Novel siRNA Delivery System Using a Ternary Polymer Complex with Strong Silencing Effect and No Cytotoxicity

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We developed a novel small interfering RNA (siRNA) delivery system using a ternary complex with polyethyleneimine (PEI) and γ-polyglutamic acid (γ-PGA), which showed silencing effect and no cytotoxicity. The binary complexes of siRNA with PEI were approximately 73–102 nm in particle size and 45–52 mV in ζ-potential. The silencing effect of siRNA/PEI complexes increased with an increase of PEI, and siRNA/PEI complexes with a charge ratio greater than 16 showed significant luciferase knockdown in a mouse colon carcinoma cell line regularly expressing luciferase (Colon26/Luc cells). However, strong cytotoxicity and blood agglutination were observed in the siRNA/Lipofectamine complex and siRNA/PEI16 complex. Recharging cationic complexes with an anionic compound was reported to be a promising method for overcoming these toxicities. We therefore prepared ternary complexes of siRNA with PEI (charge ratio 16) by the addition of γ-PGA to reduce cytotoxicity and deliver siRNA. As expected, the cytotoxicity of the ternary complexes decreased with an increase of γ-PGA content, which decreased the ζ-potential of the complexes. A strong silencing effect comparable to siRNA/Lipofectamine complex was discovered in ternary complexes including γ-PGA with an anionic surface charge. The high incorporation of ternary complexes into Colon26/Luc cells was confirmed with fluorescence microcopy. Having achieved knockdown of an exogenously transfected gene, the ability of the complex to mediate knockdown of an endogenous housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was assessed in B16-F10 cells. The ternary complex (siRNA/PEI16/γ-PGA12 complex) exhibited a significant GAPDH knockdown effect. Thus, we developed a useful siRNA delivery system.

Key words gene delivery; small interfering RNA; γ-polyglutamic acid; ternary complex

Research into RNA interference (RNAi) has advanced rapidly since it was first discovered in 1998.[1] RNAi is a natural mechanism conserved in plant and mammalian cells that results in specific silencing of a target gene by short sequences of RNA, known as small interfering RNA (siRNA).[2] siRNA is a double-stranded RNA (dsRNA) composed of 21–23 nucleotides, and its ability to induce sequence-specific RNAi-mediated down-regulation of complementary mRNA has been demonstrated, resulting in knockdown of a target gene protein at the post-transcriptional level.[3–5] Numerous studies have used siRNAs as potential therapeutic agents for treating various diseases, including cancer and other diseases as a result of genetic disorders or viral infection.[6–8]

Despite the immense therapeutic potential of this technology, effective systemic and intracellular delivery of siRNA is quite limited because of its rapid degradation by plasma nucleases and poor penetration of the plasma membrane of target cells.[9,10] Therefore, the establishment of an siRNA delivery system that enables prolonged activity without degradation by nucleases and efficient cytosolic delivery of siRNA into target cells is indispensable for the development of siRNA pharmaceutics. Both cationic and neutral lipoplexes have exhibited good efficacy with different toxicity and protein adsorption.[11–15] Previously, we also reported that lipoplexes of siRNA constructed with trimethyl[2,3-(dioleoxy)-prolyl]ammonium chloride and cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate hydrochloride showed a significant silencing effect in a mouse colon carcinoma cell line expressing luciferase regularly.[16]

On the other hand, cationic polymers are one of the most popular non-viral gene vectors due to their excellent ability to compact anionic genes, synthetic controllability, better flexibility achieved simply by varying the chemical composition, molecular weight (MW), and architecture (linear, randomly branched, dendrimer, block, and graft copolymer).[17] Cationic polymers, such as chitosan and other natural polymers, polyethyleneimine (PEI), dendrimers, and other synthetic polymers have also shown promise. In particular, PEI[18] showed high gene expression *in vitro* and *in vivo* because it strongly interacts with the cell surface, and is well taken up by cells due to its strong cationic surface. Moreover, PEI achieves efficient escape from endosomes to the cytoplasm *via* the pH-buffering effect and the gene released from the vector in the cytoplasm expresses the protein in the nucleus.

Nevertheless, the cationic surface of complexes with cationic polymers induced cytotoxicity and blood agglutination caused by its strong interaction with anionic surface of cells and erythrocytes.[19–21] Agglutination of the complexes often led to its rapid elimination and adverse effects, such as embolism and inflammatory reactions.[22–24] Therefore, there remains a need for the development of alternative less toxic vectors for siRNA delivery. Recharging cationic complexes with an anionic compound was reported to be a promising method for overcoming these toxicities.[25,26] In our previous study, we discovered anionic polymers such as γ-polyglutamic acid (γ-PGA),[27] chondroitin sulfate,[28] hyaluronic acid,[28] which decreased the toxicities of cationic complexes while maintaining high transgene efficiency. Among them, the addition of γ-PGA showed the highest gene expression. However, there is no certainty that ternary complex containing siRNA

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is effective as well as that containing plasmid DNA (pDNA) because the action site of pDNA differs from that of siRNA. Also, in the preliminary study, we could not prepare the ternary complex containing siRNA under the same condition which prepared the ternary complex containing pDNA.

In the present study, we developed a modified polyplex of siRNA with PEI by the addition of γ-PGA and investigated the efficacy and safety of this ternary complex as a novel siRNA delivery system.

MATERIALS AND METHODS

Chemicals and Reagents Bovine serum albumin (BSA) and PEI (branched form, average MW of 25000) were obtained from Sigma-Aldrich Co., LLC (St. Louis, MO, U.S.A.). The γ-PGA (average molecular weight; 55000) was provided by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). Lipofectamine RNAiMAX, Alexa Fluor 555-labeled siRNA (BLOCK-iT Alexa Fluor Red Fluorescent Oligo), and primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (GAPDH: 5′-CTC ACT CAA GAT TGT CAG CAA TG-3′ (forward) and 5′-GGC AGT GAT GGC ATG GAC TGT-3′ (reverse), β-actin: 5′-GTT CTA CAA ATG TGG CTG AGG ACT T-3′ (forward) and 5′-TTG GGA GGG TGA GGG ACT T-3′ (reverse)) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Firefly luciferase siRNA (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). The mouse GAPDH siRNA (sense: 5′-CAA GAG AGG CCCUAUCCCAdTdT-3′, antisense: 5′-UGGGAUAGGGCCUCUCUUGdTdT-3′) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Fetal bovine albumin (FBS) was obtained from Biological Industries Ltd. (Kibbutz 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-nitrophe-nyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). Fluoresceinisothiocyanate-PEI (FITC-PEI) was prepared in our laboratory. Briefly, PEI and FITC were dissolved in dimethyl sulfoxide (DMSO) and stirred overnight at room temperature in the dark. FITC-PEI was purified by gel filtration. Almost 0.73% PEI nitrogen was labeled with FITC. All other chemicals were of reagent grade.

Preparation of Complex In this study, we constructed complexes at a theoretical charge ratio of phosphate of siRNA, nitrogen of PEI, and carboxylate of γ-PGA.

To prepare binary complexes, an appropriate amount of stock PEI solution was mixed with siRNA dissolved in diethylpyrocarbonate (DEPC)-treated water (Invitrogen) (1 mg/mL) by pipetting thoroughly at charge ratios 4, 8, 12, 16, 20, and 24 of PEI to siRNA (siRNA/PEI4, 8, 12, 16, 20, and 24 complexes), and left for 30 min at room temperature. To prepare ternary complexes, γ-PGA solution was added to siRNA/PEI16 complex at charge ratios of 4, 8, 12, 16, and 20 of γ-PGA to siRNA (siRNA/PEI16/γ-PGA4, 8, 12, 16, and 20 complexes) and left for another 30 min at room temperature.

siRNA/Lipofectamine complex was prepared as a product protocol and used as a positive control.

Physicochemical Properties of Complex 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 solution containing 1 μg 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 Co., Ltd., 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., Hercules, CA, U.S.A.).

Cell Culture We used the mouse colon carcinoma cell line (Colon26) and the mouse melanoma cell line (B16-F10) which cause lung metastasis. Colon26 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). B16-F10 cells was obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). 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.

***In Vitro* Gene Silencing Experiment** Colon26/Luc or B16-F10 cells were plated on 24-well plates (Becton, Dickinson and Company, 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. The mixed solution of solution of 5% dextrose solution and DEPC-treated water, which was solvent of complexes, was added to the cells as a control (untreated cells). Then, the medium was replaced with culture medium and cells were cultured for a further 22 h at 37°C. For the luciferase assay of Colon26/Luc cells, 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 Co., Ltd., 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 the Bradford assay (Bio-Rad Laboratories, Inc.) 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).

Cytotoxicity Cytotoxicity of various complexes on Colon26/Luc cells was measured using a commercially avail-

able 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 24-well plates or 96-well plates (Becton, Dickinson and Company) at a density of 1.0×10^4 or 5.0×10^3 cells/well in the culture medium. Each complex containing 1 μg siRNA in 100 μL Opti-MEM I was added to each well and incubated for 2 h. The mixed solution of solution of 5% dextrose solution and DEPC-treated water was added to the cells as a control (untreated cells). 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 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 of untreated cells (control).

Cellular Uptake of Complex To visualize the uptake of the complexes, the cells were transfected by complexes constructed with Alexa Fluor-labeled siRNA, FITC-PEI, and γ-PGA. Fluorescence was observed with fluorescence microscopy (200× magnification; BZ-9000; KEYENCE, Osaka, Japan).

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

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 resuspended 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 30 min at room temperature. Ten microliters of these suspensions were placed on a glass plate and agglutination was observed by microscopy (200× magnification).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was isolated from B16-F10 cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Co., LLC). The concentration of RNA was measured by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Reverse transcription of mRNA was carried out using the PrimeScript RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan). Gene expression was assessed by real-time PCR using SYBR Premix Ex Taq II (TaKaRa Bio Inc.) and Stratagene Mx3000P (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). GAPDH expression was normalized to β-actin expression (GAPDH/β-actin), and the results are shown as a percentage of untreated cells (control).

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

RESULTS

Physicochemical Properties and Electrophoresis Assay of siRNA/PEI Complex The particle size and ζ-potential of siRNA/PEI complexes are shown in Table 1.

The siRNA/PEI complexes, excepted siRNA/PEI4 complex, were approximately 73–102 nm in particle size and 45–52 mV in ζ-potential.

A gel retardation assay was employed to examine the release of siRNA from siRNA/PEI complexes (Fig. 1). Naked siRNA was detected as a band on the agarose gel. On the other hand, no band was detected in the lanes of siRNA/PEI complexes.

***In Vitro* Gene Silencing Effect of siRNA/PEI Complex** Colon26/Luc cells were transfected with various complexes, and silencing effects were examined and are shown in Fig. 2A. The commercial vector, siRNA/Lipofectamine complex, showed a strong silencing effect of siRNA. The siRNA/PEI4 and siRNA/PEI8 complexes did not silence the luciferase activity of Colon26/Luc cells. The silencing effect of siRNA/PEI complexes was increased with an increase of PEI, and siRNA/PEI complexes with a charge ratio greater than 16 showed significant luciferase knockdown. No silencing effect was confirmed in siRNA/PEI16 complexes using scramble siRNA (Fig. 2B). Also, siRNA/PEI16 complexes showed no cytotoxicity under this experimental condition (Fig. 2C).

Physicochemical Properties and Electrophoresis Assay of siRNA/PEI/γ-PGA Complex In the preliminary experiment, the binary complex of siRNA with a high amount of PEI showed strong cytotoxicity. Therefore we developed a ternary complex including γ-PGA, which was reported to decrease the cytotoxicity of cationic complex without decreasing gene expression.[27] The particle size and ζ-potential of siRNA/PEI16/γ-PGA complexes are shown in Table 2. The addition of γ-PGA to the binary complex (siRNA/PEI16 complex)

Table 1. Size and ζ-Potential of siRNA/PEI Complexes

Complexes	Size (nm)	ζ-Potential (mV)
siRNA/PEI4	1388.0±451.7	16.4±0.2
siRNA/PE8	102.2±32.8	44.7±0.5
siRNA/PE12	80.8±23.0	47.8±1.3
siRNA/PE16	94.1±47.0	50.4±1.4
siRNA/PE20	87.3±16.5	52.0±1.1
siRNA/PE24	73.2±7.9	51.6±0.5

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

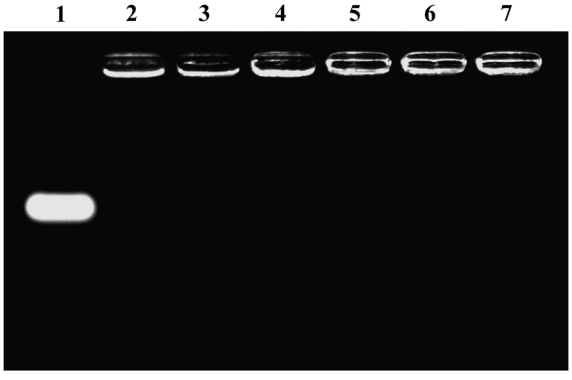

Fig. 1. Gel Retardation Assay of siRNA/PEI Complexes

The siRNA/PEI complexes 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. siRNA/PEI4 complex (lane 2), siRNA/PEI8 complex (lane 3), siRNA/PEI12 complex (lane 4), siRNA/PEI16 complex (lane 5), siRNA/PEI20 complex (lane 6), siRNA/PEI24 complex (lane 7).

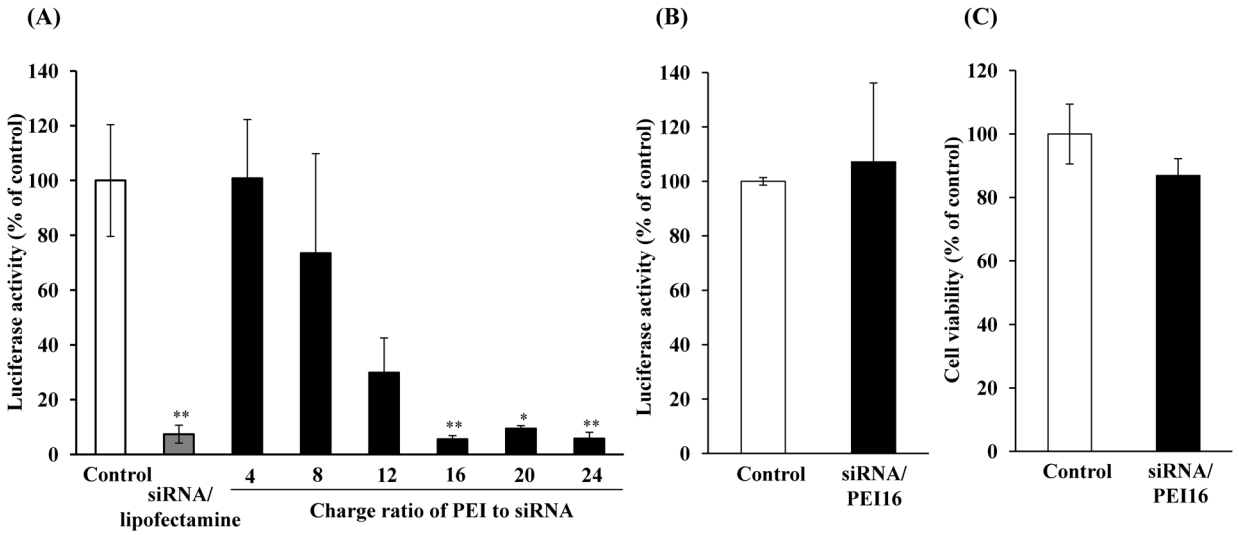

Fig. 2. *In Vitro* Gene Silencing of siRNA/PEI Complexes Using Firefly Luciferase siRNA (A) and Scramble siRNA (B) and Cytotoxicity of siRNA/PEI16 Complexes in Colon26/Luc Cells (C)

Colon26/Luc cells were incubated with siRNA/Lipofectamine complex and siRNA/PEI complexes 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. Cell viability after treatment with complexes was determined by WST-1 assay. Each bar represents the mean±S.E. (n=3). *p<0.05, **p<0.01 *vs.* control.

Table 2. Size and ζ-Potential of siRNA/PEI/γ-PGA Complexes

Complexes	Size (nm)	ζ-Potential (mV)
siRNA/PEI16/γ-PGA4	90.9±16.1	36.4±0.6
siRNA/PEI16/γ-PGA8	231.3±27.6	−9.2±0.5
siRNA/PEI16/γ-PGA12	107.6±1.8	−26.5±0.6
siRNA/PEI16/γ-PGA16	83.8±9.6	−35.5±0.5
siRNA/PEI16/γ-PGA20	85.1±65.2	−46.4±1.9

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

decreased the ζ-potential in a concentration-dependent manner. All ternary complexes (siRNA/PEI16/γ-PGA complexes), except siRNA/PEI16/γ-PGA8 complex, were approximately 41–108 nm in particle size with an anionic surface charge less than −25 mV.

A gel retardation assay was employed to examine the release of siRNA from ternary complexes (Fig. 3). Naked siRNA was detected as a band on the agarose gel. Addition of a large amount of γ-PGA to the binary complex (siRNA/PEI16 complex) showed a slight release of siRNA, although ternary complexes including less than a charge ratio of 12 for γ-PGA to siRNA showed no band of naked siRNA.

Cytotoxicity of Binary Complex and Ternary Complex The cytotoxicity of binary complexes and ternary complexes was evaluated in Colon26/Luc cells using the WST-1 assay (Fig. 4). The siRNA/Lipofectamine complex and siRNA/PEI16 complex, which had efficient silencing activity, showed significantly higher toxicity than the control. An increase of γ-PGA in ternary complexes decreased their cytotoxicity. The siRNA/PEI16/γ-PGA12 complex did not affect the cell viability of colon26/Luc cells.

***In Vitro* Gene Silencing Effect of siRNA/PEI/γ-PGA Complex** All ternary complexes (siRNA/PEI/γ-PGA complexes) were added and incubated with Colon26/Luc cells and silencing effects were determined (Fig. 5A). Strong suppression of luciferase expression in Colon26/Luc cells was observed in binary complexes (siRNA/Lipofectamine and

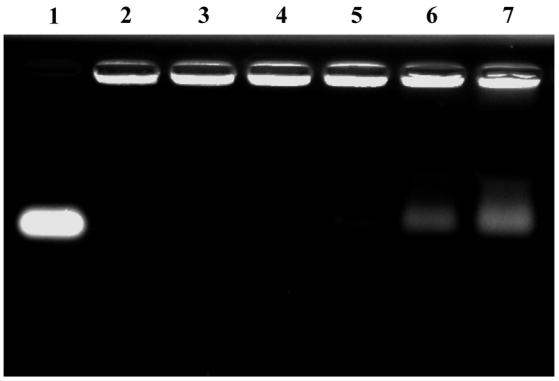

Fig. 3. Gel Retardation Assay of siRNA/PEI/γ-PGA Complexes

The siRNA/PEI/γ-PGA complexes 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. siRNA/PEI16 complex (lane 2), siRNA/PEI16/γ-PGA4 complex (lane 3), siRNA/PEI16/γ-PGA8 complex (lane 4), siRNA/PEI16/γ-PGA12 complex (lane 5), siRNA/PEI16/γ-PGA16 complex (lane 6), siRNA/PEI16/γ-PGA20 complex (lane 7).

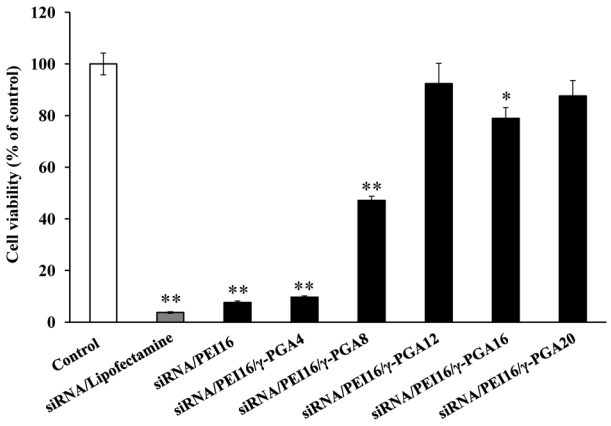

Fig. 4. Cytotoxicity of Complexes in Colon26/Luc Cells

Cell viability after treatment with complexes was determined by WST-1 assay. Each bar represents the mean±S.E. (n=8–16). *p<0.05, **p<0.01 *vs.* control.

siRNA/PEI16 complexes). Ternary complexes (siRNA/PEI16/γ-PGA complexes) also showed a significant silencing effect. On the other hand, siRNA/PEI16/γ-PGA12 complexes using scramble siRNA showed no silencing effect (Fig. 5B).

Cellular Uptake of Complex The cellular uptake of complex was visualized using Alexa Fluor 555-labeled siRNA and FITC-PEI, shown in Fig. 6. The binary complex (siRNA/PEI16 complex) showed strong signals of the red fluorescence of Alexa Fluor 555-labeled siRNA and green fluorescence of FITC-labeled PEI inside the cells. Red and green fluorescence inside the cells were also observed in siRNA/PEI16/γ-PGA12 complex. In the merged image, the complex was mostly observed as yellow signal in the cells, but red signal was also observed.

Agglutination Study The agglutination activities of the siRNA/PEI16/γ-PGA12 complex were compared to that of the siRNA/PEI16 complex in erythrocytes and are shown in Fig. 7. The siRNA/PEI16 complex showed severe agglutination, although no agglutination was observed in the siRNA/PEI16/γ-PGA12 complex.

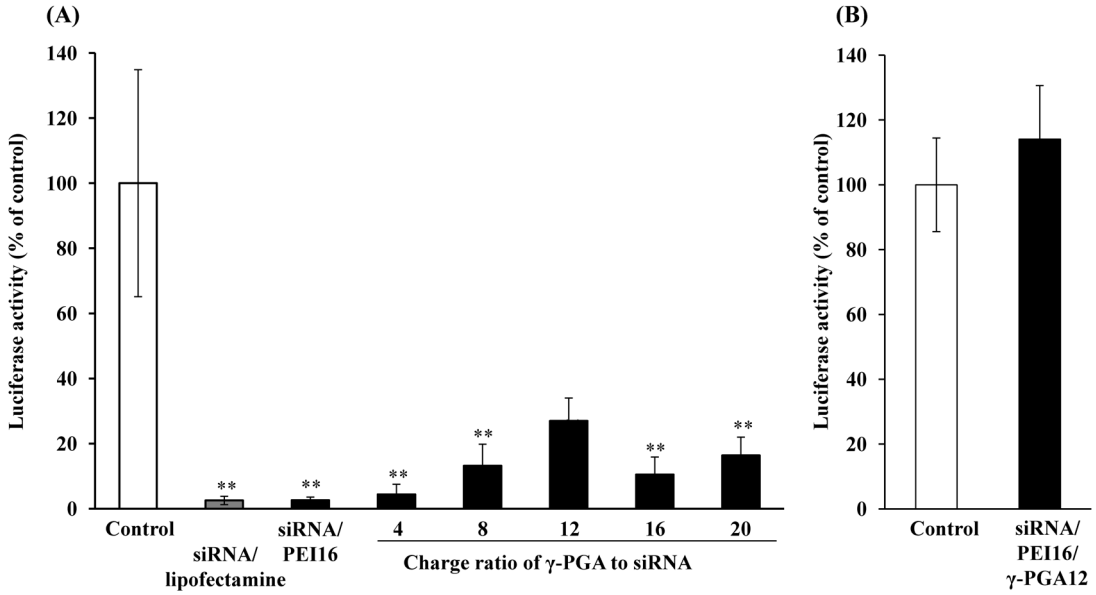

Fig. 5. *In Vitro* Gene Silencing of siRNA/PEI/γ-PGA Complexes

Colon26/Luc cells were incubated with siRNA/Lipofectamine complex, siRNA/PEI complex, and siRNA/PEI/γ-PGA complexes 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. (A): Firefly luciferase siRNA, (B): Scramble siRNA.

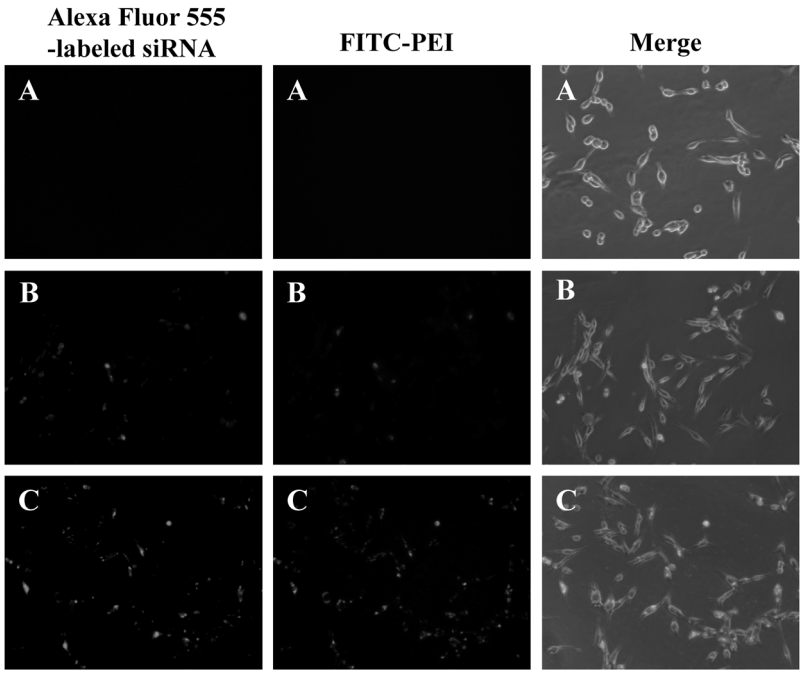

Fig. 6. Cellular Uptake of Complexes

Colon26/Luc cells were incubated with complexes containing Alexa Fluor 555-labeled siRNA and FITC-PEI, and fluorescent images were taken by fluorescence microscopy. (A): Control, (B): siRNA/PEI16 complex, (C): siRNA/PEI16/γ-PGA12 complex.

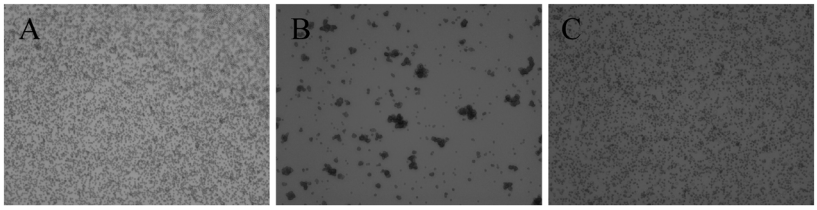

Fig. 7. Agglutination of Complexes with Erythrocytes

Each complex was added to erythrocytes, and agglutination was observed by microscopy (200× magnification). (A): Control, (B): siRNA/PEI16 complex, (C): siRNA/PEI16/γ-PGA12 complex.

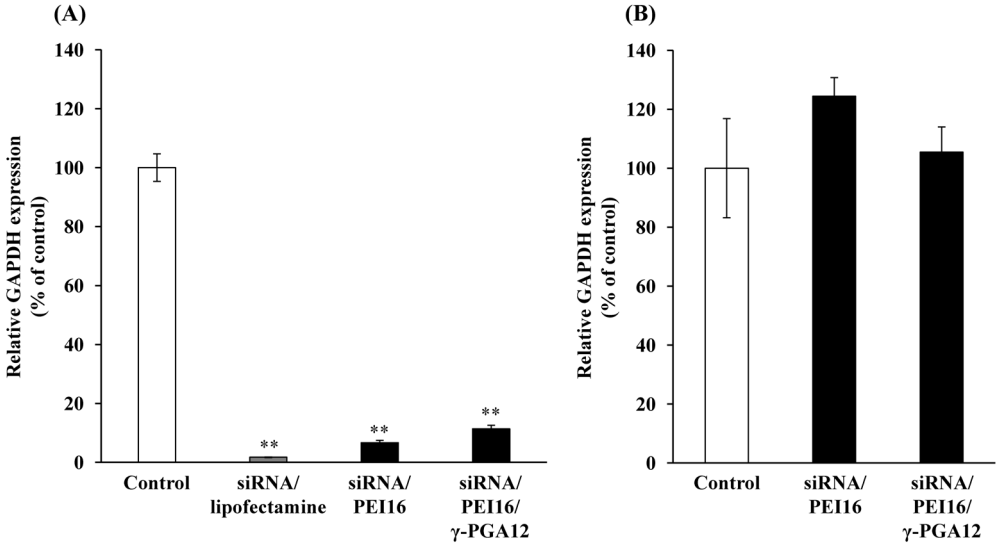

Fig. 8. GAPDH mRNA Knockdown of Complexes in B16-F10 Cells

B16-F10 cells were incubated with complexes for 2 h. After 22 h, GAPDH mRNA expression was measured using real-time RT-PCR, normalized to β-actin expression (GAPDH/β-actin), and expressed as a percentage compared to the control. Each bar represents the mean±S.E. (n=3). **$p<0.01$ *vs.* control. (A): GAPDH siRNA, (B): Scramble siRNA.

***In Vitro* GAPDH mRNA Knockdown** To evaluate the silencing effect of complexes for endogenous proteins, mRNA of a housekeeping gene, GAPDH, was determined with real-time PCR in B16-F10 cells 24 h after incubation with complexes for 2 h (Fig. 8A). Significant reduction of GAPDH expression in B16F-10 cells was observed in binary complexes (siRNA/Lipofectamine and siRNA/PEI16 complexes). The ternary complex (siRNA/PEI16/γ-PGA12 complex) also showed strong GAPDH mRNA knockdown. There were no knockdown effects in both binary complexes and ternary complexes using scramble siRNA (Fig. 8B).

DISCUSSION

siRNA formed an RNA-induced silencing complex (RISC) that consisted of an endoribonuclease. On formation of the RISC, siRNA strands direct the complex to the target complementary strand of RNA molecules, leading to destruction of the target RNA molecule in the cytoplasm.[29] siRNA technology has the potential to revolutionize the treatment of serious diseases by allowing selective silencing of oncogenic or therapeutic mRNAs and their corresponding proteins. The delivery of siRNA typically yields low levels of mRNA silencing due to barriers that include degradation by endo- and exonucleases, poor cellular uptake, and intracellular trafficking.[30] Polymer complexes have an advantage over lipid complexes in terms of stability in physiological media.[31] Polymer complexes called polyplexes can be defined as subcellular colloidal particles that entrap drug molecules, proteins, or nucleic acid.[32] Figure 2A shows the strong silencing effect of siRNA/PEI complexes on a mouse colon carcinoma cell line expressing luciferase regularly. An increase of the PEI charge ratio in the complexes increased the silencing effect. Also, the cell viability of siRNA/PEI16 complexes was almost equal to control (Fig. 2C). These results indicated that binary complexes did not induce cytotoxicity in this condition. The siRNA/PEI complexes at greater than charge ratio 12 of PEI to siRNA formed stable cationic particles of less than 100 nm in diameter and higher than 45 mV in ζ-potential (Table 1, Fig. 1). In the previous report, we demonstrated that plasmid DNA/PEI complex showed effective gene expression at greater than charge ratio 8.[27] Cationic agent-condensed plasmid DNA can exist in a variety of different morphologies depending upon the cationic condensing agent, such as spheres, toroids, and rods.[33,34] Electrostatic interactions between siRNA and cationic polymer increase the PEI amount for siRNA delivery. In contrast to plasmid DNA, siRNA cannot condense into particles of nanometric dimensions, being already a small subnanometric nucleic acid.

On the other hand, cytotoxicity and blood agglutination were induced by the cationic surface of the polyplexes.[19,20] The cationic complex was reported to bind to anionic proteoglycans on the cellular membrane, destabilizing the membrane and causing severe cytotoxicity.[35–37] The erythrocyte surface

was also negatively charged to be agglutinated with cationic complexes. Agglutination of erythrocytes by positively charged complexes can result in the occlusion of capillaries in the lungs, which could account for the high toxicity.[20,23] In fact, strong cytotoxicity and blood agglutination were observed in the siRNA/Lipofectamine complex and siRNA/PEI16 complex (Figs. 4, 7).

Recharging cationic complexes with an anionic compound was reported to be a promising method for overcoming these toxicities.[25,26] In the previous study, we successfully prepared various ternary complexes of plasmid DNA and PEI by the addition of anionic polymers such as polyadenylic acid, polyinosinic–polycytidylic acid, polyaspartic acid, polyglutamic acid, fucoidan, λ-carrageenan, xanthan gum, alginic acid, chondroitin sulfate, and hyaluronic acid.[27,28] All showed anionic surface charges with no cytotoxicity and agglutination. However, most showed no gene expression because of the lack of a strong interaction with cell and erythrocyte surfaces. Among them, we discovered high cellular uptake and high gene expression in the complexes of plasmid DNA and PEI with γ-PGA, chondroitin sulfate, or hyaluronic acid. The complex including γ-PGA showed the highest gene expression *via* a γ-PGA-specific receptor-mediated energy-dependent process.[27] Complexes including a plasmid encoding *Plasmodium yoelii* merozoite surface protein 1-C-terminus completely suppressed a lethal malaria challenge in a murine model by strong vaccination.[38]

We prepared ternary complexes of siRNA with PEI (at charge ratio 16) by the addition of γ-PGA to reduce cytotoxicity with efficient delivery. As expected, the cytotoxicity of ternary complexes decreased with an increase of γ-PGA content, which decreased ζ-potential of the complexes (Fig. 4, Table 2). siRNA/PEI16/γ-PGA12 with an anionic surface charge showed no blood agglutination. The large addition of γ-PGA to the complexes enhanced the release of siRNA, suggesting destabilization of the complexes (Fig. 3).

As projected, a strong silencing effect, comparable to the siRNA/Lipofectamine complex, was observed in ternary complexes including γ-PGA, regardless of the anionic surface charge (Fig. 5). The high incorporation of ternary complexes into Colon26/Luc cells was also confirmed with fluorescence microcopy (Fig. 6). The number of intracellular siRNA of ternary complex may be observed larger than that of binary complex. However, the silencing effect of ternary complex showed lower than that of binary complex. The endosomal escape, siRNA release from complexes, and degradation of siRNA in the cells also have an impact on the silencing effects. We will examine the endosomal escape, siRNA release from complexes, and degradation of siRNA in the cells to explain the different gene silencing effects of various complexes in the future study.

Having achieved knockdown of an exogenously transfected gene, the ability of the complex to mediate knockdown of an endogenous housekeeping gene, GAPDH, was assessed in B16-F10 cells. The binary complexes (siRNA/Lipofectamine and siRNA/PEI16 complexes) showed strong GAPDH mRNA knockdown (Fig. 8A). The ternary complex (siRNA/PEI16/γ-PGA12) with an anionic surface charge also exhibited a significant GAPDH mRNA knockdown effect (Fig. 8A).

Thus, we developed a ternary complex of siRNA with PEI by the addition of γ-PGA, showing a high silencing effect and no cytotoxicity, as a novel siRNA delivery system.

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