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Preparation of a Hydrophobized Chitosan Oligosaccharide for Application as an Efficient Gene Carrier

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Abstract: To prepare chitosan-based polymeric amphiphiles that can form nanosized core-shell structures (nanoparticles) in aqueous milieu, chitosan oligosaccharides (COSs) were modified chemically with hydrophobic cholesterol groups. The physicochemical properties of the hydrophobized COSs (COSCs) were investigated by using dynamic light scattering and fluorescence spectroscopy. The feasibility of applying the COSCs to biomedical applications was investigated by introducing them into a gene delivery system. The COSCs formed nanosized self-aggregates in aqueous environments. Furthermore, the physicochemical properties of the COSC nanoparticles were closely related to the molecular weights of the COSs and the number of hydrophobic groups per COS chain. The critical aggregation concentration values decreased upon increasing the hydrophobicity of the COSCs. The COSCs efficiently condensed plasmid DNA into nanosized ion-complexes, in contrast to the effect of the unmodified COSs. An investigation of gene condensation, performed using a gel retardation assay, revealed that COS6(M_n = 6,040 Da) containing 5% of cholesteryl chloroformate (COS6C5) formed a stable DNA complex at a COS6C5/DNA weight ratio of 2. In contrast, COS6, the unmodified COS, failed to form a stable COS/DNA complex even at an elevated weight ratio of 8. Furthermore, the COS6C5/DNA complex enhanced the *in vitro* transfection efficiency on Human embryonic kidney 293 cells by over 100 and 3 times those of COS6 and poly(L-lysine), respectively. Therefore, hydrophobized chitosan oligosaccharide can be considered as an efficient gene carrier for gene delivery systems.

Keywords: chitosan oligosaccharide (COS), cholesterol, gene delivery, hydrophobic moiety, nanoparticle.

Introduction

Chitosan, a cationic polysaccharide obtained by alkaline *N*-deacetylation of chitin, is one of the most widely used polysaccharides. The sugar backbone of chitosan consists of β-1,4-linked *D*-glucosamine. In its structure, chitosan is very similar to cellulose, except for the amino group replacing the hydroxyl group on the C-2 position.[1]

Recently, the chitosan has been widely employed in pharmaceutical and biomedical fields owing to its unique properties such as non-toxicity, biocompatibility, and biodegradability.[2-5] Although these excellent characteristics offer the chitosan for various biomedical applications such as drug delivery systems, tissue engineering fields, and gene delivery systems, it still has several disadvantages. The solubility of chitosan is primary importance. The poor solubility of chitosan in water, physiological solutions, and organic solvents, has been considered as a major obstacle for the biomedical applications. Furthermore, high molecular weight and broad molecular weight distribution inhibits the successful applications of chitosan.

To address the solubility issue, modified chitosans such as glycol chitosan and PEGylated chitosan have replaced the chitosan as soluble chitosan analogues.[6,7] Applications of small molecular weight chitosans such as chitosan oligosaccharides (COSs) and low molecular water soluble chitosan (LMWSC) could be another possible way to escape the solubility issues.[1] Due to its low molecular weight, COS and LMWSC showed superior solubility in water, physiological

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solutions, and even some organic solvents such as dimethyl sulfoxide. On the other hand, the reduction of chitosan molecular weight results in the loss of its unique properties such as DNA condensation. Furthermore, broad molecular weight distribution of the COSs may cause some problems such as reproducibility issue.

To overcome these problems, we prepared COSs with narrow molecular weight distribution by fractionation method using ultrafiltration technique, and modified the fractionated COSs with hydrophobic moiety of cholesterol. Due to its amphiphilic characteristics, the hydrophobized COSs (COSCs) may show core-shell type micro-phase separation in aqueous milieus and the resulting nanoparticles can restore the poly-valent cationic property on the COS. Here in, we investigate the physicochemical properties of the hydrophobized COSs (COSCs) by using dynamic light scattering and fluorescence spectroscopy. Then, the potential of COSCs for gene carrier were investigated by introducing them into *in vitro* gene delivery system.

Experimental

Materials. Chitosan oligosaccharide (COS, lactic acid salt) was kindly donated from Kittolife, Co. Korea. Cholesteryl chloroformate, DL-lactic acid, and pyrene were purchased from Aldrich. Ethidium bromide, and agarose were purchased from Promega (Madison, WI). The pSV-β-gal and pEGFP-N1 plasmid DNA were purchased from Fisher Scientific Co. and Clontech (Palo Alto, CA), respectively. To amplify, the plasmid DNAs were transformed into *Escherichia coli* (*E-coli*). Then, after amplification of the *E-coli*, the plasmids were isolated by using Qiagen Maxi-preps according to the manufacturers directions (Qiagen, Chatsworth, CA). Other solvents and chemicals are in reagent grade and used as received.

Preparation of Fractionated Chitosan Oligosaccharides. To obtain chitosan oligosaccharides (COSs) with various molecular weights, the crude COS was separated according to its molecular weight by using ultrafiltration technique. Briefly, the COS was dissolved in deionized water (5 w/v%), and membrane filtered through 0.45 μm pore sized membrane filter to remove water insoluble impurities. Then, the filtrate was fractionated by using ultrafiltration stirred cell (Amicon 8400, Millipore, USA) equipped with ultrafiltration disc membrane of various molecular weight cut-offs (30, 10, 3, and 1 kDa MWCO membranes). Each fraction (10~30 kDa, 3~10 kDa, and 1~3 kDa) was collected and products were obtained by freeze-drying.

Absolute molecular weights (MW) and molecular weight distributions, represented as polydispersity index (PDI), of the fractionated COS were measured by gel permeation chromatography equipped with multi-angle laser light scattering detector (GPC-MALLS, 18 angle detector, Wyatte, USA). To measure the absolute MW, the samples were dissolved in 0.5 M ammonium acetate (pH 5.5, 0.0~1 mg/mL, more than 5 different concentrations) and reflect index increments (*dn/dc*) were measured by Opt-LAB reflectometer (Wyott, USA). Then, absolute molecular weights and molecular weight distribution (PDI) of the COSs were obtained from GPC chromatogram with light scattering data

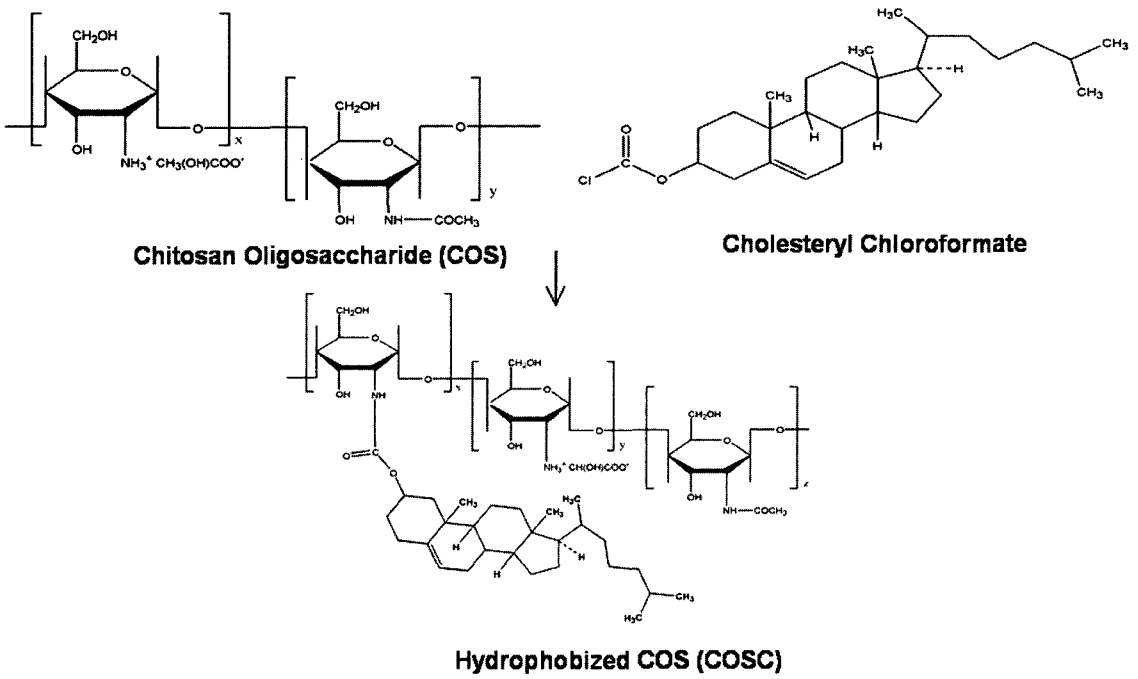

Figure 1. Synthetic schemes for cholesterol conjugation on COS.

(Debye plot regressions).

Synthesis of COS-Cholesterol Conjugates (COSCs). The hydrophobized COSs were synthesized by coupling reaction of cholesteryl chloroformate with primary amine group of COSs as shown in Figure 1. Briefly, after dissolution of the COS in DMSO/H_2O co-solvent (90/10 by vol.), predetermined amounts of cholesteryl chloroformate (2.5, 5, and 10 mole% of glucosamine subunits) were added into the solution. The reaction mixtures were stirred for 24 h at room temperature. Then, the resulting solution was precipitated into excess amount of acetone. The precipitates were recovered by centrifugation and re-suspended in water. The suspension was dialyzed against deionized water for 24 h and the final products were obtained by lyophilization. The conjugation of the cholesteryl chloroformate on COS was confirmed by ^{13}C solid NMR spectroscopy.

Preparation of Hydrophobized COS Nanoparticles. The COSC nanoparticles were prepared by diafiltration method. Briefly, after dissolution and/or suspension of COSCs in organic solvent of DMSO (ca 10 mg/mL), the solutions were dialyzed against deionized water for 24 h. After dialysis, the resulting nanoparticle solutions were sonicated for 20 sec (using probe sonicator at 80 W) and were filtered through a 0.8 μm pore sized syringe filter to remove large aggregates. Finally, the COSC nanoparticles were obtained by lyophilization. The COSC nanoparticles were characterized by using dynamic light scattering and fluorescence spectroscopy.

Fluorescence Measurements. The self-aggregation behaviors of the COSCs were investigated by fluorescence measurements (Shimadzu, RF-5301 spectrofluorophotometer) using pyrene as a hydrophobic fluorescence probe. The pyrene solution in acetone (6×10^{-5} M, prepared prior to use) was added to the deionized water to optain a pyrene concentration of 1.2×10^{-6} M, and the acetone was removed by evaporation at 40 °C for 2 h. This solution was mixed with COSC nanoparticle solution to obtain a COSC concentration ranging from 1.0×10^{-5} to 1 mg/mL, resulting in a pyrene concentration of 6.0×10^{-7} M. Fluorescence excitation spectra were measured at the emission wavelength λ_{em} = 390 nm. Slit width was set at 3 nm for the excitation. Based on the pyrene excitation spectra and red shift of the spectra with increasing COSC concentrations, the critical aggregation concentrations of the COSCs were measured.

Dynamic Light Scattering Measurements. The particle size and size distribution of COSC nanoparticles in aqueous environment were investigated by dynamic light scattering (DLS) measurement. The DLS measurements were carried out using an ELS-800 electro phoretic LS spectrophotometer (NICOMP 380 ZLS zetapotential/particle sizer), equipped with a He-Ne laser operating at 632.8 nm at 25 °C and at a fixed scattering angle of 90°. Before measurement, the COSC nanoparticles were re-dispersed in deionized water (1 mg/mL), sonicated for 30 sec, and filtered through a 0.8 μm pore sized filter. The measurements were carried out at a higher concentration of the cac, measured by fluorescence spectroscopy. The hydrodynamic diameters of the COSC nanoparticles were calculated by the Stokes-Einstein equation.

The particle sizes of COS/DNA and COSC/DNA complexes and their zeta-potentials were also measured by DLS measurements. For the measurement, the carrier/plasmid DNA complexes were prepared with various carrier/DNA weight ratios of 0.5 - 8 (20 μg/mL DNA in H_2O). Before measurements, the prepared complexes were incubated for 30 min to ensure the complex formation.

Gel Retardation Assay. Complex formation of the COSs and the COSCs with the plasmid DNA (pSV-β-gal plasmid DNA) were confirmed by gel retardation assay. COS/pSV-β-gal and COS6C5/pSV-β-gal plasmid complexes were prepared at various weight ratios ranging from 0.5 to 8 in PBS (pH 7.4, I = 0.15 M) and incubated for 30 min at room temperature to ensure complex formation. Then, the complexes were electrophoresed through a 0.8% agarose gel at 100 V for 60 min. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min, and DNA was visualized on a UV transilluminator.

Cell Culture and *in vitro* Transfection. Human embryonic kidney 293 cells (HEK 293) were purchased from American Type Culture Collection (Manassas, VA). The cells were cultivated in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were placed at a density of 6×10^4 cell/well in a 24 well plate 24 h prior to transfection.

Just before transfection, the serum containing media were removed and the cells in the 24-well plate were washed with 500 μL of serum free media. Finally, 350 μL of fresh serum-free media were added into each well. The COS6/DNA and COS6C5/DNA complexes were prepared in deionized water at a carrier/DNA weight ratio of 30 (the minimum weight ratio of COS6/DNA condensation by gel retardation assay) with the DNA concentration of 1 μg DNA/50 μL using pEGFP-N1 plasmid DNA. Then, the cell transfections were performed by adding 50 μL of the complex solution into each well. As negative and positive control group, naked DNA (1 μg DNA/well) and PLL/DNA complex (PLL MW of 21 kDa, 2/1 weight ratio, 1 μg DNA/well) were added into the cell placed well, respectively. After 4 h transfection, the transfection media were removed and the cells were further cultivated with 400 μL of normal cell culture media (containing 10% FBS) for 48 h. The gene transfection was investigated by using fluorescence microscope.

Results and Discussion

Chracterization of Fractionated COSs and Hydrophobized COSs (COSCs). Chitosan oligosaccharides (COSs)

with narrow molecular weight distribution were obtained by fractionation technique using ultrafiltration apparatus. In case of raw COS, the chitosan showed broad molecular weight distribution with number average molecular weight (M_n) of 5,300 Da and polydispersity index (PDI) value of 2.9. Three different COSs were obtained by the fractionation steps. Firstly, COS9 (M_n = 8,780 Da, PDI = 1.20) was obtained by the fractionation of the raw COS by using MWCO 30 and 10 kDa membranes. The successive fractionation, using the filtrate of the first step, by MWCO 3 and 1 kDa membranes produced COS6 (M_n = 6,040 Da, PDI = 1.19) and COS3 (M_n = 3,030 Da, PDI = 1.38). Owing to the narrow molecular distributions, the GPC chromatograms of the fractionated COSs showed standard-like features with well-defined Gaussian distribution. The GPC chromatograms of the fractionated COSs are illustrated in Figure 2.

For the hydrophobic modification, COS9 and COS6 were used. Three different amounts of cholesteryl chloroformate were conjugated on each of the COS, and the feed ratios were listed in Table I. The conjugation reactions were confirmed by ^{13}C solid NMR spectroscopy. As shown in Figure 3(A), ^{13}C solid NMR spectrum of COS6 showed broad C_1 ~ C_6 peaks around 25 ~ 105 ppm with two carbonyl peaks originated from lactic acids and *N*-acetyl-*D*-glucosamine units. As a result of conjugation reaction, several new ^{13}C NMR peaks were observed as shown in Figure 3(B). ^{13}C solid NMR spectrum of COS6C5 showed typical cholesterol originated peaks such as aliphatic carbons (12.5 and 28 ~ 50 ppm, *a* in Figure 3(B)), unsaturated carbon (122.7 and 140.8 ppm, *b* in Figure 3(B)), and urethane carbonyl peak (156.9 ppm, *c* in Figure 3(B)) which is formed by the conjugation reaction.

Physicochemical Characterization of COSC Nanoparticles. Due to the amphiphilic properties of the hydrophobized COS, the COSCs can self-associate to form micelle or micelle like self-aggregate in an aqueous environment. The mean diameters of COSC nanoparticles, prepared by the diafiltration method and measured by DLS instrument, were in the range of 450 ~ 230 nm, as listed in Table I. In case of COS9C series, there were negligible changes in particle sizes with increasing hydrophobic moiety. However, COS6C series

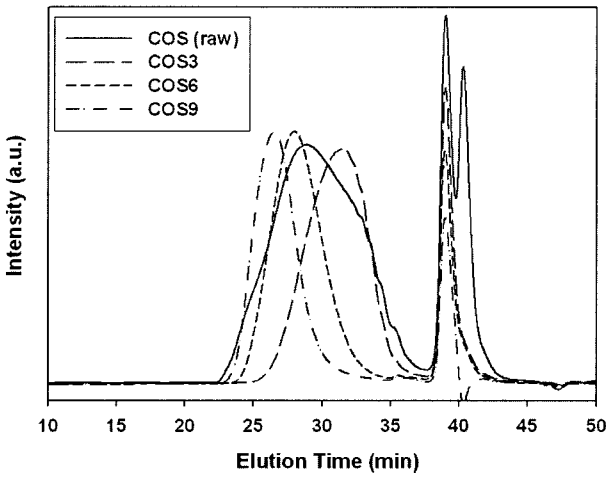

Figure 2. Aqueous GPC chromatograms of raw COS and fractionated COSs. Mobile phase: 0.5 M ammonium acetate pH 5.5, flow rate: 0.5 mL/min, injection: 0.2 mL (10 mg/mL).

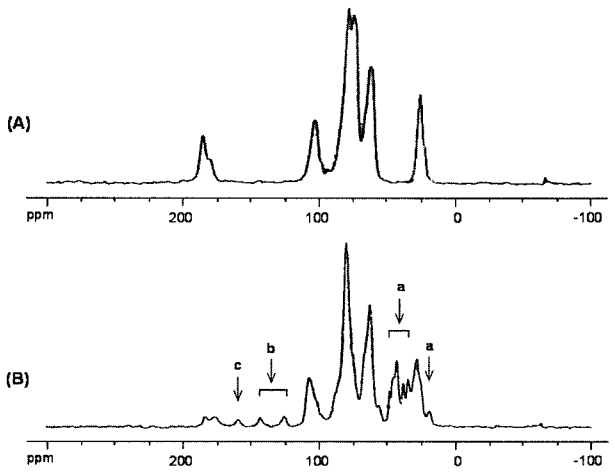

Figure 3. Solid state CP/MAS ^{13}C NMR spectra of (A) COS6 and (B) COS6C5.

Table I. Characterization Results of the Hydrophobized COSs (COSCs)

Raw Material[a]	Sample	Cholesteryl Chloroformate[b]	CAC (mg/mL)	Mean Diameter (nm)	Polydispersity Factor
COS6	COS6C2.5	2.5%	4.69×10^{-2}	349.0	0.21
	COS6C5	5%	3.25×10^{-2}	229.9	0.18
	COS6C10	10%	8.51×10^{-3}	235.7	0.14
COS9	COS9C2.5	2.5%	3.22×10^{-2}	377.8	0.17
	COS9C5	5%	1.74×10^{-2}	402.7	0.19
	COS9C10	10%	7.35×10^{-3}	446.0	0.22

[a]COS6: 3~5 kDa fraction, COS9: 10~30 kDa fraction. [b]Feed ratio per COS repeat unit (glucosamine).

showed typical trends of particle size variation. With increasing cholesterol content, the particle sizes decreased due to the strong hydrophobic interaction between hydrophobic moieties. However, the size reduction was observed between COS6C2.5 and COS6C5, and the COS6C10 did not show a significant size reduction but showed similar particle size when compared with COSC5. The trends of particle size reductions by increasing hydrophobicity are consistent with other hydrophobized polysaccharides.[6]

To investigate the self-aggregation behavior of COSCs in an aqueous milieu, pyrene was used as a hydrophobic fluorescence probe. When exposed to a polymeric micelle aqueous solution, the pyrene molecules preferably participated into the hydrophobic domains of the micelle rather than the aqueous phase. Combined with strong fluorescence illumination of pyrene in a non-polar environment, the localization of pyrene into hydrophobic domain showed different photophysical characteristics depending on the concentration of micelle forming materials.[7-9] Therefore, the self-aggregation behaviors of the COSCs in an aqueous phase were investigated by using fluorescence excitation spectra of the COSC solutions with various concentrations, in the presence of 6.0×10^{-7} M pyrene, and the results are illustrated in Figure 4. At low concentration ($C <$ cac), there were small or negligible changes in total fluorescence intensity and a shift of (0,0) band at 335 nm. As the concentration increased, a remarkable increase of the total fluorescence intensity and a red shift of the (0,0) band from 335 nm to 339 nm were observed. Figure 5 shows the intensity ratio (I_{339}/I_{335}) of the pyrene excitation spectra versus the logarithm of the COSC concentrations. Based on the intensity ratio data, the cac values of COSCs were calculated by the crossover point at low concentration ranges. The cac values of the COSCs were in the range of 0.0074 ~ 0.0469 mg/mL, with a trend of decreasing cac values with increasing in hydrophobic moiety. The cac values of COSCs were lower than those of low molecular weight surfactant (e.g., 2.3 mg/mL for SDS), similar with deoxycholic acid modified chitosans,[5] but were larger than those of other polymeric amphiphiles, such as PEG-PLA and PVP-PCL block copolymers.[10,11]

Characterization of COS/DNA and COSC/DNA Complexes. Based on the physicochemical characterizations of COSCs, COS6C5 is considered as one of the most suitable candidates for gene condensing material because of its small hydrodynamic diameter and fairly low cac value. Therefore, DNA condensation studies were carried out with COS6C5, using COS6 as a control material.

Gene compaction capacity is considered as one of the basic and most important properties of gene carriers. Due to the high molecular mass of plasmid DNA, the plasmid DNA have large hydrodynamic volume with absence of gene condensing compounds such as cationic liposome and cationic polymers.[12] Considering this large hydrodynamic volume and negatively charged character, it is difficult to transfect

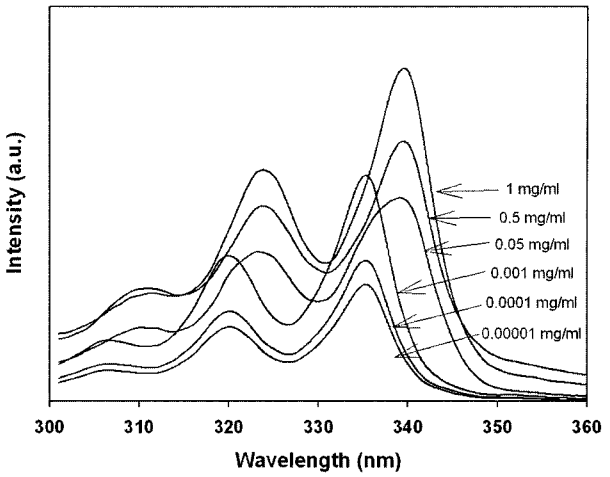

Figure 4. Pyrene excitation spectra ([Py] = 6.0×10^{-7} M) of COS6C5 nanoparticle aqueous solutions (emission wavelength of 390 nm).

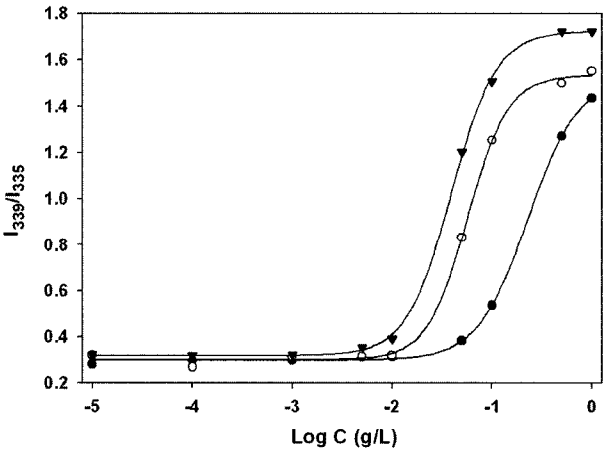

Figure 5. Plots of I_{339}/I_{335} (from pyrene excitation spectra) *vs.* log C for COS6C2.5 (●), COS6C5 (○) and COS6C10 (▼) in water.

somatic cells by using naked DNA only. Therefore, gene condensations into small sized particle with modulation of the negatively charged property of DNA are basic role for introduction of gene carriers into gene delivery systems.[5,13,14]

In this study, the self-assembled complex formations (gene condensation) of COS6 and COS6C5 with pSV-β-gal plasmid DNA were investigated by gel retardation assay. As shown in Figure 6, COS6 and COS6C5 showed different gene condensation patterns. In case of COS6, unmodified chitosan oligosaccharide, the COS6 was not successfully condensed with the plasmid DNA into small aggregates. Although large enough amount of COS6 (COS6/DNA weight ratio of 8) was mixed with plasmid DNA, the COS6/DNA complex was not retarded in the complex-loaded well and showed the eluted DNA band, as shown in Figure 6(A).

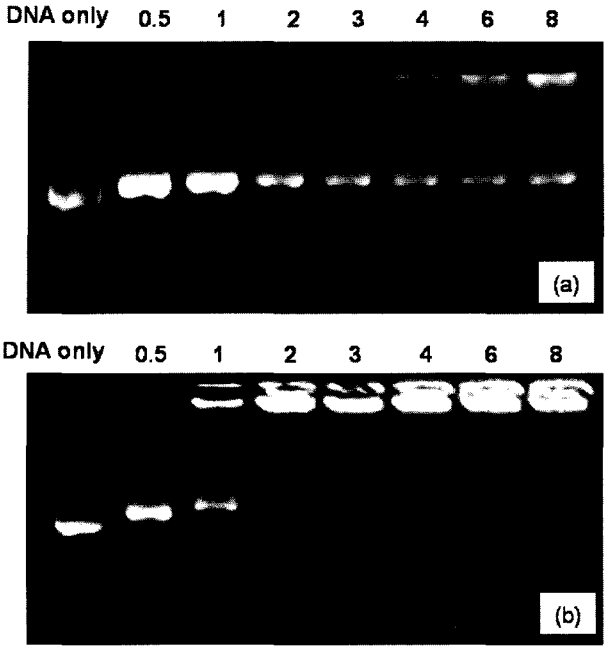

Figure 6. Gel retardation assay of (a) COS6/plasmid DNA and (b) COS6C5/plasmid DNA complexes with various carrier/DNA weight ratios. Numbers indicate the carrier/DNA weight ratio.

On the other hand, COS6C5 showed superior gene condensing capacity than its raw material of COS6. The COS6C5/DNA complexes were completely retarded when the complexes were prepared with COS6C5/DNA weight ratio of 2 or higher (Figure 6(B)). The superior gene condensing capacity of the hydrophobized COS6 may mainly result from the hydrophobic nature of the COS6C5 and its particle forming property. Unlike the COS6, the COS6C5 formed micelle like self-aggregate in an aqueous environment. Therefore, the enhanced gene condensing capacity may result from the increased amount of cationic charges and charge densities due to the nanoparticle formation of the COS6C5. Furthermore, hydrophobic interactions between cholesterol moieties in COS6C5 and charge-neutralized DNA segments were also accountable for the enhanced gene condensation capacity of COS6C5. The enhanced gene condensing capacity of COS6C5 is comparable to the high molecular weight chitosans.[15,16]

The physicochemical properties, such as hydrodynamic diameters and zeta potentials, of COS6C5/DNA complexes were also investigated with various carrier/DNA weight ratios. As shown in Figure 7, COS6C5/DNA weight ratios played a crucial role for the determination of hydrodynamic diameters of the COS6C5/DNA complexes. With low carrier/DNA ratio (the ratio of 0.5), large sized particles were formed owing to the imbalance of the charge content. With increasing carrier content, the particle sizes decreased and reached to a minimum value of 133 nm at COS6C5/DNA weight ratio of 4. Further increment of the carrier/DNA ratio did not result

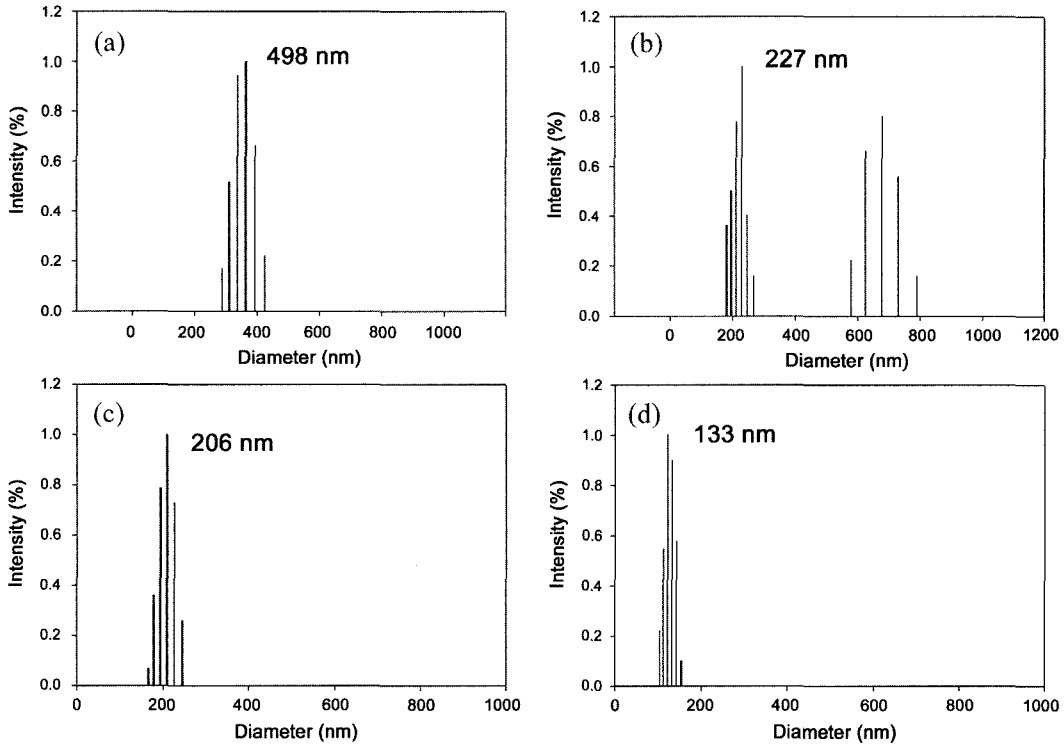

Figure 7. Size distribution of COS6C5/DNA complexes with COS6C5/DNA weight ratios. (a) 0.5, (b) 1, (c) 2, and (d) 4.

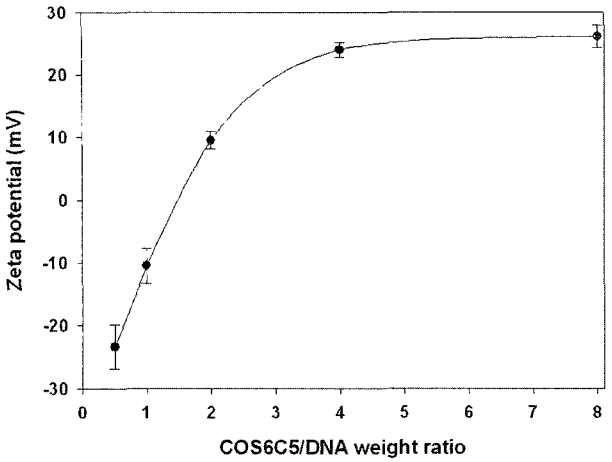

Figure 8. Zeta potential measurement of the complexes formed by COS6C5 nanoparticle and plasmid DNA with various carrier/DNA weight ratios (n=3).

in a significant effect on the complex particle size, and 175 nm sized complex was observed with COS6C5/DNA weight ratio of 8 (data not shown). The zeta potentials of COS6C5/plasmid DNA complexes with various weight ratios are shown in Figure 8. The negative zeta potential (-23.4 mV) at the weight ratio of 0.5 (carrier/DNA ratio) turned to a positive values (9.5 mV) at the weight ratio of 2, and reached their saturated value of around 25 mV at the weight ratio of 4 (24.0 mV). Further increment of the carrier/DNA weight ratio did not significantly alter the electrical properties of the carrier/DNA complex (26.1 mV at the weight ratio of 8). These trends of particle size and zeta potential variations are consistent with other polymeric gene carriers.[17,18] The physicochemical characterization of COS6C5/DNA complexes revealed that COS6C5 can neutralize plasmid DNA and effectively condense the plasmid DNA into nanoparticles with COS6C5/DNA weight ratio of two or higher.

***In vitro* Transfection Efficiencies.** A successful gene delivery system is required the high gene transfection efficiency, which is resulted from the several successive biological events such as cellular uptake of carrier/DNA complex, nuclear entry of the DNA or carrier/DNA complex, release of the DNA from the complex in cytoplasm or nucleus, and subsequent protein synthesis by gene expression.

The transfection efficiency was investigated in HEK 293 cell lines using pEGFP-N1 plasmid encoding green fluorescence protein (GFP). The gene expression was observed by fluorescence microscopy. As shown in Figure 9(A), gene transfection efficiency was dramatically enhanced by introducing hydrophobic moiety of cholesterol on the COS. The COS6C5/DNA complex showed more than 100 and 3 times enhanced transfection efficiency than that of COS6 and positive control of PLL, respectively (Figure 9(B)).

The enhanced gene transfection efficiency of the hydrophobized COS is presumably owing to the facilitated cellular

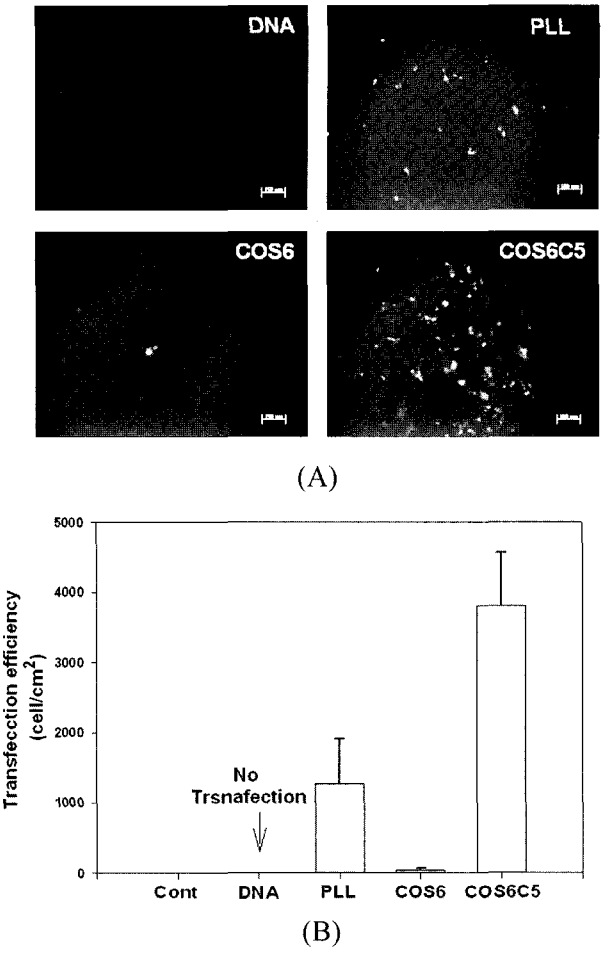

Figure 9. Gene transfection efficiency of pEGFP-N1 plasmid DNA transfected HEK 293 cells with different carriers. (A) Fluorescence microscope image of gene transfected HEK 293 cells at 48 h after transfection. (B) Gene transfection efficiencies based on transfected cell counting (n=4, mean ± SD).

uptake of the gene/carrier complex by hydrophobic interactions. Similar results of enhanced gene transfection efficiency by introduction of hydrophobic moiety on cationic polymers such as PLL, poly(ethylenimine), and chitosan were reported by several research groups.[4,5,17,19]

Conclusions

In this research, we prepared COS with narrow molecular weight distribution by ultrafiltration methods and modified the COSs with hydrophobic moiety of cholesterol. Owing to their amphiphilic character, the hydrophobized COSs formed core-shell type nanoparticles with fairly low critical aggregation concentration in aqueous milieu. The introduction of the COSCs into gene delivery system revealed that the COS6C5 showed superior gene condensing capacity and DNA neutralization by forming COS6C5/DNA ionic complex

nanoparticles contradictory to their unmodified analogue of COS6. Furthermore, the COS6C5/DNA complex showed more than 100 and 3 times enhanced transfection efficiency than that of COS6 and positive control of PLL, respectively. Therefore, the hydrophobized chitosan oligosaccharide might be considered as a potential candidate for non-viral gene carriers.

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