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Biocompatible complex coated with glycosaminoglycan for gene delivery

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Abstract

The purpose of this study was to develop a ternary complex of plasmid DNA (pDNA) electrostatically assembled with dendrigraft poly-L-lysine (DGL) and biodegradable glycosaminoglycan for effective and secure gene delivery. High gene expression of pDNA/DGL complex was confirmed with slight cytotoxicity and erythrocyte agglutination. Anionic ternary complexes of 55.4–223.8 nm were formed by the addition of a glycosaminoglycan such as chondroitin sulfate A (CS-A), chondroitin sulfate B (CS-B), chondroitin sulfate C (CS-C), or hyaluronic acid (HA). Using the cell line B16-F10, most of the ternary complexes showed only weak gene expression and little cytotoxicity, although the pDNA/DGL/CS-A complexes maintained a certain level of gene expression. In particular, the pDNA/DGL/CS-A8 complexes showed significantly higher gene expression than pDNA/DGL complexes in the presence of fetal bovine serum. Gene expression from the pDNA/DGL/CS-A8 complex was inhibited by a high concentration of CS-A and endocytosis inhibitors. After intravenous administration of the pDNA/DGL/CS-A8 complex and the pDNA/DGL complex into ddY mice, high gene expression was observed in the reticuloendothelial systems the pDNA/DGL/CS-A complex is expected to be useful for gene therapy.

Keywords : dendrigraft poly-L-lysine, chondroitin sulfate, hyaluronic acid, biodegradable, glycosaminoglycan, gene therapy, drug delivery

Abbreviations : DGL, dendrigraft poly-L-lysine; CS, chondroitin sulfate; HA, hyaluronic acid; FBS, fetal bovine serum; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; 1-methoxy PMS, 1-methoxy-5-methylphenazinium methyl sulfate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; RLU, relative light unit; CPZ, chlorpromazine

dendrigraft poly-L-lysine; glycosaminoglycan; chondroitin sulfate; hyaluronic acid; biodegradable; gene delivery; nanoparticle

1. Introduction

Gene therapy is expected to be an effective method to treat cancer, infectious diseases, and immunodeficiency^{1,2,3}. However, the success of gene therapy is highly dependent on the development of effective and secure delivery vectors, because of their large molecules and strong anionic charge of the nucleic acids involved⁴. The establishment of a gene delivery system that enables prolonged activity without degradation by nucleases and efficient cytosolic delivery of nucleic acid into target cells is indispensable for development of nucleic acid pharmaceuticals^{4,5}.

Gene delivery vectors are categorized according to their use of viral or non-viral vectors⁴. Non-viral vectors are expected to have some advantages such as safety, suitability for mass production, much lower immunotoxicity, clear structure, and easy modeling⁵⁻⁷. Among non-viral vectors, cationic polymers and cationic liposomes have often been used to form stable cationic complexes with pDNA as polyplexes and lipoplexes, respectively^{4,7}. Cationic complexes of pDNA with cationic polymers and cationic liposomes show high gene expression although they are cytotoxic and have blood component agglutination-inducing effects⁴⁻⁸. Clinical use of cationic complexes has been limited by these disadvantages. However, we have found several specific anionic polymers that when added to cationic complexes of pDNA decrease their cytotoxicity

without reducing their transgene expression efficiency⁷⁻⁹.

Glycosaminoglycans are anionic polymers consisting of long unbranched polysaccharides composed of repeating disaccharide units^{10,11}. They are useful in the body as a lubricant or shock absorber because they are highly polar and attract water. Among the glycosaminoglycans, chondroitin sulfate (CS) and hyaluronic acid (HA) have been used as therapeutic compounds for osteoarthritis, rheumatism, and dry eye^{12,13}. They are highly stable, nontoxic, hydrophilic, and biodegradable. CS and HA was often used as a vector of drug delivery^{11,14}. CS and HA are used a gene delivery carriers and there have been attempts to study the mechanism of enhancement of gene delivery efficiency^{14,15}. Previously, we firstly discovered that the ternary complex encapsulated by CS taken in the cells via CS-specific receptor and showed high gene expression without cytotoxicity and agglutination of erythrocytes⁹. Uchida et al reported that the safety and transfection efficiency of nonviral polycation-based gene carrier micelle systems was improved by adding a CS¹⁶. With regard to HA, Gu et al successfully developed ternary nanoassemblies based on HA-coating for targeted gene delivery to CD44-positive tumors¹⁷. CS is also known to target a transmembrane CD44 receptor for endocytosis that facilitates efficient receptor-mediated endocytosis^{18,19}.

On the other hand, there are several kinds (A, B, C) of CS and their characteristics

are different in each other depending on their structure difference²⁰⁻²². Characteristics of HA, one of glycosaminoglycan, is also different from CS. The ability of complexes coated by CS and HA may be affected by the difference of characteristics of CS and HA. However, there are no reports that the efficiency and safety of glycosaminoglycan-coated complex compared between several glycosaminoglycans exhaustively.

In this study, we prepared the glycosaminoglycan-coated complexes using dendrigraft poly-L-lysine (DGL), biodegradable cationic dendrimer, and four kinds of glycosaminoglycan such as chondroitin sulfate A (CS-A), chondroitin sulfate B (CS-B), chondroitin sulfate C (CS-C), and hyaluronic acid (HA) and evaluated their efficacy and safety. We previously demonstrated that cationic complex of pDNA and DGL showed high gene expression⁵. Furthermore, our complexes constructed by pharmaceutical approach, not chemical approach, would have several benefits, such as easy manufacturing, and easy sterilization.

2. Materials and methods

2.1. Chemicals

Fifth-generation DGL compounds (M.W:172300 Da, 963 lysine groups) were purchased from COLCOM S.A.S. (Montpellier, France). Fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). 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). 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 purchased from Dojindo Laboratories (Kumamoto, Japan). Chondroitin sulfate A sodium salt from bovine trachea (CS-A), chondroitin sulfate B sodium from porcine intestinal mucosa (CS-B), chondroitin 6-sulfate sodium from shark cartilage (approx. >90%; CS-C), and hyaluronic acid potassium salt from human umbilical cord (HA) were obtained from Sigma (St. Louis, MO, USA).

2.2. Preparation of pDNA, binary complexes and ternary complexes

The plasmid pCMV-Luc was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, Wisconsin,

USA) into the polylinker of a pcDNA3 vector (Invitrogen, Carlsbad, California, USA). This pDNA was amplified using an EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The pDNA was dissolved in 5% dextrose solution and stored at -80°C until analysis, for which the concentration was measured with the use of absorbance at 260 nm and adjusted to 1 mg/mL.

To prepare binary complexes, an appropriate amount of stock DGL solution (pH 7.4) and pDNA solution were mixed by pipetting thoroughly and left for 15 min at room temperature. For the preparation of ternary complexes, CS or HA was mixed with pDNA/DGL complex by pipetting and left for another 15 min at room temperature. In this study, we constructed complexes with theoretical charge ratios of phosphate of pDNA : nitrogen of DGL : sulfate of each CS = 1:6:0 (pDNA/DGL complex), 1:6:2 (pDNA/DGL/CS-A2, pDNA/DGL/CS-B2, and pDNA/DGL/CS-C2 complex), 1:6:4 (pDNA/DGL/CS-A4, pDNA/DGL/CS-B4, and pDNA/DGL/CS-C4 complex), 1:6:6 (pDNA/DGL/CS-A6, pDNA/DGL/CS-B6, and pDNA/DGL/CS-C6 complex), 1:6:8 (pDNA/DGL/CS-A8, pDNA/DGL/CS-B8, and pDNA/DGL/CS-C8 complex), 1:6:10 (pDNA/DGL/CS-A10, pDNA/DGL/CS-B10, and pDNA/DGL/CS-C10 complex), and 1:6:12 (pDNA/DGL/CS-A12, pDNA/DGL/CS-B12, and pDNA/DGL/CS-C12 complex). We also constructed other complexes with a theoretical charge ratio of phosphate of

pDNA : nitrogen of DGL : carboxylate of HA = 1:6:2 (pDNA/DGL/HA2 complex), 1:6:4 (pDNA/DGL/HA4 complex), 1:6:6 (pDNA/DGL/HA6 complex), 1:6:8 (pDNA/DGL/HA8 complex), 1:6:10 (pDNA/DGL/HA10 complex), and 1:6:12 (pDNA/DGL/HA12 complex).

2.3. Physicochemical properties of the complexes

The particle sizes and zeta-potentials of the complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., Malvern, UK). The number-fractioned mean diameter is shown. To determine complex formation, 20 μ L aliquots of complex solution containing 1 μ g pDNA were mixed with 4 μ L loading buffer (30% glycerol, 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel. 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 ethylenediaminetetraacetic acid [EDTA]) for 60 min. The retardation of pDNA was visualized using ethidium bromide staining.

2.4. In vitro gene expression and cellular uptake experiments

A mouse melanoma cell line, B16-F10 cells, was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). B16-F10 cells were

maintained in RPMI 1640 supplemented with 10% FBS and antibiotics (culture medium) in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were plated in 24-well plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at a density of 1.0×10^4 cells/well and cultivated in 500 μ L of culture medium. In the transfection experiment, the medium was replaced with 500 μ L Opti-MEM I medium after 24 h pre-incubation and each complex containing 1 μ g pCMV-Luc was added to the cells, and incubated for 2 h. In preliminary experiment, we examined the transfection efficiency of the complexes in various dosages and chose the proper dosage. After transfection, the medium was replaced with culture medium and cells were cultured for a further 22 h in a humidified atmosphere of 5% CO₂ in air at 37°C. After 22 h incubation, the cells were washed with phosphate-buffered saline (PBS) and then lysed in 100 μ L 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 each lysate sample was mixed with 50 μ L luciferase assay buffer (PicaGene; Toyo Ink Company, Ltd., Tokyo, Japan) and the light 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, California, USA) with BSA as a standard. Absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Company, Ltd., Kanagawa, Japan) at

595 nm. Luciferase activity was indicated as relative light units (RLUs) per mg protein. To visualize the uptake of the complexes, the B16-F10 cells were transfected with complexes containing of YOYO-1-labeled pCMV-Luc. After 22 h incubation, the relative levels of YOYO-1 in the cells were characterized using fluorescence microscopy.

2.5. *WST-1 assay*

Cytotoxicity tests of various complexes with B16-F10 cells were carried out using a commercially available WST-1 cell proliferation reagent. The WST-1 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, USA) just before the experiments. B16-F10 cells were plated in 96-well plates (Becton Dickinson and Company) at a density of 5.0×10^3 cells/well in culture medium. Complexes containing 1 μg pDNA in 100 μL Opti-MEM I medium were added to each well and incubated for 2 h. In preliminary experiment, we examined the cytotoxicity of the complexes in various dosages and chose the proper dosage. After incubation, the medium was replaced with 100 μL culture medium and incubated for another 22 h at 37°C. The medium was replaced with 100 μL culture medium, and 10 μL WST-1 reagent was added to each well. The cells were incubated for an additional 2 h at 37°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 relative to untreated cells.

2.6. Inhibition study

For determination of the endocytotic pathway, after 23 h pre-incubation, B16-F10 cells were treated with 0.014 mM chlorpromazine (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 treatment, pDNA/DGL complex or pDNA/DGL/CS-A8 complex was added to the medium containing each inhibitor and incubated for 1 h. For experiments using CS-A as an inhibitor, the cells were transfected with pDNA/DGL/CS-A8 complex in Opti-MEM I medium containing various concentrations of CS-A. At 2 h after transfection, the medium was replaced with culture medium, cells were cultured for a further 22 h at 37°C, and then luciferase activities were determined.

2.7. Agglutination study

Erythrocytes from mice were washed three times at 4°C by centrifugation at 5000 rpm (Kubota 3500; Kubota, Tokyo, Japan) for 5 min and re-suspended in PBS. A 2% (v/v)

stock suspension was prepared. Various complexes were added to the erythrocytes (complexes: stock suspension = 1:1). The suspensions were incubated for 15 min at room temperature. Ten microliter aliquots of these suspensions were placed on a glass plate, and agglutination was observed by microscopy (400× magnification).

2.8. Effect of fetal bovine serum on transgene efficiency

To investigate the effects of biological components, after 24 h pre-incubation, B16-F10 cells were treated with pDNA/DGL complex or pDNA/DGL/CS-A8 complex were added to medium containing various concentrations of FBS (5%, 10%, or 20%). At 2 h after transfection, the medium was replaced with culture medium, cells were cultured for a further 22 h at 37°C, and then luciferase activities were determined.

2.9. 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 several days before experiments.

2.10. In vivo study

Each complex containing 40 μg pCMV-Luc in a volume of 300 μL per mouse was injected intravenously into mice to examine the transgene efficacy. Six hours after injection, the mice were sacrificed, and the liver, kidney, spleen, heart, and lung 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 used for luciferase assays. Luciferase activity is indicated as RLU per gram of tissue.

2.11. Statistical analysis

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

3. Results

3.1. Physicochemical characteristics and gel retardation assay

We used pDNA/DGL complex at a charge ratio of 1 : 6 among various charge ratios because of the high gene expression level shown in preliminary experiments⁵. The pDNA/DGL complex was mixed with glycosaminoglycan. Their sizes and zeta-potentials are shown in Table 1, and those of the pDNA/DGL complexes were confirmed to be 59.5 ± 1.3 nm and 39.0 ± 0.4 mV, respectively. Lower zeta-potentials of particles were observed in the pDNA/DGL/CS-A complexes, pDNA/DGL/CS-B complexes, pDNA/DGL/CS-C complexes, and pDNA/DGL/HA complexes. We tried to prepare the complexes at several charge ratios of glycosaminoglycan. However, the zeta potential of complexes became a plateau at the charge ratio of >12 (data not shown). On the other hand, the complexes aggregated at the charge ratio of <2 (data not shown). Therefore, we chose these conditions (charge ratio of 2 to 12). Addition of most glycosaminoglycans did not greatly affect the sizes of the complexes, although sizes did increase slightly. The sizes of the ternary complexes were 55.4–223.8 nm.

Complex formations were examined using a gel retardation assay, as shown in Fig.

1. Naked pDNA was detected as a band on an agarose gel, but no such bands were detected in the lanes for each ternary complex.

3.2. *In vitro* gene expression

Complexes at various charge ratios were incubated with B16-F10 cells. The *in vitro* gene expression of each complex was evaluated by measurement of luciferase activity (Fig. 2). The pDNA/DGL complex showed gene expression of almost 10^{10} RLU/mg protein. Addition of glycosaminoglycan to pDNA/DGL complex decreased the luciferase activities of pDNA/DGL complex with increasing amounts of glycosaminoglycan, excepting for CS-A. The pDNA/DGL/CS-B complexes and pDNA/DGL/CS-C complexes showed low gene expression of $<10^8$ RLU/mg protein. The anionic surface particles of pDNA/DGL/HA complexes also had low gene expression of $<10^8$ RLU/mg protein. On the other hand, pDNA/DGL/CS-A complexes maintained gene expression levels close to 10^9 RLU/mg protein.

3.3. Cellular toxicity

Each complex was added to B16-F10 cells, and cell viability was evaluated using a WST-1 assay (Fig. 3). The pDNA/DGL complex displayed significant cytotoxicity compared with the control ($P < 0.01$). The pDNA/DGL/CS-B complexes and pDNA/DGL/CS-C complexes did not affect the cell viability of B16-F10 cells. The

anionic surface particles of pDNA/DGL/HA complexes showed no cytotoxicity. No cytotoxicity was also observed with pDNA/DGL/CS-A complexes with a charge ratio of 2 or more, although the pDNA/DGL/CS-A2 complex showed slight cytotoxicity.

3.4. Fluorescent microscopy

Figure 4 shows the cellular uptake of the pDNA/DGL/CS-A8 complexes, pDNA/DGL/CS-B8 complexes, pDNA/DGL/CS-C8 complexes, and pDNA/DGL/HA8 complexes using pDNA labeled with YOYO-1. Strong green fluorescence was observed in cells treated with pDNA/DGL/CS-A complexes. On the other hand, the pDNA/DGL/CS-B, pDNA/DGL/CS-C, and pDNA/DGL/HA complexes showed little fluorescence.

3.5. Influence of inhibitors on gene expression

The gene expression of DNA/DGL/CS-A8 complex in B16-F10 cells was determined in the presence of CS-A. The gene expression of the complex was significantly reduced by addition of CS-A in concentration-dependent manner, as shown in Fig. 5 (A). Endocytotic inhibitors also decreased the gene expression of the pDNA/DGL/CS-A8 complex significantly (Fig. 5 (B)). CPZ, an inhibitor of clathrin-

mediated endocytosis, and genistein, an inhibitor of caveolae-mediated endocytosis, decreased gene expression of the pDNA/DGL/CS-A8 complex to about 71.5% and 50.1%, respectively. In addition, inhibition of macropinocytosis using amiloride did not decrease gene expression of the pDNA/DGL/CS-A8 complex.

3.6. Influence of fetal bovine serum on transgene efficiency

The influence of FBS on the gene expression of the pDNA/DGL/CS-A8 complex was compared with that of pDNA/DGL complex, as shown in Fig. 6. The gene expression of pDNA/DGL complex was greatly reduced in the presence of FBS. The presence of FBS, however, did not greatly affect gene expression of the pDNA/DGL/CS-A8 complex. In the presence of FBS, expression of the pDNA/DGL/CS-A8 complex was significant higher than that of the pDNA/DGL complex.

3.7. Agglutination study

Before the in vivo study, agglutination activity of the pDNA/DGL/CS-A8 complex was compared with the pDNA/DGL complex in erythrocytes (Fig. 7). The pDNA/DGL/CS-A8 complex showed no agglutination although the pDNA/DGL complex agglutinated.

3.8. *In vivo study*

The *in vivo* gene expression was examined after intravenous administration of the pDNA/DGL complex and pDNA/DGL/CS-A8 complex into ddY male mice. The pDNA/DGL complex showed high gene expression in the lung, liver, and spleen (Fig. 8A), as did the pDNA/DGL/CS-A8 complex (Fig. 8B). In particular, the pDNA/DGL/CS-A8 complex exhibited 10-fold higher gene expression than the pDNA/DGL complex in the spleen. The gene expression of pDNA/DGL complex showed significantly higher gene expression than the pDNA/DGL/CS-A8 complex in the lung.

4. Discussion

Recently, a dendrigraft polymer composed of naturally occurring amino acid monomers has been found to be suitable for biomaterial applications^{5,6}. DGL has been examined extensively as a vector in the gene therapy field²³⁻²⁵. In the previous study, we prepared binary complexes of pDNA with DGL at various charge ratios. The positive surface charge of the binary complex was considered to interact strongly with negatively charged cellular membranes and lead to high gene expression, which was confirmed in Fig. 2. The strong interaction of the binary complex with cellular membranes also has the potential to cause cytotoxicity in B16-F10 cells and agglutination in erythrocytes, as shown in Fig. 3 and Fig. 7. We reported previously that recharging a cationic complex with several specific anionic polymers overcame their cytotoxicity without reducing their transgene expression efficiency^{4-5,7-9,26}.

In the present study, biodegradable glycosaminoglycan was added to the pDNA/DGL complex. These decreased the surface charge of the complex with an increase in additional dose, as shown in Table 1. Some glycosaminoglycans such as heparin sulfate were reported to release pDNA from the pDNA/cationic polymer complex²⁷. However, CS and HA did not push pDNA out of the complexes (Fig. 1). The pDNA/DGL/CS-A, pDNA/DGL/CS-B, pDNA/DGL/CS-C, and pDNA/DGL/HA complexes were stable as

self-assembled nanoparticles without de-complexation. These complexes hardly showed *in vitro* cytotoxicity and gene expression in B16-F10 cells because of a low level of interaction with the anionic cellular surface. Among the complexes tested, pDNA/DGL/CS-A showed relative high uptake and gene expression, as shown in Fig. 2 and Fig. 4. In addition, CS was reported to reduce cell damage by scavenging free radicals^{26,28}. These results indicated the high safety of pDNA/DGL/CS-A complexes compared with pDNA/DGL complexes.

Glycosaminoglycans have the potential to be taken up inside cells via specific receptors. Some kinds of CS and HA have been reported to bind to the CD44 receptor²⁰⁻²². CD44 is a type I transmembrane glycoprotein participating in many cellular functions, such as cell orientation, adhesion, migration, and matrix-cell signaling processes¹⁴. It is also known to be specifically overexpressed on various tumor cells^{14,29}. The gene expression of ternary complex coated with CS in ovarian cancer cells was six-times higher than in normal cells³⁰. There is also evidence that CS also binds to CD44, albeit at a 100-fold lower concentration than HA^{20,31}. Hamada et al. demonstrated that the ternary complex of pDNA/PEI coated with CS, including the murine granulocyte macrophage-colony stimulating factor gene, prolonged mouse survival compared with a complex coated with HA after intraperitoneal injections³⁰.

In the present study, the pDNA/DGL/CS-A complexes showed higher gene expression than the other pDNA/DGL/CS complexes and pDNA/DGL/HA complex. Hamada et al. reported that the gene expression of ternary complexes coated with CS varied with the kind of CS³⁰. The internalization mechanism must depend on the chemical, structure, size, charge, shape, and stability. Further study is necessary to understand the mechanism.

The intracellular distribution and dissociation of each ternary complex was visualized using YOYO-1-labeled pDNA (Fig. 4). The pDNA/DGL/CS-A8 complexes showed extremely high uptake and gene expression in B16-F10 cells. These results indicated that a specific mechanism participates in the uptake of pDNA/DGL/CS-A8 complex. There was reported that the uptake into cells of CS-coated complexes were suppressed by CS-A which is an inhibitor of CD44 receptor¹⁵. In the present study, gene expression of pDNA/DGL/CS-A8 complexes was dose-dependently inhibited by the addition of CS-A (Fig. 5A). The complex may show a similar behavior to CS-A. The gene expression of the pDNA/DGL/CS-A8 complex was significantly inhibited by CPZ and genistein (Fig. 5B), suggesting the complex was predominantly taken up by clathrin-mediated endocytosis and caveolae-mediated endocytosis. Furthermore, although FBS decreased gene expression of both complexes, the pDNA/DGL/CS-A8 complex showed significantly higher gene expression than the pDNA/DGL complex in the presence of

FBS.

The *in vivo* gene expression of pDNA/DGL complexes and pDNA/DGL/CS-A8 complexes was examined after their intravenous injection into mice. Both of them showed high gene expression in reticuloendothelial tissues such as liver, spleen, and lung. Particle size is a factor for deciding the extravasation rate of nanoparticles from the bloodstream, and small particles sized 10–200 nm are well recognized by reticuloendothelial tissues^{14,32,33}.. The pDNA/DGL and pDNA/DGL/CS-A8 complexes are within this range. The pDNA/DGL complex showed significant higher gene expression than the pDNA/DGL/CS-A8 complex in the lung, suggesting agglutination in the pulmonary vein after intravenous injection. The pDNA/DGL/CS-A8 complex had a tendency for higher gene expression in the spleen than the pDNA/DGL complex. High *in vivo* gene expression of pDNA/DGL/CS-A8 complex must be reflected by high *in vitro* gene expression in the presence of FBS.

5. Conclusion

In this study, we developed the ternary complex of pDNA electrostatically assembled with DGL and glycosaminoglycan as a novel vector. In particular, pDNA/DGL/CS-A complexes showed high *in vitro* gene expression without cytotoxicity. Furthermore, pDNA/DGL/CS-A complexes maintained a high gene expression in the presence of fetal bovine serum. The pDNA/DGL/CS-A complexes showed *in vivo* gene expression in the reticuloendothelial systems, showing no agglutination with erythrocytes. The pDNA/DGL/CS-A complexes are a useful candidate for a biodegradable gene delivery system.

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Declaration of Interest statement

The authors report no declarations of interest.

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Figure captions

Fig. 1. Electrophoresis analysis

Gel retardation assay of each glycosaminoglycan coating complex. Each complex was loaded onto an agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide. (A) pDNA/DGL/CS-A, (B) pDNA/DGL/CS-B, (C) pDNA/DGL/CS-C, and (D) pDNA/DGL/HA complexes.

Fig. 2. *In vitro* transgene efficiency

In vitro transgene efficiency of pDNA/DGL/CS-A (A), pDNA/DGL/CS-B (B), pDNA/DGL/CS-C (C), and pDNA/DGL/HA (D) in B16-F10 cells. B16-F10 cells were transfected with each complex containing pCMV-Luc. Twenty-two hours after transfection, luciferase activity was evaluated. Each bar represents the mean \pm S.E. of three experiments.

Fig. 3. Cytotoxicity

Cytotoxicity of various complexes on B16-F10 cells. Viability of cells treated with pDNA/DGL/CS-A (A), pDNA/DGL/CS-B (B), pDNA/DGL/CS-C (C), and pDNA/DGL/HA (D) complexes was measured using a WST-1 assay. Each bar represents the mean \pm S.E. (n=7). **: P <0.01, *: P <0.05.

Fig. 4. Fluorescence microscopy images of B16-F10 cells transfected with each complex

Fluorescence microscopy images of B16-F10 cells transfected with each complex. Cells were transfected with each complex labeled with YOYO-1. Twenty four hours after transfection, phase contrast image (a), the uptake of YOYO-1 (b). (200× magnification). (A) pDNA/DGL/CS-A8, (B) pDNA/DGL/CS-B8, (C) pDNA/DGL/CS-C8, and (D) pDNA/DGL/HA8 complexes.

Fig. 5. Influence of inhibitors on transgene efficiency

Influence of inhibitors on transgene efficiency. (A) Addition of CS-A was performed for the inhibition study with the pDNA/DGL/CS-A8 complex. (B) The pDNA/DGL/CS-A8 complex was transfected in medium with various endocytotic pathway inhibitors. Each bar represents the mean \pm S.E. (n=3). **: P <0.01, *: P <0.05.

Fig. 6. Effect of fetal bovine serum on transgene efficiency

pDNA/DGL (A) and pDNA/DGL/CS-A (B) complexes were transfected in medium containing various concentrations of fetal bovine serum (FBS). Twenty-four hours after transfection, luciferase fluorescence were evaluated. Each bar represents the mean \pm S.E.

(n=3). **: P <0.01, *: P <0.05 vs control.

Fig. 7. Erythrocyte agglutination

Agglutination of complexes with erythrocytes. Each complex was added to erythrocytes, and agglutination was observed by microscopy (400× magnification). (A) PBS, (B) pDNA/DGL complex, and (C) pDNA/DGL/CS-A complex *in vivo* gene silencing of complexes

Fig. 8. *In vivo* transgene efficiency

In vivo transgene efficiencies of pDNA/DGL (A) and pDNA/DGL/CS8 (B) complexes in mice. The complexes injected intravenously into mice (40 µg DNA per mouse). At 6 hours after injection, mice were sacrificed, and each organ was dissected to quantify luciferase activity. Each bar represents the mean ± S.E. (n =3).

Tables

Table 1. Particle size and zeta-potential of the complexes

| | Size (nm) | Zeta-potential (mV) | | Size (nm) | Zeta-potential (mV) |
|------------------------------|------------|---------------------|------------------------------|------------|---------------------|
| Binary complex | | | CS-C coated complexes | | |
| pDNA/DGL | 59.5±1.3 | 39.0±0.4 | pDNA/DGL/CS-C2 | 201.1±3.9 | -18.7±0.2 |
| CS-A coated complexes | | | pDNA/DGL/CS-C4 | 180.2±15.2 | -27.3±0.2 |
| pDNA/DGL/CS-A2 | 91.9±1.7 | -19.3±0.2 | pDNA/DGL/CS-C6 | 177.0±6.4 | -32.9±0.2 |
| pDNA/DGL/CS-A4 | 95.0±7.3 | -26.4±0.1 | pDNA/DGL/CS-C8 | 132.4±73.8 | -35.0±0.9 |
| pDNA/DGL/CS-A6 | 55.4±14.1 | -29.7±0.2 | pDNA/DGL/CS-C10 | 223.8±23.6 | -37.6±1.8 |
| pDNA/DGL/CS-A8 | 66.4±22.6 | -33.0±0.7 | pDNA/DGL/CS-C12 | 100.9±62.8 | -45.9±0.6 |
| pDNA/DGL/CS-A10 | 72.6±8.6 | -35.1±0.6 | HA coated complexes | | |
| pDNA/DGL/CS-A12 | 91.4±15.0 | -36.2±0.9 | pDNA/DGL/HA2 | 64.7±1.2 | 33.7±0.1 |
| CS-B coated complexes | | | pDNA/DGL/HA4 | N.D. | N.D. |
| pDNA/DGL/CS-B2 | 146.7±72.3 | -22.8±0.6 | pDNA/DGL/HA6 | 113.9±14.7 | -26.0±0.3 |
| pDNA/DGL/CS-B4 | 125.3±63.9 | -28.9±0.5 | pDNA/DGL/HA8 | 78.7±2.0 | -21.5±0.1 |
| pDNA/DGL/CS-B6 | 109.7±54.3 | -31.7±0.7 | pDNA/DGL/HA10 | 124.8±23.1 | -36.6±0.4 |
| pDNA/DGL/CS-B8 | 99.7±14.0 | -37.5±1.9 | pDNA/DGL/HA12 | 73.2±2.1 | -36.8±0.4 |
| pDNA/DGL/CS-B10 | 98.6±19.4 | -40.4±0.9 | | | |
| pDNA/DGL/CS-B12 | 118.8±21.7 | -42.5±0.7 | | | |

Each data are the mean ± S.E. (N=3). N.D.:not detect

Fig. 1

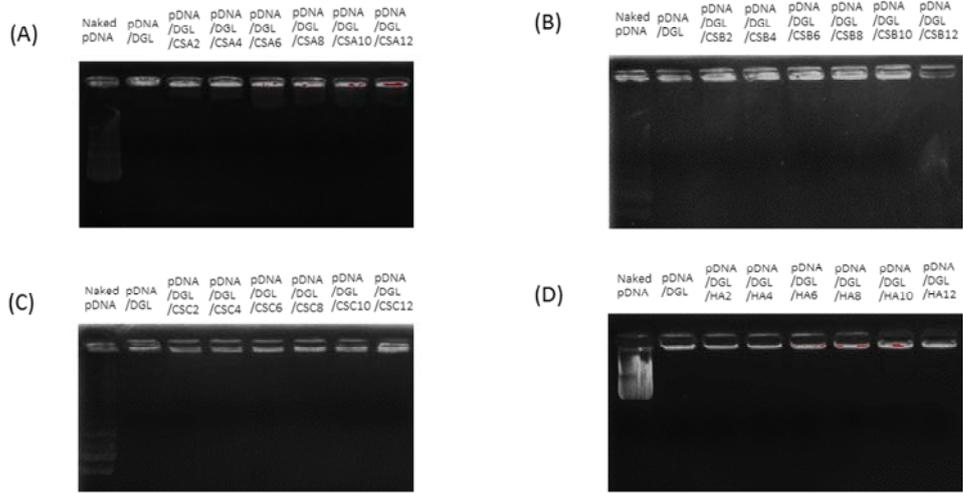


Fig. 2

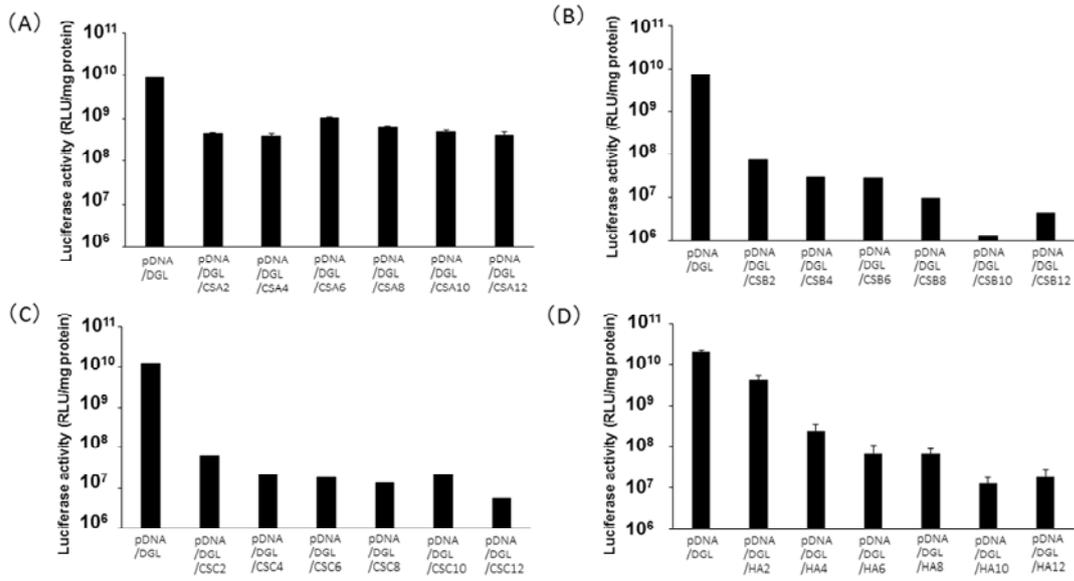


Fig. 3

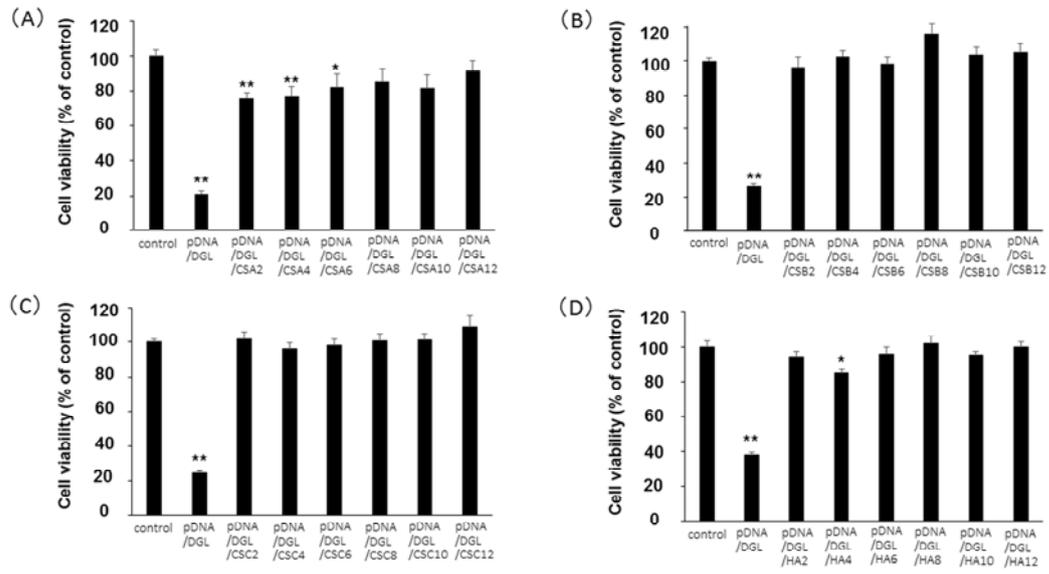


Fig. 4

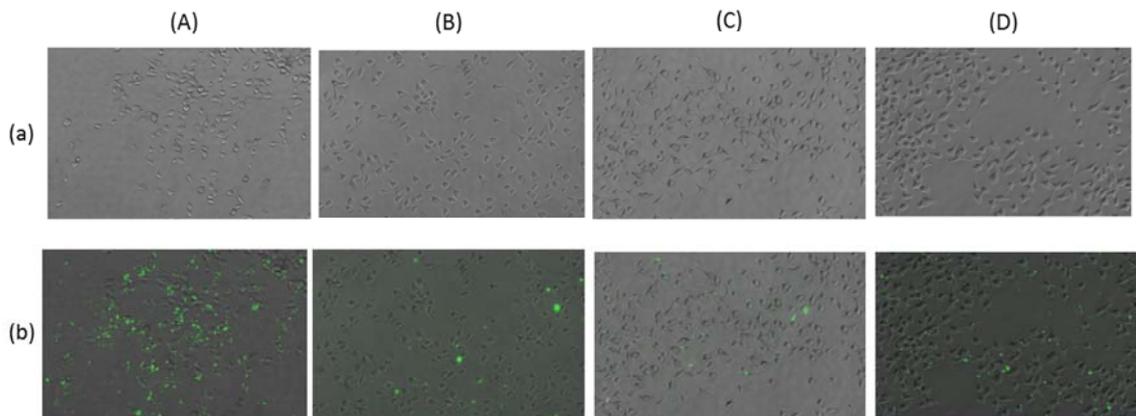


Fig. 5

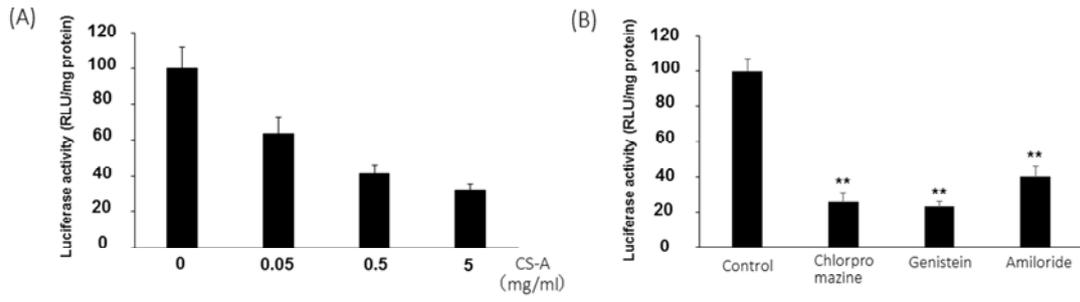


Fig. 6

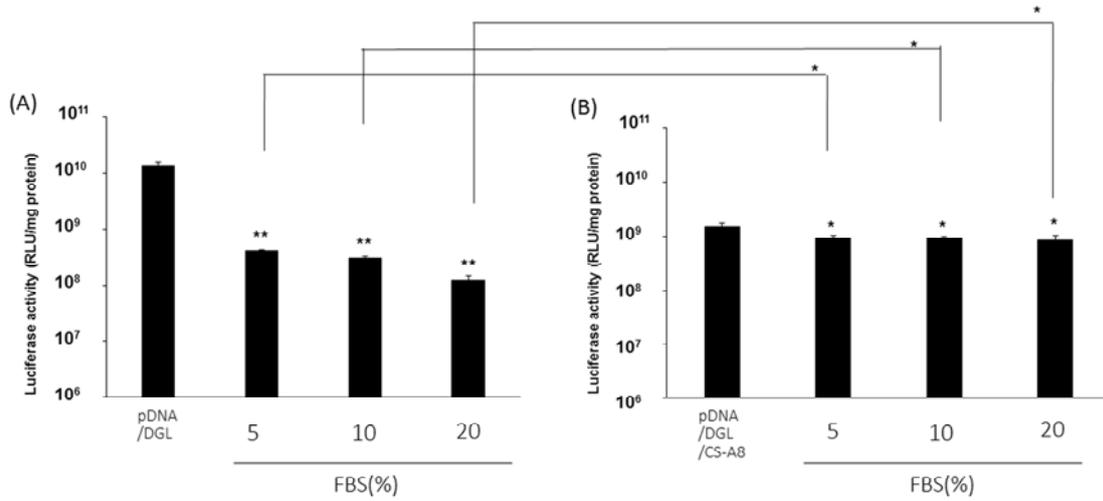


Fig. 7

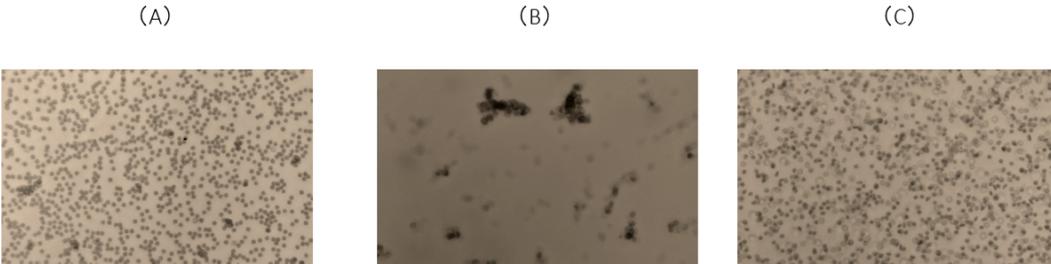


Fig. 8

