Evaluation of anionic polymer-coated siRNA lipoplexes prepared using a modified ethanol injection method

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Abstract. Cationic liposomes are useful carriers for delivering small interfering RNA (siRNA). In our previous study, a simple and efficient modified ethanol injection (MEI) method was developed for the preparation of cationic siRNA lipoplexes. However, non-specific interactions between cationic lipoplexes and biological components, including erythrocytes, induce aggregation, and thus, siRNA is not delivered to the target site after intravenous injection. In the present study, an anionic polymer coating was applied to cationic lipoplexes prepared using the MEI method, and their gene knockdown effect and biodistribution in mice were evaluated. The gene knockdown effect of cationic lipoplexes was preserved after coating with anionic polymers [hyaluronan (HA), chondroitin sulfate (CS) and polyglutamic acid (PGA)], although anionic polymer-coated lipoplexes showed lower cellular association than cationic lipoplexes. Coating of cationic lipoplexes with high-molecular-weight HA, CS and PGA reduced agglutination with erythrocytes. Following intravenous injection, CS- or PGA-coated lipoplexes exhibited lower pulmonary accumulation than cationic lipoplexes, whereas hepatic accumulation of CS- or PGA-coated lipoplexes increased. Collectively, cationic lipoplexes prepared using the MEI method were successfully coated with anionic polymers. Notably, CS or PGA coating of lipoplexes reduced non-specific interactions with erythrocytes, and CS- or PGA-coated lipoplexes could be potential vectors for *in vivo* siRNA delivery to the liver.

Introduction

Small interfering RNAs (siRNAs) have attracted attention as promising therapeutic tools because they can silence disease-related genes (1,2). However, siRNAs undergo rapid degradation by endogenous nucleases after systemic administration. Additionally, its hydrophilic nature and high molecular weight restrict penetration into target cell membranes. Therefore, developing a siRNA delivery system for target sites is crucial for inducing therapeutic effects (3). Several siRNA carriers comprising polymers or lipids have been investigated (4).

Cationic liposomes are widely used for siRNA delivery (5). The formation of siRNA lipoplexes, that is, complexes of siRNA and cationic liposomes, prevents siRNA degradation by nucleases and improves its transport into the cytoplasm. The cationic characteristics of lipoplexes induce binding to negatively charged cell surfaces, improving intracellular delivery. However, after intravenous injection, cationic lipoplexes non-specifically interact with negatively charged serum proteins or erythrocytes, forming aggregates that accumulate mainly in the lungs (6,7).

To reduce non-specific interactions and agglutination of cationic lipoplexes with blood components, polyethylene glycol (PEG) modification of lipoplexes has been explored (8,9). PEG-modified carriers reportedly improve siRNA stability and retention in the circulation by inducing steric hindrance and accumulate in the target tissue as a result of evading lung accumulation. However, PEG modification frequently inhibits cellular uptake and endosomal escape, resulting in reduced gene silencing (8,10). As an alternative to PEG modification, surface modification of cationic lipoplexes using anionic materials has been investigated. For example, coating cationic lipoplexes with anionic polymers has been reported to attenuate electrostatic interactions with blood components (11-13). Several biocompatible anionic polymers, such as hyaluronan (HA), chondroitin sulfate (CS), and polyglutamic acid (PGA), can minimize the non-specific interaction of cationic lipoplexes with biological components. Similarly, incorporation of anionic lipids into cationic lipoplexes can reduce the surface charge through charge neutralization, thereby decreasing non-specific interactions with erythrocytes as reported in our previous study (14). Compared with simple charge neutralization using anionic lipids, anionic polymer coating provides additional advantages by enabling functional modification of lipoplexes through polymer structure and functional properties, such as prolonged circulation and receptor-mediated interactions.

Previously, our group reported a simple preparation method for siRNA lipoplexes [modified ethanol injection (MEI)

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method], in which the siRNA solution was rapidly mixed with a small volume of a lipid-ethanol solution, which could be applicable for *in vitro* and *in vivo* siRNA transfections (15,16). To extend the usefulness and applicability of this preparation method, we explored the potential of anionic polymer coating on siRNA lipoplexes prepared by MEI in the current study. Biocompatible polymers, including HA, CS, and PGA, were used to coat cationic siRNA lipoplexes prepared using the MEI method. In addition, the effects of anionic polymer coating of siRNA lipoplexes on gene knockdown efficiency and biodistribution were evaluated.

Materials and methods

Materials. 1,2-Dioleoyl-3-trimethylammonium-propane methyl sulfate salt (DOTAP) was obtained from Avanti Polar Lipids (now part of Croda International Plc, Yorkshire, UK). Dimethyldioctadecylammonium bromide (DDAB; product name: DC-1-18) was obtained from Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; COATSOME® ME-8181) was purchased from NOF Corporation (Tokyo, Japan). Ultra-low-molecular-weight hyaluronan (HA-UL; molecular mass: 7.5 kDa), low-molecular-weight HA (HA-L; 16.7 kDa), medium-molecular-weight HA (HA-M; 215 kDa), and high-molecular-weight HA (HA-H; 975 kDa) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Chondroitin sulfate C sodium salt (CS; average molecular mass: 35 kDa) was purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Poly-α-L-glutamic acid sodium salt (PGA; molecular weight: 20500) was purchased from Sigma-Aldrich (now part of Merck, Darmstadt, Germany).

siRNAs. Firefly luciferase siRNA (Luc siRNA, target sequence: hLuc+, GenBank accession no. AY535007.1), non-silencing control siRNA (Cont siRNA), and cyanine 5-conjugated Cont siRNA (Cy5-siRNA) were designed as reported previously (17) and synthesized by Sigma-Aldrich (Merck). The siRNA sequences were as follows: Luc siRNA passenger strand, 5'-CCGUGGUGUUCGUGUCUAAGA-3', and guide strand, 5'-UUAGACACGAACACCACGGUA-3'; Cont siRNA passenger strand, 5'-GUACCGCACGUCAUUCGUAUC-3', and guide strand, 5'-UACGAAUGACGUGCGGUACGU-3'. For Cy5-siRNA, Cy5 dye was conjugated at the 5'-end of the passenger strand of Cont siRNA.

Preparation of anionic polymer-coated lipoplexes using the MEI method. siRNA lipoplexes were prepared using the MEI method, as reported previously (15,16). Briefly, siRNA dispersed in 5% glucose aqueous solution (500 nM siRNA) was rapidly added to a lipid-ethanol solution containing DOTAP:DOPE=1:1 (molar ratio) or DDAB:DOPE=1:1 (molar ratio) at a charge ratio (+:-) of 4:1, followed by incubation for 5 min. The charge ratio (+:-) of cationic lipids to siRNA was calculated as the molar ratio of amines in cationic lipids to siRNA phosphate. To prepare anionic polymer-coated lipoplexes, siRNA lipoplexes were mixed with an aqueous solution of anionic polymers at charge ratios (+:-) of cationic lipids to anionic polymers from 1:0.5 to 1:3, and followed by incubation for 5 min. The charge ratio (+:-) of cationic lipids to anionic polymers was calculated as the molar ratio of the carboxylic acid of HA (one negative charge per disaccharide unit), sulfate and carboxylic acid of CS (two negative charges per disaccharide unit), and carboxylic acid of PGA (one negative charge per glutamic acid).

The average particle size and ζ-potential of lipoplexes were measured using a light-scattering photometer (ELSZ-2000; Otsuka Electronics Co., Ltd., Osaka, Japan) at 25˚C after diluting the dispersion to an appropriate volume with water.

Cell culture. Human breast cancer MCF-7 cells stably expressing *firefly* luciferase (MCF-7-Luc), which were constructed by transfection of pcDNA3 plasmid containing *firefly* luciferase (hLuc+) gene (GenBank no. AY535007.1) derived from psiCHECK-2 vector (Promega Corporation), were provided by Dr. Kenji Yamato (University of Tsukuba). Cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corp.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1.2 mg/ml G418 (geneticin) sulfate (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37˚C in a humidified incubator with 5% CO_2.

Gene silencing of anionic polymer-coated lipoplexes. In brief, MCF-7-Luc cells were seeded in 6-well culture plates the day before transfection. Lipoplexes with Luc or Cont siRNA were prepared as described above, diluted in RPMI-1640 medium supplemented with 10% FBS at a 50 nM siRNA concentration, and added to the cells for 48 h. Following transfection, luciferase activity was measured as counts per second (cps)/μg protein using the luciferase assay system (PicaGene MelioraStar-LT Luminescence Reagent, Toyo B-Net Co., Ltd., Tokyo, Japan) and Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Luciferase activity (%) was calculated relative to the luciferase activity (cps/μg protein) of untreated cells, as reported previously (9).

Cellular association of siRNA lipoplexes. Flow cytometry was performed to confirm the cellular association of lipoplexes. In brief, MCF-7-Luc cells were seeded in 12-well culture plates the day before the experiment. Lipoplexes with Cy5-siRNA were prepared as described above, diluted in RPMI-1640 medium supplemented with 10% FBS at a 50 nM siRNA concentration, and added to the cells. After incubation for 3 h, the cells were washed with phosphate-buffered saline (PBS; pH 7.4), detached using TrypLE™ Express Enzyme (Gibco™, Thermo Fisher Scientific), and suspended in PBS containing 0.1% bovine serum albumin and 1 mM ethylenediaminetetraacetic acid. The association of Cy5-siRNA with cells was determined by examining the fluorescence intensity using a BD FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA, USA), as reported previously (18).

Cytotoxicity of siRNA lipoplexes. Briefly, MCF-7-Luc cells were seeded in 96-well culture plates the day before transfection. Lipoplexes with Cont siRNA were prepared as described above, diluted in RPMI-1640 medium supplemented with 10% FBS at a 50 nM siRNA concentration, and added to the cells. After incubation for 24 h, cell viability was measured using

the WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). Cell viability (%) was calculated relative to the cell viability of untreated cells.

Animals. A total of 42 female BALB/c mice (8 weeks old) were purchased from Sankyo Labo Service Corporation (Tokyo, Japan) and maintained under specific pathogen-free conditions.

All animal experiments were conducted according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Hoshi University (Permit No. P24-094).

Effect of anionic polymer-coating on erythrocyte agglutination with siRNA lipoplexes. Agglutination of erythrocytes with siRNA lipoplexes was determined as described previously (9). In brief, approximately 0.3 ml of blood was collected in a single sampling from the jugular vein of one female BALB/c mouse under inhalation anesthesia with isoflurane (5% for induction and 2% for maintenance), and the mouse was euthanized by cervical dislocation. Erythrocytes were separated by centrifugation at 300 x g for 3 min and resuspended in PBS as a 2% (v/v) suspension of erythrocyte. siRNA lipoplexes with 2 μg Cont siRNA were mixed with the prepared erythrocyte suspension. Subsequently, the mixture was placed on a glass plate and observed by microscopy.

Biodistribution of anionic polymer-coated siRNA lipoplexes. siRNA lipoplexes with 10 μg Cy5-siRNA were administered intravenously via the lateral tail vein of BALB/c mice (n=1 per siRNA lipoplex). One hour post-injection, mice were euthanized by cervical dislocation under inhalation anesthesia with isoflurane (5% for induction and 2% for maintenance), and Cy5 fluorescence imaging of tissues was performed using a NightOWL LB981 NC100 system (Berthold Technologies, Bad Wildbad, Germany), as reported previously (19). Images were analyzed using IndiGo2 software (version 2.0.1.0) provided with the *in vivo* imaging system (Berthold Technologies).

Measurement of TNF-α concentration in mice. siRNA lipoplexes with 10 μg Cont siRNA were administered intravenously via the lateral tail vein of BALB/c mice (n=3 per siRNA lipoplex). Based on previous studies (20,21), approximately 0.3 ml of blood was collected in a single sampling from the jugular vein of a mouse under inhalation anesthesia with isoflurane (5% for induction and 2% for maintenance) 2 h after administration, after which the mouse was euthanized by cervical dislocation. The blood was centrifuged at 1,000 x g for 10 min to obtain serum. The concentrations of tumor necrosis factor-α (TNF-α) in serum were determined by enzyme-linked immunosorbent assay (LBIS mouse TNF-α ELISA kit, Fujifilm Wako Shibayagi Corp. Cat # 634-44721), according to the manufacturer's instructions.

Statistical analysis. The statistical significance of differences between the means of the two groups was determined using an unpaired t-test with Welch's correction. For multiple-group comparisons, statistical significance was determined using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. GraphPad Prism 6 (GraphPad Software Inc., Boston, MA, USA) was used for the statistical analyses. Statistical significance was set at $P<0.05$.

Results

Size and ζ-potential of anionic polymer-coated lipoplexes prepared using the MEI method. Previously, cationic siRNA lipoplexes were successfully prepared using the MEI method (15,16). In this study, anionic polymers were electrostatically coated with siRNA lipoplexes to prevent the agglutination of blood components, such as erythrocytes. The size and ζ-potential of anionic polymer-coated lipoplexes prepared using the MEI method are shown in Table I. The charge ratio (+:-) of cationic lipids to anionic polymers was examined over a range from 1:0.5 to 1:3. A ratio of 1:2 was selected because not all anionic polymer-coated lipoplexes exhibited negative surface charge at ratios below 1:1, suggesting that the coating of lipoplexes may be insufficient. The average size of siRNA lipoplex composed of DOTAP/DOPE or DDAB/DOPE without polymer coating (LP-DOTAP and LP-DDAB, respectively) was 106.1 and 75.1 nm, respectively. Anionic polymer-coated lipoplexes showed comparable hydrodynamic size (84.6-141.1 nm). LP-DOTAP and LP-DDAB had positive ζ-potentials (52.8 and 36.6 mV, respectively). Each anionic polymer-coated lipoplex exhibited a negative surface charge (from -14.4 to -45.1 mV of ζ-potential), indicating that cationic lipoplexes were successfully coated with anionic polymers.

Gene knockdown effect and cellular association of anionic polymer-coated lipoplexes. To determine the effect of the anionic polymer coating on gene knockdown, MCF-7-Luc cells were incubated with lipoplexes of Luc or Cont siRNA, and their luciferase activities were evaluated (Fig. 1). Both LP-DOTAP and LP-DDAB with Luc siRNA efficiently suppressed luciferase activity in MCF-7-Luc cells. Anionic polymer-coated lipoplexes also suppressed luciferase activity to the same level as cationic lipoplexes.

Next, we evaluated the cellular association of the anionic polymer-coated siRNA lipoplexes using flow cytometry (Fig. 2). Representative flow cytometry histograms are shown in Fig. S1. The cationic lipoplexes, LP-DOTAP and LP-DDAB, exhibited a higher cellular association than the anionic polymer-coated lipoplexes. The mean fluorescence intensity of the HA-coated lipoplexes decreased slightly as the molecular weight of HA increased. CS and PGA-coated lipoplexes exhibited lower cellular association than HA-coated lipoplexes. As shown in Table I, the large negative ζ-potential of CS- and PGA-coated lipoplexes, compared with that of HA-coated lipoplexes, may influence the reduced cellular association of lipoplexes.

Collectively, these results suggested that anionic polymer-coating of siRNA lipoplexes decreased the cellular association but did not affect gene knockdown. Anionic polymer-coated siRNA lipoplexes could effectively deliver siRNAs into cells.

Cytotoxicity of anionic polymer-coated lipoplexes. The effect of the anionic polymer coating on the cytotoxicity of siRNA lipoplexes was examined (Fig. 3). The viability of MCF-7-Luc

Table I. Effect of anionic polymer coating on size and ζ-potential of small interfering RNA lipoplexes.

Lipoplexes	Lipid formulation	Polymer coating[a]	Average size[b], nm	Polydispersity index	ζ-potential[b], mV
LP-DOTAP	DOTAP/DOPE=1:1 (mol:mol)	-	106.1±2.9	0.29±0.01	52.8±4.6
LP-DOTAP/HA-UL		HA-UL	115.7±0.3	0.11±0.02	-18.0±1.4
LP-DOTAP/HA-L		HA-L	140.0±15.3	0.26±0.05	-24.1±1.4
LP-DOTAP/HA-M		HA-M	84.6±1.7	0.26±0.00	-26.2±1.7
LP-DOTAP/HA-H		HA-H	99.7±5.7	0.21±0.00	-26.3±7.0
LP-DOTAP/CS		CS	114.9±2.0	0.23±0.01	-41.4±1.9
LP-DOTAP/PGA		PGA	126.9±23.7	0.24±0.07	-45.1±1.7
LP-DDAB	DDAB/DOPE=1:1 (mol:mol)	-	75.1±5.9	0.20±0.06	36.6±8.8
LP-DDAB/HA-UL		HA-UL	96.4±0.8	0.12±0.03	-14.4±4.1
LP-DDAB/HA-L		HA-L	133.3±16.9	0.15±0.04	-26.1±0.3
LP-DDAB/HA-M		HA-M	103.1±0.6	0.24±0.01	-24.0±5.4
LP-DDAB/HA-H		HA-H	120.8±0.6	0.21±0.02	-27.8±0.6
LP-DDAB/CS		CS	141.1±8.7	0.22±0.06	-43.9±1.3
LP-DDAB/PGA		PGA	115.0±7.5	0.23±0.04	-43.2±4.2

[a]The charge ratio (+:-) of cationic lipids to anionic polymers was set at 1:2. [b]In water. Each value represents the mean ± standard deviation (n=3). CS, chondroitin sulfate; DDAB, dimethyldioctadecylammonium bromide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; HA, hyaluronan; HA-H, high-molecular-weight HA; HA-L, low-molecular-weight HA; HA-M, medium-molecular-weight HA; HA-UL, ultra-low-molecular-weight HA; LP, lipoplex; PGA, polyglutamic acid.

cells incubated with all anionic polymer-coated lipoplexes exceeded 85% of that of the untreated cells. No significant differences in cell viability were observed compared with untreated cells. These results suggested that the anionic polymer-coated lipoplexes exhibited low cytotoxicity toward MCF-7-Luc cells.

Erythrocyte agglutination with anionic polymer-coated siRNA lipoplexes. Because cationic lipoplexes have a positively charged surface, they electrostatically interact with negatively charged biological components, such as erythrocytes. We evaluated whether the anionic polymer coating of lipoplexes could prevent agglutination with erythrocytes (Fig. 4). Mixing cationic lipoplexes (LP-DOTAP or LP-DDAB) with erythrocyte suspensions resulted in large aggregates. Small aggregates were observed with HA-UL, HA-L, and HA-M-coated lipoplexes. Conversely, LP-DOTAP/HA-H, LP-DOTAP/CS, LP-DOTAP/PGA, LP-DDAB/HA-H, LP-DDAB/CS, and LP-DDAB/PGA did not induce erythrocyte agglutination. These results suggested that HA-H-, CS-, and PGA-coated lipoplexes of LP-DOTAP and LP-DDAB could prevent erythrocyte agglutination.

Since anionic polymer-coated lipoplexes prevented erythrocyte agglutination, they may have the potential to reduce toxicity *in vivo*. To evaluate the inflammatory response of lipoplexes, TNF-α concentrations in serum were determined (Fig. S2). Intravenous injection of LP-DDAB caused a slight increase in serum TNF-α, whereas no elevation was observed for LP-DDAB/HA-H or LP-DDAB/CS. Therefore, lipoplexes coated with HA-H or CS are thought to have a higher safety profile.

Biodistribution of anionic polymer-coated siRNA lipoplexes. *Ex vivo* imaging was performed to examine the biodistribution of anionic polymer-coated siRNA lipoplexes (Fig. 5). LP-DOTAP and LP-DDAB were distributed in the lungs, liver, spleen, and kidneys. As shown in Fig. 5A, LP-DOTAP/HA-UL largely accumulated in the lungs. The biodistribution of LP-DOTAP/HA-L and LP-DOTAP/HA-M was similar to that of LP-DOTAP. The lung distributions of LP-DOTAP/HA-H, LP-DOTAP/CS, and LP-DOTAP/PGA were reduced. As shown in Fig. 5B, LP-DDAB/HA-UL largely accumulated in the lungs, and other HA-coated LP-DDAB were distributed in the lungs, liver, spleen, and kidneys. The lung distribution of LP-DDAB/CS and LP-DDAB/PGA was lower than that of LP-DDAB, whereas the liver distribution of LP-DDAB/CS and LP-DDAB/PGA was higher than that of LP-DDAB.

Discussion

In a recent report, our group demonstrated that the MEI method was convenient for preparing lipoplexes of siRNA and messenger RNA (mRNA) and that these lipoplexes could exert gene transfection effects comparable to lipoplexes prepared using the conventional thin lipid film hydration method (15,22). In the MEI method, lipoplexes are prepared by mixing an RNA solution with a lipid-ethanol solution. As shown in Table I, the average size of the lipoplexes remained small (~100 nm), even after anionic polymer coating, without mechanical shearing or sonication. Previously, siRNA lipoplex prepared from cationic liposomes comprising DOTAP and cholesterol was successfully coated with CS and PGA at a charge ratio (+:-, cationic lipid:anionic polymer) of 1:1 and 1:1.5, respectively (23). In

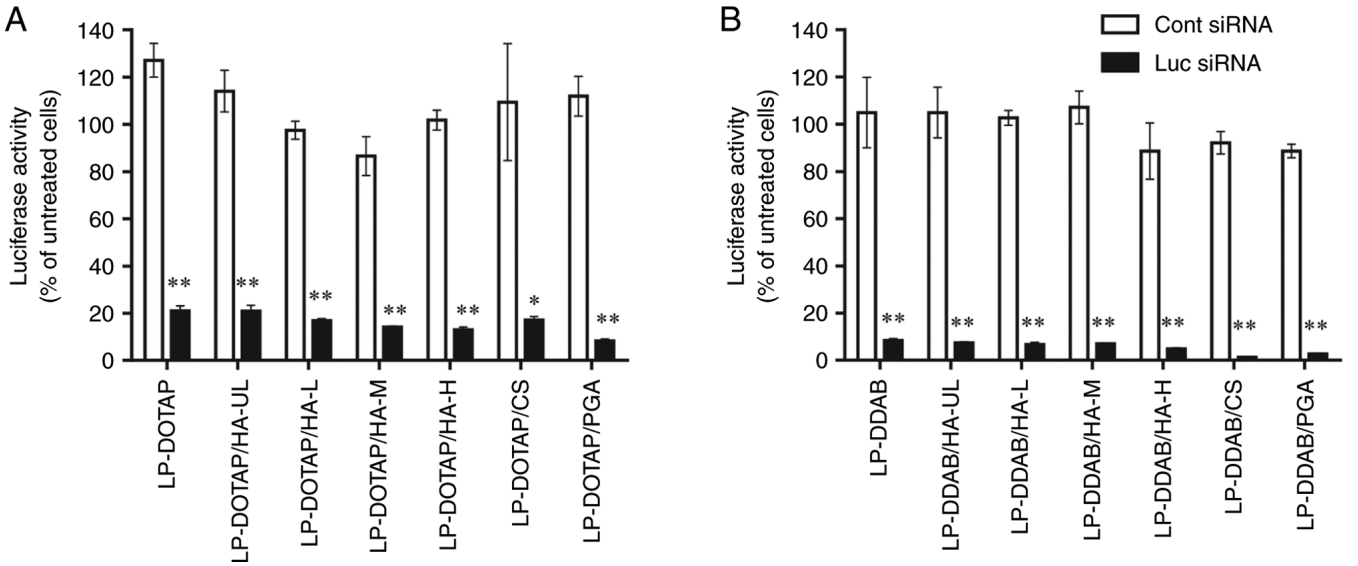

Figure 1. Effect of anionic polymer coating of siRNA lipoplexes on suppression of luciferase expression in MCF-7-Luc cells. MCF-7-Luc cells were treated for 48 h with anionic polymer-coated (A) LP-DOTAP or (B) LP-DDAB at a final Cont or Luc siRNA concentration of 50 nM. Each value represents the mean ± standard deviation (n=3). *P<0.05, **P<0.01 compared with Cont siRNA. Cont, control; CS, chondroitin sulfate; DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; HA, hyaluronan; HA-H, high-molecular-weight HA; HA-L, low-molecular-weight HA; HA-M, medium-molecular-weight HA; HA-UL, ultra-low-molecular-weight HA; Luc, luciferase; LP, lipoplex; PGA, polyglutamic acid; siRNA, small interfering RNA.

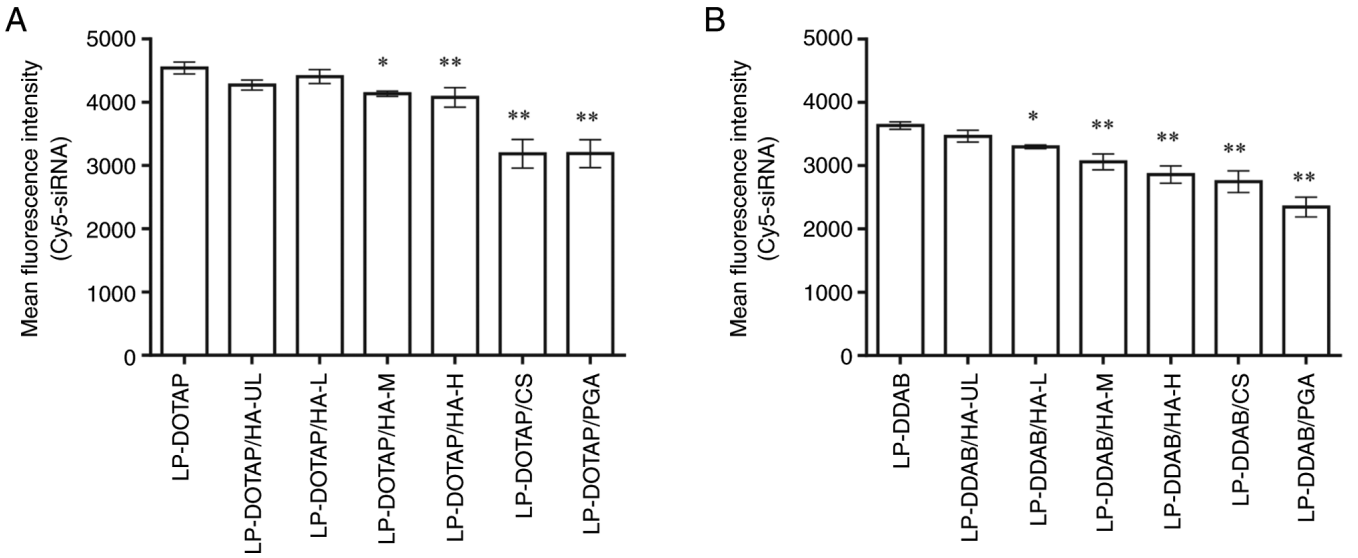

Figure 2. Cellular association of anionic polymer-coated siRNA lipoplexes. MCF-7-Luc cells were treated for 3 h with anionic polymer-coated (A) LP-DOTAP or (B) LP-DDAB at a final Cy5-siRNA concentration of 50 nM. The association between the siRNA lipoplexes and cells was determined based on Cy5 fluorescence using flow cytometry. Each value represents the mean ± standard deviation (n=3). *P<0.05, **P<0.01 compared with LP-DOTAP or LP-DDAB. CS, chondroitin sulfate; DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; HA, hyaluronan; HA-H, high-molecular-weight HA; HA-L, low-molecular-weight HA; HA-M, medium-molecular-weight HA; HA-UL, ultra-low-molecular-weight HA; LP, lipoplex; PGA, polyglutamic acid; siRNA, small interfering RNA.

the current study, we established a charge ratio (+:-, cationic lipid:anionic polymer) of 1:2 and generated lipoplexes with negative ζ-potentials.

The gene knockdown effect of anionic polymer-coated siRNA lipoplexes was comparable to that of cationic siRNA lipoplexes, although the cellular association of anionic polymer-coated siRNA lipoplexes was lower than that of cationic siRNA lipoplexes (Figs. 1 and 2). In our previous report, we observed that anionic polymer coating of cationic siRNA lipoplexes composed of DOTAP/cholesterol resulted in the disappearance of the *in vitro* gene knockdown effect (23). The discrepancy in the gene knockdown effect caused by anionic polymer coating is likely attributable to the lipid composition of the lipoplexes, since cationic complexes containing DOPE exhibited a greater gene knockdown effect despite their low cellular association (24). Namely, the *in vitro* gene knockdown effects of siRNA lipoplexes composed of DOTAP/DOPE and DDAB/DOPE were higher than those of DOTAP/cholesterol

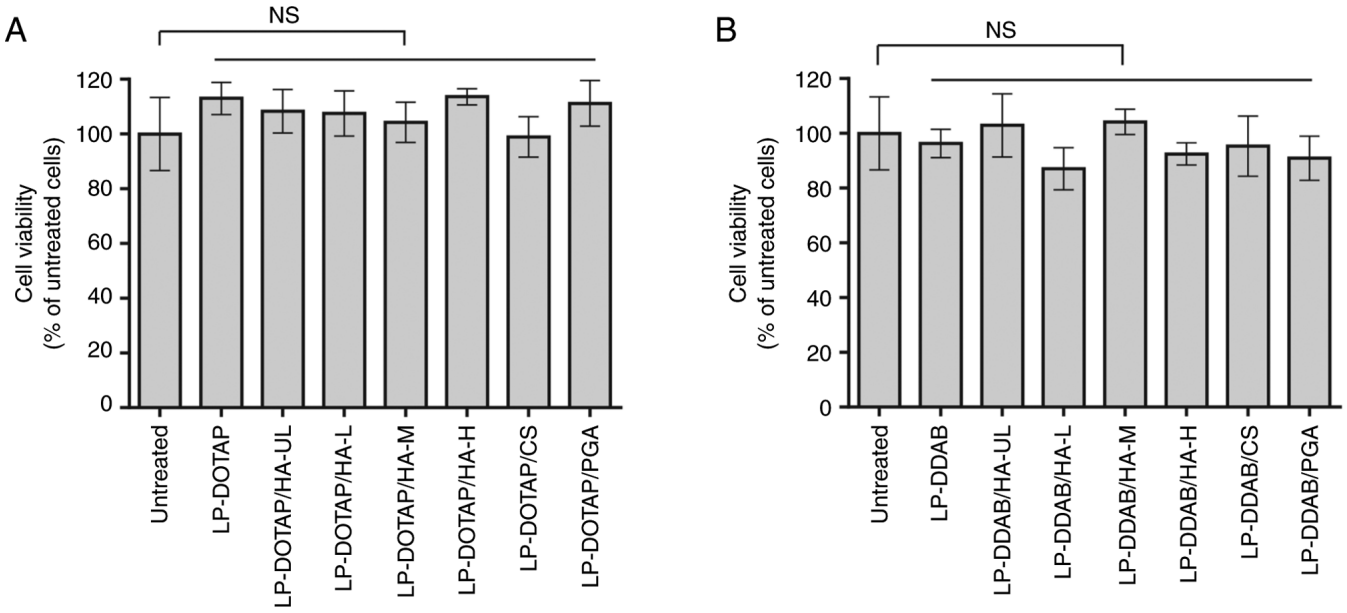

Figure 3. Effect of anionic polymer-coated siRNA lipoplexes on cell viability. MCF-7-Luc cells were treated for 24 h with anionic polymer-coated (A) LP-DOTAP or (B) LP-DDAB at a final control siRNA concentration of 50 nM. Each value represents the mean ± standard deviation (n=5-6). CS, chondroitin sulfate; DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; HA, hyaluronan; HA-H, high-molecular-weight HA; HA-L, low-molecular-weight HA; HA-M, medium-molecular-weight HA; HA-UL, ultra-low-molecular-weight HA; LP, lipoplex; NS, not significant; PGA, polyglutamic acid; siRNA, small interfering RNA.

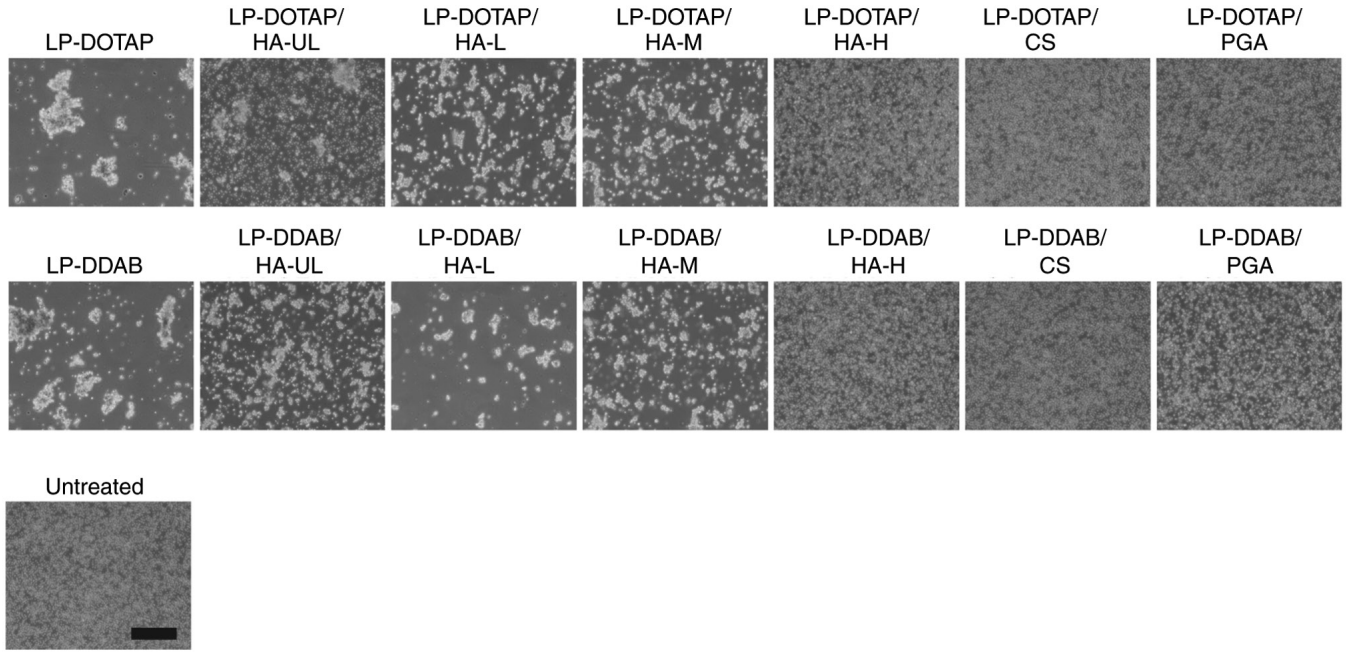

Figure 4. Erythrocyte agglutination with anionic polymer-coated siRNA lipoplexes. Anionic polymer-coated LP-DOTAP or LP-DDAB with 2 μg control siRNA were added to erythrocyte suspensions, and agglutination was observed by microscopy. Scale bar, 200 μm. CS, chondroitin sulfate; DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; HA, hyaluronan; HA-H, high-molecular-weight HA; HA-L, low-molecular-weight HA; HA-M, medium-molecular-weight HA; HA-UL, ultra-low-molecular-weight HA; LP, lipoplex; PGA, polyglutamic acid; siRNA, small interfering RNA.

and DDAB/cholesterol, although cellular associations of siRNA lipoplexes comprising DOTAP/DOPE and DDAB/DOPE were lower than those of DOTAP/cholesterol and DDAB/cholesterol (24). Recently, Nabar *et al* (25) reported that anionic polymer coating on lipid nanoparticles can alter cellular interactions and the intracellular trafficking of their cargo (mRNA and plasmid DNA). Therefore, anionic polymer-coated siRNA lipoplexes may effectively deliver siRNA into cells; however, the intracellular distribution of siRNA delivered by anionic polymer-coated lipoplexes needs to be evaluated.

After intravenous injection of cationic lipoplexes, agglutinates of cationic lipoplexes and erythrocytes are

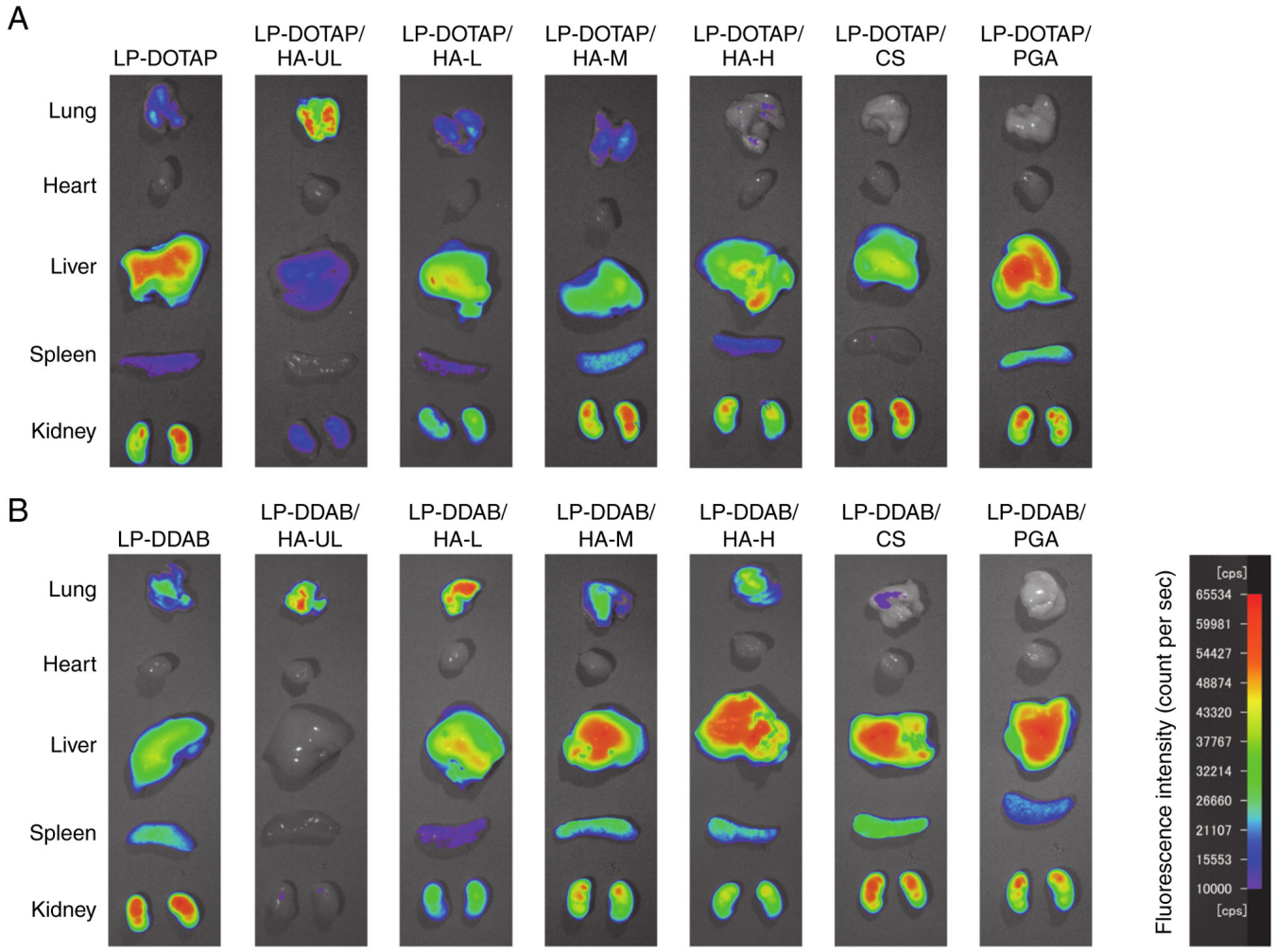

Figure 5. Biodistribution of siRNA in mice 1 h after intravenous injection of anionic polymer-coated siRNA lipoplexes. Fluorescence imaging of the tissues was performed 1 h after injection of mice with anionic polymer-coated (A) LP-DOTAP or (B) LP-DDAB with 10 μg Cy5-siRNA. Images were obtained from one mouse for each siRNA lipoplex. The fluorescence intensity was illustrated using a color-coded scale (red is the maximum, purple is the minimum). cps, count per sec; CS, chondroitin sulfate; DDAB, dimethyldioctadecylammonium bromide; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; HA, hyaluronan; HA-H, high-molecular-weight HA; HA-L, low-molecular-weight HA; HA-M, medium-molecular-weight HA; HA-UL, ultra-low-molecular-weight HA; LP, lipoplex; PGA, polyglutamic acid; siRNA, small interfering RNA.

reportedly entrapped in the highly extended lung capillary (6), which complicates delivery to organs other than the lungs. PEG modification of the cationic lipoplex surface decreases accumulation in the lung by avoiding association with blood components, but PEG-modified lipoplexes lead to low transfection efficiency (8-10). To overcome this problem, the use of PEG-lipid derivatives with cleavable linkers or releasable PEG derivatives has been explored (9,26). In the current study, we found that HA-H-, CS-, and PGA-coated lipoplexes prevented erythrocyte agglutination and exhibited transfection efficiency comparable to cationic lipoplexes (Figs. 1 and 4). These anionic polymers are biodegradable, biocompatible, and exhibit low toxicity. Their structures and charge density of polymers greatly influence the biodistribution of lipoplexes coated with them. HA has one carboxyl group per disaccharide unit, CS has a carboxyl group and a sulfate group per disaccharide unit, and PGA has one carboxyl group per glutamic acid unit. Because HA has a lower charge density than CS or PGA, it is not expected to interact strongly with lipoplexes (11); however, HA-H is likely to interact more strongly and suppress erythrocyte agglutination. These differences in charge density and structural features result in distinct performance among HA-, CS-, and PGA-coated lipoplexes, particularly in terms of coating strength, erythrocyte agglutination, and biodistribution behavior. HA and CS are known ligands for the cell surface receptor CD44, which is overexpressed in numerous cancers (27,28). The usefulness of CD44 targeting by these polymers has been reported previously (29,30). Regarding HA, negatively charged lipoplexes were formed with all molecular weights used for coating; however, the tendency to aggregate with erythrocytes varied depending on the molecular weight of HA (Fig. 4). Previous reports suggested that molecular weight and grafting density of HA on nanoparticles influenced the blood circulation time or receptor recognition of nanoparticles (31-34). Therefore, HA-H-coated lipoplexes are considered to have the potential to reduce non-specific interactions, thereby improving their stability *in vivo* while maintaining affinity for target receptors. In the case of PGA, the tumor- or liver-targeting properties have been reported previously (23,35-37). It is

important to evaluate the tumor- or liver-targeting efficacy of anionic polymer-coated lipoplexes prepared using the MEI method in the future.

Lipoplexes which form aggregates with erythrocytes tend to accumulate in the lungs, the first capillary bed they pass through (6,7). Aggregation with erythrocytes was observed for both cationic lipoplexes (LP-DOTAP and LP-DDAB) and those coated with HA-UL, HA-L or HA-M (Fig. 4), but not all lipoplexes accumulated in the lungs (Fig. 5). This discrepancy might be explained by differences in the stability of the aggregates, which may induce dissociation of lipoplexes from erythrocyte. It has been reported that the surface chemistry of nanoparticles influenced the composition of the protein corona, thereby affecting their organ tropism (38). Nanoparticles containing DOTAP have been reported to form a protein corona enriched in vitronectin, which binds to $\alpha_v\beta_3$ integrin highly expressed in lung epithelium (38,39); however, in this study, LP-DOTAP exhibited higher accumulation in the liver. It might be because the lipoplex prepared in 5% glucose solution using the MEI method has different morphology or surface properties. Further studies are needed to clarify these mechanisms.

Anionic polymer-coated lipoplexes (especially CS- or PGA-coated lipoplexes) altered the biodistribution of the siRNAs (Fig. 5). However, the accumulation of siRNA lipoplexes does not consistently correlate with the gene knockdown effect *in vivo* (24,40). Previous studies have shown that siRNA lipoplexes coated with CS or PGA accumulate in the liver, but that only PGA-coated lipoplexes can suppress the mRNA level of the hepatic target gene (23). Furthermore, it has been reported that sequential intravenous administration of CS followed by cationic lipoplexes results in siRNA accumulation in the liver and subsequent suppression of the hepatic target gene (41). A limitation of the present study is the absence of quantitative biodistribution data. Because fluorescence imaging offers qualitative visualization rather than reliable quantitative measurements (42), the biodistribution profiles of the lipoplexes could not be discussed quantitatively. Further studies are required to clarify the relationship between the biodistribution of anionic polymer-coated lipoplexes and the *in vivo* gene knockdown effects.

In conclusion, cationic siRNA lipoplexes were prepared using the MEI method and successfully coated with anionic polymers without a loss of transfection efficiency. HA-H-, CS-, and PGA-coated lipoplexes prevented erythrocyte agglutination, while decreased lung accumulation and increased liver accumulation were observed after CS or PGA coating of siRNA lipoplexes. Although further evaluation is necessary, anionic polymer-coated lipoplexes prepared using the MEI method could enable siRNA delivery to the liver or tumor tissues.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KK and YH conceived and designed the experiments. KK conducted the investigation, curated data, performed formal analysis, prepared the original draft, and wrote, reviewed and edited the manuscript. KK and YH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Hoshi University (approval no. P24-094; Shinagawa, Tokyo, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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