Size effect on transfection and cytotoxicity of nanoscale plasmid DNA/polyethyleneimine complexes for aerosol gene delivery

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Size effect on transfection and cytotoxicity of nanoscale plasmid DNA/polyethyleneimine complexes for aerosol gene delivery

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Nanoscale plasmid DNA (pDNA)/polyethyleneimine (PEI) complexes were fabricated in the aerosol state using a nebulization system consisting of a collison atomizer and a cool-walled diffusion dryer. The aerosol fabricated nanoscale complexes were collected and employed to determine fundamental properties of the complexes, such as size, structure, surface charge, and *in vitro* gene transfection efficiency and cytotoxicity. The results showed that mass ratio between pDNA and PEI should be optimized to enhance gene transfection efficiency without a significant loss of cell viability. These findings may support practical advancements in the field of nonviral gene delivery. © *2014 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4864268]

The physical instability of aqueous suspensions of nonviral vector complexes is a major limitation for their successful application in gene therapy. The suspension stability of cationic polyelectrolyte derived gene delivery complexes is very sensitive to the ionic strength of the surrounding solution.[1] This instability includes aggregation tendency and loss of transfection efficiency and thus requires fresh preparation each time directly prior to application for maximal effect.[2] To overcome this limitation, many researchers have investigated the applicability of aerosol vector fabrication for gene therapy.[3] Aerosol systems may confer many advantages including increased stability, reduced drug loss during administration, improved portability, and efficient delivery to the pulmonary targeted region.[4] Furthermore, aerosol systems also have been recently employed to fabricate nanocomposite vectors for efficient gene delivery in different cell lines.[5–7]

Nonviral aerosol gene delivery holds great therapeutical potential for many inherited and acquired pulmonary diseases.[8,9] Nucleic acid based approaches have been designed to treat both acute and chronic diseases, such as cystic fibrosis, alpha 1-antitrypsin deficiencies, surfactant protein deficiencies, acute respiratory distress syndrome, cancer, and asthma.[10] Numerous studies have demonstrated that gene condensation induced by cationic agents may minimize gene damage by shear-related forces during aerosol fabrication. One representative nonviral gene delivery agent that continues to demonstrate promise for aerosol gene delivery is the cationic polymer polyethyleneimine (PEI), which efficiently condenses plasmid DNA (pDNA) molecules to form complexes capable of mediating high-level gene expression both *in vitro* and *in vivo*.[11,12] PEI is thought to protect pDNA from degradation by buffering endosomal pH and facilitating endosomal escape through osmotic swelling and rupture.[13] Even though the size distribution and structure of pDNA/PEI complexes corresponding to their surface charge and cell cytotoxicity may be critical for efficient gene delivery,[14,15] structural investigations of aerosol fabricated pDNA/PEI complexes have not yet been reported in the literature.[16] Indeed, given the relative inefficiency of nonviral gene delivery, clinical applications for lung gene therapy are likely to require the delivery of relatively large doses of pDNA; this requirement emphasizes the need to address the issue of increasing the concentrations of complexes without compromising utilization.

In the present study, we investigated the fundamental properties of nanoscale pDNA/PEI complexes. A previous consideration makes clear that pDNA/PEI complexes should have a size smaller than 0.2 μm to ensure the uptake by pulmonary epithelial cells.[16,17] Previous studies demonstrated that the inhalative deposition of nanoscale complexes on the lung-lining fluid can follow uptake in the respiratory tract and that nanoscale complexes can reach the deepest regions of the lung alveoli.[18] Complex size, structure, and surface charge can fundamentally affect the gene transfection efficiency both *in vitro* and *in vivo* in previous works;[19] however, there are no well-organized studies corresponding to this in aerosol science. Herein, we determined that the influence of the mass ratio between the PEI and the pDNA influences complex size, structure, surface charge, and *in vitro* gene transfection efficiency (Fig. 1). Cell cytotoxicity was also evaluated with different ratios between the pDNA and the PEI; therefore, minimizing the toxicity observed with PEI based complexes remains an essential goal. Experimental details of this work are described in Fig. S1 (see supplementary material[24]).

The total number concentration (TNC), geometric mean diameter (GMD), and geometric standard deviation (GSD) of the aerosol fabricated pDNAs, which were measured using a scanning mobility particle sizer (SMPS, 3936, TSI, USA), were 2.01×10^6 particles cm^{-3}, 38.7 nm, and 1.62, respectively, as shown in Fig. 2. Most previous studies used a dynamic scattering method to measure aerosol fabricated complex sizes; however, they could not provide aerosol size distribution information of the fabricated pDNA/PEI complexes.[16] The pDNA/PEI complexes were formed by incorporating pDNA with the PEI during the collison atomization.

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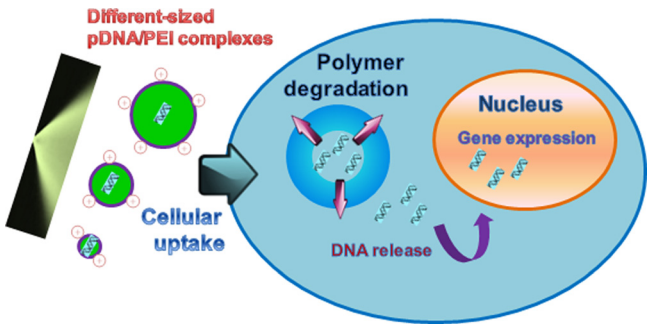

FIG. 1. Transfection of pDNA/PEI complexes into a cell.

We verified the incorporation of the pDNA with the PEI by measuring the size distributions of the PEI and pDNA/PEI complexes in the aerosol state. Table SI (see supplementary material[24]) summarizes the size distribution measurements of the pDNA/PEI complexes including pDNA and individual PEI I and PEI II. The TNC, GMD, and GSD of the pDNA/PEI I complex (0.16667 of pDNA/PEI mass ratio) were 2.03×10^6 particles cm^{-3}, 48.9 nm, and 1.64, respectively. The analogous data for individual PEI I were $1.35 \times 10^6\,cm^{-3}$, 51.1 nm, and 1.63, respectively. The size distribution of the pDNA/PEI I complex was rather similar to the PEI I compared to that of the pDNA. In addition, there was no bimodal distribution character, implying that the pDNAs were nearly quantitatively incorporated with the PEI I to form nanoscale nonviral vectors. The unimodal size distribution ($5.85 \times 10^6\,cm^{-3}$ TNC, 122.7 nm GMD, and 1.93 GSD) of the pDNA/PEI II complex (0.00167 of pDNA/PEI mass ratio) remained as they were even with PEI II; however, the distribution shifted to larger sizes, probably due to additional contact with the PEI during the aerosol fabrication.

Transmission electron microscope (TEM, JEM-3010, JEOL, Japan) images show the morphology of the pDNA, the pDNA/PEI I, and the pDNA/PEI II samples. Specimens were prepared for examination in the TEM by direct electrostatic aerosol sampling at a sampling flow of 1.01 min^{-1} and an operating voltage of 5 kV using a Nano Particle Collector (NPC-10, HCT, Korea). The TEM image (Fig. 3) reveals that the size of pDNA was ~40 nm in lateral dimension, which was consistent with the SMPS data shown in Table SI (see supplementary material[24]). TEM images of pDNA/PEI complexes hold true especially when the aerosol fabricated complexes have a smooth morphology and a spherical architecture, indicating that accidental aggregation may not be the driving force behind complex size increase. The high magnification TEM image (inset) for pDNA/PEI I complexes

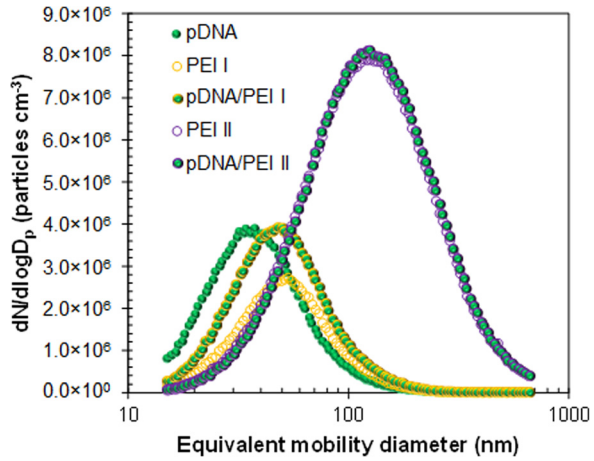

FIG. 2. Size distributions of aerosol fabricated pDNA/PEI complexes including pDNA, PEI I, and PEI II cases. Standard deviations are noted in Table SI (see supplementary material[24]).

highlights the brighter gray outer layer around the darker cores, implying the presence of a PEI moiety that completely covers a pDNA. In the case of the pDNA/PEI II complexes, pDNAs were distributed in enlarged PEI platforms, and free PEI can be seen probably due to the low mass ratio between the pDNA and the PEI.

Figure S2 (see supplementary material[24]) displays a Fourier transform infrared spectra (FTIR, IFS 66/S, Bruker Optics, Germany) of aerosol fabricated pDNA/PEI complexes. The FTIR spectrum of pDNA/PEI II complexes shows peaks at 2952 and 2862 cm^{-1} that can be attributed to asymmetric and symmetric vibrations of the CH_2 group, respectively, and the peak at 1453 cm^{-1} corresponds to inplane bending of CH_2. The peaks for the bending vibration of the NH group of PEI can be seen at 1585 cm^{-1}.[20] The intensity of the characteristic bands significantly decreased at pDNA/PEI I complexes due to the relatively larger mass ratio between the pDNA and the PEI. pDNA vibrational bands arising from base (guanine/thymidine) carbonyls (1715 cm^{-1}) and the imidazole nitrogen of guanine (1492 cm^{-1}) have not been found apparently in both pDNA/PEI complexes,[21] which implies that nearly all pDNAs were quantitatively capsulated by PEI molecules. To characterize the integrity of pDNA/PEI complexes, gel retardation assay (inset) was performed. A release of pDNAs decreased when the amount of the PEI was increased, which indicates that a higher amount of PEI could induce better condensation.

To further biophysical characterization, we investigated the transfection efficiency of aerosol fabricated pDNA/PEI complexes on A549 cells. For this, luciferase based reporter

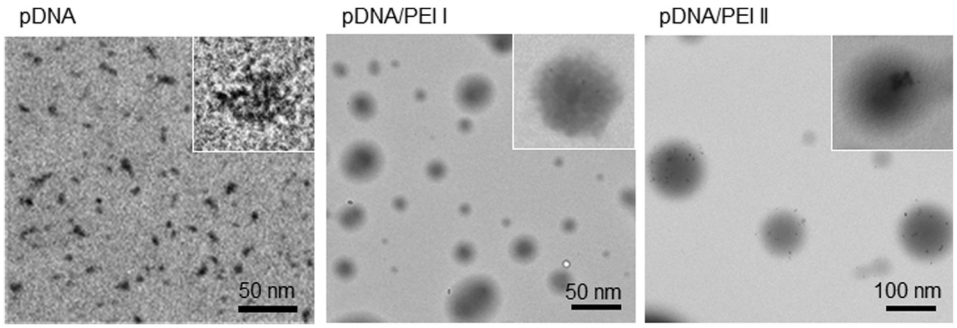

FIG. 3. TEM images of aerosol fabricated pDNAs (40 ± 7.1 nm), pDNA/PEI I complexes (58 ± 6.6 nm), and pDNA/PEI II complexes (132 ± 9.1 nm).

green fluorescent protein (GFP) expression plasmid was employed. Expression levels increased as the mass ratio between the pDNA and the PEI decreased (Fig. 4(a)), with better luciferase activity being achieved when using complexes formulated with PEI II. Instillation of a PEI-GFP expression plasmid solution formulation resulted in a similar level of luciferase activity in the cell with aerosol fabricated pDNA/PEI II complexes, while cytotoxicity increased as the mass ration between the pDNA and the PEI decreased, until finally it reached less than half at the highest mass of the transfected protein (Fig. 4(b)). The precise mechanism of PEI mediated cellular toxicity remains unknown, but it may involve adverse interactions of the highly cationic PEI molecules with anionic macromolecules within the cell.[3,12]

Figure 5 summarizes changes in gene transfection and cell cytotoxicity by different complex sizes (different mass ratios between pDNA and PEI). In order to assess the efficacy of pDNA/PEI complexes *in vitro*, complexes of GFP expression plasmid were applied to the cells by instillation (the solution state before aerosol fabrication) or aerosol fabricated complexes with different complex sizes. The decrease in mass ratio (0.16667 to 0.00167) between the pDNA and the PEI was associated with an increase in complex size (50 to 120 nm) and surface charge (10–57 mV, inset of Fig. 5). At 100 nm, in complex size, the luciferase activity was ~50 fold higher than that achieved at 50 nm. These results show that a size around 90 nm would be necessary to ensure an efficient release of pDNA from their complexes; whereas, incorporation with larger complex sizes (>100 nm) resulted in a reduction in luciferase activity. The increase of zeta potential in our case may be the consequence of additional incorporation of PEI presented in the aerosol state. At >100 nm, the transfection efficiency decreased with increasing complex sizes, which implies an incomplete release of pDNA from the complexes. This decrease in transfection efficiency may occur because of the increased binding[13] between the pDNA and the PEI, also seen in the gel retardation assay for those ratios between the pDNA and the PEI. A release of pDNA from the complexes has been previously shown to be a critical parameter for successful gene delivery.[2] Moreover, a previous study may also support a size effect of polymer pDNA complexes on gene transfection efficiency, which has been shown to be internalized more efficiently than larger sized aerosol fabricated complexes.[22] In the case of cytotoxicity, cell viability decreased as complex sizes increased, until it finally reached about 40% at 120 nm in complex size. It might have originated from adverse interactions between excessive surface charges and cell components. The number of surface charges per complex (n) can be estimated by the following equation:[23]

$$n = \frac{\sigma S_p}{e}, \tag{1}$$

where σ, S_p, and e are the surface charge densities of the complex ($=(\varepsilon_s\varepsilon_0 xkT/e)\{\exp(e\zeta/2kT) - \exp(-e\zeta/2kT) + (8/xD_p)[\exp(e\zeta/2kT) - /\exp(e\zeta/2kT) + 1]\}$, where ε_s and ε_0 are the relative permittivity of the solution and the permittivity of a vacuum, respectively, x is the Debye-Hückel parameter, k is the Boltzmann constant, T is the temperature, and ζ is the zeta potential), the surface area of the complex ($=2\pi D_p^2$), and the elementary electric charge ($+1.6 \times 10^{-19}$ C), respectively. According to the estimations, the number of charges per complex ranged 4.12×10^2–3.17×10^3 by increasing the complex size, and this may cause an increase in adverse effects from interactions between excessive cationic charges and anionic macromolecules on cell viability as well as inefficient release

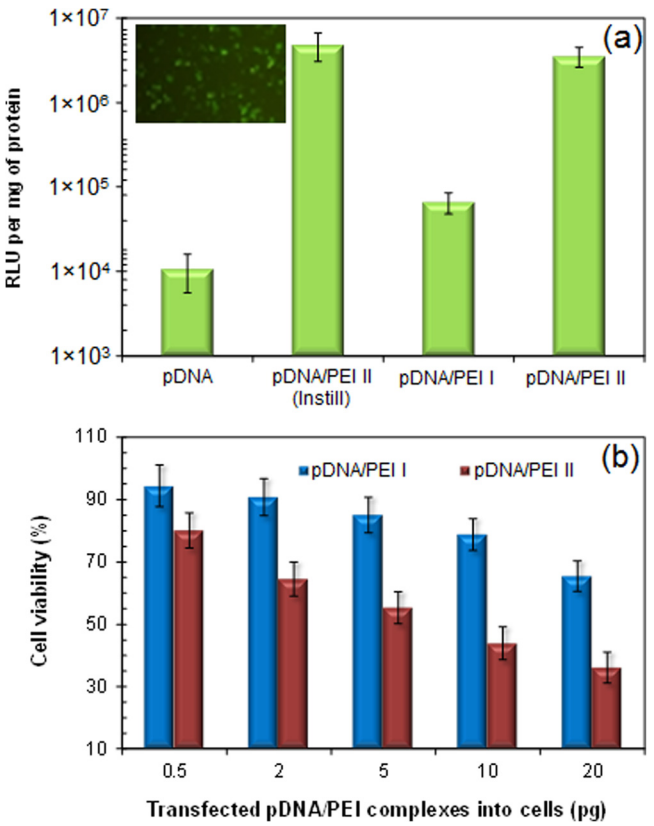

FIG. 4. (a) Transfection efficiencies of aerosol fabricated pDNA/PEI complexes into A549 cells including pDNA and instilled pDNA/PEI II complexes. Transfection fluorescence imaging of pDNA/PEI II complexes is also shown in the inset. (b) Cytotoxicity of pDNA/PEI complexes in A549 cells.

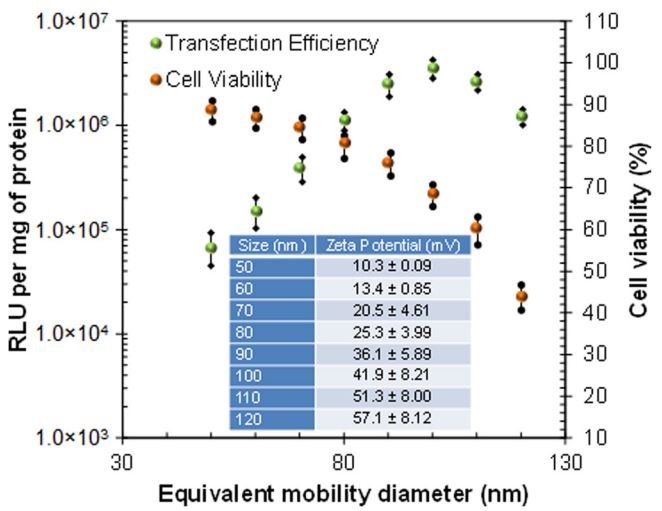

FIG. 5. Transfection efficiency and cytotoxicity of aerosol fabricated pDNA/PEI complexes with different sizes (50 ± 8.8 nm, 60 ± 6.2 nm, 70 ± 10.1 nm, 80 ± 11.8 nm, 90 ± 13.5 nm, 100 ± 15.9 nm, 110 ± 15.1 nm, and 120 ± 20.3 nm). Zeta potential data of different sized pDNA/PEI complexes are also displayed (inset).

during gene transfection, especially at >100 nm. These findings demonstrated that using optimal mass ratios between the pDNA and the PEI could improve PEI mediated gene transfection on human alveolar epithelial cells.

We reported some fundamental properties of aerosol fabricated pDNA/PEI complexes for efficient gene delivery. Different mass ratios between the pDNA and the PEI could produce different sizes of pDNA/PEI complexes through aerosol fabrication and these have affected gene transfection efficiency as well as cell cytotoxicity. These findings demonstrate that using optimal mass ratios between a pDNA and a PEI can improve PEI mediated gene transfection on human alveolar epithelial cells. Application of a composite system as an alternative carrier containing a soft core and a lesser amount of cationic component might buffer shear stress to DNA during nebulization, and the reduced surface charge might decrease cell cytotoxicity without significant decrease in gene transfection efficiency. Continued studies would ensure the development of optimized aerosol pDNA/PEI complexes and bring them a step closer to a realization of their clinical potential for gene delivery.

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[24]See supplementary material at http://dx.doi.org/10.1063/1.4864268 for details about the experimental procedure, FTIR spectra with gel retardation assay of pDNA and pDNA/PEI complex samples, and size distributions of the aerosol fabricated complexes including pDNA and individual PEI particles.