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Splenic Delivery System of pDNA through Complexes Electrostatically Constructed with Protamine and Chondroitin Sulfate

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We developed and optimized a novel gene delivery vector constructed electrostatically with an anionic biological component and a cationic biological component. Cationic binary complexes of plasmid DNA (pDNA) with novo-protamine sulfate as a medical product (PRT complexes) demonstrated high gene expression with minimal cytotoxicity, likely related with its total cationic charge. Subsequently, anionic compounds were added to the PRT complexes to form ternary complexes with neutral or anionic charges. Among the anionic compounds examined, chondroitin sulfate sodium (CS) as a medical product encapsulated the PRT complexes to produce stable ternary complexes (CS complexes) at charge ratios of ≥4 with pDNA. CS complexes exhibited high gene expression without cytotoxicity in mouse melanoma cell line, B16-F10 cells, *in vitro*. An inhibition study with endocytosis inhibitors suggested that PRT complexes were mainly taken up by caveolae-mediated endocytosis, and CS complexes were mainly taken up by clathrin-mediated endocytosis in B16-F10 cells. We found that CS complexes including pDNA encoding *Oplophorus gracilirostris* luciferase induced selective gene expression in the spleen after intravenous administration into ddY male mice. Thus, we successfully constructed useful gene vectors with biological components as medical products.

Key words protamine sulfate; chondroitin sulfate sodium; gene delivery; plasmid DNA; medical product

Currently, several nucleic acid-based compounds are being developed as medicines for refractory diseases, such as cancer and cystic fibrosis, and autoimmune diseases.[1–4] Nucleic acid-based compounds cannot cross the cell membrane spontaneously because of their anionic characteristics. Thus, the success of nucleic acid-based compound therapy highly depends on the development of an effective and secure gene delivery system. Numerous non-viral gene delivery vectors have been developed because of their low immunogenic effects, tunable size and targeting properties such as cationic polymers, lipids, and dendrimers.[5–8] However, these vectors are not sufficient for clinical use in terms of biodegradability, biocompatibility, transgene efficiency, and safety.

Protamine (PRT), a biological component, is used clinically as an antidote for heparin-induced anticoagulation.[9] PRT is a cationic peptide with a high arginine content that can condense DNA.[10] Furthermore, PRT can easily cross cell membranes and promote the internalization of associated molecules.[11,12] Thus, in addition to the products already on the market, PRT has attracted attention as a biomaterial for the design of gene delivery vectors.[13] Paradoxically, a major disadvantage of non-viral gene vectors is their cationic nature.[14] The positively charged vectors often have increased cytotoxicity, and cellular uptake may be altered by non-specific binding and binding to blood components, which inhibits cellular uptake of the particles.

The cytotoxic and blood component agglutination-inducing effects of cationic vectors can be countered by incorporating them in ternary complexes, which neutralizes their cationic charges. In a previous study, we found that coating cationic vectors with biodegradable anionic polymers decreased their toxicity without reducing their transgene efficiency.[15–18] Among them, chondroitin sulfate (CS), hyaluronic acid (HA), and glycyrrhizin (GL) are anionic biological components and are used clinically. They are known to target transmembrane proteins for endocytosis and facilitate efficient receptor-mediated endocytosis. We hypothesized that a gene delivery vector with an anionic biological component and a cationic biological component may aggregate with plasmid DNA (pDNA) and be taken up by cells for useful gene delivery. However, there are few reports on the development of safe and effective gene delivery systems using biological components. Furthermore, it is necessary to optimize the biological components in order to develop a safe and effective gene delivery system.

In the present study, we focused on novo-protamine sulfate (PRT) as a cationic compound, stronger *neo*-minophagen C (GL), sodium hyaluronate (HA) ophthalmic solution, and chondroitin sulfate sodium (CS) as anionic compounds. These compounds have regulatory approval as medical products. The binary complexes of pDNA with PRT were prepared (PRT complexes). Ternary complexes were also prepared by encapsulation of PRT complexes with GL, HA, and CS. We optimized and evaluated the usefulness of binary complexes and ternary complexes for gene vectors. We found that the optimized ternary complexes encapsulated by CS (CS complexes) demonstrated high gene expression and low cytotoxicity.

MATERIALS AND METHODS

Chemicals Protamine sulfate was purchased as novo-protamine sulfate 100 mg for I.V. Injection from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Monoammonium glycyrrhizinate was obtained as stronger *neo*-minophagen C Inj. 20 mL from Eisai Pharmaceutical Co., Ltd. (Tokyo, Japan).

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CS was purchased as chondroitin sulfate sodium 200 mg "Nichi-Iko" from Nichi-Iko Pharmaceutical Co., Ltd. (Tokyo, Japan). HA was purchased as sodium hyaluronate ophthalmic solution 0.3% "TOWA" from TOWA Pharmaceutical Co., Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and bovine serum albumin were purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel) and Sigma-Aldrich (St. Louis, MO, USA.), respectively. RPMI 1640 medium, Opti-MEM I, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and the other culture reagents were purchased from GIBCO BRL (Grand Island, NY, U.S.A.). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1), and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). Chlorpromazine (CPZ) was purchased from Nacalai Tesque (Kyoto, Japan). Amiloride and genistein were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of pDNA, Binary and Ternary Complexes pCMV-Luc (pDNA-Fl) was constructed by subcloning the *Hin*dIII/*Xba*I firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). *Oplophorus gracilirostris* luciferase encoding pNL1.1.CMV [Nluc/CMV] (pDNA-Op) was purchased from Promega (Madison, WI, U.S.A.). pDNA-Fl and pDNA-Op were amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany) before being dissolved in 5% glucose solution and stored at −80°C until experiments. The pDNA concentration was adjusted to 1 mg/mL by measuring the absorbance at 260 nm. To prepare binary complexes, pDNA solution and PRT were mixed by thorough pipetting, then left for 15 min at room temperature. The charge ratios of the pDNA-Fl-PRT complexes were 1 : 1.6 (weight ratio 1 : 1), 1 : 3.2 (weight ratio 1 : 2), 1 : 6.4 (weight ratio 1 : 4), 1 : 9.6 (weight ratio 1 : 6), 1 : 12.8 (weight ratio 1 : 8), and 1 : 16 (weight ratio 1 : 10) (PRT1.6, 3.2, 6.4, 9.6, 12.8, and 16 complexes, respectively) for the *in vitro* study. To construct ternary complexes, GL, CS, or HA were mixed with PRT12.8 complexes by pipetting to produce complexes with charge ratios of 1 : 12.8 : 4, 1 : 12.8 : 8, 1 : 12.8 : 12, 1 : 12.8 : 16, and 1 : 12.8 : 20 (GL4, 8, 12, 16, and 20 complexes, respectively), 1 : 12.8 : 2, 1 : 12.8 : 4, 1 : 12.8 : 6, and 1 : 12.8 : 8 (CS2, 4, 6, and 8 complexes, respectively), or 1 : 12.8 : 2, 1 : 12.8 : 4, 1 : 12.8 : 6, 1 : 12.8 : 8, 1 : 12.8 : 10, and 1 : 12.8 : 12 (HA2, 4, 6, 8, 10 and 12 complexes, respectively), which were then left for another 15 min at room temperature. pDNA-Op-PRT complexes (PRTM12.8 complexes) and pDNA-Op-PRT-CS complexes (CSM6 complexes) including pDNA-Op were also prepared for the *in vivo* study. The charge ratios of the PRTM12.8 complexes and CSM6 complexes were 1 : 12.8 and 1 : 12.8 : 6, respectively.

Physicochemical Properties of the pDNA-novo-protamine Sulfate Complexes The particle sizes and ζ-potentials of each complex were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, U.K.). Particle sizes are shown as the number-weighted mean diameter.

PRT12.8 complexes and CS6 complexes (5 μL) were loaded on a 200-mesh copper grid with carbon-coated plastic film (Nisshin EM, Tokyo, Japan), and negatively stained with 10 μL of uranyl acetate solution (1% (w/v)) for 10 s. The morphology of PRT12.8 complexes and CS6 complexes was observed using a JEM-1230 (JEOL Ltd., Tokyo, Japan) with an 80-kV acceleration voltage, and imaged using a 2 k×2 k Veleta CCD camera (Olympus Soft Imaging Solutions, Lakewood, CO, U.S.A.).

To assess complex formation, 20 μL aliquots of each complex solution containing 2 μg of pDNA-Fl were mixed with 4 μL of loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto 0.8% agarose gel. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 100 V in running buffer solution (40 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 40 mM acetic acid) for 30 min, and pDNA-Fl retardation was visualized using ethidium bromide staining.

***In Vitro* Gene Expression Experiments** B16-F10 cells, a mouse melanoma cell line, were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). The cells were maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) in a humidified atmosphere of 5% CO_2 in air at 37°C before being plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.) at a density of 1.0×10^4 cells/well and cultivated in 500 μL of culture medium. For the transfection experiment, the medium was replaced with 500 μL of Opti-MEM I medium after a 24-h pre-incubation, and then the cells were treated with each complex (containing 1 μg pDNA-Fl) and incubated for 2 h. After the cells were transfected, Opti-MEM I was replaced with culture medium, and the cells were cultured for a further 22 h in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were washed with phosphate-buffered saline (PBS) and then lysed with 100 μL of lysis buffer (pH 7.8; 0.1 M Tris–HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA) after a 22-h incubation. Ten-microliter lysate samples were mixed with 50 μL of luciferase assay buffer (PicaGene; Toyo Ink, Tokyo, Japan), and the amount of luminescence produced was measured immediately using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of the lysate was determined using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), in which bovine serum albumin was used as a standard, and absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Co., Ltd., Kanagawa, Japan) at 595 nm. Luciferase activity is shown as relative light units (RLUs) per mg of protein.

WST-1 Assay The cytotoxic tests for each complex in B16-F10 cells were carried out using a WST-1-based commercially available cell proliferation reagent. The reagent was prepared (5 mM WST-1 and 0.2 mM 1-methoxy PMS in PBS) and filtered through a 0.22-μm filter (Millex-GP; Millipore Co, Bedford, MA, U.S.A.) just before the experiments. B16-F10 cells were plated on 96-well plates (Becton-Dickinson) at a density of 3.0×10^3 cells/well in culture medium. Complexes containing 0.25 or 0.50 μg of pDNA-Fl in 100 μL of Opti-MEM I medium were added to each well and incubated for 2 h. Then, the medium was replaced with 100 μL of culture medium and incubated for another 22 h at 37°C. The medium was then substituted with 100 μL of culture medium, and 10 μL of WST-1 reagent was added to each well. The cells were incubated for an additional 2 h at 37°C, and the absorbance of each well was measured at a wavelength of 450 nm (reference wavelength: 630 nm) using a microplate reader. The results are shown as percentages of the value for the untreated

cells.

Hemagglutination Test Mouse erythrocytes were subjected to three rounds of centrifugation at 5000 rpm at 4°C (Kubota 3500; Kubota, Tokyo, Japan) for 5 min and then resuspended in PBS (a 2% (v/v) stock suspension was prepared). PRT12.8 complexes and CS6 complexes containing 2.5 or 5.0 μg of pDNA-Fl were added to the erythrocyte suspension (complex: stock suspension=1 : 1), and were incubated for 30 min at room temperature. Each sample was placed on a glass plate, and the extent of hemagglutination within the samples was observed by microscopy (400×magnification).

Inhibition Study To determine the endocytosis pathway used to transport the complexes into the cells, the cells were subjected to a 23-h pre-incubation before being treated with 0.014 mM CPZ, as an inhibitor of clathrin-mediated endocytosis; 0.2 mM genistein, as an inhibitor of caveolae-mediated endocytosis; or 1 mM amiloride, as an inhibitor of macropinocytosis, for 1 h. After the cells had been treated in the above-mentioned manner, PRT12.8 complexes and CS6 complexes containing 1 μg of pDNA-Fl were added to medium containing each inhibitor, and then the cells were incubated for 2 h at 37°C. Two hours after transfection, the medium was replaced with culture medium, the cells were cultured for a further 22 h at 37°C, and then their luciferase activity was examined. The results are shown as percentages of the values for the untreated cells.

***In Vivo* Study** All animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University after receiving approval from the institutional animal care and use committee. Male ddY mice (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After being delivered, the mice were allowed to acclimatize to their new environment for at least 1 d before the experiments. The PRTM12.8 complexes or CSM6 complexes containing 40 μg of pDNA-Op were injected intravenously into mice to examine the *in vivo* transgene efficiency at a volume of 200 μL per mouse. Twenty-four hours after injection, the mice were sacrificed, and the liver, kidney, spleen, heart, and lungs were dissected. The tissues were washed twice with cold saline and homogenized with lysis buffer. The homogenates were centrifuged at 15000 rpm (Kubota 3500) for 5 min and the supernatants were subjected to luciferase assays. Luciferase activity is indicated as RLU per gram of tissue.

Statistical Analysis The significance of differences between two groups was assessed using the Student's *t*-test. Multiple comparisons among the groups were performed using Dunnett's pairwise multiple comparisons *t*-test.

RESULTS

Physicochemical Properties of PRT Complexes The particle sizes and ζ-potentials of PRT complexes are summarized in Table 1. The PRT complexes had particle sizes of approximately 93–160 nm and a cationic surface charge of approximately 25–29 mV at charge ratios of ≥3.2 for pDNA-Fl. For the gel retardation assay (Fig. 1A), naked pDNA-Fl was detected as bands in the agarose gel (lane 1). Weak bands of naked pDNA-Fl were detected at a charge ratio of 1.6 (lane 2), although no bands were observed at charge ratios of 3.2, 4.8, 9.6, 12.8, or 16 (lanes 3, 4, 5, 6, and 7, respectively). The morphology of the PRT12.8 complexes was visualized using transmission electron microscopy (TEM) (Fig. 1B). PRT12.8 complexes had a spherical shape of approximately 100 nm in diameter.

Table 1. Particle Sizes and ζ-Potentials of PRT Complexes

Complexes	Charge ratio of protamine to pDNA	Sizes (nm)	ζ-Potentials (mV)
PRT1.6 complex	1.6	159.6±27.2	−9.0±0.3
PRT3.2 complex	3.2	106.4±3.9	26.5±0.4
PRT6.4 complex	6.4	95.5±24.8	25.1±0.2
PRT9.6 complex	9.6	119.6±61.4	27.3±1.3
PRT12.8 complex	12.8	93.8±9.4	28.4±1.2
PRT16 complex	16	123.6±79.9	27.1±0.5

Each value is mean±standard deviation (S.D.) (n=3).

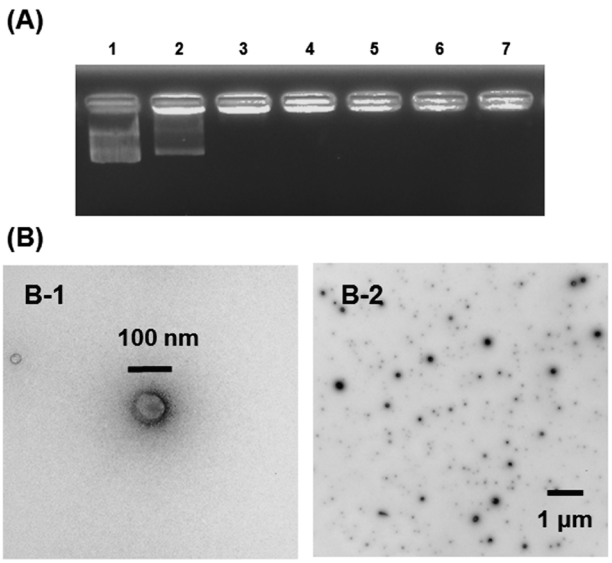

Fig. 1. Gel Retardation Assay (A) and TEM Images of PRT12.8 Complexes (B)

(A) PRT complexes were loaded onto an agarose gel, and electrophoresis was carried out. Retardation of pDNA-Fl was visualized using ethidium bromide. Naked pDNA-Fl was run in lane 1. Complexes: PRT1.6 (lane 2), PRT3.2 (lane 3), PRT6.4 (lane 4), PRT9.6 (lane 5), PRT12.8 (lane 6), and PRT16 (lane 7). (B) PRT12.8 complexes were loaded on a 200-mesh copper grid with carbon-coated plastic film, and negatively stained with 10 μL of uranyl acetate solution.

***In Vitro* Transfection Efficiency of PRT Complexes** B16-F10 cells were transfected with PRT complexes for 2 h, and their luciferase activity was then evaluated (Fig. 2). No gene expression was observed with naked pDNA-Fl. With increasing amounts of PRT to pDNA-Fl, luciferase activity increased and reached a plateau at a charge ratio of 12.8. PRT complexes at charge ratios of ≥9.6 demonstrated high gene expression ($>1.0\times10^8$ RLU/mg protein).

Physicochemical Properties of GL, CS, and HA Complexes PRT12.8 complexes were used for the preparation of ternary complexes with differing amounts of GL, CS, or HA. The particle sizes and ζ-potentials of GL, CS, and HA complexes are shown in Table 2. Addition of GL and HA to PRT12.8 complexes caused aggregation, and particle sizes could not be determined. CS complexes at charge ratios of 2 also exhibited aggregation. On the other hand, CS complexes at charge ratios of ≥4 had particulate complexes with diameters of 134–144 nm with anionic surface charges.

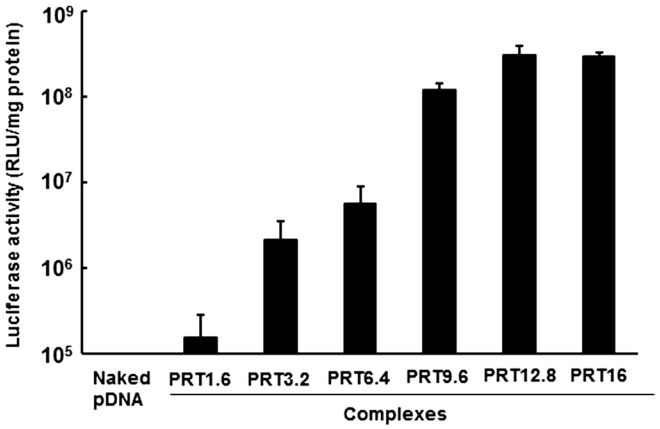

Fig. 2. Transfection Efficiency of PRT Complexes

B16-F10 cells were transfected with several complexes containing pDNA-Fl. Twenty-two hours after transfection, cells were lysed and luciferase activity was examined. Each value is the mean±standard error (S.E.) (n=3). RLU, relative light unit.

Table 2. Particle Sizes and ζ-Potentials of GL, CS, and HA Complexes

Complexes	Complexes	Charge ratio of GL CS, and HA complexes to pDNA	Sizes (nm)	ζ-Potentials (mV)
GL complex	GL4 complex	4	n.d.	0.2±0.7
	GL8 complex	8	n.d.	−6.3±0.8
	GL12 complex	12	n.d.	−6.8±4.6
	GL16 complex	16	n.d.	−13.4±3.0
	GL20 complex	20	n.d.	−22.9±7.7
CS complex	CS2 complex	2	n.d.	19.9±0.1
	CS4 complex	4	134.7±7.3	−36.2±0.7
	CS6 complex	6	136.5±4.6	−39.8±0.7
	CS8 complex	8	143.9±8.4	−41.4±1.1
HA complex	HA2 complex	2	n.d.	15.5±0.2
	HA4 complex	4	n.d.	12.9±0.3
	HA6 complex	6	n.d.	−24.8±1.0
	HA8 complex	8	n.d.	−27.3±0.7
	HA10 complex	10	n.d.	−31.1±0.9
	HA12 complex	12	n.d.	−34.3±1.4

Each value is mean±S.D. (n=3), n.d.: not detected.

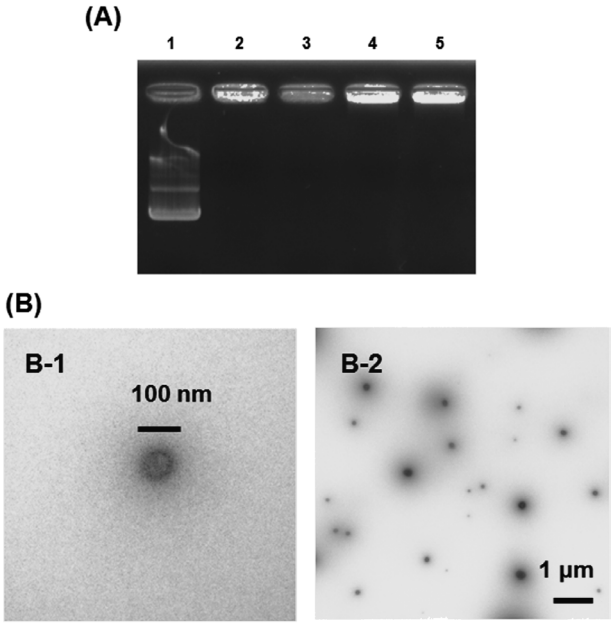

Fig. 3. Gel Retardation Assay (A) and TEM Images of CS6 Complexes (B)

(A) CS complexes were loaded onto an agarose gel, and electrophoresis was carried out. Retardation of pDNA-Fl was visualized using ethidium bromide. Naked pDNA-Fl was run in lane 1. Complexes: CS2 (lane 2), CS4 (lane 3), CS6 (lane 4), and CS8 (lane 5). (B) CS6 complexes were loaded on a 200-mesh copper grid with carbon-coated plastic film, and negatively stained with 10 μL of uranyl acetate solution.

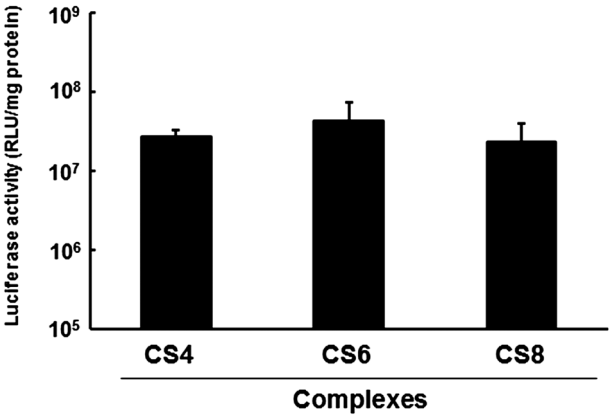

Fig. 4. Transfection Efficiency of CS Complexes

B16-F10 cells were transfected with several complexes containing pDNA-Fl. Twenty-two hours after transfection, cells were lysed and luciferase activity was examined. Each value is the mean±S.E. (n=3). RLU, relative light unit.

Gel retardation assays were performed to examine whether pDNA-Fl was released from CS complexes (Fig. 3). No bands of pDNA-Fl were detected in the lanes for the CS complexes. The morphology of CS6 complexes was visualized using TEM (Fig. 3B). CS6 complexes had a spherical shape of approximately 100 nm in diameter.

***In Vitro* Transfection Efficiency of the CS Complexes** CS4, 6, and 8 complexes were incubated with B16-F10 cells for 2 h and their luciferase activity was determined. The results are shown in Fig. 4. The CS complexes exhibited high transgene efficiency (2.4–4.4×10^7 RLU/mg protein), although PRT12.8 complexes had higher gene expression exceeding 10^8 RLU/mg protein.

Cytotoxicity CS4, 6, and 8 complexes, and PRT12.8 complexes (0.25 μg pDNA-Fl/well and 0.50 μg pDNA-Fl/well) were added to B16-F10 cells in 96-well plates for 2 h, and the cell viability was then determined using WST-1 assays. No cytotoxicity was observed with any complex at a low dose (0.25 μg pDNA-Fl/well), as shown in Fig. 5A. At a high dose (0.50 μg pDNA-Fl/well), PRT12.8 complexes and CS8 complexes demonstrated significant cellular toxicity and the cell viability of PRT12.8 complexes and CS8 complexes were 54 and 74% of control, respectively. On the other hand, CS4 and 6 complexes were not cytotoxic (Fig. 5B).

Inhibition Study The effects of endocytosis inhibitors on transgene efficiency were examined in PRT12.8 and CS6 complexes (Fig. 6). The luciferase activity of PRT12.8 complexes was significantly decreased by genistein, an inhibitor of caveolae-mediated endocytosis (<10% of control). In addition, amiloride, an inhibitor of micropinocytosis, tended to decrease the transgene efficiency of PRT12.8 complexes (40% of control). On the other hand, the luciferase activity of CS6 complexes tended to be decreased by CPZ, an inhibitor of clathrin-mediated endocytosis, (47% of control) and genistein

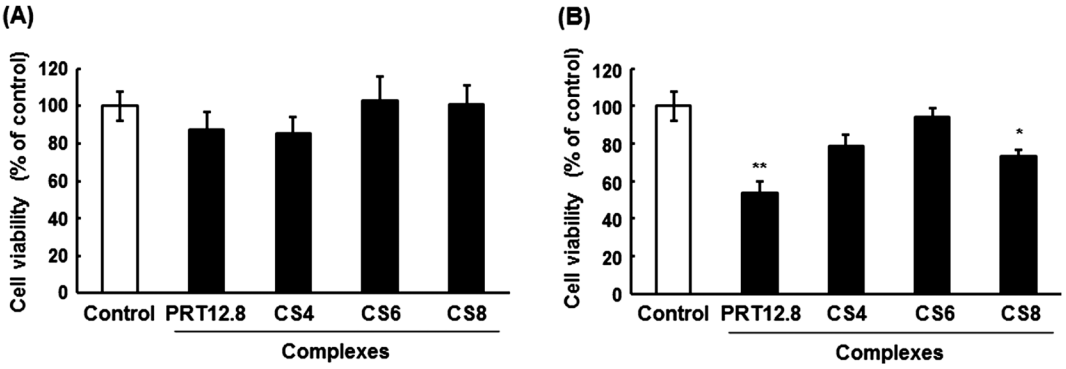

Fig. 5. Cytotoxicity of the Complexes in B16-F10 Cells

The viability of cells treated with each complex was measured using WST-1 assays. Cell viability was measured 22 h after the cells had been incubated with the complexes for 2 h. Data are the percentage to untreated cells. Each value is the mean±S.E. (n=8). *p<0.05, **p<0.01 *vs.* control. (A) pDNA-Fl 0.25 μg, (B) pDNA-Fl 0.50 μg.

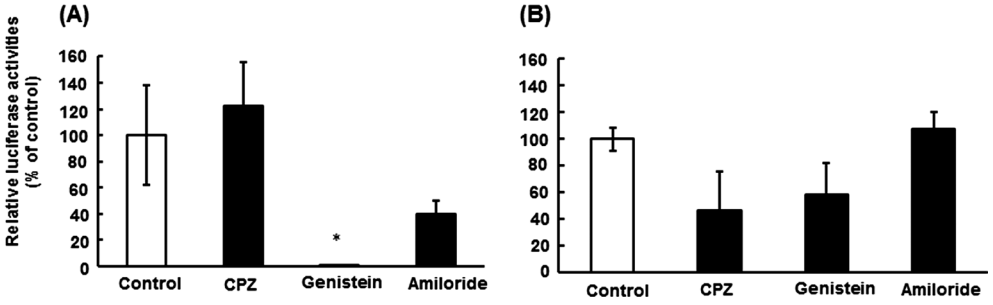

Fig. 6. Influence of Endocytosis Inhibitors on Transfection Efficiency of PRT12.8 Complexes (A) and CS6 Complexes (B)

Twenty-two hours after transfection, the luciferase activity was evaluated. Each bar represents the mean±S.E. (n=3–6) *p<0.05 *vs.* control. CPZ, chlorpromazine.

(58% of control).

Hemagglutination Test Erythrocytes were observed by microscopy 30 min after incubation with the complexes (2.5 μg of pDNA-Fl/100 μL and 5.0 μg of pDNA-Fl/100 μL). At a low concentration (2.5 μg of pDNA-Fl/100 μL), PRT12.8 complexes exhibited slight hemagglutination (Fig. 7B), although the CS6 complexes exhibited no hemagglutination (Fig. 7C). There was no hemagglutination with CS6 complexes (Fig. 7E) at even higher concentrations (5.0 μg of pDNA-Fl/100 μL). The PRT12.8 complexes increased the hemagglutination at higher concentration (Fig. 7D).

***In Vivo* Study** The transgene efficiency of PRTM12.8 and CSM6 complexes including pDNA-Op was determined in several tissues 24 h after intravenous administration into ddY male mice. PRTM12.8 complexes exhibited high gene expression (>1.0×10^6 RLU/g tissue) in all tissues except for the heart. On the other hand, CSM6 complexes induced high gene expression in the spleen (>1.0×10^7 RLU/g tissue) (Fig. 8). The gene expression in the spleen of CSM6 complex was significantly higher than that of PRTM12.8 complex. Also, the gene expression in the spleen was significantly higher than that in liver, kidney, and heart. On the other hand, the gene expression in the spleen was higher than that in lung although it was not significant.

DISCUSSION

Protamine, which is isolated from the sperm of mature fish, is a small cationic polypeptide (molecular weight 4000–4250). Protamine was reported to improve transgene efficiency mediated by non-viral vectors because the nuclear localization signal in protamine molecules can deliver DNA into the nucleus of cells.[19,20] These characteristics of protamine are appropriate for the clinical use of a non-viral vector with pDNA. Protamine has already been used clinically to reverse heparin anticoagulation following hemodialysis and in artificial heart-lung machines.[21] It has been complexed with insulin (known as NPH) and serves as a long-acting delivery system.[19]

Indeed, protamine was able to cause the compaction of pDNA to form binary complexes. The PRT1.6 complexes had a mild negative surface charge and low release of pDNA-Fl from the complexes in electrophoresis analysis (Table 1 and Fig. 1A). Strong positive charges were found in PRT complexes at weight ratios of ≥3.2, and they did not exhibit any release of pDNA-Fl, indicating strong compaction of pDNA-Fl by protamine. Protamine has an excellent ability to bind pDNA-Fl and protects it against enzyme degradation by compaction.

PRT complexes, except for PRT1.6 complexes, demonstrated high gene expression in the melanoma cell line B16-F10 (Fig. 2), a refractory cancer. Complexes with positive charges on the particle surface interacted electrostatically with the cell membrane and led to high gene expression.[22] Gene expression was increased with an increase in the protamine charge ratio, and the highest level was observed at charge ratios of 12.8 and 16 (Fig. 2). Harashima and colleagues reported that protamine/pDNA particles exhibited high gene expression at a high weight ratio after cytoplasmic microinjection.[23] At a low weight ratio, cationic amino acids contained within protamine were used to interact with pDNA. As the charge ratios of protamine increased, it was observed on the surface of particles and functioned as a nuclear localization signal. Also, the

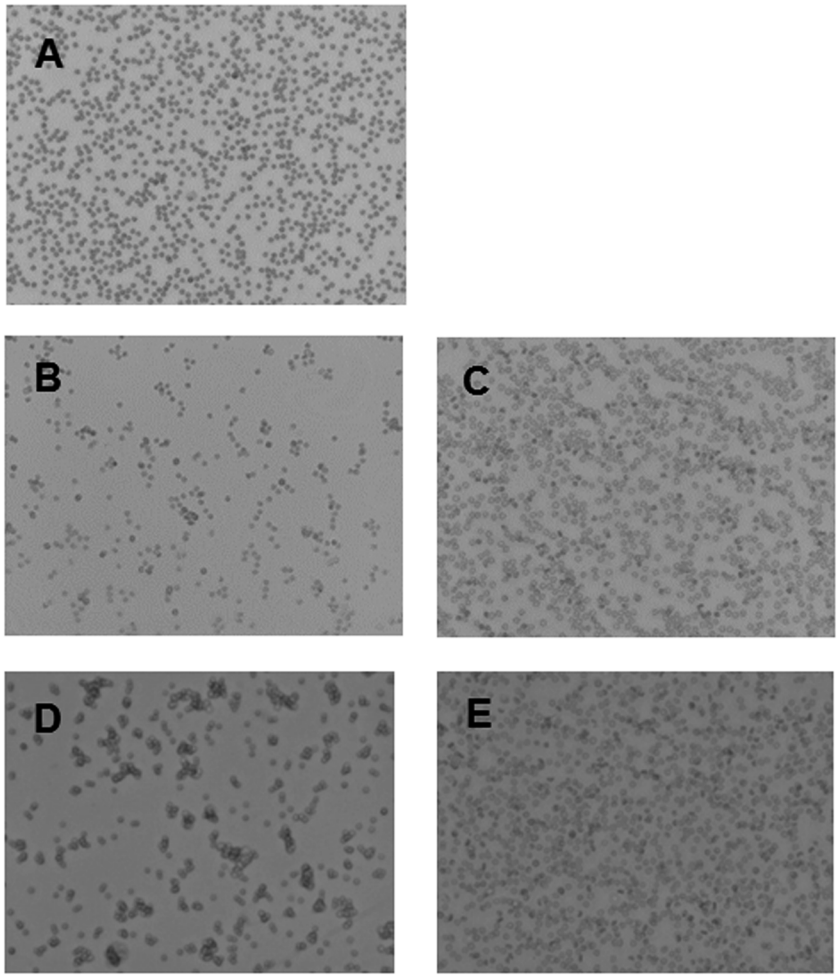

Fig. 7. Hemagglutination of Complexes with Erythrocytes

Each complex was added to erythrocytes, and hemagglutination was observed by microscopy (400× magnification). (A) Phosphate-buffered saline (PBS), (B) PRT12.8 complexes (2.5 μg of pDNA-Fl), (C) CS6 complexes (2.5 μg of pDNA-Fl), (D) PRT12.8 complexes (5.0 μg of pDNA-Fl), (E) CS6 complexes (5.0 μg of pDNA-Fl).

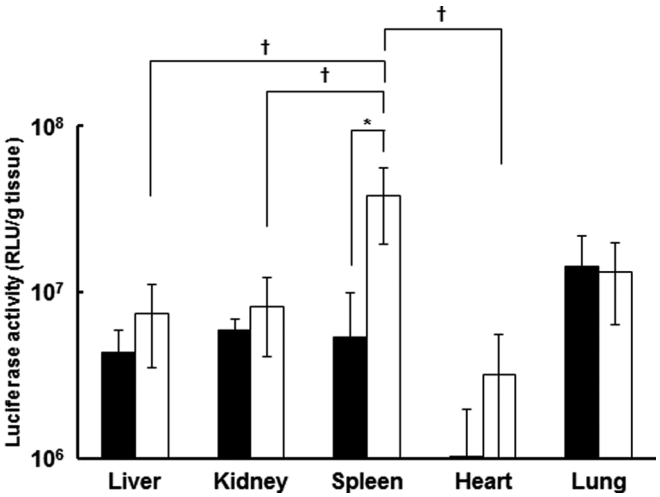

Fig. 8. *in Vivo* Transgene Efficiency of PRTM12.8 Complexes and CSM6 Complexes in Mice

The complexes were injected intravenously into mice (40 μg of pDNA-Op per mouse). Twenty-four hours after the injection, mice were sacrificed and each organ was dissected to quantify luciferase activity. Each value is the mean±S.E. (n=3). PRTM12.8 complexes (■), CSM6 complexes (□). RLU, relative light unit. *$p<0.05$ *vs.* PRTM12.8 complexes. †$p<0.05$ *vs.* other organs.

plateau of transgene efficiency at charge ratios of 12.8 may be caused by saturation of PRT coating process, uptake process, and expression process, although further study is necessary in future.

Many cationic non-viral gene vectors have been reported to cause cytotoxicity and agglutination of blood because of the strong affinity of positively charged particles for the cellular membrane. Hu and colleagues reported that binary complexes constructed with pDNA and protamine exhibited slight cytotoxicity in HEK 293 cells and MCF-7 cells.[24] PRT12.8 complexes showed cytotoxicity and hemaagglutination at a high dose in B16-F10 cells (Figs. 5B, 7B, D). The recharging of cationic complexes with anionic compounds was reported to decrease cytotoxicity.[25,26] Therefore, we prepared ternary complexes of PRT12.8 complexes with biological components such as GL, CS, and HA. The addition of GL or HA to PRT12.8 complexes induced aggregation and particle sizes were not detected (Table 2). On the other hand, CS complex particles at charge ratios of ≥4 were approximately 140 nm in size and had anionic surface charge, indicating the existence of CS on the outside of the particles (Table 2). No release of pDNA-Fl from CS complexes suggested complete compaction of pDNA-Fl with protamine (Fig. 3). The anionically charged CS6 complexes demonstrated no cytotoxicity and hemaggluti-

nation even at high doses (Figs. 5, 7C, E).

PRT12.8 and CS6 complexes both had high gene expression regardless of their different surface charges (Figs. 2, 4). Therefore, we performed an inhibition study with several endocytosis inhibitors such as CPZ for clathrin-mediated endocytosis, genistein for caveolae-mediated endocytosis, and amiloride for macropinocytosis.[27] As shown in Fig. 6A, PRT12.8 complexes were suggested to be mainly taken up by caveolae-mediated endocytosis. In general, caveolae-mediated endocytosis does not lead to lysosomal degradation[27]; therefore, this pathway may be advantageous in terms of DNA delivery. On the other hand, both clathrin-mediated and caveolae-mediated endocytosis may play a role in the incorporation of CS6 complexes into cells (Fig. 6B).

The gene delivery system constructed with biological components was demonstrated as useful by *in vitro* experiments. We also confirmed that the particle size of PRT12.8 and CS6 complexes was suitable for *in vivo* gene delivery using TEM (Figs. 1B, 3B). Thus, *in vivo* gene expression was examined after intravenous administration of PRT12.8 and CS6 complexes in mice. High gene expression, $>1.0\times10^6$ RLU/g tissue, was detected in the liver, kidneys, spleen, and lungs after administration of PRTM12.8 complexes. In addition, Delucia *et al.* reported that protamine itself is highly distributed in the kidneys, lungs, and heart after intravenous administration in rats.[14] Nanoparticles are generally distributed in the reticuloendothelial system, such as the liver, spleen, and lungs, but cannot enter the heart. CSM6 complexes had high gene expression, $>1.0\times10^7$ RLU/g tissue, in the spleen and lungs. In particular, CSM6 complexes exhibited significantly higher gene expression in the spleen than PRTM12.8 complexes (Fig. 8). Kaplan *et al.* reported that pneumococcal polysaccharide types 2 and 3 were localized in splenic macrophages, in Kupffer cells in the liver, and in inguinal lymph node macrophages.[28] The surface of CSM6 complexes may be mainly recognized by the spleen as a polysaccharide.

We constructed complexes using only medical products with regulatory-approval in this study. To the best of our knowledge, our study is the first to apply medical products with regulatory-approval to gene delivery systems and to demonstrate high gene expression *in vitro* and *in vivo*.

CONCLUSION

We successfully optimized useful gene vectors with biological components. PRT and PRTM complexes had high transgene expression efficiency both *in vitro* and *in vivo*, although PRT complexes had slight cytotoxicity and hematotoxicity. The addition of CS to the PRT complexes decreased their cytotoxicity and hematotoxicity. CS and CSM complexes exhibited high transgene expression efficiency both *in vitro* and *in vivo*.

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Conflict of Interest The authors declare no conflict of interest.

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