Aerosol-based fabrication of modified chitosans and their application for gene transfection

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ABSTRACT

Modified chitosans consisted of a chitosan backbone and an additional component [with cholesterol, poly(L-lysine) (PLL), polyethyleneimine (PEI), or poly(ethylene glycol) (PEG)]. Agarose gel retardation assays confirmed that modified chitosans could associated with plasmid DNA. Even though the cell viability of cholesterol-chitosan (Ch-Cs) showed a slight higher cytotoxicity (~90% in cell viability) than that for unmodified chitosan (Cs, ~95%), transfection [$>7.5 \times 10^5$ in relative light units (RLU) mg^{-1}] was more effective than it was for Cs (~7.6×10^4 RLU mg^{-1}). The addition of PEI onto Cs (*i.e.* Cs/PEI) enhanced the transfection efficiency (~1.3×10^6 RLU mg^{-1}) more than did the addition of PLL (*i.e.* Cs/PLL, ~9.3×10^5 RLU mg^{-1}). However it resulted in higher cytotoxicity (~86% in cell viability for Cs/PEI *vs* ~94% for Cs/PLL). The cell viability (~92%) and transfection efficiency (~1.9×10^6 RLU mg^{-1}) were complemented by further adding PEG on Cs/PEI (*i.e.* Cs/PEI-PEG).

Keywords: modified chitosans; one-step aerosol method; cationic polymers; cytotoxicity; transfection

1 INTRODUCTION

Chitosan, a natural, nontoxic, biocompatible, and biodegradable polymer, is widely used as a gene transfection reagent, in scaffolds for tissue engineering, as a drug delivery substance, and in polymeric coatings for nanoparticles [1]. Practical applications of chitosan has 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 [2]. Various modifications of chitosan have been reported that improve performance, including modification with long chain fatty acids, cholesterol, folate, peptide, or cationic polymers [3]. Among them, the addition of a lipid (e.g. cholesterol) [3,4] or cationic polymer [e.g. polyethyleneimine (PEI)] has been extensively studied and shown to enhance transfection performance [5].

The purpose of the present work is to demonstrate the fabrication of modified chitosan nanoparticles using a one–step aerosol method, and explore the effects of modification on its *in vitro* gene transfection as a nanocarrier. Chitosan was modified with cholesterol (designated throughout as Ch-Cs), and then aerosolized and subsequently collected. The details of the fabrication are noted in Supporting Inforamtion. 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. In order to enhance cytotoxicity and transfection, unmodified chitosan (Cs) particles were also modified by incorporating cationic polymers, such as PLL and PEI with or without poly(ethylene glycol) [PEG] by adding these components with the Cs solution during aerosol fabrication.

2 METHODS

The size distributions of the aerosol particles are measured using a scanning mobility particle sizer (SMPS). The SMPS system, which measures the mobility equivalent diameter, is operated at a sample flow of 0.3 L min^{-1}, a sheath flow of 1.0 L min^{-1} (measurement range: 7.91–333.8 nm). TEM (CM-100, FEI/Philips, US) images were obtained at an accelerating voltage of 19-180 kV. The zeta potential of sample/pDNA complexes was determined using a zeta potential analyzer (Nano ZS-90, Malvern Instruments, UK).

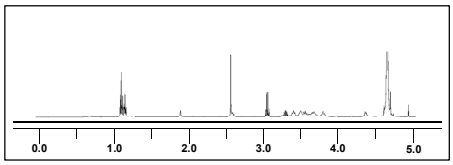

Figure 1: ^{1}H Proton NMR spectrum of Ch-Cs.

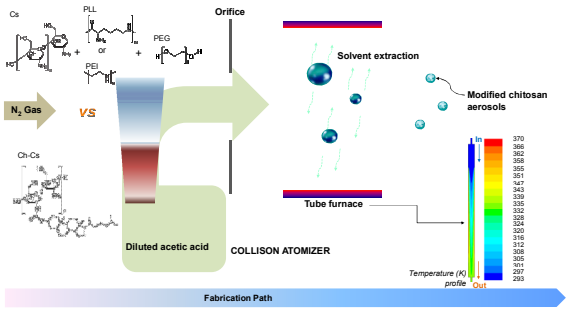

Figure 2: Schematic illustration of one-step aerosol fabrication of modified chitosans using a series connection of a collison atomizer and a heated tube reactor.

The ^{1}H NMR spectrum of Ch-Cs (Fig. 1) was consistent with that reported by Maity and Jana [6]. Schematic diagrams of the aerosol fabrication used for these experiments are shown in Fig. 2. The cytotoxicity of the modified chitosans was evaluated using HEK 293 cells by the MTS, 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, assay. The cells were cultured in 200 mL Dulbecco's modified eagle medium (DMEM, Carlsbad, US) supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO_2, and 95% relative humidity. The cells were seeded in a 96-well microtiter plate (Nunc, Germany) at densities of 1×10^5 cells well^{-1}. After 24 h, the culture media were replaced with serum-supplemented culture media containing the modified chitosans (1 mg mL^{-1}), and the cells were incubated for 24 h. Then, 30 µL of the MTS reagent was added to each well. The cells were incubated for an additional 2 h. Then, the absorbance was measured using a microplate reader (Spectra Plus, TECAN, Switzerland) at a wavelength of 490 nm. The cell viability (%) was compared with that of the untreated control cell in media without modified chitosans and calculated with $[A]_{test}$:$[A]_{control} \times 100\%$, where $[A]_{test}$ is the absorbance of the wells with modified chitosans and $[A]_{control}$ is the absorbance of the control wells.

HEK 293 cells were seeded in 24-well plates at a density of 1×10^6 cells well^{-1} in 1 mL of complete DMEM medium supplement with 10% FBS at 37°C, 5% CO_2, and 95% relative humidity, one night before transfection. The culture medium was replaced with serum free DMEM medium, and transfection complexes were added to the cells. The cells were incubated with the transfection complexes at 37°C for an additional 24 h after the medium was replaced by fresh complete medium. After incubation for 24 h, the medium was aspirated and washed with phosphate-buffered saline. The cells were trypsinized and then the transfection results were measured by fluorescence activated cell sorting. The green fluorescent protein (GFP) plasmid (Genlantis, US) expression of the modified chitosans in HEK 293 cells was observed with a fluorescent microscope (Nikon Eclipse TE2000-S, US).

3 RESULTS AND DISCUSSION

Fig. 3 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) $\times 10^6$ cm^{-3}, respectively. Analogous data for Cs atomized along with PLL (or PEI) are 110 (130) nm, 1.75 (1.85), and 1.6 (2.7) $\times 10^6$ 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) $\times 10^6$ 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.

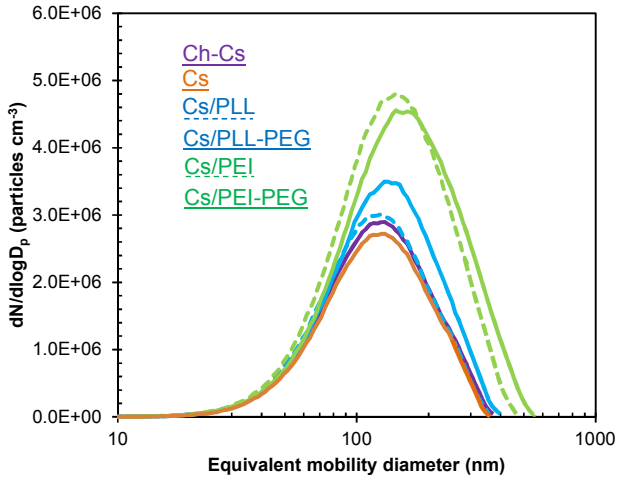

Figure 3: Aerosol size distributions of unmodified chitosan (Cs) and modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG).

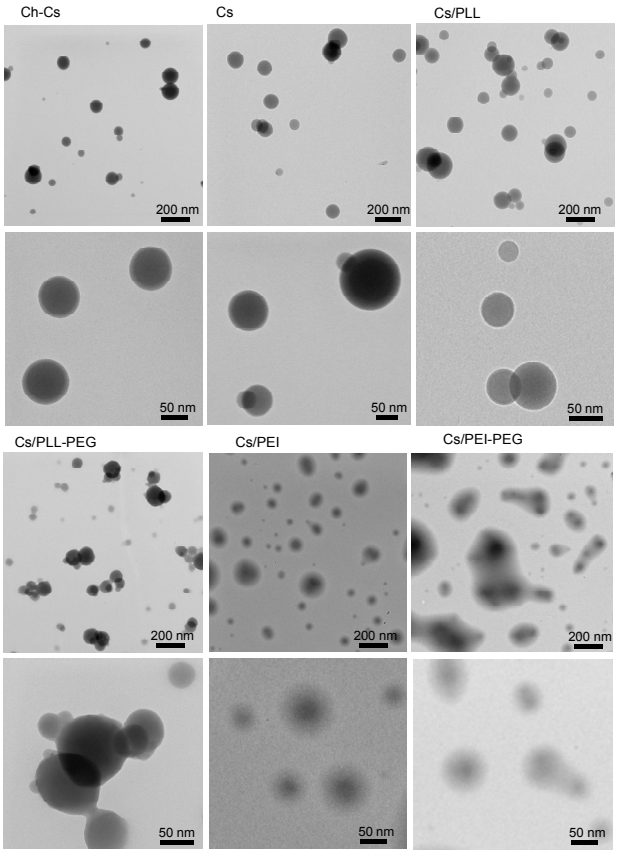

Figure 4: Low- and high-magnitude TEM images of unmodified chitosan (Cs) and modified chitosans (Ch-Cs, Cs/PLL, Cs/PEI, Cs/PLL-PEG, and Cs/PEI-PEG).

TEM images (Fig. 4) indicate that all Ch-Cs particles have similar near spherical shapes, with smooth surfaces. Particles are also well separated. The formation of dense solid particles is 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. For the present case, conditions were such that Pe << 1, ensuring that migration of solutes for the interface towards the droplet center was sufficient to keep up with convective drying, thus ensuring formation of dense solid particles. When PLL was incorporated into the Cs (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 on 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) [7]. The mean mode diameter of the Ch-Cs and Cs are 109 ± 9 and 101 ± 7 nm, respectively. The same data for Cs/PLL, /PEI, /PLL-PEG, and /PEI-PEG cases are 111 ± 10.2, 131 ± 7.9, 122 ± 11.1 and 144 ± 18.6 nm, respectively, and these data are in good agreement with the data shown in Fig. 3, which indicate that the polymer incorporation led to an increase in the Cs particle size.

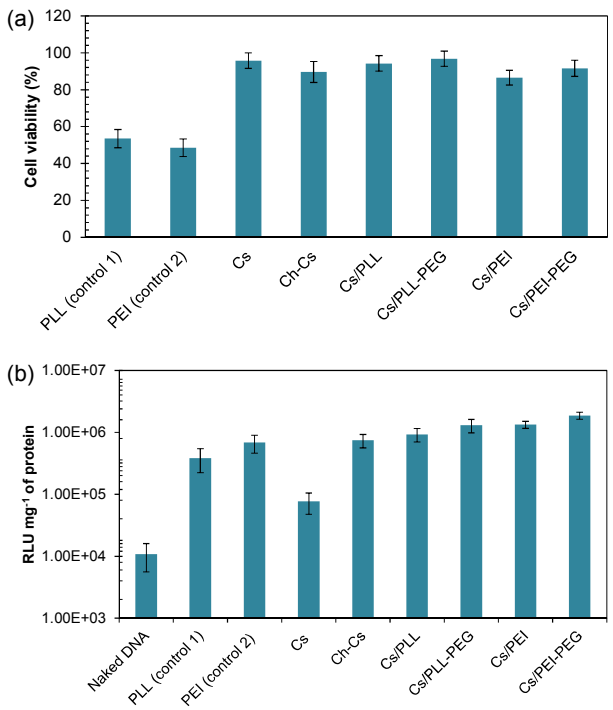

Figure 5: *In vitro* definitions of (a) cell viability and (b) gene transfection efficiency for the fabricated particles.

The cytotoxicity of the particle/pDNA complexes was evaluated using an MTS assay in HEK 293 cells. Results were compared to PLL/ and PEI/pDNA (Fig. 5a). Results show that the range of cell viability was 86%-92% for all the tested modified chitosans and was significantly higher than those from PLL (~54%) and PEI (~49%), while a slightly higher viability (~96%) was observed for Cs. This implies that modified chitosans may be non-toxic in a clinical context. 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 [8]. 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 ability of the modified chitosans to transfect HEK 293 cells using pDNA containing the luciferase and GFP gene was investigated and compared to PLL/ and PEI/pDNA complexes. From the results of the amount of luciferase protein (Fig. 5b), it is shown that naked DNA was barely transfected [~1.1×10^4 relative light units (RLU) mg^{-1}] in HEK 293 cells, whereas both unmodified (~7.6×10^4 RLU mg^{-1}) and modified (>7.5×10^5 RLU mg^{-1}) chitosans could achieve a higher intracellular transfection. Compared with PLL/pDNA (4.1×10^5 RLU mg^{-1}) and PEI/pDNA (6.8×10^5 RLU 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 (or 1.3) and 1.3 (or 1.9) $\times 10^6$ RLU mg^{-1}, respectively. In the present cases, the surface charge (2.5 to 21.4 mV) from a modification of chitosan demonstrated a key role in determining the level of transfection efficiency (0.7×10^5 to 1.9×10^6 RLU mg^{-1}) because the particle size did not show the anticipated corelation (smaller sizes commonly introduce higher transfection efficiencies) between particle size and transfection efficiency[9]. Moreover, a polymeric (*i.e.* Cs to Cs/polymers by simple addition of polymeric components during aerosolization) modification could also enhance the transfection from a synthetic (*i.e.* Cs to Ch-Cs by wet chemical reaction before aerosolization) modification (7.6×10^4 to 7.5×10^5 RLU mg^{-1}) up to 1.9×10^6 RLU mg^{-1}, even at low cytotoxicity conditions (up to ~97% in cell viability *vs* ~90% for Ch-Cs). PEG is a highly hydrophilic polymer, so the PEG addtion could enhance the transfection and reduce the interactions of a cationic polymer with the plasma membrane or other cellular compartments [10].

4 CONCLUSIONS

This work concludes that 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.

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REFERENCES

[1] N. Duceppe, M. Tabrizian, Expert Opin. Drug Delivery 2010, 7, 1191.
[2] N. Saranya, A. Moorthi, S. Saravanan, M. P. Devi, N. Selvamurugan, Int. J. Biol. Macromol. 2011, 48, 234.
[3] Z. Liu, Z. Zhong, G. Peng, S. Wang, X. Du, D. Yan, Z. Zhang, Q. He, J. Liu, Drug Delivery 2009, 16, 341.
[4] X. F. Liang, H. J. Wang, H. Luo, H. Tian, B. B. Zhang, L. J. Hao, J. I. Teng, J. Chang, Langmuir 2008, 24, 7147.
[5] F. M. Kievit, O. Veiseh, N. Bhattarai, C. Fang, J. W. Gunn, D. Lee, R. G. Ellenbogen, J. M. Olson, M. Q. Zhang, Adv. Funct. Mater. 2009, 19, 2244.
[6] A. R. Maity, N. R. Jana, J. Phys. Chem. C 2011, 115 137.
[7] Z. Xu, X. Wan, W. Zhang, Z. Wang, R. Peng, F. Tao, L. Cai, Y. Li, Q. Jiang, R. Gao, Carbohydr. Polym. 2009, 78 46.
[8] K. Ma, M.-X. Hu, Y. Qi, J.-H. Zou, L.-Y. Qiu, Y. Jin, X.-Y. Ying, H.-Y. Sun, Biomaterials 2009, 30, 6109.
[9] G. F. Liang, Y. L. Zhu, B. Sun, F. H. Hu, T. Tian, S. C. Li, Z. D. Xiao, Nanoscale Res. Lett. 2011, 6, 447.
[10] Z.-T. Li, J. Guo, J.-S. Zhang, Y.-P. Zhao, L. Lv, C. Ding, X.-Z. Zhang, Carbohydr. Polym. 2010, 80, 254.