Assessment of anionic siRNA lipoplexes prepared via modified ethanol injection for tumor cell delivery

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Abstract. Our previous study introduced a modified ethanol injection (MEI) method for preparing positively charged small interfering RNA (siRNA) lipoplexes by mixing a lipid-ethanol solution of cationic lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and poly(ethylene glycol) cholesteryl ether (PEG-Chol) with siRNA in phosphate-buffered saline (PBS). This method was adapted in the current study to develop a two-step MEI process for creating anionic siRNA lipoplexes. First, a lipid-ethanol solution comprising one of four cationic lipids [1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt (DOTAP), dimethyldioctadecylammonium bromide (DDAB), *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide (DC-1-16), or 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy) methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide (TC-1-12)], DOPE and PEG-Chol was combined with siRNA in PBS. Next, a lipid-ethanol solution of cholesteryl hemisuccinate (CHS) and DOPE was added. The gene-silencing activity of anionic siRNA lipoplexes was evaluated in human breast cancer (MCF-7) and human cervical carcinoma (HeLa) cells. Additionally, their interactions with erythrocytes were investigated. Regardless of the cationic lipid used, adding the CHS and DOPE-ethanol solution inverted the ζ-potentials of all siRNA lipoplexes from positive to negative. Notably, TC-1-12-based lipoplexes achieved strong gene silencing while minimizing interactions with erythrocytes. This study demonstrates the effectiveness of the two-step MEI method for preparing anionic siRNA lipoplexes.

Introduction

Small-interfering RNA (siRNA) therapeutics are synthetic RNA duplexes designed to target and degrade specific mRNA (1,2). Cationic liposomes, commonly used as vectors to deliver siRNA, can form complexes called siRNA lipoplexes (3,4). However, upon systemic administration, these positively charged siRNA lipoplexes bind blood components, including erythrocytes (5), forming agglutinates that are sequestered by lung capillaries (6). To avoid this, poly(ethylene glycol) modification (PEGylation) of siRNA lipoplexes is commonly applied to improve circulating stability (5,7). However, the presence of PEG on lipoplex surfaces also poses an obstacle, widely known as the 'PEG dilemma', because PEGylation reduce cellular uptake and endosomal escape, thereby compromising overall gene-silencing efficacy (8,9).

To minimize interactions between siRNA lipoplexes and blood components, their surface charge should be negative or neutral. Negatively charged ternary siRNA lipoplexes have been created using anionic liposomes composed of dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylethanolamine (DOPE) with Ca^{2+} ions and siRNA, where Ca^{2+} electrostatically binds the phosphate groups of DOPG and siRNA (10,11). Alternatively, biodegradable anionic polymer coatings, such as hyaluronic acid, chondroitin sulfate, and poly-L-glutamic acid, can convert cationic siRNA lipoplexes into anionic lipoplexes (12,13). Meanwhile, neutral or anionic siRNA lipid nanoparticles can be generated by rapidly mixing organic solvent-dissolved phospholipid, cholesterol, PEG-lipid, and ionizable cationic lipid, with aqueous solution-dissolved siRNA via microfluidics (14,15). Although these approaches prevent blood component agglutination, they require specialized preparation equipment.

A single-step modified ethanol injection (MEI) protocol was previously developed to prepare cationic siRNA lipoplexes (16,17). This method involves the rapid injection of siRNA dissolved in phosphate-buffered saline (PBS) into a lipid-ethanol solution, resulting in efficient formation of small, uniform siRNA lipoplexes without the need for specialized equipment. In the present study, a two-step MEI approach is employed to generate anionic siRNA lipoplexes. Their gene-silencing efficiency and interaction with erythrocytes are evaluated.

Materials and methods

Materials. 1,2-Dioleoyl-3-trimethylammonium-propane methyl sulfate salt (DOTAP) was obtained from Avanti Polar

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Lipids Inc. (Alabaster, AL, USA). Dimethyldioctadecylammonium bromide (DDAB; cat.no. DC-1-18), *N*-hexadecyl-*N*,*N*-dimethylhexadecan-1-aminium bromide (cat. no. DC-1-16), and 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl) propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide (cat. no. TC-1-12) were purchased from Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan). DOPE and polyethylene glycol-cholesteryl ether (PEG-Chol, average PEG molecular weight: 1,600) were purchased from NOF Co., Ltd. (Tokyo, Japan). Cholesteryl hemisuccinate (CHS) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Firefly luciferase (Luc)-specific siRNA (target sequence: hLuc$^+$; GenBank accession no. AY535007.1), firefly pGL3 Luc-specific siRNA (target sequence: Luc$^+$; GenBank accession no U47295.2), non-silencing control siRNA (Cont siRNA), and cyanine 5 (Cy5)-conjugated Cont siRNA (Cy5-siRNA) were synthesized by Sigma Genosys (Tokyo, Japan) as previously described (17-19). AlexaFluor555-labeled Allstars negative control siRNA (AF555-siRNA) was purchased from Qiagen (Valencia, CA, USA). Hoechst 33342, trihydrochloride trihydrate, was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture. Human breast cancer MCF-7-Luc cells stably expressing Luc (hLuc$^+$) were gifted by Dr. Kenji Yamato (University of Tsukuba, Ibaraki, Japan). Short tandem repeat (STR) DNA profile analysis verified that the MCF-7-Luc cells utilized in this study were identical to the MCF-7 cells registered with the Japanese Collection of Research Bioresources Cell Bank (National Institute of Biomedical Innovation, Ibaraki, Osaka, Japan). Human cervical carcinoma HeLa-Luc cells (CVCL_5J41) stably expressing Luc (Luc$^+$) were acquired from Caliper Life Sciences (Hopkinton, MA, USA).

MCF-7-Luc cells were maintained in Roswell Park Memorial Institute-1640 medium (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific) and 1.2 mg/ml G418 sulfate (Santa Cruz Biotechnology, Dallas, TX, USA) in a humidified incubator at 37˚C and 5% CO_2. HeLa-Luc cells were cultured under identical conditions in Eagle's minimum essential medium (FUJIFILM Wako Pure Chemical Industries, Ltd.) supplemented with 10% FBS and 100 μg/ml kanamycin.

Preparation of cationic and anionic siRNA lipoplexes using MEI method. To prepare cationic siRNA lipoplexes, a lipid-ethanol solution was prepared by dissolving cationic lipid (DOTAP, DDAB, DC-1-16, or TC-1-12), DOPE, and PEG-Chol in ethanol at a molar ratio of 49.5:49.5:1 (2 mg/ml concentration for the cationic lipid), following previously reported methods (17). For example, 2 mg TC-1-12, 1.53 mg DOPE, and 0.08 mg PEG-Chol were dissolved in 1 ml of ethanol. Subsequently, 50 pmol siRNA solution was transferred to a tube containing 100 μl of PBS (pH 7.4). The resulting solution was rapidly added to the lipid-ethanol solution (3.1 μl for LP-DOTAP, 2.3 μl for LP-DC116, 2.5 μl for LP-DDAB, and 3.9 μl for LP-TC112) in another tube to achieve a charge ratio (+:-) of 4:1, based on previous reports (16,17). The charge ratio (+:-) refers to the molar ratio of quaternary amines in the cationic lipid to the siRNA phosphates.

To prepare anionic siRNA lipoplexes, another lipid-ethanol solution was prepared by dissolving 2 mg CHS and 3.06 mg DOPE (molar ratio of 1:1) in 1 ml of ethanol. The lipid-ethanol solution was then mixed with a cationic lipoplex suspension containing 50 pmol siRNA. Volumes of 1.95 and 3.89 μl of the lipid-ethanol solution were used to achieve charge ratios (+:-) of 1:1 and 1:2 (1CHS and 2CHS), respectively. The charge ratio (+:-) was calculated as the molar ratio of cationic lipids to CHS.

Measurement of liposome and siRNA lipoplex sizes. Cationic and anionic siRNA lipoplexes containing 5 μg Cont siRNA were prepared using the MEI method. Cationic and anionic liposomes were prepared using the same amount of lipid-ethanol solution as the siRNA lipoplexes but without siRNA. The liposomes and siRNA lipoplexes were diluted threefold with water. Subsequently, the particle size, polydispersity index (PDI), and ζ-potential of the liposomes and siRNA lipoplexes were measured using a light-scattering photometer (ELS-Z2; Otsuka Electronics Co., Ltd., Osaka, Japan).

Evaluation of Luc knockdown efficiency in Luc siRNA lipoplexes. Anionic and cationic siRNA lipoplexes containing 50 pmol of Cont and Luc siRNAs were diluted in culture medium supplemented with 10% FBS to a final siRNA concentration of 50 nM. The siRNA lipoplexes were added to MCF-7-Luc and HeLa-Luc cells in 12-well culture plates. Luc activity [counts per second (cps)] was determined 48 h post-transfection as previously described (5). Luc activity (%) was calculated relative to that (cps/μg protein) of untreated cells.

Cytotoxicity after transfection with anionic and cationic lipoplexes. MCF-7-Luc and HeLa-Luc cells were seeded in 96-well culture plates at a density of $1x10^4$ cells/well. After 24 h of incubation at 37˚C, anionic and cationic lipoplexes containing 50 pmol Cont siRNA were prepared using the MEI method. The lipoplexes were diluted with culture medium containing 10% FBS (final siRNA concentration: 50 nM) and subsequently added to the cells (100 μl/well). Cytotoxicity was assessed 48 h post-transfection using the Cell Counting Kit-8 (Dojindo Laboratories, Inc., Kumamoto, Japan), as previously reported (20).

Cellular uptake of siRNA lipoplexes. To quantify the amount of siRNA taken up by the cells, MCF-7-Luc cells were seeded in 12-well culture plates at a density of $1x10^5$ cells/well. After 24 h of incubation at 37˚C, cationic and anionic lipoplexes containing 50 pmol AF555-siRNA in 1 ml of culture medium were added to the cells (final siRNA concentration: 50 nM). After 24-h incubation, the plates were washed with PBS to remove unbound lipoplexes, and the cells were lysed with cell lysis buffer (Pierce™ Luciferase Cell Lysis Buffer; Pierce; Thermo Fisher Scientific, Inc.), followed by centrifugation at 15,000 g for 10 sec at 4˚C. AF555-fluorescence intensity [relative fluorescence unit (RFU)] in the supernatant were then measured using a fluorescence microplate reader (GloMax® Discover system, Promega Corporation, Madison, WI, USA) at 525 nm of excitation and 580-640 nm of emission. Protein concentrations of the supernatants were determined with

bicinchoninic acid (BCA) reagent (Pierce™ BCA Protein Assay Kit; Pierce; Thermo Fisher Scientific, Inc.). The fluorescence intensity of AF555-siRNA (RFU/μg protein) in the cells was calculated by subtracting the background fluorescence (RFU/μg protein) of untreated cells.

To observe the localization of siRNA in the cells, MCF-7-Luc cells were seeded in 12-well culture plates at a density of $1x10^5$ cells/well. After 5 or 24 h of incubation at 37˚C, anionic and cationic lipoplexes containing 50 pmol Cy5-siRNA in 1 ml of culture medium were added to the cells (final siRNA concentration: 50 nM). At 24 h post-transfection, the cells were fixed with Mildform 10N (FUJIFILM Wako Pure Chemical Industries, Ltd.) for 10 min at room temperature. Subsequently, the cells were incubated with 10 μg/ml Hoechst 33342 for 10 min to stain the nuclei. Cy5-siRNA and nuclei within the cells were detected using an Eclipse TS100-F fluorescence microscope (Nikon Corporation, Tokyo, Japan) equipped with optical filters for Cy5 (excitation filter: 620/60 nm, dichroic mirror: 660 nm, absorption filter: 700/75 nm) and Hoechst 33342 (excitation filter: 365/10 nm, dichroic mirror: 400 nm, absorption filter: 400 nm).

Animal experiments. Animal experiments adhered to the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Hoshi University (Tokyo, Japan; approval number: P24-151, Research period: from December 10, 2024, to February 28, 2025). The predetermined endpoint for experimental termination was set as: the mice would be euthanized by cervical dislocation if the solid tumor volume in a tumor-bearing mouse exceeded 2,000 mm^3. However, this endpoint criterion was not applied to any mice during the course of the study.

One eight-week-old female BALB/c mouse obtained from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan) was maintained in a temperature-controlled room (24˚C) at 55% relative humidity under a 12/12-h light/dark cycle (08:00 to 20:00) with *ad libitum* access to food and water.

Hemolysis and agglutination assay. Study on erythrocyte characteristics based on donor sex shows that female donors have erythrocytes with greater resilience to storage damage and hemolysis (21). Therefore, erythrocytes from female mice were used. Total duration of the study was 1 day. Blood (0.2 ml) was collected from the jugular vein of one female BALB/c mouse under anesthesia induced by isoflurane inhalation (4% gas-air mixture for induction and 2% gas-air mixture for maintenance; FUJIFILM Wako Pure Chemical Corporation). Immediately after the blood collection, the mice were euthanized by cervical dislocation; death was confirmed by cessation of heartbeat. Erythrocytes were collected from the blood at 4˚C by centrifugation at 300 x g for 3 min and resuspended in PBS as a 2% (v/v) suspension of erythrocytes.

To assess agglutination, cationic and anionic siRNA lipoplexes containing 50 pmol Cont siRNA in 100 μl of PBS were added to 100 μl of a 2% (v/v) erythrocyte suspension. After incubating at 37˚C for 15 min, the sample was placed on a glass slide, and agglutination was observed under a light microscope (Eclipse TS100-F, Nikon Corporation).

To examine erythrocyte hemolysis induced by siRNA lipoplexes, cationic and anionic siRNA lipoplexes containing 50 pmol Cont siRNA in 100 μl of PBS were added to 100 μl of a 2% (v/v) erythrocyte suspension; the hemolysis proportion was calculated as previously reported (22). Erythrocytes suspended in water served as positive controls for complete hemolysis (100% hemolysis).

Statistical analysis. Two groups were compared using an unpaired Student's t-test. Multiple groups were compared using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. All data were analyzed using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA, USA), and statistical significance was set at $P<0.05$.

Results

Characterization of cationic and anionic siRNA lipoplexes. To prepare cationic siRNA lipoplexes, DOTAP, DC-1-16, DDAB, and TC-1-12 were used as cationic lipids, with DOPE as a neutral lipid and PEG-Chol as a PEG-lipid (Fig. 1). Cationic lipoplexes were formed by the MEI method with a 49.5:49.5:1 molar ratio of cationic lipid, DOPE, and PEG-Chol (Table I). The resulting LP-DOTAP, LP-DC116, LP-DDAB, and LP-TC112 lipoplexes measured 148, 224, 200, and 92 nm in size, respectively (PDI: 0.10, 0.08, 0.11, and 0.16, respectively), and had positive ζ-potentials (~4.6-11 mV).

To prepare anionic liposomes, CHS and DOPE were mixed at a 1:1 ratio in ethanol. Using the MEI method, LP-CHS was obtained with a particle size of 97 nm and a ζ-potential of -63 mV (Table SI), indicating a strong negative charge capable of neutralizing cationic charges.

To determine the optimal amount of CHS required to neutralize cationic liposomes, anionic liposomes were prepared using the two-step MEI method by sequentially combining a lipid-ethanol solution of cationic lipid, DOPE, and PEG-Chol with PBS without siRNA, followed by the addition of a CHS-DOPE lipid-ethanol solution. CHS was incorporated at either equimolar (1CHS) or double the molar amount (2CHS) relative to the cationic lipid. Adding CHS at twice the molar ratio consistently restored a negative charge to the liposomes (Table SI).

Anionic siRNA lipoplexes were prepared using a two-step MEI method: a lipid-ethanol solution containing cationic lipids, DOPE, and PEG-Chol was rapidly mixed with siRNA in PBS, followed by the addition of a CHS-DOPE ethanol solution (Fig. 2). LP-DOTAP-1CHS, LP-DC116-1CHS, and LP-DDAB-1CHS lipoplexes had near-neutral ζ-potentials (-4.8 to 0.3 mV), and increased sizes (230, 374, and 214 nm, respectively) compared to lipoplexes without CHS (Table I). Meanwhile, their 2CHS counterparts exhibited strongly negative ζ-potentials (-16.2 to -26.5 mV) and smaller sizes (207, 229, and 162 nm, respectively). LP-TC112-1CHS and LP-TC112-2CHS lipoplexes measured 156 and 146 nm with ζ-potentials of -12.8 and -19.5 mV, respectively.

Luc activity suppression by cationic and anionic Luc siRNA lipoplexes. Cationic and anionic lipoplexes containing Luc siRNA were transfected into MCF-7-Luc and HeLa-Luc cells, with Luc activity measured 48 h post-transfection. Cationic siRNA

Table I. Size and ζ-potential of cationic and anionic siRNA lipoplexes prepared using modified ethanol injection method.

Lipoplex	Liposomal formulation (molar ratio)	Size[a], nm	PDI	ζ-potential[a], mV
LP-DOTAP	DOTAP/DOPE/PEG_{1600}-Chol (49.5:49.5:1.0)	147.8±1.5	0.10±0.01	10.9±3.2
LP-DOTAP-1CHS	DOTAP/DOPE/CHS/PEG_{1600}-Chol (24.9:49.7:24.9:0.5)	229.2±4.3	0.06±0.01	-2.2±1.8
LP-DOTAP-2CHS	DOTAP/DOPE/CHS/PEG_{1600}-Chol (16.6:49.8:33.2:0.3)	206.5±1.6	0.12±0.02	-26.5±1.8
LP-DC116	DC-1-16/DOPE/PEG_{1600}-Chol (49.5:49.5:1.0)	223.5±1.6	0.08±0.01	11.1±0.6
LP-DC116-1CHS	DC-1-16/DOPE/CHS/PEG_{1600}-Chol (24.9:49.7:24.9:0.5)	374.4±4.0	0.23±0.01	0.3±0.7
LP-DC116-2CHS	DC-1-16/DOPE/CHS/PEG_{1600}-Chol (16.6:49.8:33.2:0.3)	228.5±0.5	0.30±0.01	-16.2±4.6
LP-DDAB	DDAB/DOPE/PEG_{1600}-Chol (49.5:49.5:1.0)	199.9±0.9	0.11±0.01	4.6±0.0
LP-DDAB-1CHS	DDAB/DOPE/CHS/PEG_{1600}-Chol (24.9:49.7:24.9:0.5)	213.6±2.3	0.19±0.01	-4.8±0.1
LP-DDAB-2CHS	DDAB/DOPE/CHS/PEG_{1600}-Chol (16.6:49.8:33.2:0.3)	161.8±1.5	0.25±0.01	-17.8±2.8
LP-TC112	TC-1-12/DOPE/PEG_{1600}-Chol (49.5:49.5:1.0)	91.9±0.4	0.16±0.02	8.0±1.3
LP-TC112-1CHS	TC-1-12/DOPE/CHS/PEG_{1600}-Chol (24.9:49.7:24.9:0.5)	156.0±1.7	0.10±0.01	-12.8±0.5
LP-TC112-2CHS	TC-1-12/DOPE/CHS/PEG_{1600}-Chol (16.6:49.8:33.2:0.3)	145.7±2.8	0.08±0.02	-19.5±1.5

[a]siRNA lipoplexes suspended in PBS were diluted 3 times with water. Each value represents the mean ± standard deviation (n=3). LP, liposome; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl) amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; CHS, cholesteryl hemisuccinate.

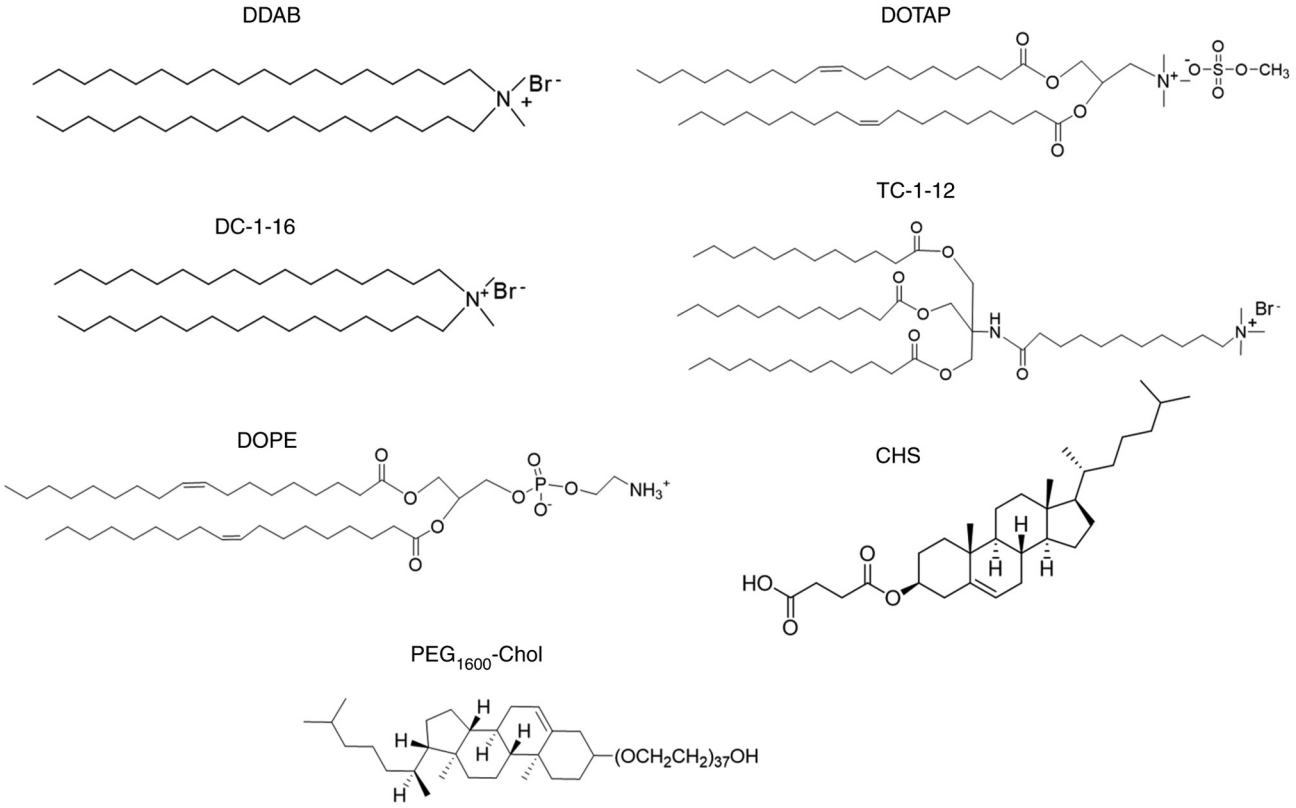

Figure 1. Structures of cationic lipids, anionic lipid, neutral lipid and PEG-lipid. DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; CHS, cholesteryl hemisuccinate; PEG-Chol, poly(ethylene glycol) cholesteryl ether.

lipoplexes exhibited strong gene-silencing activity in both cell lines, regardless of the cationic lipid type (Fig. 3A and B). However, for DOTAP, DC116, and DDAB formulations, increasing CHS reduced silencing activity. Notably, LP-DC116-2CHS and LP-DDAB-2CHS showed no silencing in MCF-7-Luc cells. In contrast, LP-TC-112-1CHS and LP-TC112-2CHS lipoplexes did not exhibit decreased gene silencing activity in either cell type compared with LP-TC-112 lipoplexes.

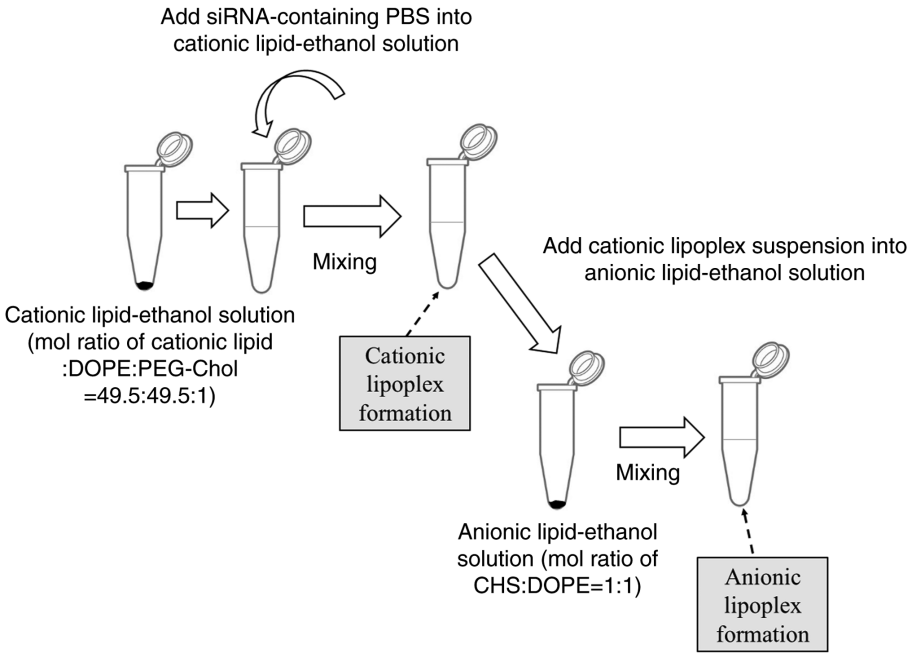

Figure 2. Schematic diagram of anionic siRNA lipoplex preparation. To prepare cationic siRNA lipoplexes, a 100 μl PBS solution (pH 7.4) containing 50 pmol siRNA was rapidly added to a lipid-ethanol solution of cationic lipid, DOPE and PEG-Chol at a molar ratio of 49.5:49.5:1. To prepare anionic lipoplexes, the cationic lipoplex suspension was added to a lipid-ethanol solution of CHS and DOPE at a molar ratio of 1:1, achieving charge ratios (+:-) of 1:1 or 2:1 for cationic lipid:CHS. siRNA, small interfering RNA; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PEG-Chol, poly(ethylene glycol) cholesteryl ether; CHS, cholesteryl hemisuccinate.

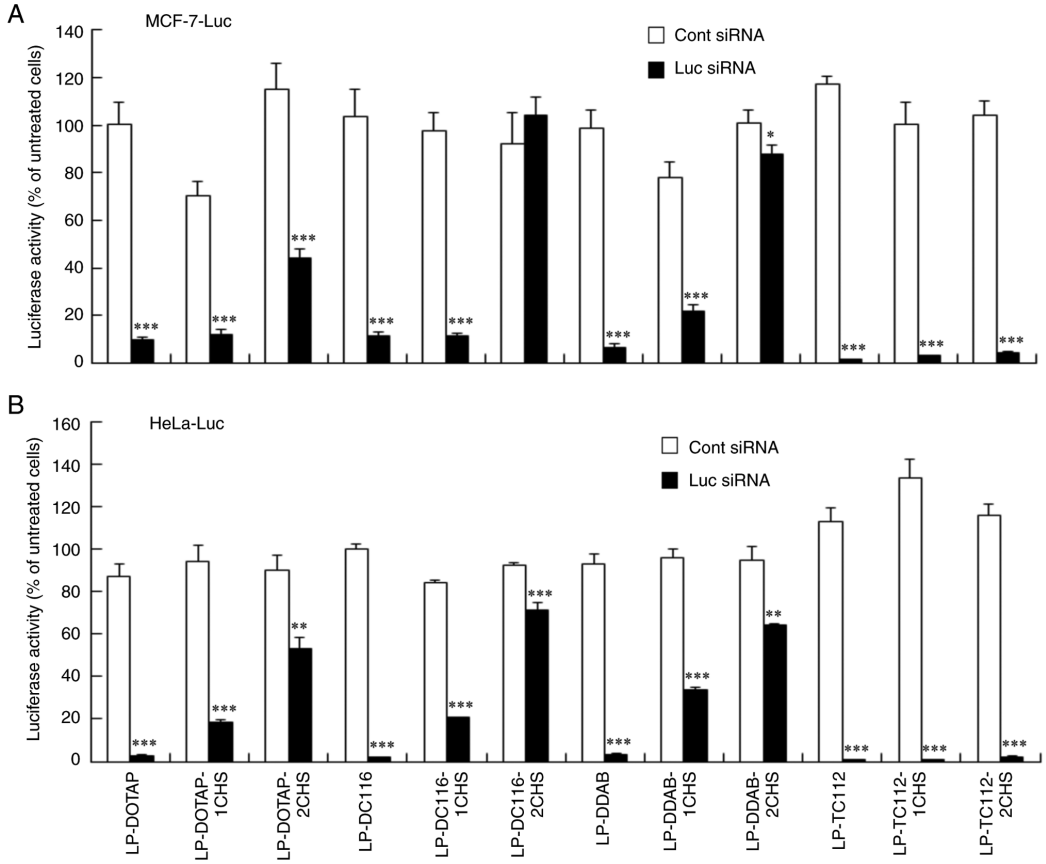

Figure 3. Luc activity suppression by cationic and anionic siRNA lipoplexes. Cationic and anionic lipoplexes containing Cont and Luc siRNAs were prepared using the modified ethanol injection method. These lipoplexes were then added to (A) MCF-7-Luc and (B) HeLa-Luc cells at a final siRNA concentration of 50 nM. Luc activity was measured 48 h post-transfection. Data are represented as the mean + standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. Cont siRNA. Luc, luciferase; siRNA, small interfering RNA; Cont, control; LP, liposome; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; CHS, cholesteryl hemisuccinate.

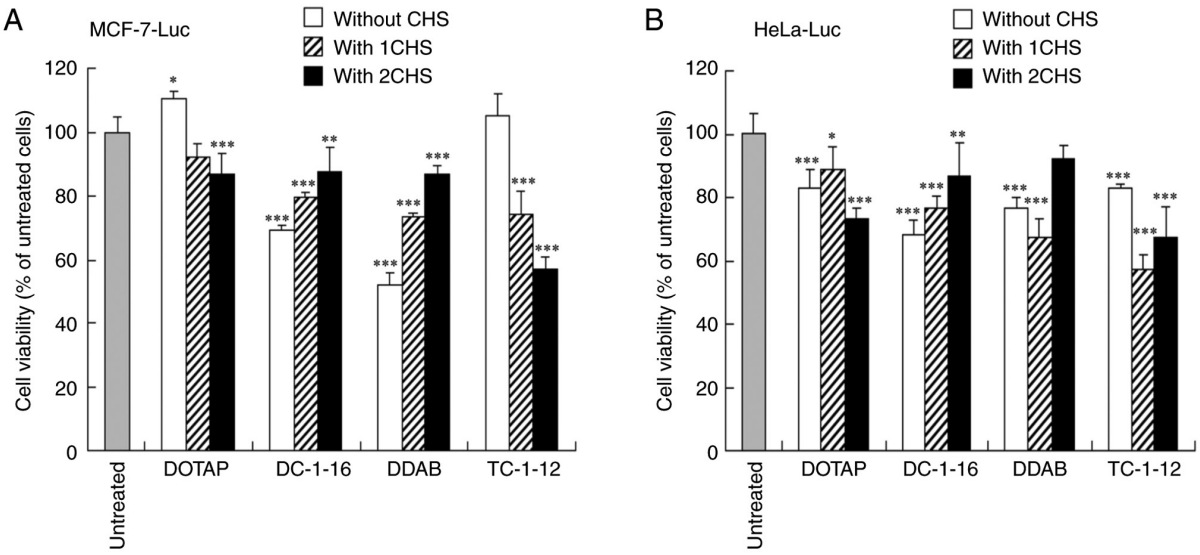

Figure 4. Cell viability after transfection with cationic and anionic siRNA lipoplexes. Cationic and anionic lipoplexes containing Cont siRNA were prepared by the modified ethanol injection method and subsequently added to (A) MCF-7-Luc and (B) HeLa-Luc cells at a final siRNA concentration of 50 nM. Cell viability was assessed 48 h after transfection. Data are represented as the mean + standard deviation (n=5). *P<0.05, **P<0.01 and ***P<0.001 vs. untreated cells. Luc, luciferase; siRNA, small interfering RNA; Cont, control; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; CHS, cholesteryl hemisuccinate.

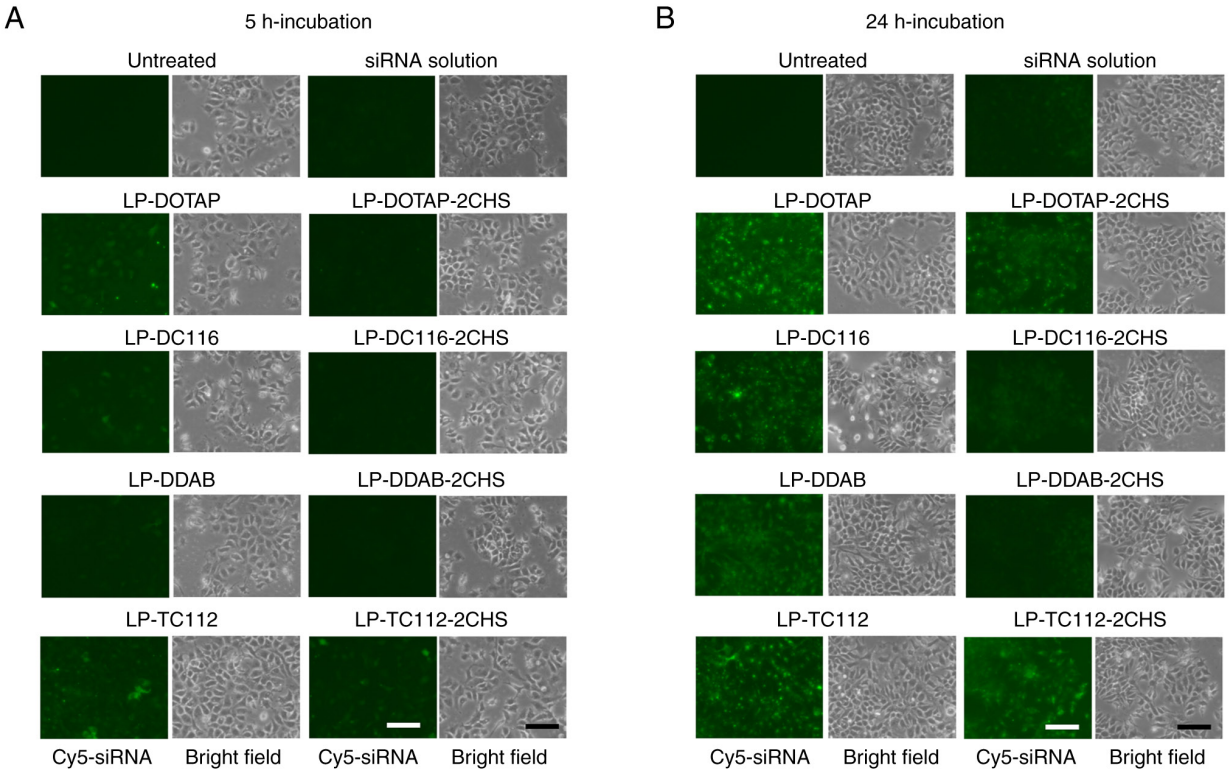

Figure 5. Cellular uptake of siRNA by MCF-7-Luc cells after transfection with cationic and anionic lipoplexes. Cationic and anionic lipoplexes containing Cy5-siRNA were prepared using the modified ethanol injection method and added to MCF-7-Luc cells at a final siRNA concentration of 50 nM. As a control, Cy5-siRNA solution was added to the MCF-7-Luc cells. Localization of Cy5-siRNA (green) was observed at (A) 5 and (B) 24 h post-incubation, respectively. Scale bar, 100 μm. siRNA, small interfering RNA; Luc, luciferase; LP, liposome; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; CHS, cholesteryl hemisuccinate.

Cytotoxicity of cationic and anionic siRNA lipoplexes. Cationic and anionic siRNA lipoplexes were transfected into MCF-7-Luc and HeLa-Luc cells, with viability measured after 48 h. Cytotoxicity of DOTAP and TC-1-12 formulations increased in both cell types with more CHS added to LP-DOTAP and LP-TC-1-12 lipoplexes, respectively

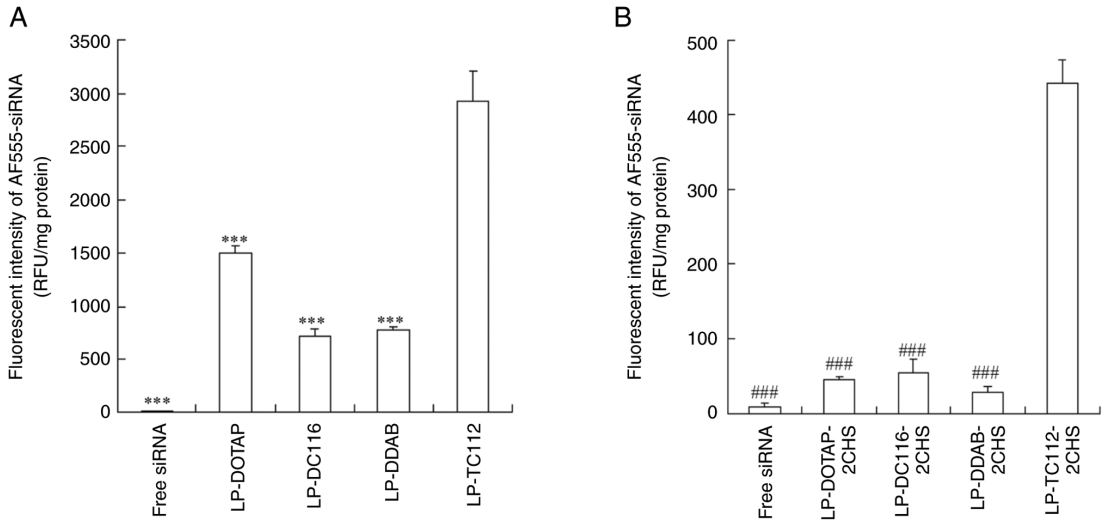

Figure 6. Cellular uptake of siRNA by MCF-7-Luc cells after transfection with cationic and anionic lipoplexes. (A) Cationic and (B) anionic lipoplexes containing AF555-siRNA were prepared using the modified ethanol injection method and added to MCF-7-Luc cells at a final siRNA concentration of 50 nM. As a control, AF555-siRNA solution (free siRNA) was added to the MCF-7-Luc cells. After 24 h, the cells were lysed, and the amount of AF555-siRNA within the cells was measured using a fluorescence plate reader. Data are represented as the mean + standard deviation (n=3). ***P<0.001 vs. LP-TC112; ###P<0.001 vs. LP-TC112-2CHS. LP, liposome; siRNA, small interfering RNA; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; CHS, cholesteryl hemisuccinate.

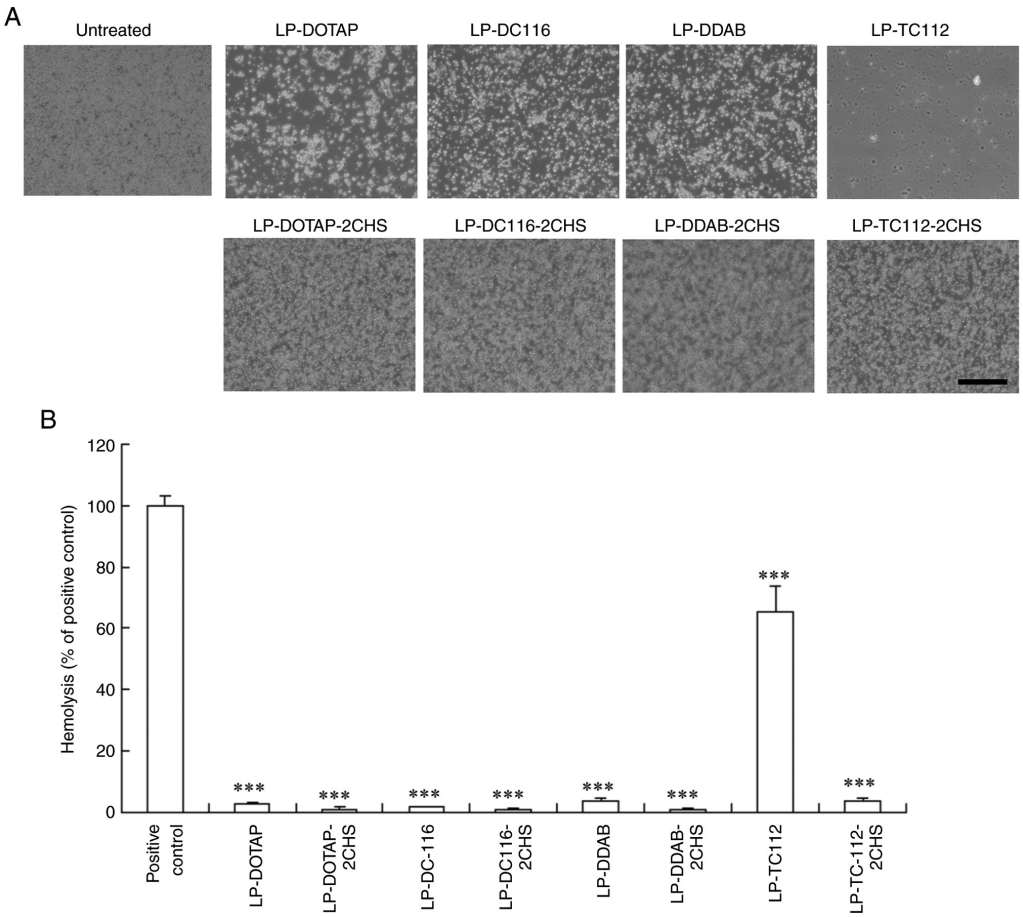

Figure 7. Hemolysis and erythrocyte agglutination induced by cationic and anionic siRNA lipoplexes. Cationic and anionic lipoplexes containing 50 pmol of Cont siRNA were prepared using the modified ethanol injection method and incubated with erythrocyte suspensions at 37˚C for 15 min. (A) Erythrocyte agglutination after mixing with the siRNA lipoplexes. Scale bar, 200 μm. (B) Hemolysis (%) calculated relative to the absorbance of the hypotonic solution and presented as the mean + standard deviation (n=3). Erythrocytes suspended in a hypotonic solution (water) serve as a positive control for 100% hemolysis. ***P<0.001 vs. positive control. LP, liposome; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane methyl sulfate salt; DDAB, dimethyldioctadecylammonium bromide; DC-1-16, *N*-hexadecyl-*N*, *N*-dimethylhexadecan-1-aminium bromide; TC-1-12, 11-((1,3-bis(dodecanoyloxy)-2-((dodecanoyloxy)methyl)propan-2-yl)amino)-*N*,*N*,*N*-trimethyl-11-oxoundecan-1-aminium bromide; CHS, cholesteryl hemisuccinate.

(Fig. 4A and B). Conversely, the cytotoxicity of DC-1-16 and DDAB formulations decreased with increased CHS added to LP-DC116 and LP-DDAB lipoplexes, respectively.

When anionic siRNA lipoplexes with 2CHS were transfected into the cells, the culture medium contained 7.8 μg/ml of CHS. To examine the cytotoxicity of CHS, CHS was added into MCF-7-Luc and HeLa-Luc cells. CHS was found to be more toxic to MCF-7-Luc cells than to HeLa-Luc cells (Fig. S1A). Furthermore, when cationic and anionic liposomes without siRNA were added into both cell lines, LP-TC112-2CHS increased cytotoxicity in MCF-7-Luc cells compared to LP-TC112 (Fig. S1B, C).

siRNA cellular uptake after transfection with cationic and anionic lipoplexes. siRNA uptake in MCF-7-Luc cells was examined 5 or 24 h post-transfection with cationic and anionic lipoplexes. Among the cationic lipoplexes, LP-TC112 lipoplexes exhibited the highest siRNA uptake, showing approximately twofold higher uptake than LP-DOTAP lipoplexes and fourfold higher uptake than both LP-DDAB and LP-DC116 lipoplexes (Figs. 5A, B, 6A). In contrast, all anionic lipoplexes showed reduced siRNA uptake compared to their cationic counterparts (Fig. 5A, B). However, LP-TC112-2CHS lipoplexes exhibited higher siRNA uptake compared to the other anionic lipoplexes (Figs. 6B, S2).

Hemolysis and erythrocyte aggregation by cationic and anionic lipoplexes. Considering that positively charged lipoplexes can disrupt erythrocytes through electrostatic interactions, hemolysis and aggregation of mouse erythrocytes were investigated following incubation with cationic and anionic siRNA lipoplexes. LP-DOTAP, LP-DC116, and LP-DDAB lipoplexes caused marked erythrocyte aggregation (Fig. 7A) but minimal hemolysis (<5%; Fig. 7B). Notably, LP-TC112 lipoplexes induced high levels of hemolysis (65%). In contrast, LP-DOTAP-2CHS, LP-DC116-2CHS, LP-DDAB-2CHS, and LP-TC112-2CHS lipoplexes did not cause erythrocyte aggregation or hemolysis (<5%).

Discussion

This study presents a two-step MEI method to prepare anionic siRNA lipoplexes. CHS served as the anionic lipid to neutralize the positive charge of siRNA lipoplexes. Adding a lipid-ethanol solution of CHS and DOPE to the cationic siRNA lipoplex suspensions reversed the surface potential from positive to negative. It is hypothesized that either CHS and DOPE directly integrate into the lipid membrane of the cationic siRNA lipoplexes, forming anionic siRNA lipoplexes, or pre-formed CHS/DOPE anionic liposomes bind and fuse electrostatically with cationic lipoplexes to yield anionic siRNA lipoplexes.

Neutral charged siRNA lipoplexes (LP-DOTAP-1CHS, LP-DC116-1CHS, and LP-DDAB-1CHS) were larger than their negatively charged counterparts (LP-DOTAP-2CHS, LP-DC116-2CHS, and LP-DDAB-2CHS), likely due to reduced electrostatic repulsion in the neutral charged lipoplexes. At a cationic lipid to CHS ratio of 1:2, all siRNA lipoplexes exhibited strong negative ζ-potentials (-16 to -27 mV), regardless of the cationic lipid used, indicating that twice the molar ratio of CHS relative to the cationic lipid is required to form anionic siRNA lipoplexes.

LP-TC112-2CHS lipoplexes with Luc siRNA achieved strong gene silencing in both MCF-7-Luc and HeLa-Luc cells, whereas LP-DOTAP-2CHS lipoplexes had a moderate effect. LP-DC116-2CHS and LP-DDAB-2CHS lipoplexes were less effective. However, LP-TC112-2CHS lipoplexes exhibited increased cytotoxicity in MCF-7-Luc cells. CHS is an amphipathic cholesterol ester with anticancer activity against MCF-7 cells (23). It was found that CHS was more toxic to MCF-7-Luc cells than to HeLa-Luc cells. In addition, LP-TC112-2CHS without siRNA also exhibited higher cytotoxicity than LP-TC112 without siRNA in MCF-7-Luc cells. These suggest that the LP-TC112-2CHS lipoplexes were efficiently delivered into MCF-7 cells, and the CHS might induce cytotoxicity.

All anionic lipoplexes reduced siRNA uptake in MCF-7-Luc cells compared to their cationic counterparts, indicated that the negative charge on the surface of the lipoplexes may have weakened their interaction with cells. However, intracellular siRNA was detected at high levels following transfection with LP-TC112-2CHS lipoplexes, indicating better cellular uptake and gene silencing. The reduced gene-silencing efficacy of LP-DOTAP-2CHS, LP-DC116-2CHS and LP-DDAB-2CHS lipoplexes is likely due to poor cellular uptake. However, the precise mechanisms underlying the differential internalization of these lipoplexes remain unclear. In our study, siRNA uptake after transfection with cationic lipoplexes was highest in the order of LP-TC112 >LP-DOTAP >LP-DDAB=LP-DC116 lipoplexes. It has been reported that the linker structure of a cationic lipid significantly impacts the cellular uptake of lipoplexes, thereby affecting transfection efficiency (24,25). TC-1-12 and DOTAP have a biodegradable ester linker, while DDAB and DC-1-16 have a non-biodegradable linker. This suggests that the difference in linker structure may affect the interaction between the cell membrane and the siRNA lipoplexes. In addition, TC-1-12, a lipid with trialkyl chains, exhibits high cell membrane fusion activity (20). Koulov et al. reported that vesicles with cationic trialkyl chains promote greater membrane fusion than those with structurally analogous cationic dialkyl or monoalkyl chains (26). Therefore, LP-TC112-2CHS lipoplexes may enhance cellular uptake and gene silencing due to their strong membrane fusion activity, even though they have a negative charge. Future studies should assess how different cationic lipids used in anionic siRNA lipoplexes impact cellular uptake.

LP-DOTAP, LP-DC116, and LP-DDAB lipoplexes caused erythrocyte agglutination without hemolysis, while LP-TC112 lipoplexes exhibited strong hemolytic activity due to their fusogenic properties. However, adding 2CHS prevented agglutination after incubation with LP-DOTAP-2CHS, LP-DC116-2CHS, or LP-DDAB-2CHS lipoplexes, whereas LP-TC112-2CHS lipoplexes did not cause agglutination or hemolysis. Notably, LP-TC112-2CHS maintained high cellular uptake and gene-silencing efficiency while suppressing erythrocyte interaction. Positively charged siRNA lipoplexes interact with erythrocytes and form agglutination in the bloodstream, leading to their entrapment in highly vascularized lung capillaries. However, negatively charged lipoplexes

may reduce siRNA accumulation in the lungs by avoiding interaction with erythrocytes in the bloodstream, resulting in improved blood stability. Further research is warranted to assess its biodistribution and *in vivo* gene-silencing effects. Erythrocytes from female mice were utilized in this study. Nonetheless, male erythrocytes are reportedly more susceptible to hemolysis than female ones (21), suggesting that differences in hemolytic potential across species should be considered carefully.

Previous studies have described the preparation of cationic 3β-[*N*-(*N'*,*N'*-dimethylaminoethyl)carbamoyl] cholesterol (DC-Chol)/DOPE and anionic CHS/DOPE liposomes separately via thin-film hydration, followed by neutralizing positively charged DC-Chol/DOPE-plasmid DNA lipoplexes through incubation with CHS/DOPE liposomes (27). However, this requires specialized equipment, including evaporators, sonicators, and extruders. In the current study, a simple two-step MEI method was developed for preparing anionic siRNA lipoplexes without the need for evaporation or sonication. Overall, the results suggest that TC-1-12-based anionic siRNA lipoplexes prepared using lipid-ethanol solutions may be suitable for siRNA transfection.

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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

YH conceptualized the study, developed the methodology, conducted the investigation, curated the data, performed the formal analysis, prepared the original draft, and wrote, reviewed and edited the manuscript. AK and MS conducted the study. YH and AK confirmed the authenticity of the raw data. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of Hoshi University (approval no. P24-151).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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