Safe and Effective Delivery of Small Interfering RNA with Polymer- and Liposomes-Based Complexes

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We developed binary and ternary complexes based on polymers and liposomes for safe and effective delivery of small interfering RNA (siRNA). Anti-luciferase siRNA was used as a model of nucleic acid medicine. The binary complexes of siRNA were prepared with cationic polymers and cationic liposomes such as polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimer, poly-L-arginine (PLA), trimethyl[2,3-(dioleoxy)-propyl]ammonium chloride (DOTMA), and cholesteryl 3*β*-*N*-(dimetylaminnoethyl)carbamate hydrochloride (DC-Chol). The ternary complexes were constructed by the addition of *γ*-polyglutamic acid (*γ*-PGA) to the binary complexes. The complexes were approximately 54–153 nm in particle size. The binary complexes showed a cationic surface charge although an anionic surface charge was observed in the ternary complexes. The polymer-based complexes did not show a silencing effect in the mouse colon carcinoma cell line expressing luciferase regularly (Colon26/Luc cells). The binary complexes based on liposomes and their ternary complexes coated by *γ*-PGA showed a significant silencing effect. The binary complexes showed significant cytotoxicity although the ternary complexes coated by *γ*-PGA did not show significant cytotoxicity. The ternary complexes coated by *γ*-PGA suppressed luciferase activity in the tumor after their direct injection into the tumors of mice bearing Colon26/Luc cells. Thus, we have newly identified safe and efficient ternary complexes of siRNA for clinical use.

Key words gene delivery; small interfering RNA; cancer; *γ*-polyglutamic acid; cationic liposome; cationic polymer

Small interfering RNA (siRNA) is a class of double-stranded RNA molecules, 20–25 base pairs in length, which is able to induce RNA interference (RNAi). siRNA is incorporated into the RNAi-induced silencing complex (RISC) and is a guide for cleavage of the complementary target mRNA in the cytoplasm.[1)] Gene silencing using siRNA has prospective applications in the treatment of various diseases including cancer,[2)] viral infection,[3)] and genetic disorders.[4)]

The therapeutic effect of siRNA *in vivo* is limited by poor cellular uptake due to the large molecular weight and negative phosphate charge, rapid degradation by nucleases, and rapid renal clearance.[5,6)] Clinical use of siRNA is largely dependent on adequate delivery systems that can efficiently protect and accumulate siRNA molecules in target cells and tissues.

The promising approach is chemical modifications of siRNA. Several chemical approaches have been reported to greatly increased resistance to nuclease degradation without reduction of gene silencing activity[7,8)]; however, there was a report that the increase in stability did not translate into enhanced or prolonged inhibitory activity of target gene reduction in mice.[9)] Another promising approach is to use the vectors for siRNA to be protected from degradation and be delivered into target cells.

We have reported many effective vectors for plasmid DNA, binary complexes based on cationic polymers and cationic liposomes.[10–16)] We have also demonstrated the high efficiency and low toxicity of ternary complexes constructed with binary complexes and anionic components such as *γ*-polyglutamic acid (*γ*-PGA)[10,13)] These vectors are useful carriers of siRNA, which is the same nucleic acid treatment as plasmid DNA. Therefore, in the present study, we developed binary and ternary complexes based on polymers and liposomes for siRNA delivery and evaluated their silencing efficiencies and toxicities. Binary complexes of siRNA were prepared with cationic polymer and cationic liposomes such as polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimer, poly-L-arginine (PLA), trimethyl[2,3-(dioleoxy)-propyl]ammonium chloride (DOTMA), and cholesteryl 3*β*-*N*-(dimetylaminoethyl)-carbamate hydrochloride (DC-Chol). Ternary complexes were constructed by the addition of *γ*-PGA to the binary complexes.

MATERIALS AND METHODS

Chemicals and Reagents PEI (branched form, average molecular weight (MW) of 25000) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). *γ*-PGA was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). PAMAM dendrimer based on an ethylenediamine core of the fifth generation (MW 28826 Da, 128 N-terminal amines) and PLA were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). DC-Chol was obtained from Sigma Aldrich. DOTMA was purchased from Toyo Chemical Industry Co., Ltd. (Tokyo, Japan). The 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was obtained from Nippon Oil and Fats Co. (Tokyo, Japan). Lipofectamine RNAiMAX and Alexa Fluor 555-labeled siRNA (BLOCK-iT Alexa Fluor Red Fluorescent Oligo) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Firefly luciferase siRNA

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(sense: 5′-CUUACGCUGAGUACUUCGAdTdT-3′, antisense: 5′-UCGAAGUACUCAGCGUAAGdTdT-3′) and scramble siRNA (sense: 5′-CUUACGCUGUCAUGAUCGAdTdT-3′, antisense: 5′-UCGAUCAUGACAGCGUAAGdTdT-3′) were obtained from GeneDesign, Inc. (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Sigma Aldrich. Fetal bovine albumin (FBS) was obtained from Biological Industries Ltd. (Kibbuts Beit Haemek, Israel). For the cell culture, RPMI 1640, Opti-MEM I and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL) were purchased from GIBCO BRL (Grand Island, NY, U.S.A.). The antibiotic G418 solution was obtained from Roche Diagnostics (Indianapolis, IN, U.S.A.). The 2-(4-iodophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade.

Preparation of Polymer-Based Complexes In this study, we constructed complexes at a theoretical charge ratio: phosphate of siRNA : nitrogen of PEI, PAMAM, PLA, DC-Chol or DOTMA : carboxylate of γ-PGA.

To prepare binary complexes based on the polymer, an appropriate amount of stock polymer solution was mixed with siRNA dissolved in diethylpyrocarbonate (DEPC)-treated water (Invitrogen) (1 mg/mL) by pipetting thoroughly at a charge ratio of 1 : 8 (siRNA/PEI8 complex, siRNA/PAMAM8 complex, and siRNA/PLA8 complex), and left for 30 min at room temperature. To prepare ternary complexes based on the polymer, γ-PGA solution was added to each binary complex at a charge ratio of 1 : 8 : 6 (siRNA/PEI8/γ-PGA6 complex and siRNA/PAMAM8/γ-PGA6 complex) or 1 : 8 : 15 (siRNA/PLA8/γ-PGA15 complex), and left for another 30 min at room temperature.

Preparation of Liposomes-Based Complexes DC-Chol and DOPE, DOTMA and DOPE (1 : 1 molar ratio) were dissolved in chloroform and dried as a thin film in each test tube using an evaporator at room temperature, and then vacuum-desiccated for approximately 3 h. The film was hydrated in 5% sterile dextrose overnight. After hydration, the solutions were sonicated at 100 W (Sonicator; Ohtake Works Co., Tokyo, Japan) for 3 min on ice. The solutions were then extruded 11 times through a double-stacked 100 nm polycarbonate membrane filter. To prepare binary complexes based on liposomes, an appropriate amount of stock liposome solution was mixed with a siRNA (1 mg/mL) by pipetting thoroughly, and left for 30 min at room temperature. The charge ratio was 1 : 6 for siRNA/DC-Chol-DOPE6 complex or 1 : 2 for siRNA/DOTMA-DOPE2 complex. To prepare ternary complexes based on liposomes, γ-PGA solution was added to each binary complex at a charge ratio of 1 : 6 : 10 (siRNA/DC-Chol-DOPE6/γ-PGA10 complex) or 1 : 2 : 6 (siRNA/DOTMA-DOPE2/γ-PGA6 complex). siRNA/Lipofectamine complex was prepared as a product protocol and used as a positive control.

Physicochemical Properties of Complexes The particle size and ζ-potential of complexes were measured with Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, U.K.). The number-fractioned mean diameter is shown.

To determine the complex formation, 20 μL aliquots of complex solutions containing 1 μg of siRNA were mixed with 4 μL loading buffer (30% glycerol and 0.2% bromophenol blue) and loaded on a 2% agarose gel. Electrophoresis (i-Mupid J; Cosmo Bio, Tokyo, Japan) was carried out at 100 V in running buffer solution (40 mM Tris–HCl, 40 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)) for 20 min. The retardation of siRNA was visualized with ethidium bromide staining using a Gel Doc EZ System (Bio-Rad Laboratories, Inc., Tokyo, Japan).

Cell Culture The mouse colon carcinoma cell line expressing luciferase regularly (Colon26/Luc cells) was prepared in our laboratory. Briefly, to establish Colon26/Luc cells, Colon26 cells were transfected with plasmid DNA encoding luciferase reporter gene (pCMV-Luc) and selected by G418. The pCMV-Luc 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 the pcDNA vector (Invitrogen).

***In Vitro* Gene Silencing Experiment** The Colon26/Luc cells were 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 culture medium. In the silencing experiment, after 24 h pre-incubation, the medium was replaced with 500 μL Opti-MEM I (transfection medium) and each complex containing 1 μg siRNA was added to the cells and incubated for 2 h. Then, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37°C. After 22 h incubation, the cells were washed with phosphate-buffered saline (PBS) and then lysed in 100 μL lysis buffer (pH 7.8 and 0.2 M Tris–HCl buffer containing 2 mM EDTA and 0.05% Triton X-100). Ten microliters of lysate samples were mixed with 50 μL luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) and the light produced was immediately measured using a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). The protein content of lysate was determined by a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) using BSA as a standard. Absorbance was measured using a microplate reader (Sunrise RC-R; Tecan Japan Co., Ltd., Kanagawa, Japan) at 595 nm. Luciferase activity was recorded as relative light units (RLU) per mg protein and the results are shown as a percentage of untreated cells (control).

Cellular Uptake of siRNA To visualize the uptake of the complexes, the cells were transfected with complexes containing Alexa Fluor-labeled siRNA. Fluorescence was observed with fluorescence microscopy (200× magnification; BZ-9000; KEYENCE, Osaka, Japan). The tone of each image was adjusted and overlapped to give a merged picture by digital processing.

Cytotoxicity Cytotoxicity of various complexes on Colon26/Luc cells was measured using commercially available WST-1 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, U.S.A.) just before the experiments. Colon26/Luc cells were plated on 96 well plates (Becton-Dickinson) at a density of 5.0×10^3 cells/well in the culture medium. Each complex containing 0.5 μg of siRNA in 100 μL Opti-MEM I was 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. The medium was replaced with 100 μL culture medium, and then 10 μL of the WST-1 reagent was added to each well. The cells were incubated for

Table 1. Size and ζ-Potential of Complexes

		Size (nm)	ζ-Potential (mV)
Polymer-based complexes	siRNA/PEI8	86.6±55.0	48.5±1.5
	siRNA/PAMAM8	67.7±9.1	47.3±1.5
	siRNA/PLA8	54.3±60.2	47.8±1.1
	siRNA/PEI8/γ-PGA6	70.3±28.2	−35.2±0.8
	siRNA/PAMAM8/γ-PGA6	55.1±18.4	−39.6±2.2
	siRNA/PLA8/γ-PGA15	137.8±3.8	−17.0±1.0
Liposomes-based complexes	siRNA/Lipofectamine	86.8±3.5	45.8±0.3
	siRNA/Dc-Chol-DOPE6	67.2±10.1	40.8±0.6
	siRNA/DOTMA-DOPE2	124.5±4.8	48.7±0.6
	siRNA/Dc-Chol-DOPE6/γ-PGA10	152.7±12.4	−32.1±0.1
	siRNA/DOTMA-DOPE2/γ-PGA6	113.8±8.0	−39.8±0.8

Each value represents the mean±S.D. (n=3).

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 of untreated cells (control).

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. Female BALB/c mice (4 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for at least one day before experiments.

***In Vivo* Gene Silencing Experiment** The transfected Colon26/Luc cells (5×10^5 cells per mouse) suspended in 100 μL PBS were injected intracutaneously into the flank of BALB/c mice.

To evaluate the *in vivo* silencing effect of the siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex, when the volume of the tumors reached 100 mm^3, each complex containing 10 μg siRNA and naked siRNA that targeted luciferase was injected directly into the tumors. After 24 and 48 h, mice were sacrificed and the tumors were dissected. The tumor tissues were homogenized in lysis buffer, and the homogenates were centrifuged at 15000 rpm for 5 min (Kubota 3500; Kubota, Tokyo, Japan). The supernatants were used for the luciferase assay, as described above. Luciferase activity was described as RLU per g of tissue and the results are shown as a percentage of untreated cells (control).

Tumor volume was estimated according to the formula: tumor volume (mm^3)=(smallest diameter)^2×(longest diameter)/2.

Statistical Analysis Statistical significance among groups was identified by Dunnett's pairwise multiple comparison test.

RESULTS

Physicochemical Properties of Complexes The particle size and ζ-potential of complexes are shown in Table 1. The polymer-based complexes were approximately 54–138 nm in particle size. In the polymer-based complexes, the binary complexes showed a cationic surface charge although an anionic surface charge was observed in the ternary complexes because of the addition of γ-PGA. The liposomes-based complexes were approximately 67–153 nm in particle size. In the liposomes-based complexes, the binary complexes showed a cationic surface charge although an anionic surface charge was observed in the ternary complexes because of the addition of γ-PGA.

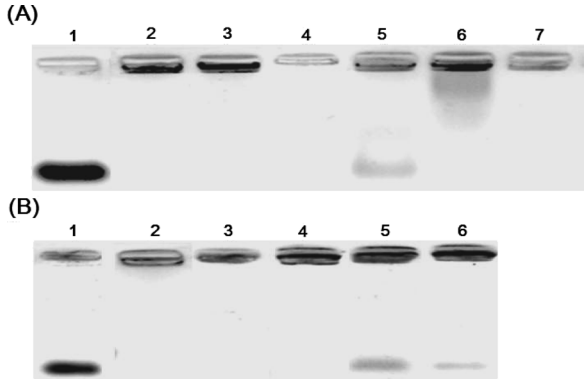

Fig. 1. Gel Retardation Assay

Polymer-based complexes (A) and liposomes-based complexes (B) were loaded onto agarose gel, and electrophoresis was carried out. Naked siRNA was run in lane 1. The siRNA was visualized with ethidium bromide staining. (A) siRNA/PEI8 complex (lane 2), siRNA/PAMAM8 complex (lane 3), siRNA/PLA8 complex (lane 4), siRNA/PEI8/γ-PGA6 complex (lane 5), siRNA/PAMAM8/γ-PGA6 complex (lane 6), and siRNA/PLA8/γ-PGA15 complex (lane 7). (B) siRNA/Lipofectamine complex (lane 2), siRNA/DC-Chol-DOPE6 complex (lane 3), siRNA/DOTMA-DOPE2 complex (lane 4), siRNA/DC-Chol-DOPE6/γ-PGA10 complex (lane 5), and siRNA/DOTMA-DOPE2/γ-PGA6 complex (lane 6).

To examine the complex formation, a gel retardation assay was employed (Fig. 1). Naked siRNA was detected as a band on the agarose gel. In polymer-based complexes (Fig. 1A), the binary complexes showed no band of naked siRNA. On the other hand, siRNA/PEI8/γ-PGA6 complex and siRNA/PAMAM8/γ-PGA6 complex showed a slight band, although no band was observed in the siRNA/PLA8/γ-PGA15 complex. In liposomes-based complexes (Fig. 1B), the binary complexes showed no band of naked siRNA, although a slight band was observed in ternary complexes.

***In Vitro* Gene Silencing Effect** Colon26/Luc cells were transfected with various complexes, and silencing efficiencies were evaluated (Fig. 2). The polymer-based complexes did not inhibit luciferase activity of Colon26/Luc cells, although siRNA/PEI8 complex showed a slightly silencing effect (Fig. 2A). In the liposomes-based complexes, the commercial vector, siRNA/Lipofectamine complexes, showed a strong silencing effect. The binary and ternary complexes including γ-PGA also showed significant luciferase knockdown (Fig. 2B). No silencing effect was confirmed in all complexes using scramble

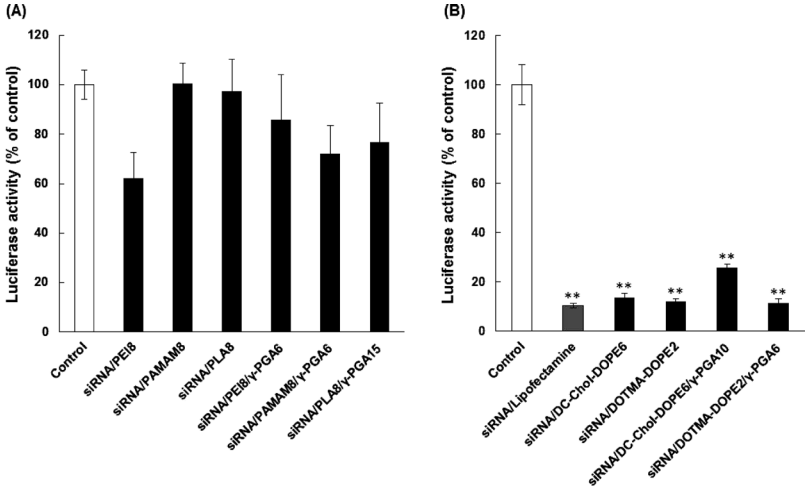

Fig. 2. *In Vitro* Gene Silencing of Complexes

Colon26/Luc cells were incubated with polymer-based complexes (A) and liposomes-based complexes (B) for 2 h. After 22 h, luciferase activity of cells was analyzed, normalized with the protein concentration and expressed as a percentage of luminescence intensity compared to the control. Each bar represents the mean±S.E. (n=3). $^{**}p<0.01$ *vs.* control.

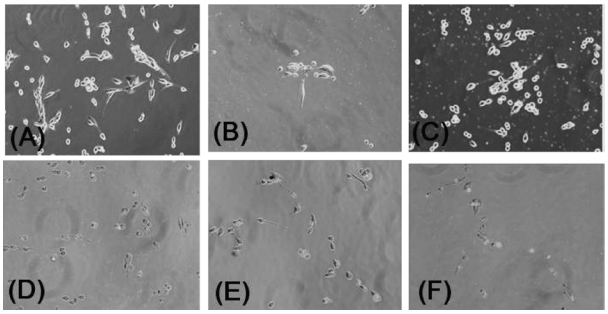

Fig. 3. Cellular Uptake of siRNA

Colon26/Luc cells were incubated with complexes, and fluorescent images (red fluorescence of Alexa Fluor 555 labeled siRNA) were taken by fluorescence microscopy. Control (A), siRNA/Lipofectamine complex (B), siRNA/DC-Chol-DOPE6 complex (C), siRNA/DOTMA-DOPE2 complex (D), siRNA/DC-Chol-DOPE6/γ-PGA10 complex (E), and siRNA/DOTMA-DOPE2/γ-PGA6 complex (F). (Color images were converted into gray scale.)

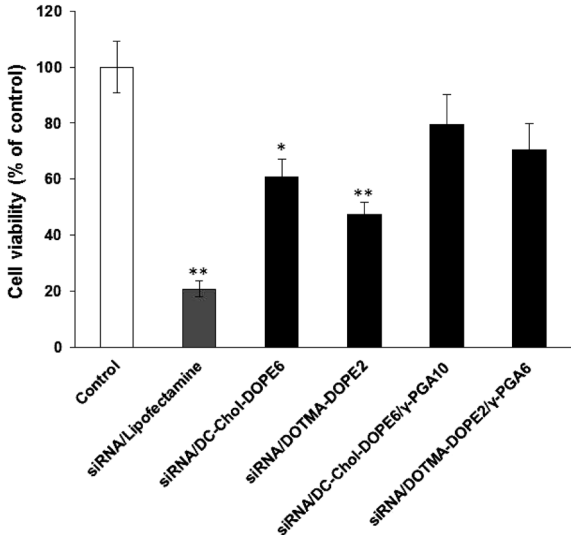

Fig. 4. Cytotoxicity of Complexes in Colon26/Luc Cells

Cell viability after treatment with complexes was determined by WST-1 assay. Each bar represents the mean±S.E. (n=8). $^{*}p<0.05$, $^{**}p<0.01$ *vs.* control.

siRNA (data not shown).

Cellular Uptake of siRNA The cellular uptake of siRNA was observed with fluorescent microscopy in siRNA/Lipofectamine complex, siRNA/DC-Chol-DOPE6 complex, siRNA/DOTMA-DOPE2 complex, siRNA/DC-Chol-DOPE6/γ-PGA10 complex, and siRNA/DOTMA-DOPE2/γ-PGA6 complex, which showed a significant silencing effect. As compared to the control, the red fluorescence signals of Alexa Fluor 555-labeled siRNA were strongly observed in cells treated with liposomes-based complexes (Fig. 3).

Cytotoxicity The cytotoxicity of liposomes-based complexes such as siRNA/Lipofectamine complex, siRNA/DC-Chol-DOPE6 complex, siRNA/DOTMA-DOPE2 complex, siRNA/DC-Chol-DOPE6/γ-PGA10 complex, siRNA/DOTMA-DOPE2/γ-PGA6 complex was evaluated with the WST-1 assay in Colon26/Luc cells (Fig. 4). The siRNA/Lipofectamine complex had the highest cellular toxicity and the binary complexes also showed significant toxicity. On the other hand, ternary complexes such as siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex did not show significant cytotoxicity.

***In Vivo* Gene Silencing Effect** *In vivo* gene silencing effect of siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex was evaluated in mice bearing Colon26/Luc cells. These ternary complexes showed an *in vitro* gene silencing effect without cytotoxicity. Each complex was directly injected into the tumors. Mice were treated with naked siRNA (targeted luciferase) as negative controls. The tumors were dissected and their luciferase activities were measured with a luminometer. Naked siRNA did not show a significant silencing effect (Fig. 5). On the other hand, the siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex significantly suppressed the luciferase activity after 24 h (Fig. 5A). However, both complexes did not show a significant silencing effect after 48 h (Fig. 5B).

DISCUSSION

Sequence-specific gene silencing with siRNA has trans-

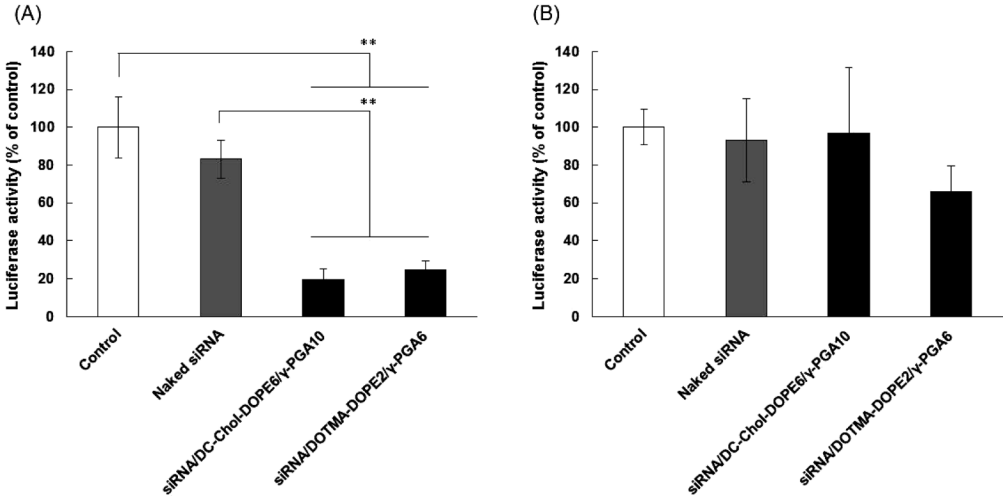

Fig. 5. *In Vivo* Gene Silencing of Complexes

The complexes were injected directly into the tumors of mice (10 μg siRNA per mouse). After 24 (A) and 48 (B) h, mice were sacrificed and tumors were dissected to measure their luciferase florescence. Each bar represents the mean±S.E. (n=6). **p<0.01.

formed basic science research, and the efficacy of siRNA therapeutics for a variety of diseases is now being evaluated in pre-clinical and clinical trials.[17] Despite its potential value, the highly negatively charged siRNA has the classic delivery problem of requiring transport across cell membranes to the cytosol. Several vehicles for delivery of siRNA are currently being tested for their efficacy in animal studies, including liposomes,[18] cyclodextrin,[19] polymers such as PEI,[20] peptides,[21] micelles,[22] siRNA conjugates,[23] antibody-protamine fusion carriers[24] and polyconjugates[25]; however, there are no clinically proven effective carriers of siRNA.

We developed polymer-based and liposomes-based complexes for siRNA delivery. In previous reports, the highest gene expression of plasmid DNA complexes was about 8 and 2 charge ratio of cationic polymer and liposomes to plasmid DNA, respectively.[10,13,16] Some liposomes (DC-Chol-DOPE) aggregated with siRNA at a charge ratio of less than 6. On the basis of preliminary results, binary complexes were constructed with a polymer to siRNA at a charge ratio of 8. Binary complexes based on liposomes were constructed with DC-Chol-DOPE and DOTMA-DOPE to siRNA at charge ratios of 6 and 2, respectively. To prepare ternary complexes, γ-PGA was added to binary complexes until ζ-potential reached the steady state of the surface negative charge.

The polymer-based complexes and liposomes-based complexes were approximately 54–153 nm in particle size. The binary complexes had a cationic surface charge and showed no band of naked siRNA in the gel retardation assay, indicating the formation of stable particles. On the other hand, an anionic surface charge was observed in the ternary complexes because of the addition of γ-PGA, suggesting that the outside of particles was coated with an anionic component. The large size of siRNA/PLA8/γ-PGA15 might be explained by a weak affinity of γ-PGA to PLA, which may be caused by steric hinderance. The ternary complexes, except for the siRNA/PLA8/γ-PGA15 complex, showed a slight release of siRNA from the complexes. We reported that the ternary complexes of plasmid DNA, which was constructed at the same charge ratio as siRNA ternary complexes, showed no release of plasmid DNA from the complexes.[10,13,16] The intensity of electrical interaction with cationic polymers and liposomes of siRNA may be weaker than that of plasmid DNA because siRNA is a low molecular nucleic acid compared with plasmid DNA. On the other hand, γ-PGA has many carboxylates and strongly interacts with cationic polymers and liposomes. Thus, γ-PGA may push siRNA out partly by electrical interaction.

Colon26/Luc cells was prepared in our laboratory. *In vitro* gene silencing of the complexes, including anti-luciferase siRNA, was evaluated (Fig. 2). The commercial vector, siRNA/Lipofectamine complex, showed a strong silencing effect.

Generally, PEI and PAMAM dendrimer have shown efficacy as carriers of siRNA. Schiffelers *et al.* developed self-assembling PEGylated PEI siRNA nanoparticles targeting tumor angiogenesis.[26] Waite *et al.* reported the highest silencing effect of PAMAM dendrimer at a charge ratio of 20 to siRNA and high degrees of acetylation of PAMAM reduced the gene silencing effect.[27] In the present study, siRNA/PEI8 complex showed a slight but not significant silencing effect. The polymer-based complexes did not silence the luciferase activity of Colon26/Luc cells (Fig. 2A). The charge ratio of cationic polymers might be not enough to have a large silencing effect; however, an excess amount of cationic components in the complexes is associated with increased toxicity.

Nanoparticles including siRNA are taken up by cellular endocytosis. Once released by nanoparticles into the cytosol, siRNA is incorporated into RISC, a protein-RNA complex that separates the strands of the RNA duplex and discards the sense strand.[17] The present polymer-based complexes may release little siRNA into the cytosol. On the other hand, binary complexes based on liposomes showed significant luciferase knockdown (Fig. 2B). The ternary complexes including γ-PGA showed significant luciferase knockdown. We and Peng *et al.* demonstrated the high cellular uptake of complexes including γ-PGA by a specific mechanism.[10,16,28] High cellular uptake of labeled siRNA, which was observed in over 80% of cells in the visual field, was confirmed in the complexes showing a significant silencing effect, which included siRNA/Lipofectamine complex, siRNA/DC-Chol-DOPE6 complex, siRNA/DOTMA-DOPE2 complex, siRNA/DC-Chol-DOPE6/

γ-PGA10 complex, and siRNA/DOTMA-DOPE2/γ-PGA6 complex (Fig. 3). These uptake efficiencies are in good agreement with the silencing efficiencies of complexes *in vitro*. Further study including siRNA release from complex is necessary to explain the different gene silencing effects of various complexes.

Cellular toxicity was evaluated with the WST-1 assay in Colon26/Luc cells in the complexes showing a significant silencing effect (Fig. 4). The siRNA/Lipofectamine complex showed the highest cellular toxicity and significant toxicity was observed in binary complexes; however, ternary complexes such as siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex did not show significant cytotoxicity. The strong positive charge of complexes was associated with cellular toxicity.[29,30] Anionic surface charges of the ternary complexes decrease cytotoxicity by reducing cellular interactions.

In vivo gene silencing of siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex was evaluated in mice bearing Colon26/Luc cells. These complexes, showing a significant gene silencing effect and no cytotoxicity *in vitro*, significantly suppressed luciferase activity in tumors 24 h after direct injection into the tumors of mice, although naked siRNA did not significantly decrease luciferase activity (Fig. 5A). Silencing effect of both complexes disappeared at 48 h after administration (Fig. 5B). The siRNA/DC-Chol-DOPE6 complex and siRNA/DOTMA-DOPE2 complex were not administrated because of their strong cytotoxicity and blood agglutination.

Thus, we newly found that siRNA/DC-Chol-DOPE6/γ-PGA10 complex and siRNA/DOTMA-DOPE2/γ-PGA6 complex showed *in vitro* and *in vivo* silencing effects without cytotoxicity. The γ-PGA is known to be biocompatible and produced by microbial species typified by *Bacillus subtilis*.[31] Synthesized γ-PGA showed little toxic effect on the human B-cell line EHRB, even at high concentration, 100 mg/L. It also showed no toxic effect on mice following the injection of 1 mg γ-PGA and was not caused by immunoreactions and inflammatory reactions.[32,33] They are expected to have clinical use, although further study is necessary.

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