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The development of a gene vector electrostatically assembled with a polysaccharide capsule

Tomoaki Kurosaki[a], Takashi Kitahara[a], Shigeru Kawakami[b], Koyo Nishida[c], Junzo Nakamura[c], Mugen Teshima[a], Hiroo Nakagawa[a], Yukinobu Kodama[a], Hideto To[a], Hitoshi Sasaki[a*]

[a] Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan; [b] Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshidashimoadachi-cho, Sakyo-ku, Kyoto, 606-8501, Japan; [c] Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

Fax +81-95-819-7251

Telephone +81-95-819-7245

e-mail sasaki@nagasaki-u.ac.jp

Correspondence: H Sasaki, Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

Abstract

The purpose of this study was to develop a gene vector electrostatically assembled with a polysaccharide capsule. We used pDNA/polyethylenimine (PEI) complexes as efficient non-viral vectors. The pDNA/PEI complex was electrostatically encapsulated with various polysaccharides such as fucoidan, λ-carrageenan, xanthan gum, alginic acid, hyaluronic acid, and chondroitin sulfate (CS). The pDNA/PEI complex was shown as nanoparticles with positive ζ-potential, although the ternary complexes encapsulated with polysaccharides were shown as nanoparticles with negative ζ-potential. The pDNA/PEI complex showed high agglutination activity and cytotoxicity, although the ternary complexes encapsulated with polysaccharides had no agglutination activities and lower cytotoxicities. The pDNA/PEI complex showed high uptake and high transgene efficiency in B16-F10 cells. On the other hand, most of the ternary complexes show little uptake and gene expression. The ternary complex encapsulated by CS, however, showed comparable transgene efficiency to the pDNA/PEI complex. The uptake and gene expression of the ternary complex encapsulated by CS were significantly inhibited by hypothermia and the addition of CS, suggesting that the ternary complex was taken by CS-specific receptor-mediated energy-dependent process.

1. Introduction

Polysaccharides consist of monosaccharides linked together by O-glycosidic linkages, and diversification of their monosaccharides yield a variety of properties. They are found in abundance, widely available, inexpensive, and able to select some properties according to their monosaccharides [1]. Furthermore, they are also highly stable, safe, nontoxic, hydrophilic, and biodegradable, which suggests their use in targeted drug delivery systems [2, 3].

Some glucosides, such as asialoglycoproteins and galactosides, were known to be suitable for the receptor mediated drug delivery systems. It was reported that the mannosylated, fucosylated, and galactosylated liposomes showed high accumulation in the liver via each specific receptor [4]. The polysaccharides also have the potential to be taken by the cells via specific receptors. Actually, it was also reported that hyaluronic acid (HA) was taken by hyaluronic acid-specific receptor mediated endocytosis, and HA was suitable for the targeted drug delivery systems via their specific receptor [5, 6].

In the present study, we investigated the ternary complexes of pDNA/PEI encapsulated by some polysaccharides such as fucoidan, λ-carrageenan (CGN), xanthan gum (XG), alginic acid (AA), HA, and chondroitin sulfate (CS) for the effective and safe gene delivery. Among them, we firstly discovered that the ternary complex encapsulated by CS taken in the cells via CS-specific receptor and showed highest gene expression without cytotoxicity and agglutination of erythrocytes.

2. Materials and Methods

2.1. Chemicals

PEI (branched form, average molecular weight of 25,000) and rhodamine B isothiocyanate were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Fucoidan, xanthan gum from *Xanthomonas campestris,* alginic acid, hyaluronic acid, and chondroitin sulfate A sodium salt were obtained from Sigma (St. Louis, MO, USA). The λ-carrageenan was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 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)-2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were obtained from Dojindo Laboratories (Kumamoto, Japan). YOYO-1 and Hoechst 33342 were purchased from Molecular Probes (Leiden, The Netherlands). Rhodamine-PEI (Rh-PEI) was prepared in our laboratory. Briefly, PEI and rhodamine B isothiocyanate were dissolved in dimethyl sulfoxide (DMSO) and stirred overnight at room temperature in the dark. Rh-PEI was purified by gel filtration. Almost 1.5% of PEI nitrogen was labeled with rhodamine B. All other chemicals were of the highest purity available.

2.2. Preparation of pDNA and Ternary Complexes

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 the pDNA (pEGFP-C1) was purchased from Clontech (Palo Alto, CA, USA). The 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 ^{o}C until analysis. The pDNA concentration was measured at 260 nm absorbance and adjusted to 1 mg/mL. For fluorescent labeling, pDNA was mixed with the intercalating nucleic acid stain YOYO-1 using a molar ratio of 1 dye molecule per 300 base pairs for 30 minutes at room temperature in the dark.

For the preparation of ternary complexes, pDNA solution and PEI solution (pH 7.4) were mixed by pipetting thoroughly and left for 15 min at room temperature, and then each polysaccharide was mixed with pDNA/PEI complex by pipetting and left for another 15 min at room temperature. In this study, we constructed ternary complexes at a theoretical charge ratio: phosphate of pDNA: nitrogen of PEI: sulfate or carboxylate of polysaccharide = 1: 8: 6.

2.3. Physicochemical Property of Ternary Complexes

The particle sizes and ζ-potentials of ternary complexes were measured using a Zetasizer Nano ZS (Malvern Instruments, Ltd., United Kingdom). The number-fractioned mean diameter is shown.

To determine complex formations, 10 μL aliquots of ternary complex solution containing 1 μg of pDNA were mixed with 2 μL of loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded onto a 0.8% agarose gel containing 0.5 μg/mL of ethidium bromide. Electrophoresis (i-Mupid J®; Cosmo Bio, Tokyo, Japan) was carried out at 35 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, and 1 mM EDTA) for 80 min. The retardation of the pDNA was visualized using a FluorChem Imaging Systems (Alpha Innotech, CA, USA).

2.4. Agglutination study

Erythrocytes from mice were washed three times at 4 °C by centrifugation at 5,000 rpm (Kubota 3700, Kubota, Tokyo, Japan) for 5 min and resuspension 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. The 10 μL suspensions were placed on a glass plate and agglutination was observed by microscopy (400× magnification).

2.5. WST-1 Assay

The 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) under a

humidified atmosphere of 5% CO_2 in air at 37 °C. Cytotoxicity tests of various complexes on B16-F10 cells were carried out using a WST-1 commercially available cell proliferation reagent. 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 on 96-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 3.0×10^3 cells/well in the culture medium. Ternary complexes containing 1 μg of 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. Medium was replaced with 100 μL culture medium and 10 μL of the 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 (Multiskan Spectrum, Thermo Fisher Scientific, Inc., Wyman Street Waltham, MA, USA). The results are shown as a percentage of untreated cells.

2.6. Transfection Experiments

B16-F10 cells were plated on 24-well plates (Becton-Dickinson, Franklin Lakes, NJ, USA) at a density of 1.5×10^4 cells/well and cultivated in 0.5 mL culture medium. In the transfection experiment, after 24 h pre-incubation, the medium was replaced with 0.5 mL Opti-MEM I medium and each complex containing 1 μg of pDNA 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 °C.

2.7. Determinations of Uptake of Ternary Complexes and Gene Expressions

To visualize the uptake of the ternary complexes and gene expressions, B16-F10 cells were transfected by various complexes constructed with pEGFP-C1, Rh-PEI, and polysaccharides as described above. After 22 h incubation, the relative levels of Rh-PEI and GFP expression were characterized using fluorescent microscopy (200× magnification).

To determine the uptake of ternary complexes, B16-F10 cells were transfected by various complexes containing Rh-PEI as described above. After 22 h incubation, cells were washed with PBS and then lysed in 300 μL of lysis buffer (pH 7.8 and 0.1 M Tris/HCl buffer containing 0.05% Triton X-100 and 2 mM EDTA). The lysates were placed onto 96-well plates, and the fluorescence of Rh-PEI was measured at an emission wavelength of 590 nm with an excitation wavelength of 540 nm, using a fluorometric microplate reader (Fluostar OPTIMA, BMG LABTECH, Offenburg, Germany).

To determine gene expression, B16-F10 cells were transfected by various complexes containing pCMV-Luc, PEI, and polysaccharides as described above. After 22 h incubation, the cells were washed with PBS and then lysed in 100 μL of lysis buffer. Ten microliters of lysate samples were mixed with 50 μL of 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 a Bradford assay using BSA as a standard. Absorbance was measured using a microplate reader at 570 nm. Uptake of Rh-PEI was indicated as μg per mg protein, and luciferase activity was indicated as relative light units (RLU) per mg protein.

2.8. Evaluations of Intracellular Distribution of Ternary Complexes

To evaluate the intracellular distribution of complexes, B16-F10 cells were transfected as described above with the pDNA/PEI complex and pDNA/PEI/CS complex containing YOYO-1 labeled pCMV-Luc and Rh-PEI. At 21.5 h after transfection, cells were incubated with culture medium containing Hoechst 33342 for 30 min to visualize nuclei and then medium was replaced with PBS, and fluorescence distributions of YOYO-1 labeled pCMV-Luc, Rh-PEI, and Hoechst 33342 were observed with fluorescent microscopy (400× magnifications). The tone of each image was adjusted and overlapped to give a merged picture by digital processing.

2.9. Inhibition Study

For hypothermal experiments, B16-F10 cells were plated as described above and pre-incubated for 23.5 h, and the cells were incubated at 4 ^{o}C for 30 min in Opti-MEM I medium prior to the addition of complexes to the cells. After incubation, pDNA/PEI/CS complex was added to the well and incubated for a further 2 h at 4 ^{o}C. After transfection,

medium was replaced with culture medium and the cells were cultured for a further 22 h at 37 ^{o}C. For experiments using CS as inhibitors, the cells were transfected by pDNA/PEI/CS complex in Opti-MEM I medium containing various concentrations of CS. After transfection, medium was replaced with culture medium and cells were cultured for a further 22 h at 37 ^{o}C, and then the uptake of Rh-PEI and luciferase activities were determined as described above.

2.10. Statistical Analysis

Statistical significance among groups was identified by Dunnett's pairwise multiple comparison *t* test.

3. Results

3.1. Physicochemical Characteristics and Electrophoresis Assay

We determined the ζ-potentials of ternary complex at various charge ratios (Fig. 1). The addition of polysaccharides to the pDNA/PEI complex decreased its ζ-potential and reached a plateau at a charge ratio 1:8:6 of phosphate of pDNA: nitrogen of PEI: sulfate or carboxylate of polysaccharide; therefore, the ternary complex at a charge ratio of 1:8:6 was used throughout this study. The sizes and ζ-potentials of various complexes at a charge ratio of 1:8:6 were shown in Table 1. The pDNA/PEI complex showed 72.0 ± 11.1 nm particle size and +48.6 ± 0.6 mV ζ-potential. Ternary complexes, however, showed anionic charges and had significantly lower ζ-potentials than the pDNA/PEI complex ($P < 0.01$), although addition of polysaccharide did not greatly affect the size of the complex.

A gel retardation assay was performed for determination of the complex formations (Fig. 2). In the lane of naked pDNA, the pDNA was migrated in the gel, and a band was showed. The other lanes loaded various complexes, however, pDNA was not migrated in the gel and stayed in the wells.

3.2. Agglutination Study

Agglutination activities of various complexes were evaluated (Fig. 3). The pDNA/PEI complex agglutinated a lot of erythrocytes. On the other hand in the ternary complexes, any agglutination was not observed.

3.3. Cellular Toxicities

Each complex was added to B16-F10 cells and cell viability was evaluated by WST-1 assay (Fig. 4). The pDNA/PEI complex significantly decreased cell viability ($P < 0.01$). On the other hand, the cell viabilities were not decreased by the additions of ternary complexes.

3.4. Fluorescent Microscopy

The various complexes containing Rh-PEI and pEGFP-C1 were added to the B16-F10 cells for the observation of fluorescent microscopy (Figs. 5A and 5B). The bright red and green fluorescence were highly observed in the cells transfected with pDNA/PEI complex. On the other hand, no fluorescence was observed in the cells transfected with the ternary complexes encapsulated by fucoidan, CGN, and XG. In the cells transfected with the pDNA/PEI/AA complex, slight uptake and gene expression (arrowhead) could be observed. The bright red and green fluorescence were highly observed in the cells transfected with the ternary complexes encapsulated by HA and CS. The pDNA/PEI/CS complex showed comparable fluorescence to the pDNA/PEI complex.

3.5. Determination of Uptake and Transfection Activities

Each complex containing pCMV-Luc and/or Rh-PEI was added to the B16-F10 cells to

quantify uptake and gene expression (Figs. 6A and 6B). The ternary complexes encapsulated by fucoidan, CGN, XG, AA, and HA showed significantly lower uptakes than pDNA/PEI complex ($P < 0.01$, Fig. 6A). On the other hand, the pDNA/PEI/CS complex, however, showed significantly higher uptake than the pDNA/PEI complex ($P < 0.01$).

Gene expression of each complex was evaluated by luciferase activity (Fig. 6B). The pDNA/PEI complex showed extremely high gene expression exceeded 10^{10} RLU/mg protein. On the other hand, all ternary complexes were significantly lower than the pDNA/PEI complex ($P < 0.01$). The pDNA/PEI/CS complex, however, showed high gene expression exceeded 10^{10} RLU/mg protein; it dramatically higher than the commercial transfection reagent, lipofectin, showed only 2.52×10^8 RLU/mg protein under the same conditions.

3.6. Intracellular Distribution of Ternary Complexes

To visualize the intracellular distributions of pDNA/PEI and pDNA/PEI/CS complexes, YOYO-1 labeled pCMV-Luc, Rh-PEI, and Hoechst 33342 were applied (Figs. 7A and 7B). In the both complexes, green dots of YOYO-1-labeled pCMV-Luc were located mainly in the cytoplasm of most cells together with red dots of Rh-PEI. Synchronized green dots with red dots were seen as orange dots in merged images as shown in Figs. 7Av and 7Bv. On the other hand, a few dots were observed in nuclei.

3.7. Inhibition Study

Hypothermia and addition of CS were performed for the inhibition study. Figure 8A shows the uptake of the pDNA/PEI/CS complex. Uptake of the complex significantly decreased at 4 ^{o}C incubation ($P < 0.05$), and CS reduced the uptake of pDNA/PEI/CS complex concentration-dependently. Figure 8B shows the gene expression of the pDNA/PEI/CS. The same inhibitions were shown in gene expression experiment.

4. Discussion

The gene delivery vector is categorized into viral and non-viral vector. The viral vector is highly effective and has been used in clinical trials, although some severe adverse events such as immunogenicity and their oncogenic potential were of great concern for its safety [7-9]. In light of these concerns, non-viral gene delivery has emerged as a promising alternative, because non-viral vectors have advantages such as much lower immunotoxicity, a clear structure, and easy modeling [10, 11].

Polyethylenimine (PEI) is a popular cationic polymer as shown by high gene expression in *in vitro* and *in viv*o gene delivery, because of their specific mechanisms such as condensing pDNA by electrostatic interaction, binding to the cell surface, take up by the endocytotic pathway, and release of pDNA into the cytoplasm, via the so-called 'proton sponge mechanism' [12-16]. On the other hand, PEI was known to cause some severe adverse effects such as cytotoxicity and agglutination by its strong cationic charge [17].

In the present study, we prepared some ternary complexes of pDNA/PEI complex encapsulated by anionic polysaccharides for overcoming those disadvantages of PEI. Because of their highly stable, safe, nontoxic, and biodegradable, polysaccharides are useful biomaterials in targeted drug delivery systems. Some polysaccharides such as fucoidan, CGN, XG, AA, HA, and CS were used in this experiment. Among them, we newly discovered that a ternary complex encapsulated by CS was taken by the CS-specific receptor mediated pathway.

Physicochemical properties such as the sizes and ζ-potentials of these complexes were determined and are shown in Table 1. The polysaccharides changed the surface charge of pDNA/PEI complex cationic to anionic without much effect on the particle size. The concentrated capsules of the anionic polysaccharides on the complex surface were suggested, because those ternary complexes showed apparently anionic surface charge regardless of their total charge ratio +1. It was reported that the ternary complex, encapsulated pDNA/PEI complex with HA and AA, showed much large particle sizes [18, 19]; however, large particles often lead adverse effect such as microinfarctions. In this experiment, the ternary complexes showed less than 150 nm particle size, and such nano-particles should show high biocompatibility.

Some anionic polysaccharides, such as heparin sulfate and heparan sulfate, have known to release pDNA from pDNA/PEI complex [20, 21]. We performed the agarose gel electrophoresis experiment. In the any complexes, the band of pDNA was not detected, when the naked pDNA was detected as a band on agarose gel as shown in Fig. 2. These results support that stable self-assembled nano-particles were formed.

Many cationic non-viral gene vectors have been reported to show agglutination activities and cytotoxicities [17, 22]. We evaluated agglutination activities and cytotoxicities of those complexes (Figs. 3 and Fig. 4). The pDNA/PEI complex agglutinated a lot of erythrocytes, and a huge agglomeration was observed. In contrast, all ternary complexes showed no agglutination activities (Fig. 3). The basic cytotoxicities of complexes were evaluated in

the B16-F10 cells by WST-1 assay. The pDNA/PEI complex showed extremely high cytotoxicity, as shown in Fig. 4. On the other hand, all ternary complexes showed little cytotoxicity. In the previous reports, the ternary complexes, pDNA/PEI and pDNA/protamine coated with anionic polymer such as alginic acid and anionic PEG derivatives, were demonstrated to reduce agglutination and cytotoxicity [19, 23]. In the same way, the negative charges of the polysaccharides on the surface must abate the toxicities of their cationic core.

Generally, anionic complexes can not be taken up well by cells because they repulse the cellular membrane electrostatically. We evaluated the uptake of these complexes and their gene expression efficiency; the complexes loading Rh-PEI and pEGFP-C1 were added to B16-F10 cells, and the fluorescent microscopy was performed (Figs. 5A and 5B). Any uptake and gene expression were not shown in the ternary complexes encapsulated by fucoidan, CGN, and XG. Amazingly, uptake of the complex and GFP expression were observed in the ternary complexes encapsulated by AA, HA, and CS even if it had anionic surface charge. Furthermore, uptake of these complexes and their gene expression efficiency was quantified in B16-F10 cells by using the complexes loading pCMV-Luc and/or Rh-PEI (Figs. 6A and 6B). The pDNA/PEI/CS complex showed extremely high uptake and gene expression in the cells, although, the other ternary complexes showed significantly lower uptake and gene expressions than the pDNA/PEI complex ($P < 0.01$). The cationic pDNA/PEI complex was reported to be taken by cells through endocytosis according to

electrostatic interaction with cell membrane [24], however, anionic complex could not be taken by cells in the same manner. These results indicate that the specific mechanism participates in the uptake of the pDNA/PEI/CS complex with a negative charge. So, the intracellular distribution and complex dissociation of the pDNA/PEI/CS complex and the pDNA/PEI complex were visualized by using Rh-PEI, YOYO-1-labeled pDNA, and Hoechst 33342 (Figs. 7A and 7B). We were able to confirm that pDNA/PEI/CS complexes were located in the cytoplasm without dissociations and a few complexes were observed in the nuclei. It was also reported that some efficient non-viral vectors were reported to be mostly located in the cytoplasm, not nuclei [25].

It is valuable to note that pDNA/PEI/CS complex have strong gene expression regardless of its anionic surface charge. Therefore, we evaluated the inhibition effects of the hypothermia and CS on the uptake and gene expression of pDNA/PEI/CS complex (Figs. 8A and 8B). Hypothermia and the addition of CS significantly inhibited the uptake and gene expression of pDNA/PEI/CS by the cells. These results strongly indicate that an energy-dependent process and CS-specific receptor-mediated pathway concerned the uptake of pDNA/PEI/CS complex. It was reported that clearance of CS from lymph and blood in mammals is mediated by the HA receptor for endocytosis (HARE), which is present in the sinusoidal endothelial cells of liver, spleen, and lymph nodes [26, 27]. Furthermore, it was also reported that HARE was substantially colocalized with clathrin [28]. In the present study, the pDNA/PEI/CS complex might be recognized by HARE and taken by the cells via

clathrin-mediated endocytosis and released pDNA to cytoplasm from endosome by proton sponge mechanisms of PEI; and then the complex showed high gene expressions. On the other hand, the ternary complex encapsulated with HA showed lower uptake and gene expression than the complex with CS. The particular mechanisms may exist, and the further study should be performed.

5. Conclusion

We developed a non-viral vector of pDNA/PEI complex encapsulated by CS. This vector consisted of stable particles with apparently negative ζ-potential and low toxicities. On the other hand, pDNA/PEI/CS complex was highly taken by the cells via CS-specific receptor-mediated pathway and showed high gene expressions. Further studies are necessary to examine the in detailed uptake mechanism.

Figures

Fig. 1.

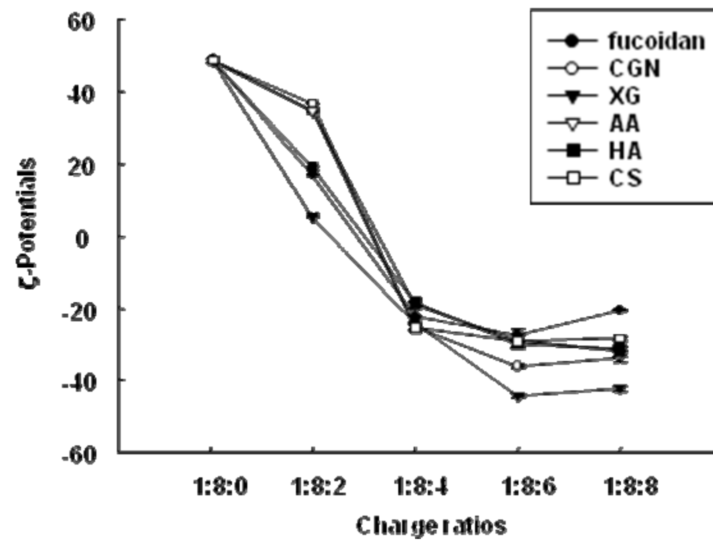


Fig. 2.

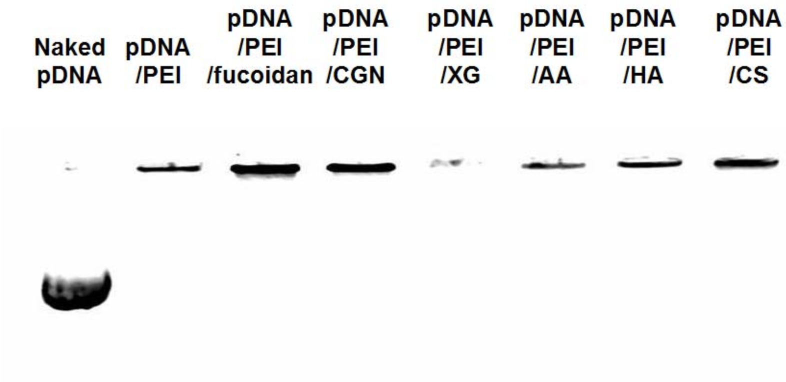


Fig. 3.

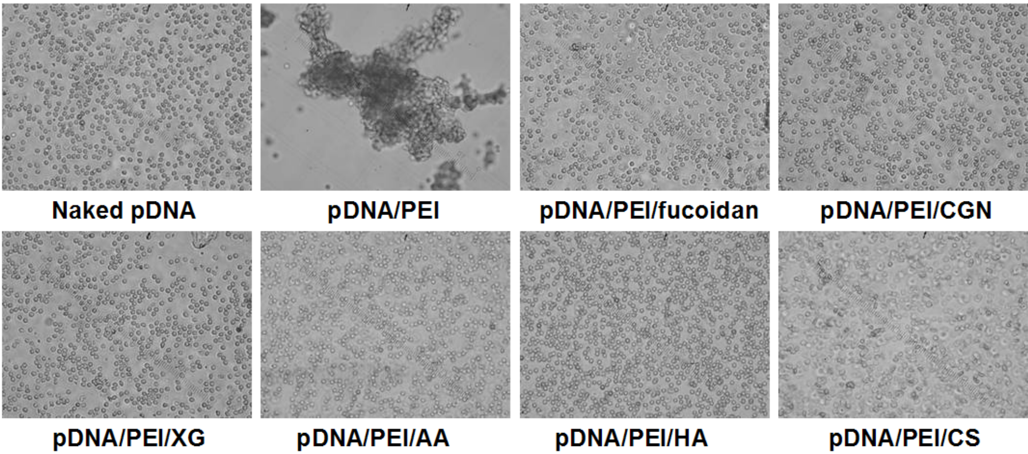


Fig. 4.

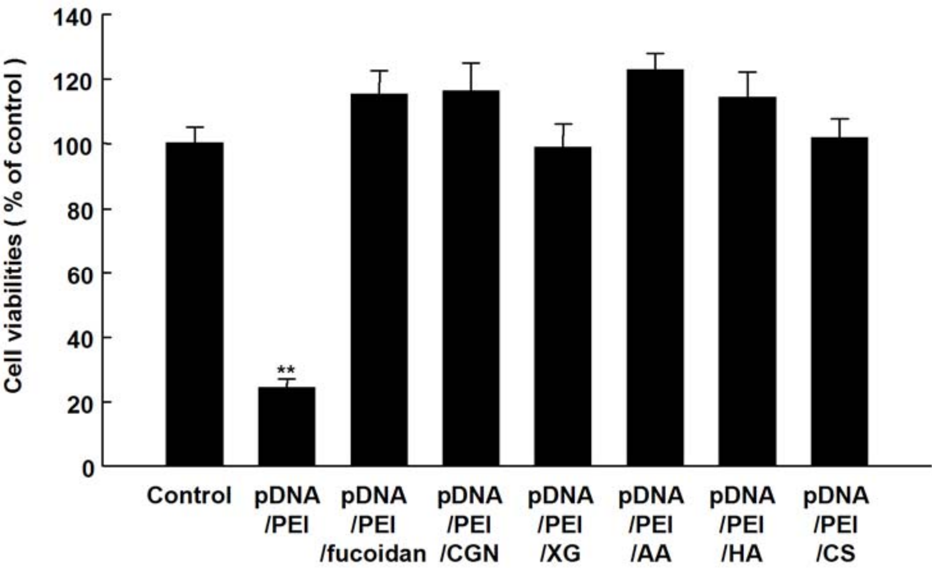


Fig. 5.

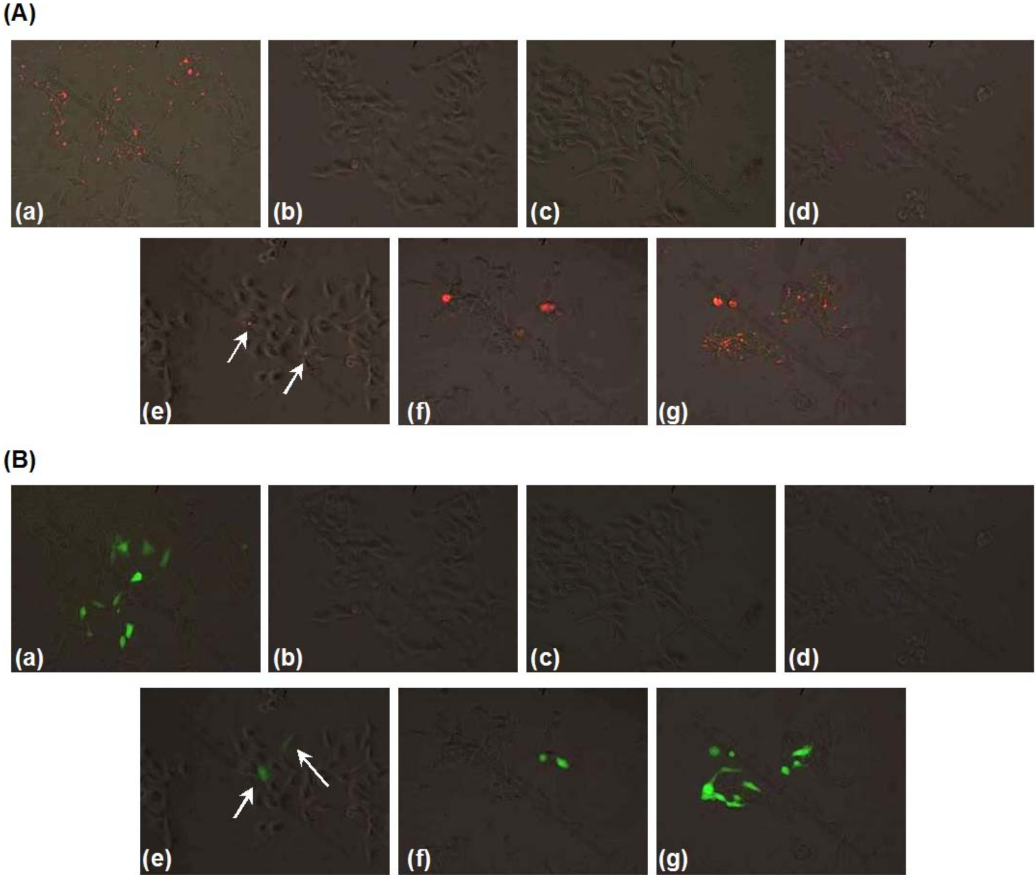


Fig. 6.

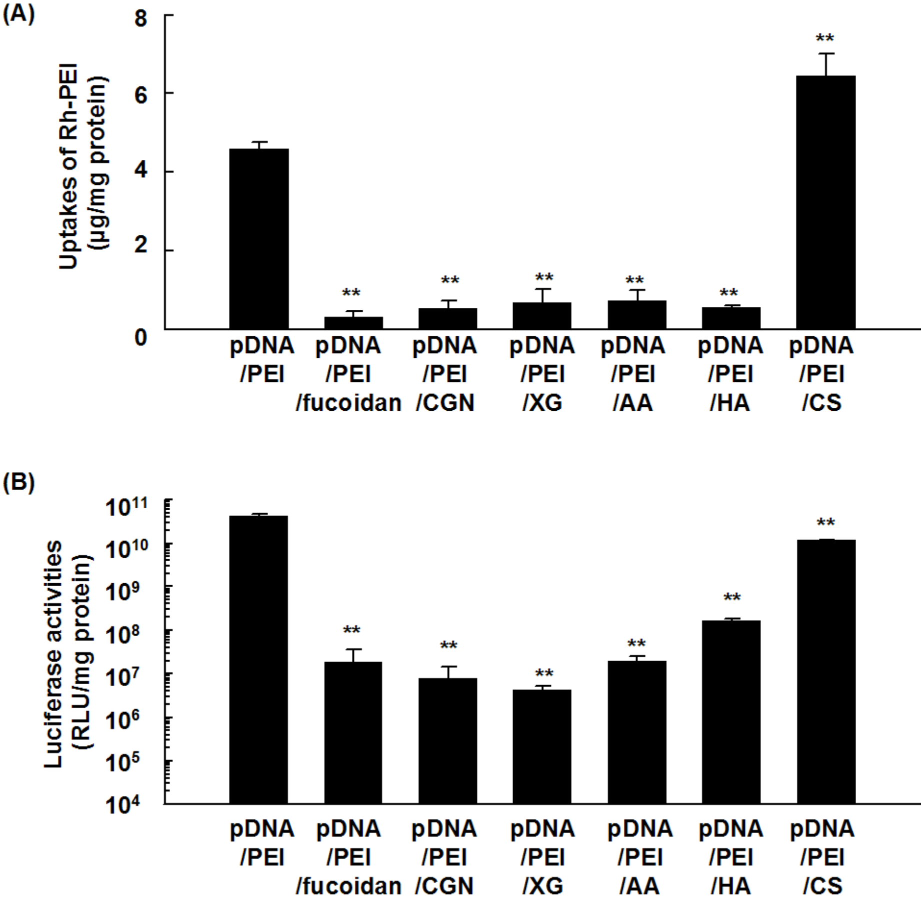
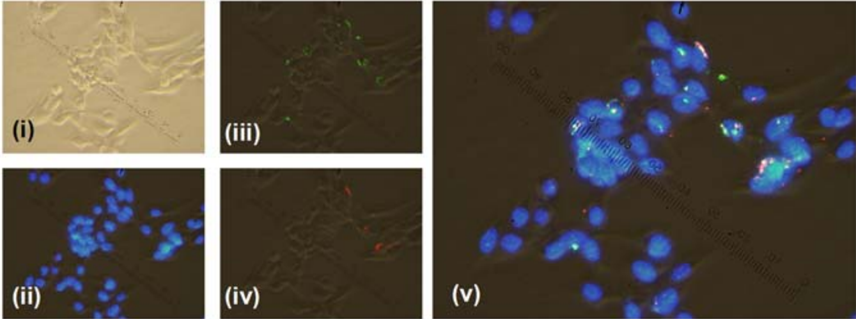


Fig. 7.

(A)



(B)

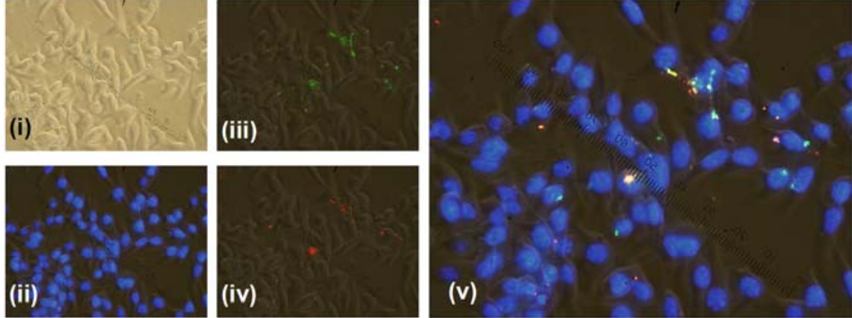


Fig. 8.

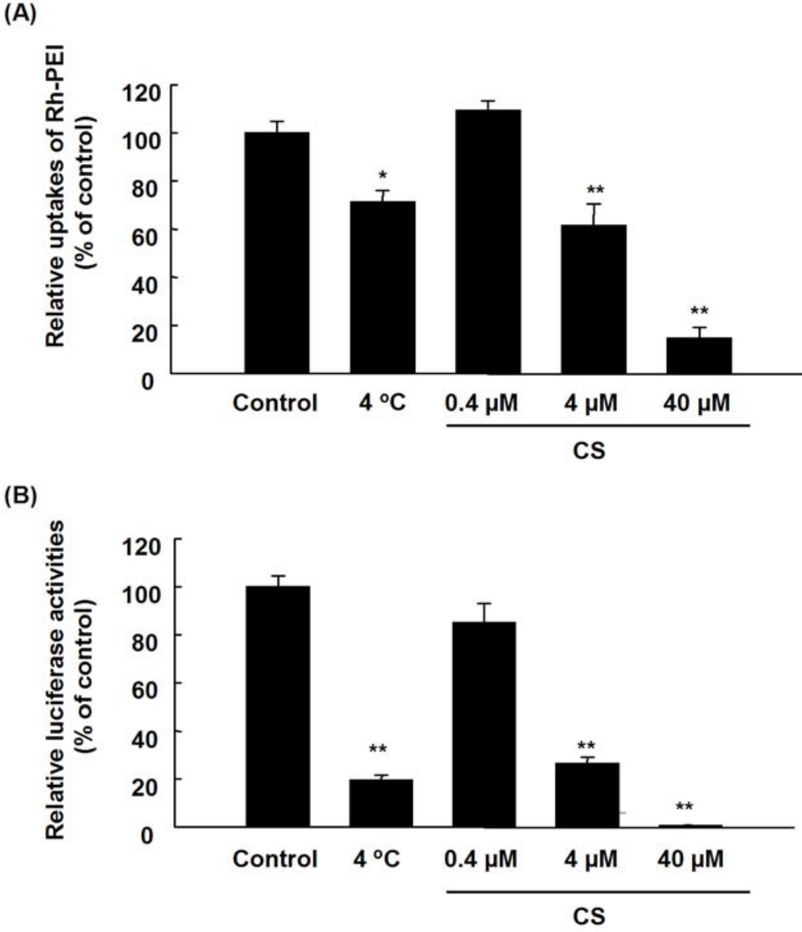


Figure captions and tables

Fig. 1. Effect of each polysaccharide on ζ-potentials of pDNA/PEI complex.

Each polysaccharide was added to the pDNA/PEI complex at various charge ratios, and ζ-potentials of the complexes were evaluated. Each value represents the mean ± S.E. of three experiments.

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

	Size	ζ-Potential
pDNA/PEI	72.0 ± 11.1	+48.6 ± 0.6
pDNA/PEI/fucoidan	71.9 ± 3.2	-27.4 ± 0.4**
pDNA/PEI/CGN	92.3 ± 33.2	-35.9 ± 0.4**
pDNA/PEI/XG	132.7 ± 35.3	-44.2 ± 0.6**
pDNA/PEI/AA	26.7 ± 6.1	-29.0 ± 1.9**
pDNA/PEI/HA	95.0 ± 8.3	-29.9 ± 0.9**
pDNA/PEI/CS	77.0 ± 3.2	-29.2 ± 0.2**

Each data was represent the mean ± S.E. (n=3).
**; $P < 0.01$ vs pDNA/PEI

Fig. 2. Effect of each polysaccharide on electrophoretic migration of pDNA through an agarose gel.

Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide.

Fig. 3. Agglutination with erythrocytes.

Each complex was added to erythrocytes, and agglutinations were assessed. Agglutination was observed by phase microscopy (400× magnification).

Fig. 4. Cytotoxicity tests of various complexes on B16-F10 cells.

Cell viability of cells treated with each complex was measured by WST-1 assay. Cells were incubated with each complex for 2 h and cell viability was measured at 24 h after treatment. Data represent the percentage to untreated cells. Each bar represents the mean ± S.E. of sixteen experiments. **: $P < 0.01$ vs control.

Fig. 5. Fluorescent microscopy images of B16-F10 cells transfected with each complex.

Cells were transfected with each complex containing pEGFP-C1 and Rh-PEI. Twenty-four hours after transfection, the uptake of Rh-PEI (A) and the expression of GFP (B) were monitored (200× magnification). (a): pDNA/PEI; (b): pDNA/PEI/fucoidan; (c): pDNA/PEI/CGN; (d): pDNA/PEI/XG; (e): pDNA/PEI/AA; (f): pDNA/PEI/HA; (g): pDNA/PEI/CS.

Fig. 6. Uptake efficiency (A) and transgene efficiency (B) of each complex.

B16-F10 cells were transfected with each complex containing pCMV-Luc and/or Rh-PEI. Twenty-four hours after transfection, fluorescence of Rh-PEI (A) and luciferase activity (B) were evaluated. Each bar represents the mean ± S.E. of three experiments. **: $P < 0.01$ vs pDNA/PEI complex.

Fig. 7. Intracellular distribution of pDNA/PEI complex (A) and pDNA/PEI/CS complex (B).

Cells were transfected with each complex containing YOYO-1-labeled pDNA and Rh-PEI. Twenty-four hours after transfection, phase contrast image (i), nuclei staining with Hoechst 33342 (ii), YOYO-1-labeled pDNA (iii), Rh-PEI (iv), and merged image (v) are indicated (400× magnification).

Fig. 8. Effect of Inhibitors on uptake efficiency (A) and transgene efficiency (B) of pDNA/PEI/CS complex. pDNA/PEI/CS complex was transfected in medium which was at 4 °C or contained various concentrations of CS. Twenty-four hours after transfection, fluorescence of Rh-PEI (A) and luciferase activity (B) were evaluated. Each bar represents the mean ± S.E. of three experiments. *: $P < 0.05$, **: $P < 0.01$ vs control.

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