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ORIGINAL RESEARCH

Salt ions and related parameters affect PEI–DNA particle size and transfection efficiency in Chinese hamster ovary cells

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Abstract Transfection efficiency is directly associated with the expression level and quantity of recombinant protein after the transient transfection of animal cells. The transfection process can be influenced by many still-unknown factors, so it is valuable to study the precise mechanism and explore these factors in gene delivery. Polyethylenimine (PEI) is considered to have high transfection efficiency and endosome-disrupting capacity. Here we aimed to investigate optimal conditions for transfection efficiency by setting different parameters, including salt ion concentration, DNA/PEI ratio, and incubation time. We examined the PEI–DNA particle size using a Malvern particle size analyzer and assessed the transfection efficiency using flow cytometry in Chinese hamster ovary-S cells. Salt ions, higher amounts of PEI tended to improve the aggregation of PEI–DNA particles and the particle size of PEI–DNA complexes and the transfection efficiency were increased. Besides, the particle size was also found to benefit from longer incubation time. However, the transfection efficiency increased to maximum of 68.92 % at an incubation time of 10 min, but decreased significantly thereafter to 23.71 %, when incubating for 120 min ($P < 0.05$). Besides, PEI–DNA complexes formed in salt-free condition were unstable. Our results suggest DNA and PEI incubated in 300 mM NaCl at a ratio of 1:4 for 10 min could achieve the optimal transfection efficiency. Our results might provide guidance for the optimization of transfection efficiency and the industrial production of recombinant proteins.

Keywords Transfection efficiency · Salt ions · PEI–DNA complexes · Particle size · CHO cells

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Introduction

Recently, transient transfection of mammalian cells has been extensively used in the rapid production of recombinant proteins for biochemical, biophysical, and pre-clinical studies (Shi et al. 2005; Baldi et al. 2005; Sharma et al. 2005). Different expression vectors, both viral and non-viral, have been developed to transfer extracellular genes into the cellular nucleus. Among various non-viral gene delivery systems, cationic polymers have been shown as the most promising carriers (Garnett 1999). Polycation-DNA complexes are more stable than other non-viral gene delivery systems and can overcome some technical

bottlenecks and biological barriers during the DNA delivery process. A number of biocompatible cationic polymers, such as polyethylenimine (PEI) (Jordan et al. 1996; Meissner et al. 2001), polysaccharides (Mao et al. 2001), and poly(beta-amino ester)s (Jere et al. 2008), have been reported as gene carriers. Among them, PEI belong to one of the most efficient family of cationic compounds for delivery of plasmid DNA into mammalian cells (Kichler et al. 2001). It was reported that PEI–DNA particles are stable for more than 1 month at 4 °C in terms of size and transfection activity (Chen et al. 2007).

Transfection with PEI involves condensing and releasing of the DNA cargo (McBain et al. 2007). Thus, the transfection efficiency using PEI is closely associated with DNA binding and dissociation of the polymer. PEI-condensed DNA can be used to transfect a broad range of murine and human tumor cell lines in vitro in the presence of fetal calf serum (Kichler et al. 2001). Chinese hamster ovary (CHO) cells are able to confer appropriate posttranslational modifications and can be adapted to growth in serum-free environments. Therefore, these cells are used predominantly in commercial biopharmaceutical protein production. CHO cells offer a post-translationally modified product and thus a more accurate in vitro rendition of the natural protein because they provide stable and accurate glycosylation (Wang et al. 2012; Tait et al. 2004). Thus, in our study, we also used CHO cells to investigate the optimized conditions for aggregation of PEI–DNA particles in vitro.

Free PEI has a certain degree of cytotoxicity; therefore, several parameters should be optimized, including the DNA amount, the DNA-to-PEI ratio, the incubation time and solution for complex formation (Derouazi et al. 2004; Goula et al. 1998). Additionally, many factors, including temperature, surfactants, complex concentration, ionic strength, viscosity, and pH, can greatly impact the aggregation process. Complexes can be stabilized by reducing particle collisions, increasing electrostatic repulsion, preventing particle aggregation, and reducing hydrophobicity (Goula et al. 1998). Here, we investigated the optimization of a robust process for PEI-mediated transient transfection of CHO-S cells using plasmid-encoded reporter genes. The linear 25 kDa PEI, which was reported to exhibit the highest transfection efficiency, resulting in the highest level of gene expression in CHO cells (Derouazi et al. 2004; Goula et al. 1998), was used in this study. In light of the importance of the aggregation of the PEI–DNA complexes to transfection efficiency, we investigated the effects of critical parameters in aggregation, such as ionic strength, ratio of DNA to PEI, and incubation time. We hope this study provides experimental evidence and theoretical support for researchers in optimizing transfection efficiency and the industrial production of recombinant proteins.

Materials and methods

Preparation of PEI–DNA complexes

Linear 25 kDa PEI (Polysciences, Eppenheim, Germany) was dissolved in distilled water to a final concentration of 1 mg/mL (pH 7.0) and stored at − 80 °C after 0.22 μm filtration. The vector peGFP-N1 expressing the enhanced green fluorescence protein (GFP) under the control of the human cytomegalovirus (CMV) promoter was purchased from ClonTech (Mountain View, CA, USA). Plasmid DNA was purified by a commercial purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The absorbance at 260 and 280 nm was measured spectrophotometrically to ensure A260/A280 ratios between 1.8 and 2.0. Purified plasmids were checked for purity on agarose gels (0.7 % (w/v), 50 V constant voltage).

For each milliliter of solution, 2.5 μg plasmid DNA and varying amounts of PEI were separately diluted in microcentrifuge tubes containing 50 μL NaCl (300 mM). DNA/NaCl solution was then added to PEI/NaCl solution. After being vortexed for 30 s, the mixture was incubated in room temperature to obtain PEI–DNA complexes.

Measurement of particle size

Particle sizes of the PEI–DNA complexes were measured by photon correlation spectroscopy, using a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA, USA). Size measurement was performed at room temperature at a 90° scattering angle and a 50 mW laser ($\lambda = 676$ nm). Independent experiments were performed in triplicate.

Cell culture and transient transfection

CHO-S cells (1.5×10^5 per well) were seeded in Costar 12-well tissue culture plates supplemented with Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA), containing 10 % heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA) at 37 °C in a humidified atmosphere of 5 % CO_2. When cells reached ~80 % confluency, transfection with PEI–DNA complexes was performed as described previously with some modifications (Tait et al. 2004). Briefly, the medium was replaced with fresh RPMI 1,640 medium containing 25 mM HEPES (pH 7.1) prior to transfection. Various concentrations of PEI/NaCl solution was added to the DNA-NaCl solution and incubated for the indicated times at room temperature. Subsequently, a 10 μL aliquot of the PEI–DNA complexes was added to each well (Swanson and Watts 1995) and incubated at 37 °C in an atmosphere containing 5 % CO_2 at 95 % relative humidity. After 4 h, 1 mL fresh medium was added to each well for post transfection culture. Unless otherwise stated, the concentration of DNA in the transfection medium before dilution was kept constant at 2.5 μg/mL.

Evaluation of transfection efficiency

Photographs were taken with a fluorescence microscope and transfection efficiency was examined 3 days thereafter by the fluorescence of the translational product of GFP. CHO-S cells were first treated with 0.25 % trypsin, and harvested by centrifugation. After washing twice with 1 × PBS for 2 min each, cells were resuspended in pre-chilled PBS and the expression of GFP was examined on a FACScan flow cytometer (BD biosciences, Mountain View, CA, USA). Independent experiments were performed in triplicate.

Statistical analysis

Experiments were performed routinely with three samples per group, and values are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Student's *t* test for unpaired data or ANOVA. $P < 0.05$ and $P < 0.01$ were considered to indicate statistical significance.

Results

Salt ions increase the particle size of PEI–DNA complexes and transfection efficiency

To study the role of NaCl concentration on the aggregation of PEI–DNA complexes, 2.5 μg/mL DNA and 10 μg/mL PEI (DNA/PEI = 1:4) were diluted in solutions with different concentrations of NaCl (0, 150, 300 and 600 mM) and incubation for various times. The average particle size of PEI–DNA complexes increased roughly along with the increase of NaCl concentration and incubation time (Fig. 1a). Generally, the higher the NaCl concentration is, the larger is the size of the PEI–DNA complexes, although particles incubated in 300 mM NaCl for 30–120 min were smaller than those formed in 150 mm NaCl. Particles incubated in 600 mM NaCl for 60–120 min were significantly larger than those in the other solutions. At 120 min, the particle sizes were 2,134, 2,190 and 2,972 nm in solutions with 150, 300 and 600 mM NaCl, respectively. On the other hand, in the NaCl-deprived condition, the PEI–DNA particle size reached to a maximum of 1,106 nm at 15 min, and then decreased thereafter to a minimum value of 350 nm at 120 min. Furthermore, the particle size had 2–3 peaks in the salt-free solution, suggesting that the complexes were unstable (Fig. 1c). These data suggest that salt ions promote the aggregation of PEI–DNA complexes and that particles might be benefit from longer incubation time.

To study the effects of salt ions on transfection efficiency, PEI–DNA complexes (DNA/PEI = 1:4) incubated for 10 min were transfected into CHO-S cells. Flow cytometry analysis showed that the transfection efficiencies in 0 and 150 mM NaCl were comparable as well as those in 300 and 600 mM NaCl. Besides, the transfection efficiencies of the later two groups (73.82 and 72.15 %, respectively) were significantly higher than those in the former two groups ($P < 0.05$; Fig. 1b). This finding suggests that higher NaCl concentration contributes to better transfection efficiency. Considering particle size, stability and transfection efficiency, we suggest that a solution/

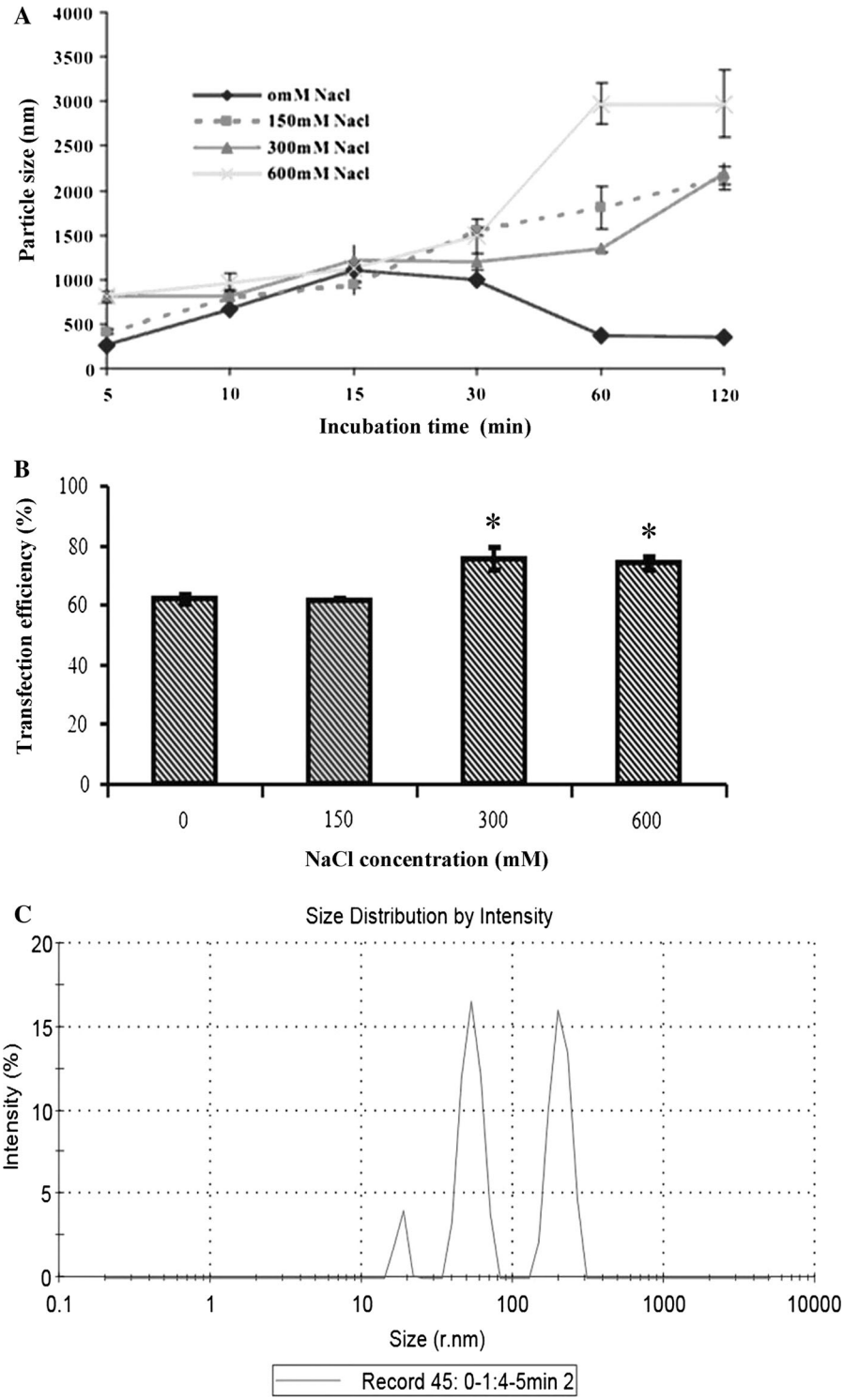

Fig. 1 NaCl increases the particle size of PEI–DNA complexes and transfection efficiency **a** DNA and PEI were diluted with different concentrations of NaCl solution (0, 150, 300, and 600 mM NaCl). The particle size of PEI–DNA complexes increased roughly with the increasing concentration of NaCl and the incubation time in NaCl containing condition and the particle size increased first and decreased after 15 min in salt-free condition. **b** The PEI–DNA complexes (DNA/PEI = 1:4) were incubated in 0, 150, 300, or 600 mM NaCl for 10 min, and then transfected into CHO-S cells. The transfection efficiency in 300 mM NaCl solution was higher than that in the other groups. *Compared with the salt-free solution, $P < 0.05$. **c** This diagram shows that 2–3 peaks of intensity appeared in the particle size of PEI–DNA complexes in the salt-free condition

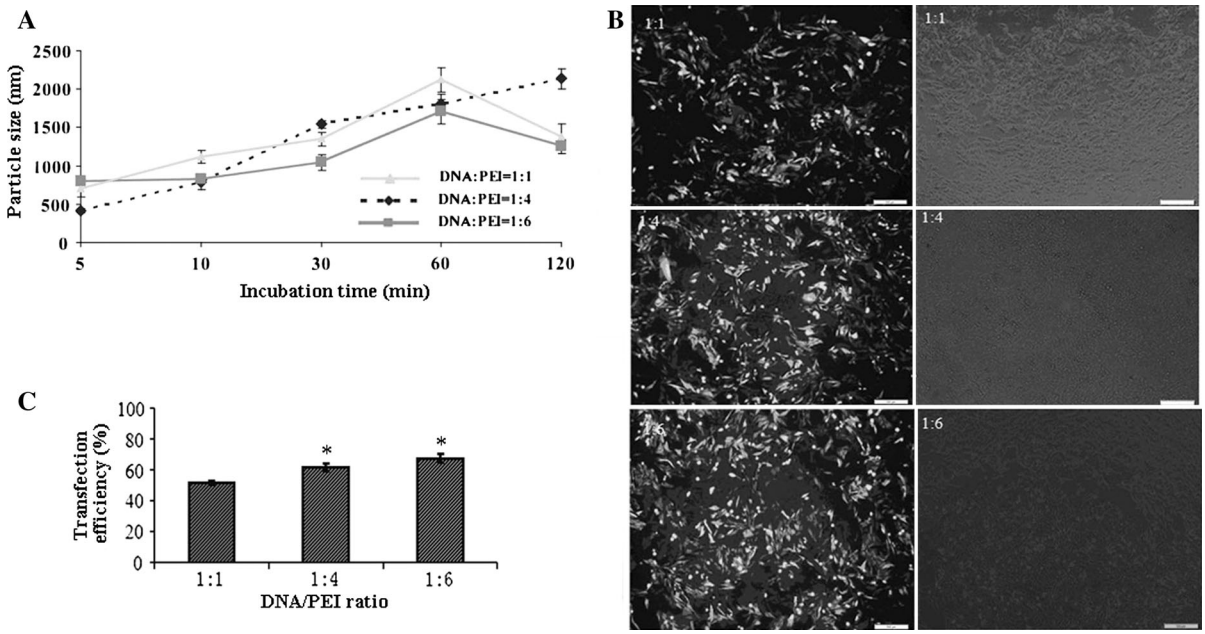

Fig. 2 DNA/PEI ratio affects the particle size of PEI–DNA complexes and transfection efficiency in CHO-S cells **a** DNA and different amounts of PEI were diluted in 300 mM NaCl solution. After incubating for different times (5, 10, 30, 60, and 120 min), the particle sizes of the complexes were measured. At 10 min, the particle size was smaller when the ratio of DNA to PEI was 1:4 or 1:6. **b** The PEI–DNA complexes were transfected into CHO-S cells and photographs were taken with a fluorescence microscope in *dark* field (*left*) and *bright* field (*right*). There were more fluorescence-labeled cells at DNA/PEI ratio of 1:4 and 1:6 than at DNA/PEI ratio of 1:1. *Scale bars* = 500 μm. **c** Statistical analysis showed transfection efficiency was significantly higher when DNA/PEI was 1:4 or 1:6 than when DNA/PEI ratio was 1:1. *Compared with DNA/PEI ratio of 1:1, $P < 0.05$

buffer with 300 mM NaCl is optimal for preparing PEI–DNA complexes.

Ratio of DNA to PEI affects the particle size of PEI–DNA complexes and transfection efficiency in CHO-S cells

DNA/PEI ratio was considered important to the optimization of DNA compaction (Godbey et al. 1999). Various amounts of PEI were mixed in the solution with a constant amount of DNA yielding a final DNA concentration of 2.5 μg/mL. At DNA/PEI ratios of 1:1, 1:4 and 1:6, particle size and transfection efficiency were determined at 5, 10, 30, 60 and 120 min post transfection. As shown in Fig. 2a, the particle size of PEI–DNA complexes increased from 5 min to 60 min post transfection at all three DNA/PEI ratios, and the particle size generally became smaller within 60 min post transfection with increasing amounts of PEI. At 10 min, the particle size was 1,117 nm at DNA/PEI ratio of 1:1 and the particle sizes were similar at DNA/PEI ratio of 1:4 and 1:6. Maximal particle size of PEI–DNA complexes were observed by incubation for 60 min at DNA/PEI ratio of 1:1 and incubation for 120 min at DNA/PEI ratio of 1:4.

From Fig. 2b, there were fewer fluorescently labeled cells when the amounts of DNA and PEI were equal, and the number of fluorescently labeled cells increased obviously when the ratio of DNA to PEI decreased to 1:4 or 1:6. Statistical analysis of the results (Fig. 2c) showed that the transfection efficiency at DNA/PEI ratio of 1:4 (63.71 %) or 1:6 (67.55 %) was significantly higher than that at DNA/PEI ratio of 1:1 (50.35 %; $P < 0.05$). These findings demonstrate that the amount of PEI can affect the particle size of PEI–DNA complexes and further determine the transfection efficiency. As the transfection efficiencies were not significantly different in DNA/PEI ratios of 1:4 and 1:6, we considered the 1:4 ratio optimal because this process required a lower mass of PEI, which is toxic.

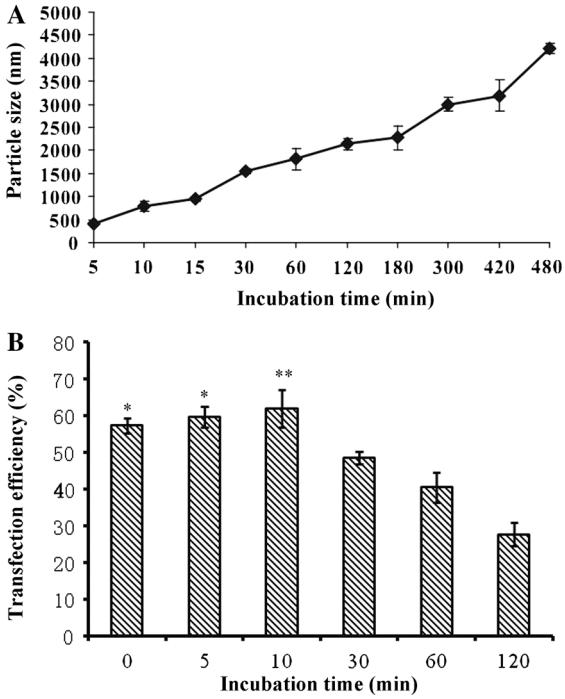

Fig. 3 Longer incubation time increases particle size and decreases transfection efficiency **a** The DNA-PEI complexes (DNA/PEI = 1:4) was incubated in 300 mM NaCl for different times. The particle sizes of PEI–DNA complexes increased gradually with incubation time. At incubation time of 480 min, the particle size was the largest. **b** The PEI–DNA complexes were incubated for 0, 5, 10, 30, 60, or 120 min and then transfected into CHO-S cells. At 10 min, the transfection efficiency was significantly higher than that at any other time point. The values are expressed as mean ± SD from three independent assays. * $P < 0.05$, ** $P < 0.01$, compared with 30, 60, 120 min incubations

Longer incubation time increases particle size and decreases transfection efficiency

To further examine the effect of incubation time on stability of PEI–DNA complexes, we mixed the DNA and PEI in 300 mM NaCl at DNA/PEI ratio of 1:4 and measured the particle sizes and transfection efficiency after different incubation times. As shown in Fig. 3a, the particle size increases in a time-dependent manner. At 10 min post transfection, the particle size was 813 nm and it increased to 4,206 nm when incubation time was 8 h. On the other hand, the transfection efficiency increased to maximum of 68.92 % at incubation time of 10 min, but decreased significantly thereafter, to 23.71 %, when incubating for 120 min ($P < 0.05$, Fig. 3b). These results show that PEI–DNA complexes aggregate with time, but the transfection efficiency would be reduced beyond a certain time. Therefore, we suggest 10 min as optimal incubation time for preparing PEI–DNA complexes.

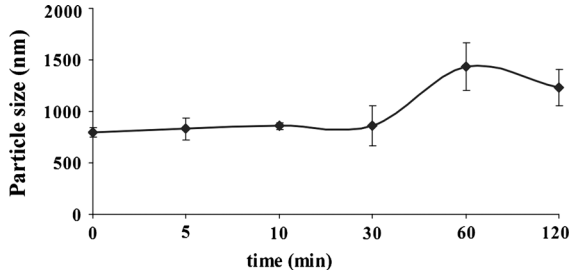

Fig. 4 Stability of PEI–DNA complexes in culture medium. DNA/PEI (1:4) was diluted in 300 mM NaCl solution, and then basic medium was added. The particle size of PEI–DNA complexes was measured at different time intervals (0, 5, 10, 30, 60, and 120 min). At 60 min, the particle size was the largest

Culture medium affects the stability of PEI–DNA complexes

To examine the effects of the culture medium on the stability of PEI–DNA complexes, DNA/PEI (1:4) was diluted in 300 mM NaCl solution, and then basic medium (DMEM, Gibco) was added after incubation. From Fig. 4, the particle size of PEI–DNA complexes were maintained at the same level (around 800 nm) within 30 min, and sharply increased to almost 1,500 nm and then decreased gradually. These results suggest that the PEI–DNA complexes were stable for 30 min in basic medium and aggregated further at a certain time point.

Discussion

Transient gene expression has become an increasingly popular approach because of the rapid generation of recombinant proteins in mammalian cells. Expression levels of exogenous genes depend on the type of solute, concentration of plasmid DNA, amount of PEI, PEI-to-DNA ratio, and incubation time. PEI belongs to one of the most effective families of cationic compounds for the delivery of plasmid DNA into mammalian cells. High transfection efficiencies can be obtained even in the absence of endosomolytic agents, such as fusogenic peptides or chloroquine, in contrast to most other cationic polymers (Kunert and Vorauer-Uhl 2012; Fitzsimmons and Uludag 2012). Although PEI can be used in transfecting many types of mammalian cells, it

remains a challenge to achieve high transfection efficiencies generally. In this study, we experimentally determined the optimal basic conditions for PEI-mediated transfection of CHO-S cells with linear 25 kDa PEI. Our results suggest that DNA and PEI incubated in 300 mM NaCl at a ratio of 1:4 for 10 min could achieve the optimal transfection efficiency.

Particle size is an important factor that influences cellular uptake of particles across the cytoplasmic membrane (Zauner et al. 2001). Two factors might contribute to the increase in particle size: particle aggregation results from colloidal instability and particle swelling due to charge screening (Ren et al. 2010). In this study, we investigated the effect of NaCl concentration on particle size of PEI–DNA complexes and transfection efficiency. Results showed that the average particle size of PEI–DNA complexes and transfection efficiency increased roughly with the increase of NaCl concentration and the complexes are unstable in salt-free condition and the PEI–DNA complexes generated under salt-free conditions had low transfection efficiency in vitro. Our results suggest that NaCl was important in the particle aggregation and stability of PEI–DNA complexes, which was consistent with a previous study (Wightman et al. 2001). NaCl solution has been reported to support polyplex formation (Kircheis et al. 2001). The complexes were unstable without salt ions, which might be attributable to Na^+ and Cl^- rearranging DNA and PEI by an electrostatic process. When PEI–DNA complexes were prepared in NaCl solutions of increasing salt concentration, the charges on PEI and DNA macromolecules of the complexes are screened, cooperative binding becomes weaker and dissociation of the complexes occurs (Neu et al. 2005). However, our results showed that high ionic strength could condense DNA more effectively and therefore increase the transfection efficiency. In addition, the stability of PEI–DNA complexes during transfection are prerequisites for their effective in vivo transfection. Therefore, we studied the stability of the complexes in DMEM. Result showed that the complexes maintain their stability within 30 min, suggesting that some ingredients in the basic medium might interact with PEI–DNA complexes and influence their stability. The endocytosis of the complexes into mammalian cells was complete within 10 min, delivering the particle into the CHO cell (Remy-Kristensen et al. 2001; Coupin et al. 1999).

A previous study showed that the optimum size of PEI–DNA complex particles was smaller than 1 μm; otherwise, the complex is difficult to depolymerize, which reduces the transfection efficiency in HEK293 cells (Sharma et al. 2005). As expected, the sizes of the PEI–DNA complexes incubated in different concentrations of NaCl for 10 min were all smaller than 1 μm in our study. At higher PEI concentrations, these complexes can be further compacted into PEI–DNA nanoparticles. It has been reported that DNA is condensed in the presence of PEI and that the compression depends on the ratio of DNA to PEI (Godbey et al. 1999), salt ions concentration and the aggregation of stable PEI–DNA complexes. However, the optimal ratio of DNA to PEI is still controversial, varying from 1:3 to 1:20 (Derouazi et al. 2004; Boussif et al. 1995; Galbraith et al. 2006). The condensed DNA anneals to the cationic polymers, thereby forming the polyplex (PEI–DNA complexes) with an overall positive charge, which supports entry via the negatively charged cell membrane (Lungwitz et al. 2005). Therefore, the transfection efficiency is in proportion to the amounts of PEI. In our study, the transfection efficiency reached a peak at DNA/PEI ratio of 1:6. However, statistical analysis suggest that there was no significant difference between the transfection efficiency of DNA/PEI ratio of 1:4 and that of 1:6. As PEI was reported to be cytotoxic to endothelial cells (Godbey et al. 2001), we suggest DNA/PEI ratio of 1:4 to be optimal condition.

Here, we have made a basic examination of the particle size of complexes and transfection efficiency. Moreover, membrane proteins, membrane potential, and ionic channels in different cell types may affect the endocytosis of PEI–DNA complexes, leading to variation in transfection efficiency. This study provides a foundation for the study of PEI–DNA complexes as carriers for gene delivery in vivo and in vitro, and examining these questions will undoubtedly aid in gaining a better understanding of the underlying mechanisms in PEI transfection. It could also provide experimental evidence for more effective gene delivery in the future.

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Conflict of interest None.

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