Supporting Information for Publication

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

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METHODS

- *Aerosol Fabrication of Modified Chitosans*

Ch-Cs was synthesized before aerosolization by the previously described method of Maity and Jana.[1] Briefly, chitosan oligosaccharide lactate (1.2 g, Sigma-Aldrich, US) in dimethyl sulfoxide (20 mL) was added to triethylamine (2.4 mL) in dichloromethane (30 mL) and the reaction mixture was stirred for 30 min. Cholesteryl chloroformate (340 mg) in dichloromethane (10 mL) was added to the mixture, and the reaction mixture was stirred at room temperature for 3 days. The mixture was concentrated under reduced pressure, and the product was precipitated by centrifugation. After washing (5 times) with dichloromethane (30 mL), the product was dispersed in water by sonication and dialyzed (using a membrane with molecular weight cut-off 3000) for 3 days to produce Ch-Cs. The ^{1}H NMR spectrum of Ch-Cs (**Figure S1**) was consistent with that reported by Maity and Jana.[1] Schematic diagrams of the aerosol fabrication used for these experiments are shown in **Figure S2**. A nitrogen (>99.99% purity) flow, which was controlled by a mass flow controller (3810DS, Kofloc, Japan), had a flow rate of 3 L min^{-1}, and was used as the operating gas for atomizing a solution containing Ch-Cs dissolved in 100 mL of diluted acetic acid [1% (*v/v*)]. To fabricate Cs-polymer mixtures, polymer (purchased from Sigma-Aldrich, US) added Cs particles, Cs (Mw: 15 kDa, Polysciences, US) was first dissolved in diluted acetic acid, and then mixed with 4×10^{-6} mol dm^{-3} of PLL [Mw: 150-300 kDa, 0.1% (w/v) in H_2O], PEI (Mw: 25 kDa), PLL-PEG, or PEI-PEG before atomization. The droplets then passed through a heated tubular flow reactor operating at 90^{o}C wall temperature (refer to the temperature profile in **Figure S2**, calculated using Fluent, a commercial computational fluid dynamics program) to drive solvent from the droplets. The condition for complete evaporation was estimated by considering the time required for the evaporation of the droplets and comparing it with the appropriate residence time in the tubular flow reactor. The characteristic time to saturate the gas with vapor from evaporating droplets, τ_d, is given *via* the equation,[2]

$$\tau_d = \frac{1}{2\pi D_d \delta_v C_n(D_d)} \quad \text{(S1)}$$

where D_d, δ_v, and $C_n(D_d)$ are the droplet diameter, vapor diffusion coefficient, and the droplet number concentration, respectively. D_d can be estimated *via* the equation,

$$D_d = \left(\frac{\rho_s w + \rho_p (1-w)}{\rho_s w} D_p^3 \right)^{1/3} \quad \text{(S2)}$$

where ρ_s and ρ_p are the densities of the solvent and the solid particle, respectively, w is the weight fraction of solid in liquid, and D_p is the size of solid particle. The estimated and applied residence times were 16.8 and 17.6 sec, respectively, which implies that experimental conditions were sufficient to drive off all of the solvent.

- *Instrumentation*

The size distributions of the aerosol particles were measured using a scanning mobility particle sizer (SMPS), consisting of an electrostatic classifier (3085, TSI, US), ultrafine condensation particle counter (3776, TSI, US), and a soft X-ray charger (4530, HCT, Korea). The SMPS system, which measured the mobility equivalent diameter, was operated at a sample flow of 0.3 L min^{-1}, a sheath flow of 1.0 L min^{-1}, and a scan time of 135 sec (measurement range: 7.91-333.8 nm). The mass (m) of modified chitosans was measured using a microbalance (DV215CD, Ohaus, Switzerland) and also confirmed *via* the following equation:

$$m = Q \cdot t_s \int_0^\infty \eta(D_p) C_m(D_p) dD_p \ ) \quad \text{(S3)}$$

where Q is the flow rate of nitrogen gas, t_s is the sampling time, $\eta(D_p)$ is the fractional collection efficiency, and $C_m(D_p)$ is the mass concentration of particles.

Transmission electron microscope (TEM, CM-100, FEI/Philips, US) images were obtained at an accelerating voltage range of 46-180 kV. Specimens were prepared for examination in the TEM by

direct electrostatic aerosol sampling at a sampling flow of 0.5 L min^{-1} and an operating voltage of 5 kV using a nano particle collector (NPC-10, HCT, Korea).

The ^{1}H nuclear magnetic resonance (NMR) spectrum of a Ch-Cs sample in D_2O was determined using a Bruker Avance 400 NMR spectrometer. For Fourier transform infrared (FTIR) spectroscopy analysis, samples were prepared using polytetrafluoroethylene media substrate (0.2 μm pore size, 47 mm diameter, 11807-47-N, Sartorius, Germany) by physical filtration (*i.e.* mechanical filtration mainly by diffusion, of particles on the surfaces of the substrate), and the spectra were recorded on a Nicolet 6700 FTIR spectrometer (Thermo Electron, US). The spectra were taken for samples in the range of 4000-400 cm^{-1} in absorbance mode.

The zeta potential of modified chitosan/plasmid DNA (pDNA) complexes was determined using a zeta potential analyzer (Nano ZS-90, Malvern Instruments, UK). The modified chitosans were mixed with pDNA, and incubated at room temperature for 30 min. Then, the complexes were diluted with double de-ionized water to an appropriate concentration. Measurements of the zeta potential were carried out at 25^{o}C and calculated using the manufacturer's supplied software.

- *Agarose Gel Retardation Assay*

The pDNA condensation ability of the modified chitosans under different weight ratios were analyzed by 1% agarose gel electrophoresis using tris-acetate-ethylenediaminetetraacetic acid buffer (242 g Tris, 57.1 mL glacial acetic acid, and 0.5 mM ethylenediaminetetraacetic acid, pH 8.0) containing 0.5 μg mL^{-1} ethidium bromide. Complexes containing modified chitosans and pDNA with different weight ratios were prepared by mixing, vortexing, and incubating them at room temperature for 30 min. Approximately 100 ng of each complex was loaded on agarose gels. A gel loading dye blue (New England BioLabs, US) was added to each well and agarose gel electrophoresis was carried out at a constant voltage of 80 V for 50 min. The pDNA bands of the resultant gels were then visualized under a ultraviolet transilluminator at a wavelength of 365 nm.

- *In Vitro Cytotoxicity and Transfection*

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 (1mg 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. The absorbance was then 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 GFP plasmid expression of the modified chitosans in HEK 293 cells was observed with a fluorescent microscope (Nikon Eclipse TE2000-S, US).

All experiments were performed in triplicate, and the results were reported as average values and standard deviations.

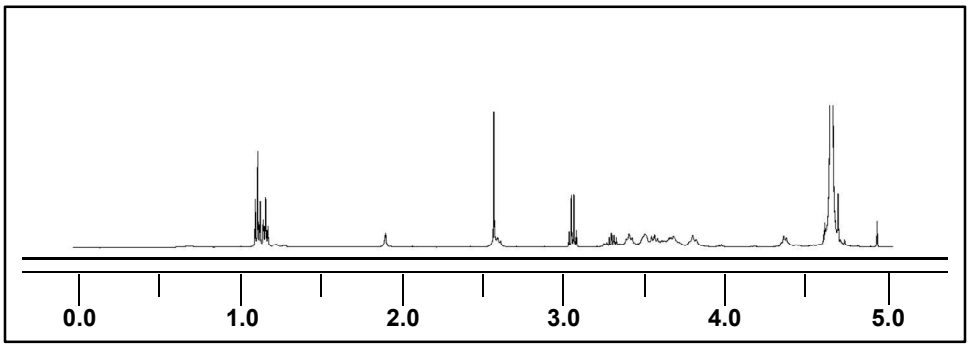

Figure S1. ^{1}H Proton NMR spectrum of Ch-Cs.

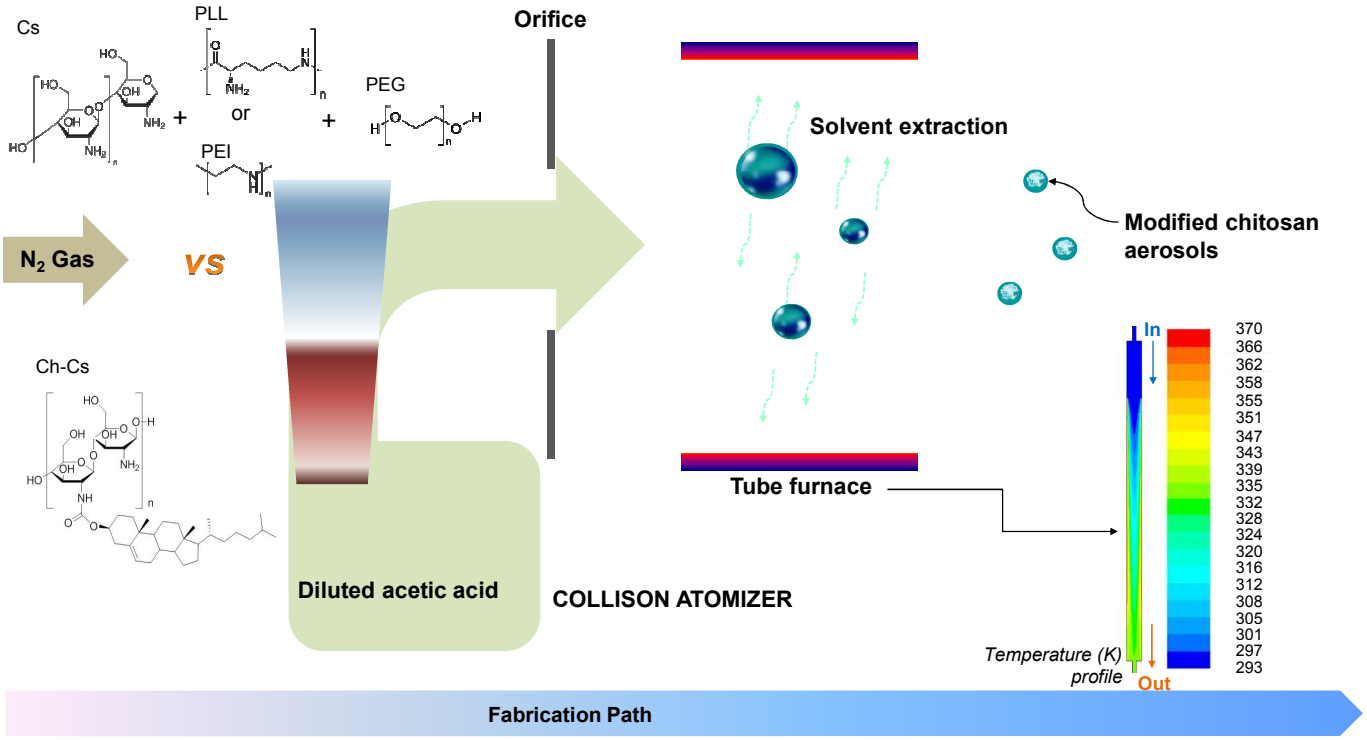

Figure S2. Schematic illustration of one-step aerosol fabrication of modified chitosans using a series connection of a collison atomizer and a heated tube reactor.

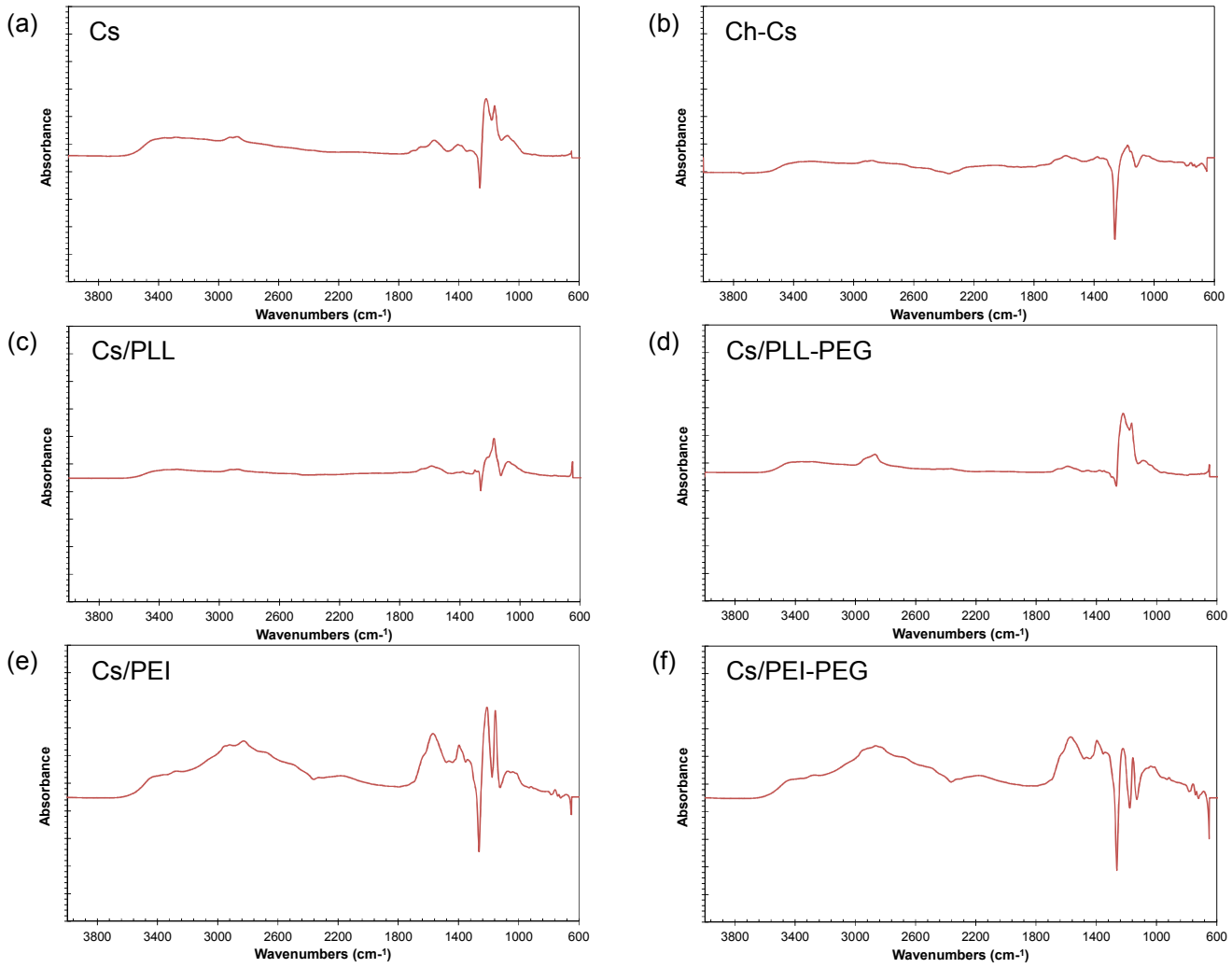

Figure S3. FTIR spectra of (a) unmodified chitosan (Cs), (b) synthetic modified chitosan (Ch-Cs), and further copolymeric modified chitosans of (c) Ch-Cs/PLL, (d) Ch-Cs/PEI, (e) Ch-Cs/PLL-PEG, and (f) Ch-Cs/PEI-PEG.

The Cs IR spectrum (**Figure S3**a) exhibited characteristic bands of 1,664 (amide I), 1,580 (amide II), and 1,380 cm^{-1} (amide III). The absorption bands at 1,160 cm^{-1} (asymmetric stretching of C-O-C bridge) and 1,033 cm^{-1} (C-O stretching) were characteristics of its saccharine structure.[3] N-H and O-H stretching

vibrations were characterized by the broad band in the region of 3,200-3,500 cm^{-1}, and bands at 2,946 and 2,960 cm^{-1} were characteristics of the CH_2-bend and CH_3-bend, respectively. The Ch-Cs spectrum (**Figure S3**b) shows that the intensity of the amide groups of Cs decreased, and this corresponded to the Cs being partially linked with cholesterol.[4] The C-O-C bridge and C-O stretching were relatively intensive owing to a cholesterol molecule loading onto the Cs backbone. After adding PLL onto Ch-Cs (**Figure S3**c), the intensity of the amide groups was weakened further due to further linking with PLL (forming a composite of free amide groups), while the peaks of the C-O-C bridge and C-O stretching were intensified. These modifications imply that there were hydrogen bonding changes between Ch-Cs and PLL during aerosol fabrication.[5] The characteristic peaks at 1,280 and 947 cm^{-1} were newly generated by further adding PEG (**Figure S3**d), and the peaks of the C-O-C bridge and C-O stretching were further intensified. For PEI addition onto Ch-Cs (**Figure S3**e), the broad band in the region of 3,200-3,500 cm^{-1} grew in intensity, indicating that N-H groups were enhanced after PEI loading.[S6] The other significant enhancement of amide groups was also due to a superposition of amide groups from PEI, and new peaks appeared at 1,468 and 814 cm^{-1} attributed to the absorption of the $–CH_2CH_2NH-$ moiety.[S7] For further addition of PEG (**Figure S3**f), even though the spectrum is nearly same as Ch-Cs/PEI, the characteristic PEG peaks at 947 and 843 cm^{-1} further intensified, indicating that additional PEG linkage was successfully performed. In short, the changed spectra from copolymeric modifications were evidence for linking cationic components onto Ch-Cs, and it could be inferred that the pDNA could be complexed individually with the modified chitosans by different electrostatic interactions. The zeta potential of modified chitosan/pDNA complexes is described in **Table 2**, and the net positive charge of the complexes varied with different surface chemistry. Moreover, these copolymeric modifications of Ch-Cs may cause changes in particle formation behavior, and have consequently induced the formation of particles with different morphologies, as depicted in **Figure 2**.

Table S1. Zeta Potential Data of Modified Chitosan Nanoparticles (Tested after Synthesized 30, 90, and 180 Days)

Case	Zeta potential		
	30 days	90 days	180 days
Ch-Cs	10.7 ± 2.77	6.2 ± 2.58	3.6 ± 2.94
Cs/PLL-PEG	37.0 ± 3.52	33.0 ± 2.98	34.9 ± 3.17
Cs/PEI-PEG	34.6 ± 3.14	35.7 ± 2.55	35.7 ± 4.01

Table S2. Results of Transfection Efficiency of Wet Chemically Modified Chitosans (Ch-Cs, Ch-Cs/PLL, and Ch-Cs/PEI) in HEK 293 Cells for 24 h

Wet chemically synthesized	Ch-Cs	Ch-Cs/PLL	Ch-Cs/PEI
Gene transfection efficiency	$8.6 \pm 1.72 \times 10^5$	$1.8 \pm 0.33 \times 10^6$	$2.8 \pm 0.47 \times 10^6$

SUPPORTING REFERENCES

(1) Maity, A. R.; Jana, N. R. Chitosan-cholesterol-based cellular delivery of anionic nanoparticles. *J. Phys. Chem. C* **2011,** *115,* 137-144.

(2) Byeon, J. H.; Kim, H.-K.; Roberts, J. T. Monodisperse poly(lactide-co-glycolic acid)-based nanocarriers for gene transfection. *Macromol. Rapid Commun.* **2012**, *33*, 1840-1844.

(3) Bhattarai, N.; Ramay, H. R.; Gunn, J.; Matsen, F. A.; Zhang, M. PEG-grafted chitosan as an injectable thermosensitive hydrogel for sustained protein release. *J. Control. Release* **2005**, *103*, 609-624.

(4) Chen, M.; Liu, Y.; Yang, W.; Li, X.; Liu, L.; Zhou, Z.; Wang, Y.; Li, R.; Zhang, Q. Preparation and characterization of self-assembled nanoparticles of 6-O-cholesterol-modified chitosan for drug delivery. *Carbohydr. Polym.* **2011**, *84*, 1244-1251.

(5) Zheng, Z.; Zhang, L.; Kong, L.; Wang, A.; Gong, Y.; Zhang, X. The behavior of MC3T3-E1 cells on chitosan/poly-L-lysine composite films: effect of nanotopography, surface chemistry, and wettability. *J. Biomed. Mater. Res. Part A* **2008**, *89A*, 453-465.

(6) Chatterjee, S.; Chatterjee, T.; Woo, S. H. Influence of the polyethyleneimine grafting on the adsorption capacity of chitosan beads for Reactive Black 5 from aqueous solutions. *Chem. Eng. J.* **2011**, *166*, 168-175.

(7) Li, Z.-T.; Guo, J.; Zhang, J.-S.; Zhao, Y.-P.; Lv, L.; Ding, C.; Zhang, X.-Z. Chitosan-graft-polyethylenimine with improved properties as a potential gene vector. *Carbohydr. Polym.* **2010**, *80*, 254-259.