Chondroitin Sulfate Capsule System for Efficient and Secure Gene Delivery

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Received, May 19, 2010; Revised, August 30, 2010; Accepted, August 31, 2010; Published, September 3, 2010.

ABSTRUCT- Purpose. In this study, we developed various ternary complexes of encapsulated polyplexes and lipoplexes using chondroitin sulfate (CS) and investigated their universal usefulness for gene delivery. **Methods.** To prepare the cationic complexes, pDNA was mixed with some cationic vectors such as poly-L-arginine, poly-L-lysine, N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA)-cholesterol liposomes, and DOTMA-dioleylphosphatidylethanolamine (DOPE) liposomes. CS was added to the cationic complexes for constructions of ternary complexes. We examined *in vitro* transfection efficiency, cytotoxicity, hematotoxicity, and *in vivo* transfection efficiency of the ternary complexes. **Result.** The cationic polymers and cationic liposomes bound to pDNA and formed stable cationic polyplexes and lipoplexes, respectively. Those cationic complexes showed high transgene efficiency in B16-F10 cells; however, they also had high cytotoxicity and strong agglutination with erythrocytes. CS could encapsulate the polyplexes and lipoplexes and form stable anionic particles without disrupting their structures. The ternary complexes encapsulated by CS showed high transgene efficiency in B16-F10 cells with low cytotoxicity and agglutination. As the result of animal experiments, the polyplexes had little transgene efficiency after intravenous administration in mice, whereas polyplexes encapsulated by CS showed specifically high transgene efficiency in the spleen. The capsulation of CS, however, reduced the high transgene efficiency of the lipoplexes. **Conclusion.** These results indicate that CS can contribute to polyplex-mediated gene delivery systems for effective and safe gene therapy.

INTRODUCTION

Non-viral gene delivery has been drawn keen attention as a promising vector with very low immunotoxicity, a clear structure, and easy modeling (1-3). As non-viral vectors, various cationic polymers and cationic lipids have been developed in previous reports (3-7). Cationic polymers and cationic lipids can electrostatically bind to DNA to form polyplexes and lipoplexes, and show high transgene efficiency under *in vitro* and *in vivo* conditions; however, cationic vectors bind non-specifically to negatively charged proteoglycans on cell membranes and agglutinate with blood components, such as erythrocytes and serum albumin, and these agglutinations often lead to adverse events, such as rapid elimination, embolization, and inflammatory reactions (8-10).

One promising approach for overcoming the disadvantages of cationic vectors is capsulation of the cationic vector with anionic polymer electrostatically (11, 12). In the previous study, we discovered a capsule, chondroitin sulfate (CS), which could encapsulate plasmid DNA/polyethylenimine (pDNA/PEI) polyplex electrostatically, and the pDNA/PEI/CS complex showed not only high gene expression but also extremely low toxicity (13). CS is known to be a highly biocompatible polymer for tablets, eye drops, cosmetics, and medical applications (14-17).

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CS capsulations of cationic systems should therefore be useful to develop novel biocompatible vectors with high effectivity and safety.

In the present study, we investigated the universal utility of CS capsulation for efficient and secure gene delivery. Cationic vectors were constructed with pDNA and cationic polymers: poly-L-arginine (PLA) and poly-L-lysine (PLL) or cationic liposomes: N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA)-cholesterol (Chol) liposomes and DOTMA-dioleylphosphatidylethanolamine (DOPE) liposomes. CS capsulation markedly decreased the toxicity of those cationic vectors and significantly increased the *in vivo* transgene efficiency of polyplexes in the spleen.

MATERIALS AND METHODS

Chemicals

DOTMA was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Chol was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). DOPE was purchased from Nippon Oil and Fats Co. (Tokyo, Japan). PLA (average molecular weight 22,500), PLL (average molecular weight 10,000), chondroitin sulfate A, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biosource International Inc. (Camarillo, CA, USA). RPMI 1640, Opti-MEM I, antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL), and other culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-*2H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of the highest purity available.

Construction of pDNA

pCMV-luciferase (pCMV-Luc) was constructed by subcloning the *Hind* III/*Xba* I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). Enhanced green fluorescence protein (GFP) encoding pDNA (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, USA). pDNA was amplified using an EndoFree® Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany), dissolved in 5% dextrose solution to 1 mg/mL and stored at -80 ^{o}C until analysis.

Preparation of Cationic Liposomes

DOTMA-Chol (1: 1 molar ratio) and DOTMA-DOPE (1: 1 molar ratio) liposomes were prepared according to a previous report (18). Lipids were dissolved in chloroform. Mixtures of each lipid were dried as a thin film in a test tube using an evaporator at 25 ^{o}C, and then vacuum-desiccated for approximately 4 h. The film was resuspended in 5% sterile dextrose. After hydration, dispersions were sonicated at 100 W for 3 min on ice. The resulting liposomes were extruded 11 times through doublestacked 100 nm polycarbonate membrane filters.

Preparation of Complexes

The theoretical charge ratio of cationic polymers and liposomes to pDNA was calculated as the molar ratio of their nitrogen to pDNA phosphate. To prepare binary complexes, an appropriate amount of stock polymer solution or liposomes solution was mixed with a diluted solution of pDNA by pipetting thoroughly at a charge ratio of 8 for polyplexes (pDNA/PLA and pDNA/PLL) and 2 for lipoplexes (pDNA/DOTMA-Chol and pDNA/DOTMA-DOPE), and left for 15 min at room temperature. To prepare ternary complexes, CS solution was added to each binary complex at a charge ratio of 6 (pDNA/PLA/CS, pDNA/PLL/CS, pDNA/DOTMA-Chol/CS, and pDNA/ DOTMA-DOPE/CS), and left for another 15 min at room temperature. The theoretical charge ratio of CS to pDNA was calculated as the molar ratio of CS sulfate to pDNA phosphate.

Physicochemical Property and Gel Retardation

The particle size and ζ-potential of the complexes were measured with a Zetasizer Nano ZS (Malvern Instruments, Ltd., United Kingdom). The number-fractioned mean diameter is shown.

To determine complex formations, 10 µL aliquots of the complex solution containing 1 µg pDNA were mixed with 2 µL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel containing 0.1 µg/mL ethidium bromide. Electrophoresis (i-Mupid J®; Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA) for 60 min. The retardation of the pDNA was visualized using a FluorChem Imaging System (Alpha Innotech, CA, USA).

Transfection Experiments

The mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University. B16-F10 cells were maintained in culture medium (RPMI 1640 supplemented with 10% FBS and antibiotics) under a humidified atmosphere of 5% CO_2 in air at 37 ^{o}C. B16-F10 cells were plated on 24-well collagen-containing plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5×10^4 cells/well and cultivated in 1.0 mL culture medium.

In the transfection experiment, after 24 h pre-incubation, the medium was replaced with 1 mL Opti-MEM I medium and each complex containing 1 µg pEGFP-C1 was added to the cells and incubated for 2 h. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37 ^{o}C. After incubation, the relative levels of GFP expression were characterized using fluorescent microscopy (200× magnifications).

To quantify gene expressions of CS ternary complexes, B16-F10 cells were transfected with complexes containing 1 µg pCMV-Luc as described above. After 22 h incubation, the cells were washed with PBS and then lysed in 100 µL lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). Ten microliters of lysate samples were mixed with 50 µL luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of the lysate was determined by the Bradford assay using BSA as a standard. Absorbance was measured using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 570 nm. Luciferase activity was indicated as relative light units (RLU) per mg protein.

WST-1 Assay

Cytotoxicity tests of the complex on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. B16-F10 cells were plated on 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 3.0×10^3 cells/well in the culture medium. Complexes containing 1 µg pDNA in 100 µL Opti-MEM I medium were added to each well and incubated for 2 h. After incubation, the medium was replaced with 100 µL culture medium and incubated for another 22 h. Then, medium was replaced with 100 µL culture medium and 10 µL WST-1 reagent (4.95 mM WST-1 and 0.2 mM 1-methoxy PMS) was added to each well. The cells were incubated for an additional 2 h at 37 ^{o}C, and absorbance was measured at a wavelength of 450 nm with a reference wavelength of 630 nm, using a microplate reader. The results are shown as a percentage of untreated cells.

Animals

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval from the Institutional Animal Care and Use Committee. Male ddY mice (5-6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one week before the experiments.

Interaction with Erythrocytes

Erythrocytes from mice were washed three times at 4 ^{o}C by centrifugation at 5000 rpm (Kubota 3700; Kubota, Tokyo, Japan) for 5 min and resuspended in PBS. A 2% (v/v) stock suspension of erythrocytes was prepared for agglutination study. The complexes were added to the erythrocyte suspension and incubated for 15 min at room temperature. A 10 µL sample was placed on a glass

plate and agglutination was observed by microscopy (400× magnification). For hemolysis study, 5% stock suspensions of erythrocytes were prepared. The complexes were added to erythrocytes and incubated for 1 h at room temperature. After incubation, the suspensions were centrifuged at 5000 rpm for 5 min, and supernatants were taken. Hemolysis was quantified by measuring the absorbance of hemoglobin at a wavelength of 545 nm using a microplate reader. Lysis buffer was added to erythrocytes and used as the 100% hemolysis sample.

In Vivo Gene Expression Experiment

Each complex, including 40 μg pCMV-Luc, was prepared for *in vivo* gene expression experiments. A 300 μL aliquot of complexes was injected into mice via the tail vein. The liver, kidney, spleen, heart, and lung of mice were dissected 6 h after the injection. The tissues were washed twice with cold saline and homogenized with lysis buffer, homogenates were centrifuged at 15000 rpm for 5 min, and the supernatants were used for luciferase assays. Luciferase activity is indicated as RLU per gram of tissues.

Statistical Analysis

Statistical analyses were performed by unpaired Student *t*-test following the F-test for *in vitro* studies and the Mann-Whitney U test for *in vivo* studies. Multiple comparisons were carried out by Scheffe's test with ANOVA. P value <0.05 was considered significant.

RESULTS

Physicochemical Properties and Electrophoresis Assay

The particle sizes and ζ-potentials of various complexes are shown in Table 1. The polyplexes and lipoplexes had 50.3–133.7 nm particle size and +34.6–50.8 mV ζ-potential. On the other hand, ternary complexes encapsulated by CS had significantly lower ζ-potentials than each binary complexes ($P < 0.01$), although CS did not greatly affect the size of the complexes.

To assess complex formations, the gel retardation assay was employed (Fig. 1). Naked pDNA was detected as a band on agarose gel. At the same time, bands of pDNA were not detected in any complexes.

Table1. Particle size and z-potential of various complexes.

Complex	Particle size (nm)	ζ-Potential (mV)
pDNA/PLA	50.3 ± 11.5	+42.5 ± 0.5
pDNA/PLA/CS	69.0 ± 16.0	-26.4 ± 0.3**
pDNA/PLL	67.4 ± 4.9	+34.6 ± 1.5
pDNA/PLL/CS	93.1 ± 1.4**	-22.4 ± 0.1**
pDNA/DOTMA-Chol	108.1 ± 22.5	+50.8 ± 0.8
pDNA/DOTMA-Chol/CS	124.0 ± 17.6	-41.2 ± 0.3**
pDNA/DOTMA-DOPE	133.7 ± 4.1	+42.5 ± 0.4
pDNA/DOTMA-DOPE/CS	90.4 ± 14.8*	-41.7 ± 0.4**

Each value represents the mean with S.E. (n=3).
*: $P < 0.05$, **: $P < 0.01$ vs. each binary complex.

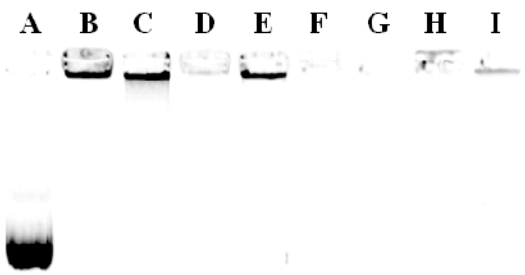

Figure 1. Gel retardation assay of the various complexes. (A): Naked DNA, (B): pDNA/DOTMA-Chol, (C): pDNA/DOTMA-Chol/CS, (D): pDNA/DOTMA-DOPE, (E): pDNA/DOTMA-DOPE/CS, (F): pDNA/PLA, (G): pDNA/PLA/CS, (H): pDNA/PLL, (I): pDNA/PLL/CS.

Evaluation of Transfection Efficiencies

B16-F10 cells were transfected with complexes containing pEGFP-C1, and GFP expression was observed with fluorescent microscopy (Fig. 2). Not only polyplexes and lipoplexes with cationic charges but also ternary complexes encapsulated by CS with anionic charges showed GFP expression. To quantify the gene expression of each complex, luciferase activity was determined by a sensitive chemiluminescent technique after transfection of B16-F10 cells with complexes containing pCMV-Luc (Fig. 3).

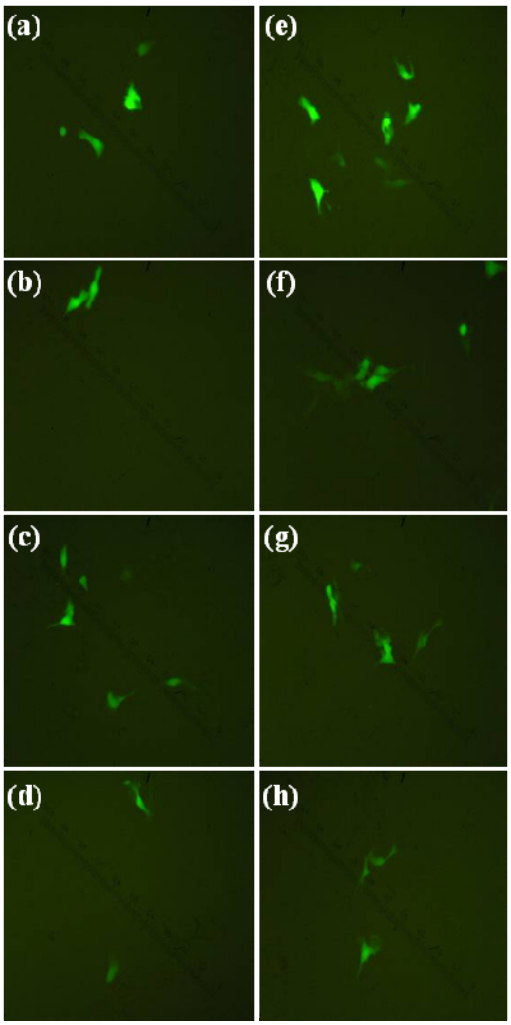

Figure 2. Fluorescent microscopy images of B16-F10 cells transfected with polyplexes, lipoplexes, and ternary complexes containing pEGFP-C1. Cells were transfected with each complex for 2 h and the expression of GFP was monitored at 22 h after transfection (200× magnification). (a): pDNA/PLA, (b): pDNA/PLL, (c): pDNA/DOTMA-Chol, (d): pDNA/DOTMA-DOPE, (e): pDNA/PLA/CS, (f): pDNA/PLL/CS, (g): pDNA/DOTMA-Chol/CS, (h): pDNA/DOTMA-DOPE/CS.

pDNA/PLA, pDNA/DOTMA-Chol, and pDNA/DOTMA-DOPE had a high gene expression but pDNA/PLL gene expression was lower. CS capsulation did not have much effect on the transgene efficiency of polyplexes and pDNA/DOTMA-DOPE lipoplex, whereas in pDNA/DOTMA-Chol lipoplexes, CS capsulation decreased transgene efficiency.

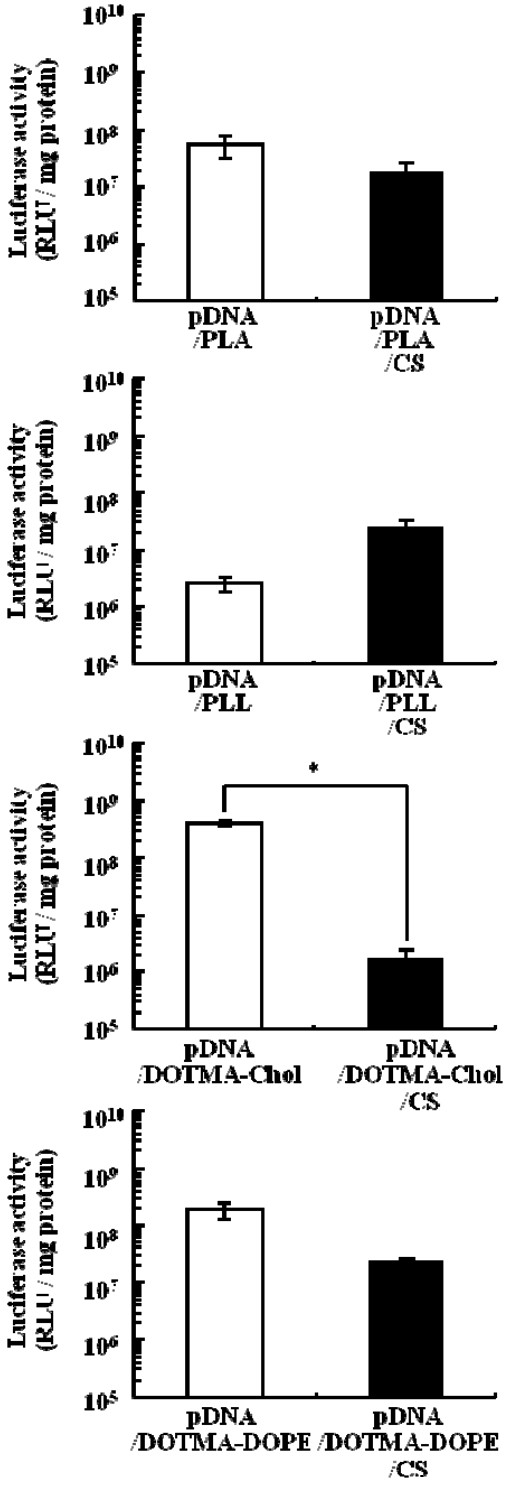

Figure 3. Gene expression of B16-F10 cells transfected with polyplexes, lipoplexes, and ternary complexes containing pCMV-Luc. Cells were transfected with each complex for 2 h and luciferase activity was evaluated at 22 h after transfection. Each value represents the mean with S.E. (n = 3-6). *: $P < 0.05$.

Cytotoxicity

The complexes were added to B16-F10 cells and cell viability was evaluated by WST-1 assay (Fig. 4). The polyplexes and lipoplexes showed significantly higher cytotoxicity than the control ($P < 0.01$). The ternary complexes encapsulated by CS, however, had significantly lower cytotoxicity than each binary complex ($P < 0.01$).

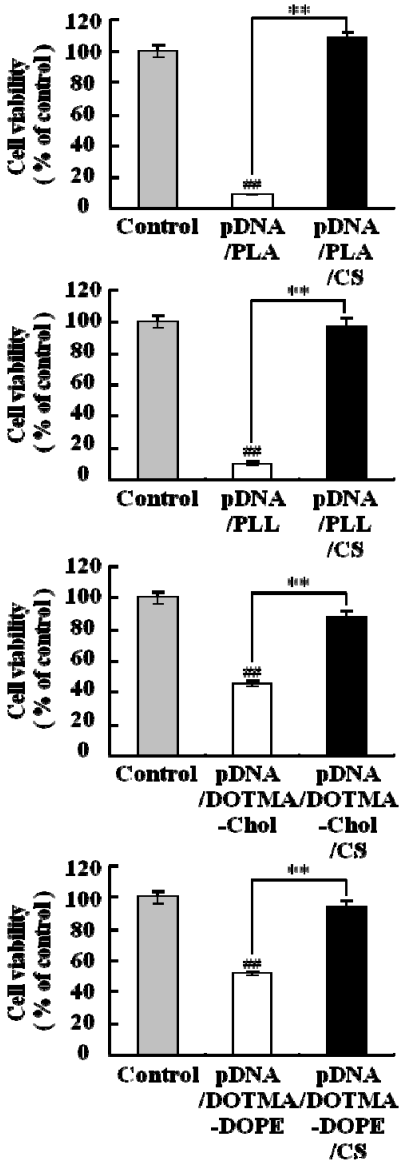

Figure 4. Cytotoxicity of polyplexes, lipoplexes, and ternary complexes on B16-F10 cells. Cell viability of cells treated with each complex was measured by WST-1 assay. Cells were incubated with the complexes for 2 h and cell viability was measured at 22 h after treatment. Data represents the percentage to the untreated cells. Each value represents the mean with S.E. (n = 12). ##: $P < 0.01$ vs. Control, **: $P < 0.01$.

Interaction with Erythrocytes

Figure 5 shows a photomicrograph of erythrocytes after addition of the complexes. Erythrocyte agglutination was observed in polyplexes and lipoplexes, which showed not only agglutination but also hemolysis. CS capsulation markedly decreased the agglutination activity of each binary complex, and no agglutination was observed.

Figure 6 shows hemolysis activities of the complexes. High hemolysis was recognized in lipoplexes. By forming a ternary complex encapsulated by CS, hemolysis activities were significantly decreased ($P < 0.05$). The polyplexes and their ternary complexes encapsulated by CS did not show hemolysis of erythrocytes.

***In Vivo* Transgene Efficiency**

In vivo transgene efficiencies of the complexes were examined in ddY male mice (Fig. 7). Luciferase activities in several tissues were evaluated 6 h after intravenous administration of the complexes. In polyplexes, transgene efficiency was significantly increased in the spleen by capsulation of CS ($P < 0.05$), whereas only significant low levels of transgene efficiency with ternary complex pDNA/PLL/CS were shown in the lung. On the other hand, in lipoplexes, decreased transgene efficiency was observed by capsulation of CS, especially in the spleen and lung ($P < 0.05$).

DISCUSSION

Gene delivery utilizing non-viral approaches has been widely developed as a basic tool for intracellular gene entry and gene therapy (1, 2). As non-viral vectors, many cationic polymers and liposomes have been developed (3, 19-24). Among cationic polymers, PLA and PLL are known to be biodegradable peptides with high transgene efficiency (25-28). In previous studies, cationic liposomes DOTMA-DOPE and DOTMA-Chol were widely used (29-32). DOTMA-DOPE liposomes are commercially supplied as a transfection reagent "lipofectin" which is reported to have high potential for *in vitro* and *in vivo* gene transfection.

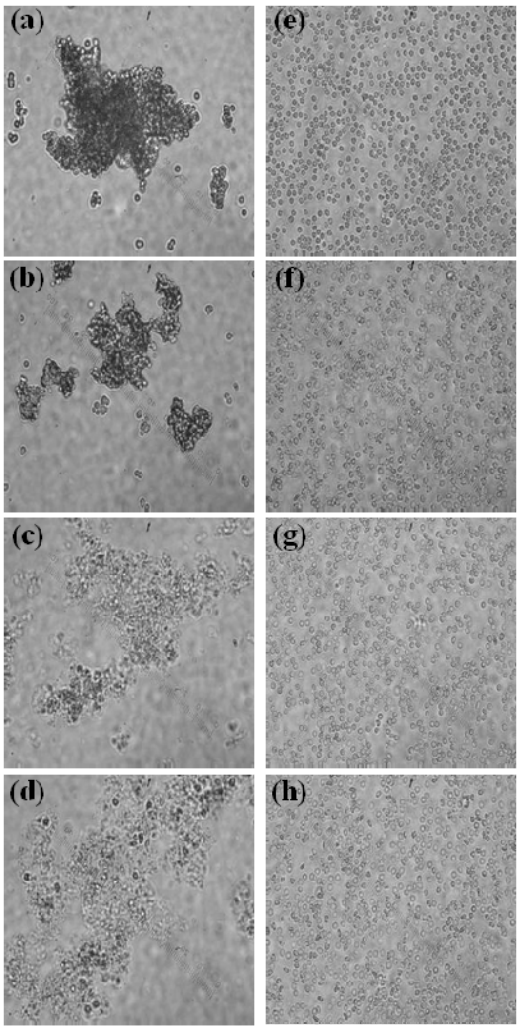

Figure 5. Agglutinations of polyplexes, lipoplexes, and ternary complexes with erythrocytes. Each complex was added to erythrocytes and agglutination was observed by phase microscopy (400× magnification). (a): pDNA/PLA, (b): pDNA/PLL, (c): pDNA/DOTMA-Chol, (d): pDNA/DOTMA-DOPE, (e): pDNA/PLA/CS, (f): pDNA/PLL/CS, (g): pDNA/DOTMA-Chol/CS, (h): pDNA/DOTMA-DOPE/CS.

DOTMA-Chol liposomes have often been used for *in vivo* studies because of their higher stability and transgene efficiency than lipofectin in *in vivo* conditions. In this experiment, we prepared two polyplexes, pDNA/PLA and pDNA/PLL, and two lipoplexes, pDNA/DOTMA-Chol and pDNA/DOTMA-DOPE, for the core ternary complexes.

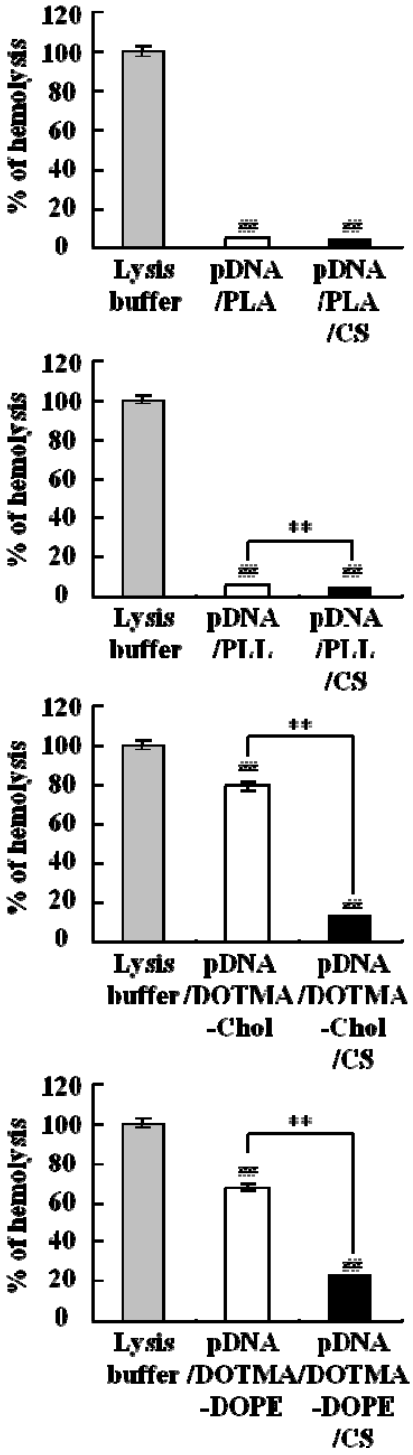

Figure 6. Hemolysis activities of polyplexes, lipoplexes, and ternary complexes with erythrocytes. Each complex was added to erythrocytes and hemolysis activities were determined by measuring hemoglobin release at 545 nm. Each value represents mean with S.E. (n = 3). ##: $P < 0.01$ vs. Lysis buffer, **: $P < 0.01$.

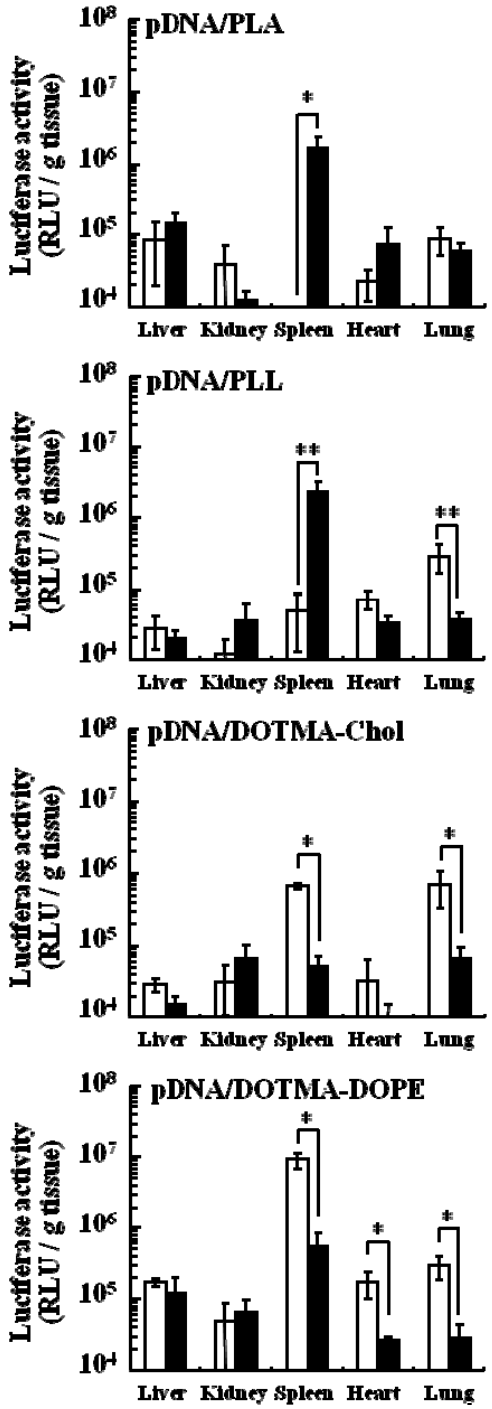

Figure 7. *In vivo* transgene efficiency of polyplexes, lipoplexes, and ternary complexes. Each complex was injected intravenously in mice (40 μg DNA per mouse). At 6 h after administration, mice were sacrificed and each organ was dissected for quantification of luciferase activity. Each value represents the mean with S.E. (n = 3-6). *: $P < 0.05$, **: $P < 0.01$. □: binary complex and ■: ternary complex capsulated by CS.

As shown in Table 1 and Fig. 1, the cationic polymers and liposomes bound to pDNA and could form stable particles charged positively. The capsulation of CS changed the positive ζ-potential of polyplexes and lipoplexes to negative without much effect on the particle size. Furthermore, capsulations of CS did not release pDNA from cationic complexes, suggesting that the anionic capsule of CS was concentrated on the complex surface. We then confirmed the CS could encapsulate the cationic complex without disrupting the complexes and formed stable anionic nano-particles.

In cationic gene delivery systems, the positive charge on the particle surface ensures their binding to the negatively charged cellular membrane; the polyplexes and lipoplexes showed high transgene efficiency in B16-F10 cells (Figs. 2 and 3). The ternary complexes, such as pDNA/PLA/CS, pDNA/PLL/CS, and pDNA/DOTMA-DOPE/CS, also showed high transgene efficiency, regardless of their anionic surface charges. pDNA/DOTMA-Chol/CS, however, showed significantly lower transgene efficiency than pDNA/DOTMA-Chol. Capsulation of CS may affect the intracellular distribution and dissociation of pDNA/DOTMA-Chol.

Cationic vectors often lead to the cytotoxicity and agglutination of erythrocytes by the strong affinity of positively charged particles to the cellular membrane (8, 9). In the present study, cationic complexes showed high cytotoxicity and agglutination, as shown in Figs. 4 and 5. Furthermore, the lipoplexes showed not only agglutination but also hemolysis, and it suggests that lipid components interact with the cellular membrane (Fig. 6). In the previous study, we revealed that the ternary complexes of pDNA/PEI encapsulated by biocompatible anionic polysaccharides showed low cytotoxicity and inhibited agglutination of erythrocytes (13). Anionic CS capsulation successfully reduced the cytotoxicity of polyplexes and lipoplexes, and inhibited their agglutination of erythrocytes and hemolysis, as shown in Figs. 4, 5, and 6. These results must be due to the negatively charged surface of ternary complexes, indicating that CS may overcome some of the problems associated

with the use of positively charged complexes *in vivo*.

We evaluated the *in vivo* transgene efficiency of ternary complexes encapsulated by CS in mice (Fig. 7); polyplexes showed little transgene efficiency after intravenous administration. Biodegradable polymers may be unstable and release pDNA into the bloodstream. It was reported that serum proteins such as albumin are able to release DNA from polyplexes containing polylysine (10); however, CS capsulation significantly enhanced the transgene efficiency of polyplexes in the spleen. Kaplan *et al*. reported that pneumococcal polysaccharide types 2 and 3 were localized in splenic macrophages, Kupffer cells in the liver, and inguinal lymph node macrophages (33). Hill *et al.* also demonstrated that *Haemophilius influenzae* type b capsular polysaccharide was accumulated in Kupffer cells of liver sinusoids, in macrophages of the red pulp of the spleen, and the sinuses of lymph nodes (34). CS capsulation may increase the intensity of these polyplexes and be mainly recognized by the spleen as a polysaccharide. On the other hand, CS decreased the transgene efficiency of pDNA/PLL in the lung. This result might be explained by agglutination with the complexes and blood components, because the large particles are known to be embolized in the first capillary vascular bed of the lung (8-10). The high agglutination activity of pDNA/PLL was reduced by CS and should decrease the high transgene efficiency in the lung.

Lipoplexes showed high transgene efficiency in the spleen and lung. Stable lipoplexes with a positive charge may be able to reach the spleen and high transgene efficiency is present non-specifically. In contrast, the ternary complexes of lipoplexes encapsulated by CS showed decreased transgene efficiency in the spleen. Specific delivery of ternary complexes to the spleen may be lower than the non-specific delivery of positively charged lipoplex. The slight transgene efficiency of pDNA/DOTMA-Chol/CS in the spleen must be reflected by its low *in vitro* transgene efficiency in B16-F10 cells. The high transgene efficiency of lipoplexes in the lung was reduced by CS capsulation. These results may be explained by decreased agglutination.

The process of gene transfection has been reported to be influenced by condensing pDNA by electrostatic interaction, binding to the cell surface, being taken up by the endocytotic pathway, and releasing pDNA into the cytoplasm (35). CS capsulation must be predominantly related to particle stability in the blood and its uptake by cells. The differences between polyplexes and lipoplexes encapsulated by CS may be caused by different intracellular trafficking. In particular, in the process of endosomal escape, there is a big difference between polyplexes and lipoplexes; therefore, modification of the core must be important for developing useful ternary complexes encapsulated by CS.

In this experiment, we investigated the universal utility of CS capsulation for efficient and secure gene delivery. CS capsulation decreased the toxicity of polyplexes and lipoplexes. At the same time, ternary complexes encapsulated by CS showed high transgene efficiency in B16-F10 cells regardless of their anionic surface charges. In the *in vivo* study, ternary complexes encapsulating polyplexes by CS led to high transgene efficiency in the spleen after intravenous administration. These results indicated that CS capsulation of polyplexes is an outstanding method for effective and safe gene delivery. Further study should be performed to examine the detailed mechanisms and further applications.

ACKNOWLEDGEMENT

This study was supported in part by the Global COE Program, Nagasaki University, Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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