plasmids were kindly provided by Inbiopro Solutions Pvt Ltd. (Bangalore, India). All plasmids were purified using commercial plasmid purification kits (Qiagen, Hilden, Germany). A stock solution of salmon sperm DNA (Sigma Aldrich) was made in TE buffer before purification. 45 µg salmon sperm DNA was treated with RNase for 1 h at 37 °C and purified through QIAquick column (Qiagen, Hilden, Germany) resulting in average purified DNA yield of 23 µg. This purified DNA was used as a carrier DNA for transfections where indicated. 25 kDa branched PEI was obtained from Sigma Aldrich. 1 mg/ml stock solution was prepared in water; pH was adjusted to 7 and the solution was sterilized by filtration through a 0.2 µm membrane.

DNA:PEI complex size measurement

DNA:PEI complex sizes were measured to study the aggregation behavior of complexes over time in two commercially available medium compositions. Particle size measurements were carried out by dynamic light scattering using a Brookhaven BI-MAS particle size analyzer accompanied with BIC Particle sizing software with a 90° scattering angle and 15 mW solid state laser ($\lambda = 670$ nm). For complex formation, 3 µg plasmid DNA and 3.9 µg PEI were mixed separately in 75 µl of 150 mM NaCl. PEI solution was added to plasmid DNA containing solution and incubated for 10 min at room temperature. Subsequently, this mixture was added to 3 ml of CD CHO medium (chemically defined medium) or CHO-S-SFM II (serum free medium). Particle size measurements were performed at 10, 30 and 60 min after

addition of DNA:PEI mixture to medium. Effective particle diameter is reported.

Transfection of CHO cells

Cells were seeded into CHO-S-SFM II medium at a density of 0.5×10^6 cells/ml prior to transfection, unless otherwise indicated. PEI/DNA complexes were formed at a N/P molar ratio of 10 for all transfections as it has been shown to be the optimal ratio for transfection of CHO-S cells adapted to grow in CHO-S-SFM II medium (Tait et al. 2004). We observed decreased cell viability at higher N/P ratios. The plasmid DNA concentration in the culture during transfection is 1 µg/ml, unless otherwise indicated. In the case of transfection with IgG heavy and light chain genes containing plasmids, each plasmid was used at a concentration of 0.5 µg/ml. Briefly, plasmid DNA and PEI were mixed separately in CHO-S-SFM II medium at 2.5% of culture volume to be transfected. PEI solution was added to DNA containing solution, mixed thoroughly, incubated at room temperature for 10 min and added to the culture. Five hours after transfection, an equal volume of medium was added to the transfected culture.

Measurement of transfection efficiency

Transfection efficiency was calculated after transfection with plasmid encoding β -galactosidase followed by staining for β -galactosidase. For staining, cells were washed twice with 1.5 ml phosphate buffered saline (PBS, pH 7.4) and incubated with 1 ml cell fixative (containing formaldehyde, glutaraldehyde and PBS) at room temperature for 15 min. After fixation, cells were again washed twice with 1.5 ml PBS and incubated with 300 µl histochemical stain (containing potassium ferrocyanide, potassium ferricyanide, MgCl₂, PBS and X-gal in dimethylformamide) for 18 h.

Effect of transient pH excursion before transfection

Prior to transfection, cells were seeded in shake flasks at viable cell density of 0.5×10^6 cells/ml in CHO-S-SFM II medium which was previously equilibrated with 10% CO₂ in the incubator for 1.5 h resulting in a pH of 7.2, and in CHO-S-SFM II medium which was not pre

equilibrated with CO₂ resulting in a pH ~7.8. Cells were transfected according to the procedure described above. Five hours after transfection, cultures were diluted by adding an equal volume of pre-equilibrated or non-equilibrated medium, respectively. Cells were sampled daily to measure cell density and IgG concentration using sandwich ELISA. Briefly, the standard sandwich ELISA format included coating a 96-micro-well plate with affinity purified rabbit anti-human IgG for capture of IgG, followed by application of culture supernatant sample and detection with a rabbit HRP conjugated anti-human IgG. TMB was added for enzymatic oxidation by HRP, and the intensities were read at 450 nm. Concentrations were calculated based on a standard curve between 4 and 150 ng/ml. All samples were analyzed in duplicates. IgG concentration was seen to peak 3 days after transfection. The concentrations on the 3rd day of the culture with or without medium pre-equilibration were compared.

DNA:PEI complex size was also measured in CHO-S-SFM II medium at pH 7.2 and 7.8. pH was adjusted to 7.2 by addition of HCl. DNA:PEI complex was prepared at pH 7.8 in non-equilibrated CHO-S-SFM II medium to simulate conditions during actual complex formation for transfection, and after 10 min incubation at room temperature, was diluted either with medium at pH 7.2 or 7.8. Particle size measurements were performed at 10, 30 and 60 min after complex formation as described previously.

Effect of DNA concentration on protein production

CHO-S cells were seeded at viable cell densities of 0.5, 1, 2.5 and 5×10^6 cells/ml in a 24 well plate and transfected with 1, 2 and 3 µg/ml total plasmid DNA. Transfection was performed with equal amounts of heavy chain and light chain plasmids. Transfected cultures were diluted threefold by addition of pre-equilibrated medium after 5 h of transfection. Cells were harvested 28 h after transfection to avoid confounding due to nutrient limitation, and IgG concentration was estimated using ELISA as described above.

Effect of adding non coding DNA to increase total DNA concentration on protein production

Cells were transfected at viable cell densities of 1 and 2.5×10^6 cells/ml in a 24 well plate. Total DNA

concentration of 2 µg/ml (1 µg/ml plasmid + 1 µg/ml salmon sperm DNA) or 3 µg/ml (1 µg/ml plasmid + 2 µg/ml salmon sperm DNA or 2 µg/ml plasmid + 1 µg/ml salmon sperm DNA) were used for transfection. 1 and 2 µg/ml plasmid DNA were used as controls. Transfected cultures were diluted threefold 5 h after transfection by addition of medium and cells were harvested 28 h after transfection as noted previously.

The effect of addition of carrier DNA was also evaluated in shake flasks. Cells were seeded at 1×10^6 cells/ml, and transfected with 1 µg/ml plasmid, 1 µg/ml plasmid + 1 µg/ml salmon sperm DNA and 1 µg/ml plasmid + 2 µg/ml salmon sperm DNA at a N:P ratio of 10:1. Five hours post transfection, the culture was diluted threefold by addition of medium. Cultures were sampled daily to measure cell density and IgG concentration.

Statistical test

A Student's *t* test was used for statistical comparison. Equal variance in both groups was assumed, except where it is indicated that a paired *t* test was used.

Results

Optimization of operating conditions for transfection

To rationally guide the choice of operating conditions for transfection, we tested the effect of medium composition on the size distribution of the DNA:PEI complex and hence their ability to penetrate the cell membrane. We then used the medium that led to reduced aggregation for further optimization. We next tested the effect of a transient drop in pH and the effect of cell age to establish limits on these conditions that yield the best productivity.

Dynamic light scattering was used to assess the size distribution of DNA:PEI particles in CD CHO (chemically defined medium) and CHO-S-SFM II (serum free medium). DNA:PEI complex was allowed to form in 150 mM NaCl at a N:P ratio of 10:1, which has previously been reported to be optimum for transfection of CHO cells (Tait et al. 2004; Tait et al. 2005). Figure 1a shows that DNA:PEI complex size increased significantly with time in CD-CHO medium

Fig. 1 **a** Effective particle size of DNA:PEI complexes in CD CHO (circle) and CHO-S-SFM II (square) media measured by dynamic light scattering. **b** Transfection efficiency of cells when transfected in CHO-S-SFM II medium pre equilibrated with 10% CO₂ (pH ~7.2, triangle) and non equilibrated medium (~pH 7.8, square). Error bars show one standard deviation

above and below the average value. Number of replicates = 2 **c** IgG expression level on the 3rd day and transfection efficiency after transfection as a function of cell age measured in number of generations starting from the CHO-S cells purchased from Invitrogen Corporation. Experiments were performed across different vial thaws indicated by the different marker symbols

indicating aggregation behavior. In comparison, the particle size increase in CHO-S-SFM II medium was less. CHO-S-SFM II medium was then used in further transfections.

We then evaluated the effect of transient changes in medium pH immediately before transfection, to establish medium equilibration requirements with CO₂ for a generic transfection protocol for transient protein expression.

The CHO-S-SFM II medium used in this study had a pH of ~7.8 when not appropriately equilibrated with CO₂. In the case of the shake flasks used in this study, visual observation of change in color of the pH indicator in the medium indicated that approximately 1.5 h were required to achieve complete equilibration with CO₂ resulting in appropriate pH value. The effective particle size of complexes in CHO-S-SFM II medium at pH 7.2 or 7.8 was not significantly different (data not shown).

To test the effect of such a short-term pH excursion, cells were seeded at the time of transfection in CHO-S-SFM II medium which was pre equilibrated with 10% CO₂ in the incubator for 1.5 h (pH 7.2) and in the non-equilibrated medium which was slightly alkaline (~pH 7.8). Figure 1b shows that the difference in transfection efficiency of the cells was not statistically significant. To evaluate the effect of a short-term pH excursion on the transient expression of a recombinant protein, cells were also transfected with heavy chain and light chain genes for IgG. We observed that short term alkaline pH excursion due to non equilibrated medium prior to transfection reduced the overall IgG production by $23 \pm 10\%$ (95% CI). A paired *t* test comparing the IgG expression levels in the two conditions resulted in a *p* value of 0.003. Thus, pre equilibrated medium should be used for transfection to reduce the level of variation. Though the decrease in the protein expression is not large, this highlights the

importance of appropriate handling procedures prior to transfection to reduce variability.

To establish limits on cell age, we evaluated protein expression at different numbers of generations of cells, counted starting from the vial of cells purchased from Invitrogen Corporation (CA, USA) which were expanded and stored frozen in our laboratory. Four vials of frozen cells thawed at different instances were compared at different cell ages. Figure 1c shows the transfection efficiency and IgG expression levels on day 3 when cells were transfected at different numbers of generations across the 4 different thaws. We found no significant trend in the protein expression levels when transfections were carried out over 110 generations, at 1.3 mg/l branched PEI concentration. A recent report (Bollin et al. 2011) also has shown no difference in antibody titers at 1.5 mg/l linear PEI concentration at 4 and 70 passages, with no data reported in between. The number of cell generations corresponding to 70 passages by Bollin et al. (2011) cannot be calculated from that report, but the general trend in this study is consistent with those results. Bollin et al. report a decrease in expression at higher linear PEI concentrations, but this was not evaluated in our study due to higher toxicity of branched PEI at higher N:P ratios.

Thus, for all further experiments, cells were used below 110 generations age with complete equilibration of CHO-S-SFM II medium with CO₂ prior to inoculation of cells for transfection.

Effect of addition of carrier DNA on protein expression

Effect of varying cell density and DNA concentration at transfection

We first evaluated the effect of increasing plasmid DNA concentration at different cell densities on protein expression using branched PEI to guide the choice of cell density for testing effect of carrier DNA addition.

Cells were seeded at 0.5, 1, 2.5 and 5 × 10⁶ cells/ml in a 24 well plate and transfected with IgG heavy and light chain gene containing plasmids at total DNA concentrations of 1, 2 and 3 µg/ml (Fig. 2). It was observed that cell growth and viability decreases with increasing DNA concentration at lower seeding cell densities (0.5 and 1 × 10⁶ cells/ml) (Fig. 2a, b). In general, IgG expression increased when the plasmid DNA concentration was increased from 1 to 2 µg/ml (significance, *p* < 0.05) at all cell densities. An increase in DNA concentration from 2 to 3 µg/ml resulted in a significant increase in IgG concentration only at 5 × 10⁶ cells/ml. These experiments indicated that an equal per cell availability of DNA did not result in equal protein expression (for example, in Fig. 2 compare 2.5 × 10⁶ cells/ml at 1 µg/ml DNA to 5 × 10⁶ cells/ml at 2 µg/ml DNA). Transfecting cells at higher densities, albeit with higher plasmid DNA

Fig. 2 Effect of cell density and plasmid DNA concentration on IgG expression. CHO-S cells were transfected at different viable cell densities between 0.5 × 10⁶ and 5 × 10⁶ cells/ml with varying DNA concentrations 1 µg/ml (diagonal lines), 2 µg/ml (dashed lines) and 3 µg/ml (dots). Culture was diluted threefold by medium addition 5 h post transfection. Effect on **a** number of population doublings, **b** cell viability and **c** IgG

expression level was evaluated after 28 h. 4 replicate experiments were carried out at different instances and each condition was tested in duplicate during each experiment. The protein expression values obtained using 2 and 3 µg/ml plasmid were, respectively, compared with those obtained using 1 µg/ml plasmid using a *t* test. All comparisons were significant at a 0.05 significance value

Fig. 3 Effect of supplementing plasmid DNA with salmon sperm DNA on IgG expression and transfection efficiency. Cells were transfected in 24 well plates at cell densities of **a** 1×10^6 cells/ml, **b** 2.5×10^6 cells/ml. 0 µg/ml (indicated as 0 s), 1 µg/ml (1 s) and 2 µg/ml (2 s) of salmon sperm DNA was supplemented to the indicated plasmid DNA concentration, 1 µg/ml (1p) and 2 µg/ml (2p), at both cell densities. Culture was diluted threefold by medium addition 5 h post transfection.

IgG expression was evaluated 28 h after transfection. Experiments were carried out in duplicate at different instances and carrier DNA transfection condition was tested in triplicates during each experiment. **c** Transfection efficiency when cells were transfected at the density of 1×10^6 cells/ml in a 6 well plate with 1 µg/ml plasmid DNA supplemented with 1 and 2 µg/ml carrier DNA. Experiment was performed in duplicate

concentrations did lead to higher expression at equivalent DNA per cell availability. Since cell pelleting was observed at 5×10^6 cells/ml, and in order to avoid any effects of nutrient limitation during batch culture in CHO-S-SFM II medium, we further evaluated the effect of carrier DNA addition at 1×10^6 and 2.5×10^6 cells/ml.

Effect of carrier DNA on IgG production

Salmon sperm DNA was used as non coding DNA to supplement plasmid DNA. Transfections were performed at two different seeding cell densities, 1×10^6 and 2.5×10^6 cells/ml, in a 24 well plate, with and without addition of carrier DNA to 1 and 2 µg/ml plasmid DNA. Figure 3 shows that the addition of carrier DNA led to higher IgG expression for the same plasmid DNA concentration. Table 1 shows the average IgG expression in all conditions, along with the significance value obtained from a Student's *t* test comparing IgG expression levels with carrier DNA addition to that without any carrier DNA addition, for the same plasmid DNA concentration in both conditions. At both seeding cell densities, 1 µg/ml plasmid DNA + carrier DNA produced greater IgG as compared to when only 1 µg/ml plasmid DNA was used (significance, $p < 0.05$). The addition of carrier DNA to 2 µg/ml plasmid DNA led to a significant increase

in IgG expression only at 2.5×10^6 cells/ml. The transfection efficiencies for cells transfected at 1×10^6 cells/ml with varying carrier DNA concentrations are shown in Fig. 3c, and show a small increase with the addition of carrier DNA.

The effect of addition of carrier DNA was further evaluated in shake flasks at cell seeding densities of 1×10^6 cells/ml (Fig. 4), and showed an average 1.7-fold increase in final IgG yield. A paired *t* test comparing the IgG concentration of the cultures with and without carrier DNA resulted in significance values less than 0.05 on all 3 days. Thus the results demonstrate that the use of carrier DNA results in a higher amount of expressed protein per unit plasmid DNA used for transfection.

Discussion

Since it has been shown that the amount of DNA delivered to the nucleus is amplified at increasing extracellular DNA concentration (Glover et al. 2010; Tachibana et al. 2002), we hypothesized that a higher intranuclear DNA concentration might be achieved by adding non-coding DNA to increase total extracellular DNA concentration, which could finally lead to a concomitant increase in protein expression. We showed that the use of carrier DNA in the form of

Table 1 Effect of carrier DNA addition on IgG expression level

Seeding cell density (cells/ml)	Plasmid DNA conc. (μg/ml)	Average IgG expression level (ng/ml)			<i>p</i> value compared to control	
		Carrier DNA = 0 μg/ml	Carrier DNA = 1 μg/ml	Carrier DNA = 2 μg/ml	Carrier DNA = 1 μg/ml	Carrier DNA = 2 μg/ml
1×10^6	1	322	999	899	0.001	8.7×10^{-5}
	2	964	1,273	ND	0.094	ND
2.5×10^6	1	329	779	1,463	7×10^{-4}	3.6×10^{-6}
	2	1,389	2,265	ND	4×10^{-4}	ND

Average IgG expression shown in all conditions, along with the significance value obtained from a Student's *t* test comparing IgG expression levels with carrier DNA addition as compared to those without any carrier DNA addition (control), for the same value of plasmid DNA concentration in both conditions

ND not determined

Fig. 4 Effect of addition of carrier DNA on IgG yield. Cells were seeded into shake flasks at a density of 1×10^6 cells/ml, and transfected with only 1 μg/ml plasmid DNA (control) or 1 μg/ml plasmid DNA + 1 μg/ml salmon sperm DNA (open bars) or 1 μg/ml plasmid DNA + 2 μg/ml salmon sperm DNA (solid bars). IgG concentration in culture supernatant was estimated using ELISA. Fold increase in IgG concentration with carrier DNA addition as compared to control culture. Experiment was performed in triplicate at different instances. A paired *t* test comparing IgG yields was significant at a *p* value of 0.05 on all 3 days

salmon sperm DNA to increase the total DNA concentration during transfection led to increased IgG expression.

Tait et al. (2004) have previously reported IgG yields of ~ 4.5 μg/ml (~ 1 μg/ml 1 day post transfection) when transfecting CHO cells at a density of 10^6 cells/ml with branched PEI at a plasmid DNA concentration of 5 μg/ml when cells were arrested in G2/M phase of the cell cycle using the microtubule depolymerizing agent nocodazole to enhance protein expression. Thus, the IgG yield per unit plasmid DNA concentration was ~ 0.9 μg IgG/μg plasmid DNA.

With the use of carrier DNA in this study, we observed an average IgG yield of 2.5 μg/ml (1 μg/ml 1 day post transfection) when 10^6 cells/ml were transfected with 1 μg/ml plasmid DNA along with 1 μg/ml carrier DNA. Thus the average IgG yield per unit plasmid DNA concentration is 2.5 μg IgG/μg plasmid DNA with the use of carrier DNA. Galbraith et al. (2006) reported an IgG yield of ~ 1 μg/ml (~ 0.2 μg/ml 1 day post transfection similar to the levels achieved in control condition in this study), when transfecting CHO cells at a density of 10^6 cells/ml with branched PEI using a plasmid DNA concentration of 1.25 μg/ml. They showed that treatment with LR3-IGF resulted in a yield of ~ 2.5 μg/ml, which is similar to the yields obtained in this study at a lower plasmid DNA concentration of 1 μg/ml with the use of carrier DNA. They also showed that hypothermia and LR3-IGF acted synergistically to increase product titer 11-fold. Similar use of hypothermia and LR3-IGF can also be evaluated in conjunction with the use of carrier DNA to further enhance protein expression per unit plasmid DNA.

The rate of reporter protein expression has been shown to follow saturation kinetics as a function of the amount of plasmid DNA delivered to the nucleus (Tachibana et al. 2002; Takahashi et al. 2011). The use of carrier DNA is hence not likely to be useful at high plasmid DNA concentrations where the cellular protein expression machinery is already saturated. Nonetheless, it may be a useful approach to increase protein expression when plasmid DNA availability is limiting. Though our original plan to assess the effect of supplementation with non coding DNA was motivated by a possibility of amplification of DNA delivery with

increased extracellular DNA concentration causing increased protein expression, there could also be other mechanisms resulting in this effect. The increased expression with addition of carrier DNA could also be due to an unknown effect of linear mammalian DNA on DNA unpacking from the DNA:PEI complex.

Acknowledgments The authors gratefully acknowledge gift of IgG plasmids from Inbiopro Solutions Pvt Ltd. (Bangalore, India). We thank Dr Suresh Bhat for help with DLS measurements. This work was supported by a grant to MG from the Department of Science and Technology, Government of India.

References

- Backliwal G, Hildinger M, Hasija V, Wurm FM (2008) High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. *Biotechnol Bioeng* 99:721–727
- Baldi L, Muller N, Picasso S, Jacquet R, Girard P, Huy PT, Derow E, Wurm FM (2005) Transient gene expression in suspension HEK-293 cells: application to large-scale protein production. *Biotechnol Prog* 21:148–153
- Bollin F, Dechavanne V, Chevalet L (2011) Design of experiment in CHO and HEK transient transfection condition optimization. *Protein Expr Purif* 78:61–68
- Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci USA* 92:7297–7301
- Derouazi M, Girard P, Van Tilborgh F, Iglesias K, Muller N, Bertschinger M, Wurm FM (2004) Serum-free large-scale transient transfection of CHO cells. *Biotechnol Bioeng* 87:537–545
- Galbraith DJ, Tait AS, Racher AJ, Birch JR, James DC (2006) Control of culture environment for improved polyethylenimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. *Biotechnol Prog* 22:753–762
- Geisse S, Fux C (2009) Chapter 15 recombinant protein production by transient gene transfer into mammalian cells. In: Richard RB, Murray PD (eds) *Methods in enzymology*, vol 463. Academic Press, London, pp 223–238
- Girard P, Derouazi M, Baumgartner G, Bourgeois M, Jordan M, Jacko B, Wurm FM (2002) 100-Liter transient transfection. *Cytotechnology* 38:15–21
- Glover DJ, Leyton DL, Moseley GW, Jans DA (2010) The efficiency of nuclear plasmid DNA delivery is a critical determinant of transgene expression at the single cell level. *J Gene Med* 12:77–85. doi:10.1002/jgm.1406
- Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG (2000) Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J Biomed Mater Res* 51:321–328
- Grosjean F, Batard P, Jordan M, Wurm FM (2002) S-phase synchronized CHO cells show elevated transfection efficiency and expression using CaPi. *Cytotechnology* 38:57–62
- Kawaura C, Noguchi A, Furuno T, Nakanishi M (1998) Atomic force microscopy for studying gene transfection mediated by cationic liposomes with a cationic cholesterol derivative. *FEBS Lett* 421:69–72
- Liu C, Dalby B, Chen W, Kilzer JM, Chiou HC (2008) Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. *Mol Biotechnol* 39:141–153. doi:10.1007/s12033-008-9051-x
- Männistö M, Rönkkö S, Mättö M, Honkakoski P, Hyttinen M, Pelkonen J, Urtti A (2005) The role of cell cycle on polyplex-mediated gene transfer into a retinal pigment epithelial cell line. *J Gene Med* 7:466–476
- Meissner P, Pick H, Kulangara A, Chatellard P, Friedrich K, Wurm FM (2001) Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnol Bioeng* 75:197–203
- Muller N, Derouazi M, Van Tilborgh F, Wulhfard S, Hacker DL, Jordan M, Wurm FM (2007) Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. *Biotechnol Lett* 29:703–711
- Ogris M, Steinlein P, Kursu M, Mechtler K, Kircheis R, Wagner E (1998) The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther* 5:1425–1433. doi:10.1038/sj.gt.3300745
- Pham P, Kamen A, Durocher Y (2006) Large-Scale transfection of mammalian cells for the fast production of recombinant protein. *Mol Biotechnol* 34:225–237. doi:10.1385/mb.34:2:225
- Schlaeger EJ, Christensen K (1999) Transient gene expression in mammalian cells grown in serum-free suspension culture. *Cytotechnology* 30:71–83
- Schlaeger EJ, Kitas EA, Dorn A (2003) SEAP expression in transiently transfected mammalian cells grown in serum-free suspension culture. *Cytotechnology* 42:47–55
- Sharma VK, Thomas M, Klibanov AM (2005) Mechanistic studies on aggregation of polyethylenimine-DNA complexes and its prevention. *Biotechnol Bioeng* 90:614–620. doi:10.1002/bit.20444
- Sun X, Hia HC, Goh PE, Yap MG (2008) High-density transient gene expression in suspension-adapted 293 EBNA1 cells. *Biotechnol Bioeng* 99:108–116. doi:10.1002/bit.21537
- Susa T, Kato T, Kato Y (2008) Reproducible transfection in the presence of carrier DNA using FuGENE6 and Lipofectamine2000. *Mol Biol Rep* 35:313–319. doi:10.1007/s11033-007-9088-0
- Tachibana R, Harashima H, Ide N, Ukitsu S, Ohta Y, Suzuki N, Kikuchi H, Shinohara Y, Kiwada H (2002) Quantitative analysis of correlation between number of nuclear plasmids and gene expression activity after transfection with cationic liposomes. *Pharm Res* 19:377–381
- Tait AS, Brown CJ, Galbraith DJ, Hines MJ, Hoare M, Birch JR, James DC (2004) Transient production of recombinant proteins by Chinese hamster ovary cells using polyethylenimine/DNA complexes in combination with microtubule disrupting anti-mitotic agents. *Biotechnol Bioeng* 88:707–721. doi:10.1002/bit.20265
- Tait A, Hoare M, Birch J, Galbraith D, Hines M, Brown C, James D (2005) Optimisation of PEI-mediated transient

- expression in chinese hamster ovary cells. In: Gòdia F, Fussenegger M (eds) *Animal cell technology meets genomics*, vol 2. ESACT Proceedings. Springer, Netherlands, pp 655–657. doi:[10.1007/1-4020-3103-3_133](https://doi.org/10.1007/1-4020-3103-3_133)
- Takahashi Y, Nishikawa M, Takiguchi N, Suehara T, Takakura Y (2011) Saturation of transgene protein synthesis from mRNA in cells producing a large number of transgene mRNA. *Biotechnol Bioeng*. doi:[10.1002/bit.23179](https://doi.org/10.1002/bit.23179)
- Takeuchi K-i, Ishihara M, Kawaura C, Noji M, Furuno T, Nakanishi M (1996) Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. *FEBS Lett* 397:207–209