

# Aerosol-Based Fabrication of Modified Chitosans and Their Application for Gene Transfection

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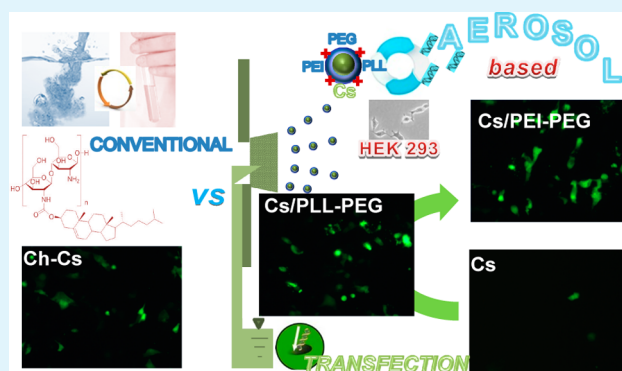
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## Supporting Information

**ABSTRACT:** Modified chitosan nanoparticles were conveniently obtained by a one-step aerosol method, and their potential for gene transfection was investigated. Droplets containing modified chitosans were formed by collision atomization, dried to form solid particles, and collected and studied for potential use as nanocarriers. Modified chitosans consisted of a chitosan backbone and an additional component [covalently attached cholesterol; or blends with poly(L-lysine) (PLL), polyethyleneimine (PEI), or poly(ethylene glycol) (PEG)]. Agarose gel retardation assays confirmed that modified chitosans could associate with plasmid DNA. Even though the average cell viability of cholesterol-chitosan (Ch-Cs) showed a slightly higher cytotoxicity ( $\sim 90\%$  viability) than that for unmodified chitosan (Cs,  $\sim 95\%$ ), transfection ( $>7.5 \times 10^5$  in relative light units (RLU)  $\text{mg}^{-1}$ ) was more effective than it was for Cs ( $\sim 7.6 \times 10^4$  RLU  $\text{mg}^{-1}$ ). The blending of PEI with Cs (i.e., a Cs/PEI) to produce transfection complexes enhanced the transfection efficiency ( $\sim 1.3 \times 10^6$  RLU  $\text{mg}^{-1}$ ) more than did the addition of PLL (i.e., a Cs/PLL,  $\sim 9.3 \times 10^5$  RLU  $\text{mg}^{-1}$ ); however, it also resulted in higher cytotoxicity ( $\sim 86\%$  viability for Cs/PEI vs  $\sim 94\%$  for Cs/PLL). The average cell viability ( $\sim 92\%$ ) and transfection efficiency ( $\sim 1.9 \times 10^6$  RLU  $\text{mg}^{-1}$ ) were complemented further by addition of PEG in Cs/PEI complexes (i.e., a Cs/PEI-PEG). This work concludes that gene transfection of Cs can be significantly enhanced by adding cationic polymers during aerosol fabrication without wet chemical modification processes of Cs.

**KEYWORDS:** modified chitosans, one-step aerosol method, cationic polymers, cytotoxicity, transfection



## INTRODUCTION

Engineered nanoparticles intended for biomedical purposes must generally be in the size range 20–500 nm diameter. Such nanoparticles have relatively large surface areas, enhancing their ability to bind, adsorb, and carry other compounds, including drugs, probe molecules, and proteins for interaction with molecules, cells, and tissues.<sup>1,2</sup> Engineered nanoparticles thus have the potential to lead to improvements in therapeutic responses or diagnostic imaging. The delivery of nanoparticles into cells is often necessary for their application in cellular and subcellular targeting, labeling and imaging.<sup>3,4</sup> It is generally observed that cationic polymers and lipid-based nonviral carriers are readily taken up by cells, due to strong electrostatic interactions with the anionic cell membrane.<sup>5,6</sup> Among them, polyethyleneimine (PEI) is one of the most extensively investigated cationic polymers for gene delivery, because of its superior transfection in many different types of cells resulting from its so-called “proton sponge” effect, allowing endosomal escape and the transfer of deoxyribonucleic acid (DNA) to the nucleus.<sup>7,8</sup> Nevertheless, PEI has not advanced to clinical evaluation, mainly because of its cytotoxicity.

Chitosan, a natural, nontoxic, biocompatible, and biodegradable polymer, is widely used as a gene transfection reagent,<sup>9</sup> in scaffolds for tissue engineering,<sup>10</sup> as a drug delivery substance,<sup>11</sup> and in polymeric coatings for nanoparticles.<sup>12</sup> Practical applications of chitosan have mainly made use of the unmodified form. However, modified chitosans could potentially be used in a wide range of biomedical applications, including the interaction and intracellular delivery of genetic material.<sup>13,14</sup> Various modifications of chitosan have been reported that improve performance, including modification with long chain fatty acids,<sup>12</sup> cholesterol,<sup>15</sup> folate,<sup>16</sup> peptide,<sup>17</sup> or cationic polymers.<sup>18</sup> Among them, the addition of a lipid (e.g., cholesterol)<sup>11,15</sup> or cationic polymer (e.g., PEI)<sup>18,19</sup> has been extensively studied and shown to enhance transfection performance.

Many formulations of polymer-modified chitosans exist as colloidal liquids, usually synthesized using time-consuming batch wet chemical processes and generally stable only for short

Received: November 13, 2013

Accepted: March 14, 2014

Published: March 14, 2014

periods of time. Also, some polymer systems are specifically designed to be gradually degradable by hydrolysis, making long-term storage in liquid form not a viable option.<sup>20,21</sup> In addition, many formulations are unstable as liquid suspensions for a variety of reasons, including degradation of the carrier and/or active substance, formation of insoluble aggregates, and unwanted loss of bioactivity.<sup>22,23</sup> One approach for overcoming such stability limitations is to formulate and store dry powders.

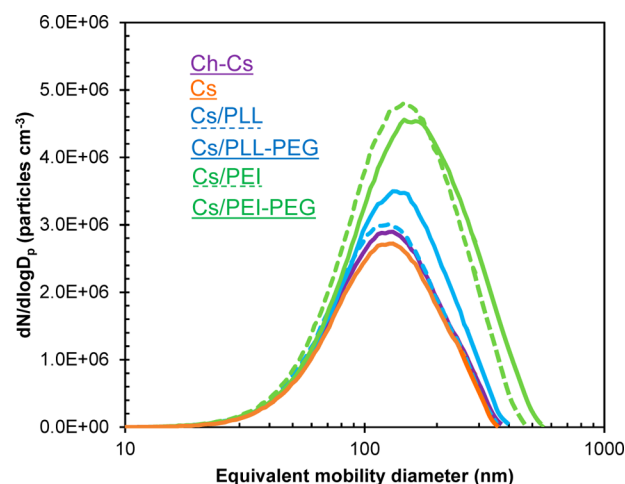
In contrast to classical wet chemical methods, aerosol processing involves a much more limited number of preparation steps. It also produces material continuously, allowing for straightforward collection of powders and generating low waste.<sup>24</sup> The field of therapeutic aerosol bioengineering, driven originally by the goal of developing inhalable insulin, is now expanding to address medical needs ranging from respiratory to systemic diseases.<sup>25</sup> Aerosol processing typically consists of spraying liquid formulations into droplets dispersed in a carrier gas.<sup>26</sup> Dry particles can be formulated in the aerosol state by atomizing liquid solutions in an appropriate carrier gas and removing solvent from the resulting particles.<sup>27</sup> The method is mostly used in the pharmaceutical industry to generate spherical microparticles for treating pulmonary diseases,<sup>28</sup> although it also can be used to generate a variety of nanosized polymeric particles,<sup>29</sup> some of which have been proven to be effective therapeutic carriers.<sup>30,31</sup>

The purpose of the present work is to demonstrate the fabrication of modified chitosan nanoparticles using a one-step aerosol method, and to explore the effects of modification on its *in vitro* gene transfection as a nanocarrier. Chitosan was modified covalently with cholesterol (designated throughout as Ch-Cs), and then aerosolized and subsequently collected. The details of the fabrication are noted in the Supporting Information. All the tested particles were aerosolized to compare their transfection efficiencies without size effect between the particles although Ch-Cs was fabricated by wet batch chemical processes since our previous report showed significant differences in transfection efficiency between different sized particles.<sup>31</sup> Cytotoxicity and transfection measurements were made using human embryonic kidney HEK 293 cells, and compared to analogous measurements on poly(L-lysine) [PLL] and PEI particles. To enhance cytotoxicity and transfection, we also prepared modified chitosan particles by atomizing mixtures of pure chitosan (Cs) particles and the cationic polymers PLL and PEI, both with and without poly(ethylene glycol) (PEG).

## RESULTS AND DISCUSSION

Figure 1 summarizes the size distribution measurements of particles formed from the collision-atomization of aqueous solutions of Ch-Cs and Cs. The geometric mean diameter (GMD), geometric standard deviation (GSD), and total number concentration (TNC) of the Ch-Cs (or Cs) particles are 110 (107) nm, 1.75 (1.76), and  $1.6 (1.5) \times 10^6 \text{ cm}^{-3}$ , respectively (Table 1). Analogous data for Cs atomized along with PLL (or PEI) are 110 (130) nm, 1.75 (1.85), and  $1.6 (2.7) \times 10^6 \text{ cm}^{-3}$ , respectively, and for Cs with PLL-PEG (or PEI-PEG) are 120 (145) nm, 1.75 (1.86), and  $1.9 (2.7) \times 10^6 \text{ cm}^{-3}$ , respectively. The particle distributions for modified particles were larger than they were for Cs. This implies that added components were incorporated into the Cs particles.

TEM images (Figure 2) indicate that Ch-Cs particles have near-spherical shapes, with smooth surfaces. Particles are also well separated. The formation of dense solid particles is



**Figure 1.** Aerosol size distributions of unmodified chitosan (Cs) and modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG).

**Table 1.** Aerosol Size Distributions of Unmodified Chitosan (Cs) and Modified Chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG)

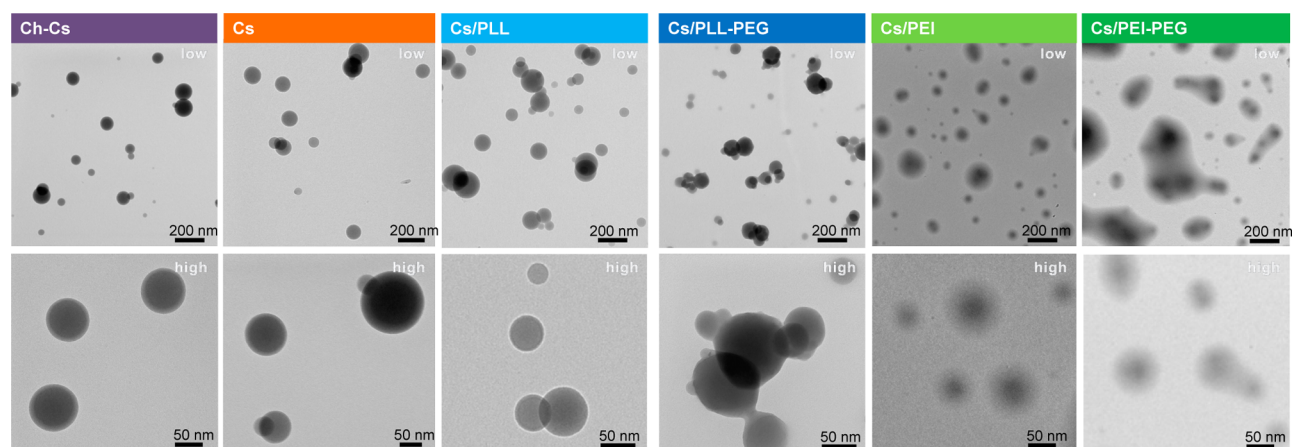
case	GMD (nm)	GSD	TNC (particles $\text{cm}^{-3}$ )
Ch-Cs	110	1.75	$1.6 \times 10^6$
Cs	107	1.76	$1.5 \times 10^6$
Cs/PLL	110	1.75	$1.6 \times 10^6$
Cs/PLL-PEG	120	1.75	$1.9 \times 10^6$
Cs/PEI	130	1.85	$2.7 \times 10^6$
Cs/PEI-PEG	145	1.86	$2.7 \times 10^6$

facilitated by slow convective drying, where the time for the liquid to evaporate is greater than the time required for supersaturated particles at the liquid–vapor interface to migrate back toward a droplet center. The Peclet number,  $Pe$ , is a dimensionless number that expresses the relative time-scales for diffusion ( $D_d^2/4\delta_v$ ) and convective drying ( $\tau_d$ ).

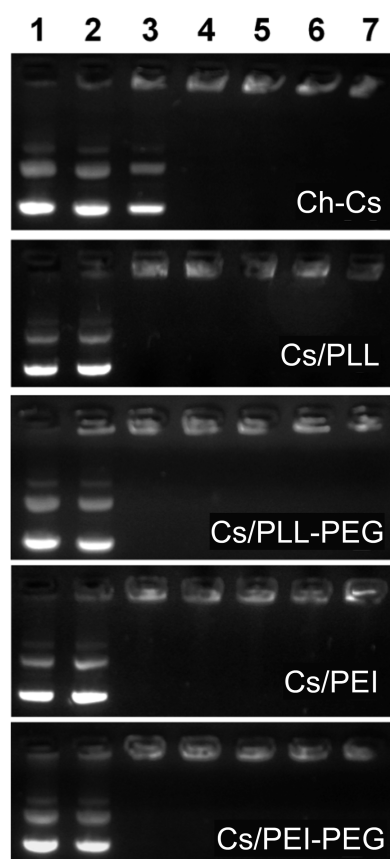
$$Pe = \frac{D_d^2}{4\tau_d\delta_v} \quad (1)$$

For the present case, conditions were such that  $Pe \ll 1$ , ensuring that migration of solutes for the interface toward the droplet center was sufficient to keep up with convective drying, thus ensuring formation of dense solid particles. When PLL was incorporated into the particles (forming particles designated Cs/PLL), the particle boundaries were vaguer than that for pure Cs, perhaps due to a linkage between separate Cs and PLL particles. It was also harder to verify the boundaries when PEI was incorporated (i.e., Cs/PEI). With further addition of PEG to Cs/PLL (i.e., Cs/PLL-PEG) or Cs/PEI (i.e., Cs/PEI-PEG), the phenomenon was even more pronounced, and the morphology was changed further, as reported by Xu et al. (2009).<sup>32</sup> The mean mode diameter of the Ch-Cs and Cs are  $109 \pm 9$  and  $101 \pm 7$  nm, respectively. The same data for Cs/PLL, /PEI, /PLL-PEG, and /PEI-PEG cases are  $111 \pm 10.2$ ,  $131 \pm 7.9$ ,  $122 \pm 11.1$ , and  $144 \pm 18.6$  nm, respectively, and these data are in good agreement with the data shown in Figure 1 and Table 1, which indicate that the polymer incorporation led to an increase in the Cs particle size.

Agarose gel retardation assays (Figure 3) were carried out to confirm whether pDNA would associate with the modified



**Figure 2.** Low- and high-magnitude TEM images of unmodified chitosan (Cs) and modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG).

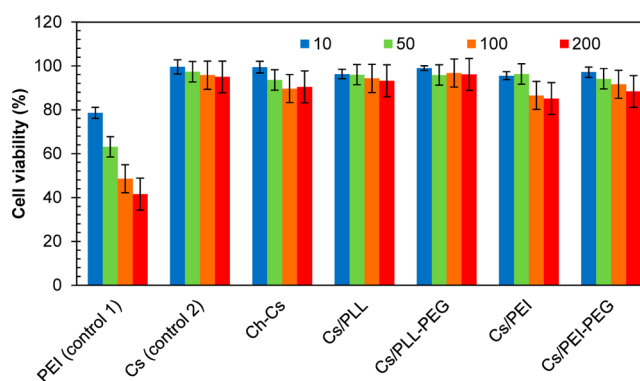


**Figure 3.** Gel retardation assay of modified chitosan/pDNA complexes. The complexes are composed of modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG). Lane 1 is pDNA, and lanes 2–7 are modified chitosan/pDNA complexes with the weight complex ratios (modified chitosan to pDNA) of 0.2, 1.0, 5.0, 10.0, 20.0, and 50.0.

chitosans, and to qualitatively investigate the optimal weight ratio (0.2, 1.0, 5.0, 10.0, 20.0, and 50.0) of modified chitosan to pDNA for binding efficiency. Particles were detached from the polytetrafluoroethylene substrate by immersing samples in water and subjecting them to ultrasound treatment for 10 s. At a ratio of 10.0 or above, almost all DNA combined with modified chitosan, with little free DNA visibly escaping. Thus,

the complexes prepared with a ratio of 10.0 were selected for further *in vitro* studies.

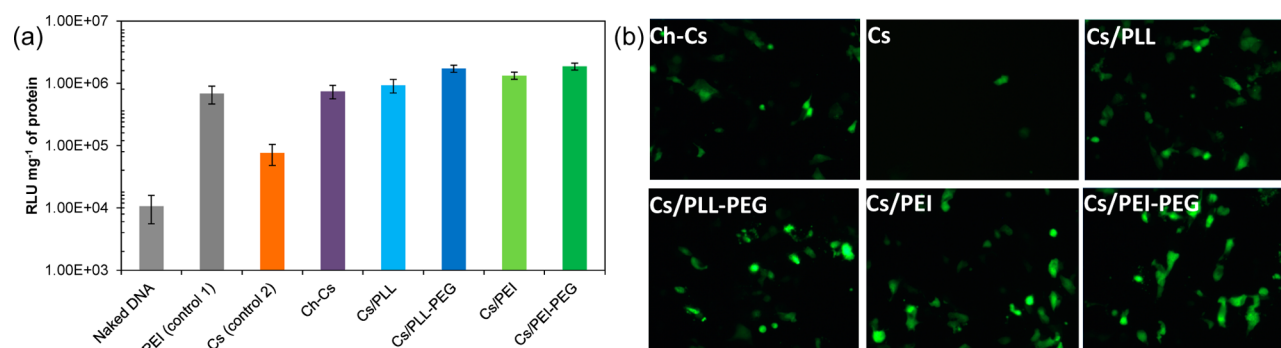
The cytotoxicity of the particle/pDNA complexes was evaluated using an MTS assay in HEK 293 cells. Results were compared to PLL/pDNA and PEI/pDNA (Figure 4). Results



**Figure 4.** Cytotoxicity of unmodified chitosan (Cs) and modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG) in HEK 293 cells in comparison with PLL and PEI.

show that the range of average cell viability with different mass concentrations of 10–200  $\mu\text{g mL}^{-1}$  was  $86 \pm 3.97\%$  to  $92 \pm 4.38\%$  for all the tested modified chitosans and was significantly higher than those from collision atomized PLL ( $54 \pm 5.21\%$ ) and PEI ( $49 \pm 4.74\%$ ), while a slightly higher viability ( $96 \pm 4.88\%$ ) was observed for Cs. This implies that modified chitosans may be nontoxic under standard cell culture conditions. In addition, there were no significant differences of cytotoxicity between the unmodified and modified chitosans. The slightly higher cytotoxicity of the modified chitosans was considered to be a consequence of damage from interactions with plasma membranes or other cellular compartments.<sup>33</sup> Therefore, the fact that the cell viability of all modified chitosans was slightly lower suggested that the charge density of modified chitosans was acceptable *in vitro*.

Differences in the transfection ability between the modified chitosans were further confirmed by luciferase assays. The kinetics of the gene transfection was first confirmed via measurements of the efficiency at different duration times (1, 6, 24, and 36 h). A significant uptake (reaching 55%) occurred 1–6 h into the duration time and reached a plateau at 24 h,



**Figure 5.** Results of (a) transfection efficiency, and (b) fluorescence images of pDNA with unmodified chitosan (Cs) and modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG) in HEK 293 cells for 24 h.

implying that 24 h may be the most suitable for comparison studies in gene transfection. In the case of GFP expression, the intensity lasted about 20 h after showing the maximum expression around 24 h during the 72 h of monitoring. The ability of the modified chitosans to transfect HEK 293 cells using pDNA containing the luciferase and green fluorescent protein (GFP) gene was investigated and compared to PLL/pDNA and PEI/pDNA complexes. From the results of the amount of luciferase protein (Figure 5a), it is shown that naked DNA was barely transfected ( $1.1 \pm 0.52 \times 10^4$  relative light units (RLU)  $\text{mg}^{-1}$ ) in HEK 293 cells, whereas both unmodified ( $7.6 \pm 2.85 \times 10^4$  RLU  $\text{mg}^{-1}$ ) and modified ( $>7.5 \times 10^5$  RLU  $\text{mg}^{-1}$ ) chitosans could achieve a higher degree of transfection. The transfection efficiency for wet chemically synthesized Ch-Cs particles before their aerosolization was  $8.6 \pm 1.72 \times 10^5$  RLU  $\text{mg}^{-1}$  (see Table S2 in the Supporting Information, also contains the efficiencies from other wet chemically modified chitosans, i.e. Ch-Cs/PLL and Ch-Cs/PEI). There was no significant difference between the Ch-Cs particles from different methods, which implies that aerosolization did not significantly affect their ability in gene transfection. Compared with PLL/pDNA ( $4.1 \pm 1.47 \times 10^5$  RLU  $\text{mg}^{-1}$ ) and PEI/pDNA ( $6.8 \pm 2.19 \times 10^5$  RLU  $\text{mg}^{-1}$ ) complexes, the transfection of Cs/PLL (or PEI)/ and Cs/PLL-PEG (or PEI-PEG)/pDNA complexes still exhibited a higher expression of up to  $0.9 \pm 0.23$  (or  $1.3 \pm 0.17$ ) and  $1.7 \pm 0.23$  (or  $1.9 \pm 0.24$ )  $\times 10^6$  RLU  $\text{mg}^{-1}$ , respectively. Figure 5b shows fluorescence images of HEK 293 cells for the chitosan/pDNA complexes derived from GFP expression, which further confirmed the transfection and differences between the complexes. In the present cases, the surface charge (2.5 to 21.4 mV, refer Table 2) from a modification of chitosan demonstrated a key role in

**Table 2. Zeta Potential of pDNA Complexes with Unmodified Chitosan (Cs) and Modified Chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG)**

samples/pDNA polyplexes	zeta potential (mV)
Ch-Cs	$2.5 \pm 0.89$
Cs	$-1.59 \pm 0.60$
Cs/PLL	$16.7 \pm 3.36$
Cs/PLL-PEG	$19.4 \pm 3.65$
Cs/PEI	$19.8 \pm 3.91$
Cs/PEI-PEG	$21.4 \pm 3.52$

\*12.7 mV for Ch-Cs, 4.1 mV for Cs, 28.8 mV for Cs/PLL, 33.3 mV for Cs/PLL-PEG, 30.1 mV for Cs/PEI, and 36.8 mV for Cs/PEI-PEG before pDNA complexing.

determining the level of transfection efficiency ( $0.7 \times 10^5$  to  $1.9 \times 10^6$  RLU  $\text{mg}^{-1}$ ) because the particle size did not show the anticipated correlation (smaller sizes commonly introduce higher transfection efficiencies)<sup>34,35</sup> between particle size and transfection efficiency. Moreover, a polymeric (i.e., Cs to Cs/polymer) by simple addition of polymeric components during aerosolization) modification could also enhance the transfection relative to a synthetic modification (i.e., Cs to Ch-Cs by wet chemical reaction before aerosolization,  $7.6 \times 10^4$  to  $7.5 \pm 1.83 \times 10^5$  RLU  $\text{mg}^{-1}$ ) up to  $1.9 \times 10^6$  RLU  $\text{mg}^{-1}$ , even at low cytotoxicity conditions (up to  $\sim 97\%$  in cell viability vs  $\sim 90\%$  for Ch-Cs). PEG is a highly hydrophilic polymer, so the PEG addition could enhance the transfection and reduce the interactions of a cationic polymer with the plasma membrane or other cellular compartments.<sup>8,36</sup> Furthermore, the surface charge data of the modified chitosan nanoparticles are measured by zeta potential analyzer (Nano-7, Malvern, UK), with different storage days (see Table S1 in the Supporting Information). The results show that the zeta potential for Ch-Cs decreased [ $+12.7$  (initial state) to  $+3.6$  mV at 180 days] with increasing storage time while the zeta potential for Cs/PLL-PEG and Cs/PEI-PEG particles remained as their initial states even for their sizes [cf. for Ch-Cs suspension in water (110 nm, initial  $\rightarrow$  347 nm, after 180 days)], and there are no significant differences between the storage days. This implies that the aerosol-modified chitosan nanoparticles have stability that may be suitable in a long-term storage.

## CONCLUSIONS

For the first time, aerosol fabrication of modified chitosans has been used in *in vitro* cytotoxicity and transfection. This showed that modified chitosans could achieve a higher intracellular transfection ( $>7.5 \times 10^5$  RLU  $\text{mg}^{-1}$ ) than Cs ( $\sim 1.1 \times 10^4$  RLU  $\text{mg}^{-1}$ ) in HEK 293 cells, which was first performed with Ch-Cs (from synthetic modification before aerosol fabrication), and also enhanced with Cs/polymer (from copolymeric modification during aerosol fabrication). Compared with PLL ( $4.1 \times 10^5$  RLU  $\text{mg}^{-1}$ ) or PEI ( $6.8 \times 10^5$  RLU  $\text{mg}^{-1}$ ) alone, the transfections with Cs/PLL (or PEI)/ and Cs/PLL-PEG (or PEI-PEG) still introduced higher expressions of up to 0.9 (or 1.3) and 1.7 (or 1.9)  $\times 10^6$  RLU  $\text{mg}^{-1}$  even at low cytotoxicity conditions (up to  $\sim 97\%$  in cell viability), respectively. This work demonstrates that the gene transfection of Cs can be enhanced by adding cationic polymers during aerosol fabrication without wet chemical processes. These results provide some useful evidence for aerosol fabrication which is efficient, green, scalable fabrication, which is generalizable to an

extraordinarily broad range of exogenous genes and therapeutic agents.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

The methods, FTIR results, and zeta potential data. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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### Author Contributions

†Authors J.H.B. and H.-K.K. contributed equally to this paper.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was partially supported by NSF grant CHE-0924431.

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