Regular Article

siRNA Delivery into Tumor Cells by Cationic Cholesterol Derivative-Based Nanoparticles and Liposomes

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Previously, we reported that cationic nanoparticles (NP) composed of diamine-type cholesteryl-3-carboxamide (OH-Chol, *N*-(2-(2-hydroxyethylamino)ethyl)cholesteryl-3-carboxamide) and Tween 80 could deliver small interfering RNA (siRNA) with high transfection efficiency into tumor cells. In this study, we synthesized new diamine-type cationic cholesteryl carbamate (OH-C-Chol, cholesteryl (2-((2-hydroxyethyl)-amino)ethyl)carbamate) and triamine-type carbamate (OH-NC-Chol, cholesteryl (2-((2-((2-hydroxyethyl)-amino)ethyl)amino)ethyl)carbamate), and prepared cationic nanoparticles composed of OH-C-Chol or OH-NC-Chol with Tween 80 (NP-C and NP-NC, respectively), as well as cationic liposomes composed of OH-C-Chol or OH-NC-Chol with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (LP-C and LP-NC, respectively) for evaluation of their possible use as siRNA delivery vectors. LP-C and LP-NC/siRNA complexes (lipoplexes) exhibited larger gene silencing effects than NP-C and NP-NC/siRNA complexes (nanoplexes), respectively, in human breast tumor MCF-7 cells, although the NP-C nanoplex showed high association with the cells. In particular, LP-NC lipoplex could induce strong gene suppression, even at a concentration of 5 nM siRNA. From these results, cationic liposomes composed of OH-NC-Chol and DOPE may have potential as gene vectors for siRNA transfection to tumor cells.

Key words cationic nanoparticle; cationic liposome; small interfering RNA (siRNA) delivery; transfection; cholesteryl carbamate

RNA interference (RNAi) is a powerful gene-silencing process that holds great promise in the field of cancer therapy.[1)] Synthetic small interfering RNAs (siRNAs), which are small double-stranded RNAs, are substrates for the RNA-induced silencing complex. siRNA suppresses the expression of a target gene by triggering specific degradation of the complementary mRNA sequence.[2)] In siRNA delivery, non-viral vectors such as cationic liposomes or nanoparticles have been more commonly used than viral vectors.[3,4)] For efficient siRNA delivery into cells by cationic liposome, many different cationic lipids have been synthesized. In particular, cholesterol- and glycerol-based cationic lipids have been extensively used for cationic liposome-mediated gene delivery.

For gene delivery with cationic liposomes, cationic cholesterol derivatives are useful due to their high transfection efficiency and low toxicity.[5–7)] Cationic cholesterol derivatives are composed of three distinct parts: a cholesteryl skeleton, a cationic amino group and a linker arm between the cholesteryl skeleton and the cationic amino group. Previously, we reported that cationic nanoparticles (NP) composed of *N*-(2-(2-hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol) and Tween 80 could efficiently deliver siRNA into tumor cells without any helper lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol.[8,9)] OH-Chol is a cationic cholesterol derivative with a hydroxyethyl group at the amino terminal and a carboxamide-type linker. However, to obtain a large gene silencing effect by NP, the presence of sodium chloride in the formulation of NP/siRNA complex (nanoplex) was needed, but it often induced the formation of a large complex. For *in vivo* application, injectable-sized nanoplexes showing a large gene silencing effect must be prepared.

For efficient gene silencing in tumor cells, the intracellular trafficking of siRNA after endocytosis must be controlled. Polyethylenimines (PEIs) are also used as siRNA vectors and have been shown to have the ability to escape the endosome by buffering capacity.[10)] Therefore, to facilitate the endosomal escape of siRNA transfected by cationic nanoparticles or liposomes, we designed new cationic cholesterol derivative, triamine-type cholesteryl carbamate (OH-NC-Chol, cholesteryl (2-((2-((2-hydroxyethyl)amino)ethyl)amino)ethyl)carbamate), which has 2 units of aminoethylene between the amino head group and the cholesterol skeleton. In this study, we synthesized cationic triamine-type cholesteryl carbamate (OH-NC-Chol) and diamine-type carbamate (OH-C-Chol, cholesteryl (2-((2-hydroxyethyl)amino)ethyl)carbamate), and examined the formulation of cationic liposomes and nanoparticles for siRNA delivery. We prepared cationic nanoparticles composed of OH-C-Chol or OH-NC-Chol with Tween 80 (NP-C and NP-NC, respectively) and cationic liposomes composed of OH-C-Chol or OH-NC-Chol with DOPE (LP-C and LP-NC, respectively), and evaluated their possible use as siRNA delivery vectors.

MATERIALS AND METHODS

Synthesis of Cationic Cholesterol Derivatives *N*-(2-(2-Hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol) (Fig. 1) was synthesized as previously described.[11)]

Diamine-type cholesteryl carbamate **7**, cholesteryl (2-((2-hydroxyethyl)amino)ethyl)carbamate (OH-C-Chol), was synthesized as shown in Fig. 2. To a mixture of commercially

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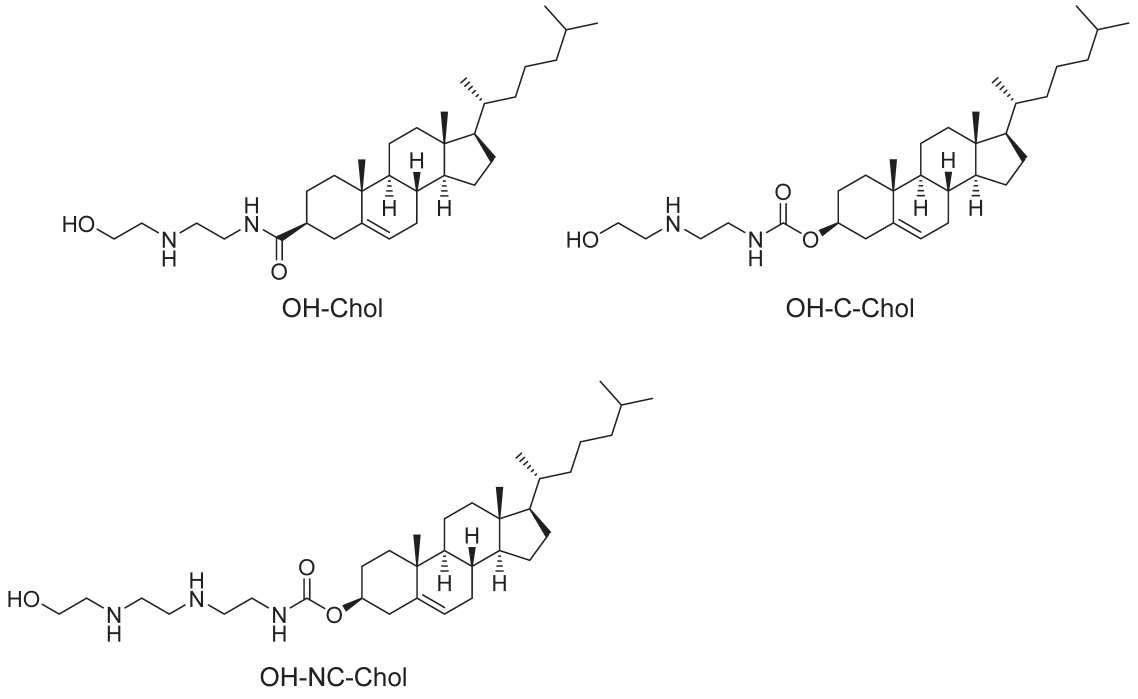

Fig. 1. Structure of Cationic Cholesterol Derivatives: (3*S*)-*N*-(2-(2-Hydroxyethylamino)ethyl)cholesteryl-3-carboxamide (OH-Chol); Cholesteryl (2-((2-Hydroxyethyl)amino)ethyl)carbamate (OH-C-Chol); Cholesteryl (2-((2-((2-Hydroxyethyl)amino)ethyl)amino)ethyl)carbamate (OH-NC-Chol)

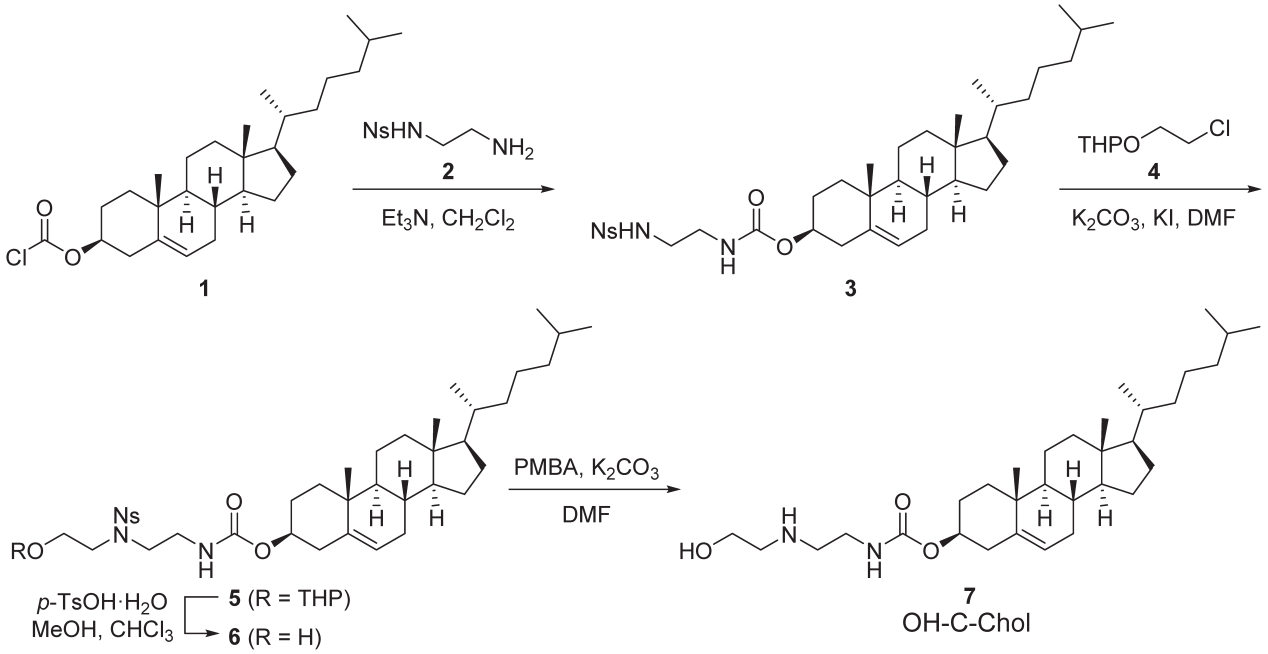

Fig. 2. Synthetic Scheme for Diamine-Type Cationic Cholesteryl Carbamate: Cholesteryl (2-((2-Hydroxyethyl)amino)ethyl)carbamate (OH-C-Chol)
Ns, 2-Nitrobenzenesulfonyl; THP, Tetrahydropyranyl; PMBA, *p*-Mercaptobenzoic acid.

available cholesteryl chloroformate (**1**) (2.56 g, 5.7 mmol) and mono-nosylated ethylenediamine (**2**)[12] (1.4 g, 5.7 mmol) in CH_2Cl_2 (28.5 mL) was added Et_3N (1.03 mL, 7.41 mmol) at room temperature. The mixture was stirred overnight at this temperature. The mixture was diluted with $CHCl_3$ (50 mL) and H_2O (50 mL). The aqueous phase was extracted with $CHCl_3$ (50 mL, twice), and the combined organic phases were washed with brine (50 mL), dried over $MgSO_4$ and evaporated to give crude **3**. Purification of the crude sample by column chromatography over silica gel (hexane/EtOAc) gave pure **3** (2.64 g, 70% yield).

A mixture of **3** (2.64 g, 4.02 mmol), commercially available 2-(2-chloroethoxy)tetrahydro-2*H*-pyran (**4**) (0.79 g, 4.82 mmol), K_2CO_3 (1.11 g, 8.04 mmol) and KI (0.13 g, 0.80 mmol) in *N*,*N*-dimethylformamide (DMF) (40 mL) under argon was stirred overnight at 90°C. After cooling to room temperature, the mixture was diluted with $CHCl_3$ (200 mL) and H_2O (200 mL). The aqueous phase was extracted with $CHCl_3$ (100 mL, twice), and the combined organic phases were washed with brine (50 mL), dried over $MgSO_4$ and evaporated to give crude **5**.

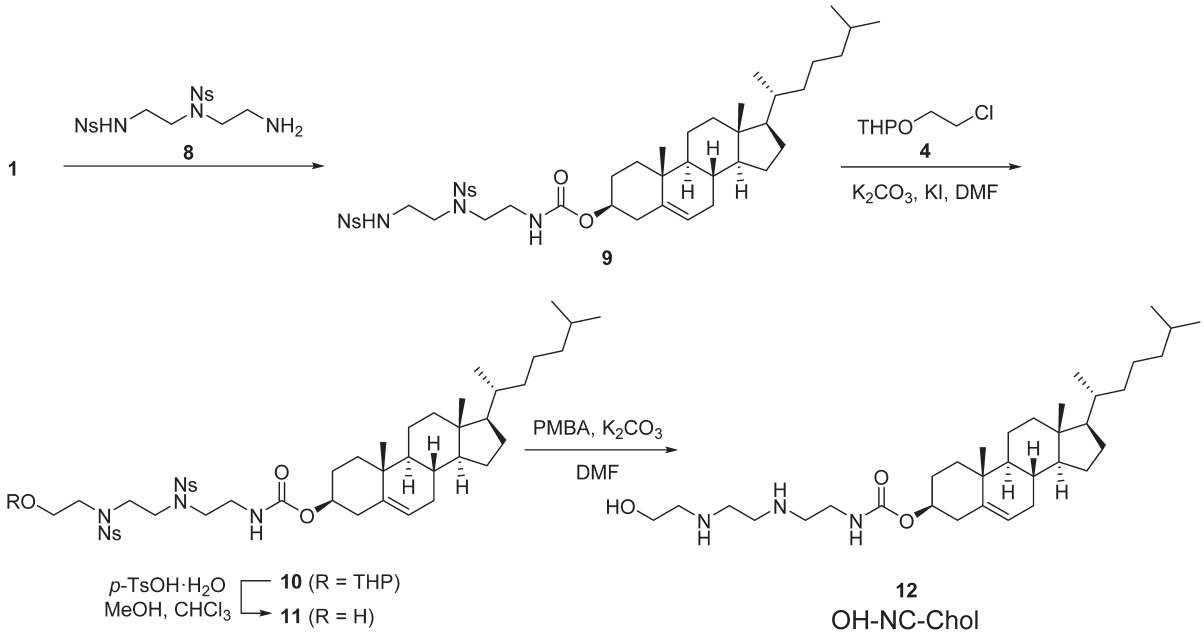

Fig. 3. Synthetic Scheme for Triamine-Type Cationic Cholesteryl Carbamate: Cholesteryl (2-((2-((2-Hydroxyethyl)amino)ethyl)amino)ethyl)carbamate (OH-NC-Chol)

Purification of the crude sample by column chromatography over silica gel (hexane/EtOAc) gave pure **5** (1.22 g, 39% yield).

To a mixture of **5** (1.22 g, 1.55 mmol) in MeOH (5.85 mL) and $CHCl_3$ (1.9 mL) was added *p*-TsOH·H_2O (3.0 mg, 0.016 mmol) at room temperature under stirring. After completion of the reaction monitored by TLC (SiO_2, hexane/EtOAc=1/1), the reaction was quenched by the addition of saturated $NaHCO_3$ (5 mL). Concentration of the mixture gave residual oil, which was diluted with $CHCl_3$ (70 mL) and saturated $NaHCO_3$ (60 mL). The aqueous phase was extracted with $CHCl_3$ (60 mL, twice), and the combined organic phases were dried over $MgSO_4$ and evaporated to give crude **6**. Purification of the crude sample by column chromatography over silica gel (hexane/EtOAc) gave pure **6** (964 mg, 89% yield).

A mixture of **6** (964 mg, 1.375 mmol), *p*-mercaptobenzoic acid (PMBA) (233 mg, 1.51 mmol) and K_2CO_3 (418 mg, 3.03 mmol) in DMF (13.8 mL) under argon was stirred overnight at 60°C. Further PMBA (233 mg, 1.51 mmol), K_2CO_3 (418 mg, 3.03 mmol) and DMF (6.9 mL) were added to the mixture, and the mixture was stirred for one day. After cooling, the mixture was diluted with $CHCl_3$ (50 mL), 2 N NaOH (10 mL) and H_2O (10 mL). The aqueous phase was extracted with $CHCl_3$ (50 mL, twice), and the combined organic phases were dried over $MgSO_4$ and evaporated to give crude **7**. Purification of the crude sample by column chromatography over NH-silica gel ($CHCl_3$/MeOH, twice) gave pure **7** (294 mg, 41% yield).

Triamine-type cholesteryl carbamate **12**, cholesteryl (2-((2-((2-hydroxyethyl)amino)ethyl)amino)ethyl)carbamate (OH-NC-Chol), was synthesized as shown in Fig. 3. To a mixture of cholesterol chloroformate (**1**) (1.66 g, 3.71 mmol) and bis-nosylated diethylenetriamine (**8**) (1.76 g, 3.71 mmol) in CH_2Cl_2 (18.6 mL) was added Et_3N (616 μL, 4.45 mmol) at room temperature. The protected diethylenetriamine derivative **8** was prepared according to a reported procedure with a slight modification (2 eq of Et_3N were used for bis-nosylation).[13] The mixture was stirred overnight at this temperature. The mixture was diluted with $CHCl_3$ (20 mL) and H_2O (20 mL). The aqueous phase was extracted with $CHCl_3$ (40 mL, twice), and the combined organic phases were washed with brine (20 mL), dried over $MgSO_4$ and evaporated to give crude **9**. Purification of the crude sample by column chromatography over silica gel (hexane/EtOAc) gave pure **9** (3.02 g, 87% yield).

By using a procedure identical to that described for the preparation of **5**, **9** (3.02 g, 3.4 mmol) was converted to **10** (1.12 g, 32% yield) by the reaction with **4** (0.67 g, 4.08 mmol), K_2CO_3 (1.08 g, 7.8 mmol) and KI (0.13 g, 0.78 mmol) in DMF (34 mL).

By using a procedure identical to that described for the preparation of **6**, **10** (1.12 g, 1.1 mmol) was converted to **11** (777 mg, 76% yield) by the reaction with *p*-TsOH·H_2O (2.1 mg, 0.011 mmol) in MeOH (4.1 mL) and $CHCl_3$ (1.38 mL).

By using a procedure identical to that described for the preparation of **7**, **11** (777 mg, 0.835 mmol) was converted to **12** (134 mg, 29% yield) by the reaction with PMBA (566 mg, 3.68 mmol) and K_2CO_3 (1.01 g, 7.36 mmol) in DMF (12.5 mL).

siRNA siRNAs targeting nucleotides of *firefly* pGL3 luciferase (Luc siRNA), Cy5.5-labeled Luc siRNA (Cy5.5-siRNA) and nonsilencing siRNA (Cont siRNA) as a negative control were synthesized by Sigma Genosys (Tokyo, Japan). The siRNA sequences of the Luc siRNA were as follows: sense strand: 5′-GUGGAUUUCGA GUCGUCUUAA-3′, and antisense strand: 5′-AAG ACG ACUCGA AAUCCA CAU-3′. In Cy5.5-siRNA, Cy5.5 dye was conjugated at the 5′-end of the sense strand. The siRNA sequences of the Cont siRNA were as follows: sense strand: 5′-GUACC GCA CGUCAUUCGUAUC-3′, and antisense strand: 5′-UACG AAUGAC GUGCG GUACGU-3′. Alexa Fluor®488-labeled AllStars Negative Control siRNA (AF-siRNA) was obtained from Qiagen (Valencia, CA, U.S.A.).

Preparation of Nanoparticles and Nanoplexes The cationic cholesterol derivative-based nanoparticles, NP, NP-C and NP-NC, were prepared from OH-Chol/Tween 80, OH-C-Chol/Tween 80 and OH-NC-Chol/Tween 80 at a molar ratio of 95/5 by a thin-film hydration method, respectively.[13]

Cationic liposomes, LP, LP-C and LP-NC, were prepared from OH-Chol/DOPE, OH-C-Chol/DOPE and OH-NC-Chol/DOPE at a molar ratio of 3/2 by a thin-film hydration method, respectively. The nanoparticle/siRNA complex (nanoplex) or the liposome/siRNA complex (lipoplex) at charge ratios (+/−) of 1/1, 3/1, 4/1, 7/1 and 10/1 of cationic lipid to siRNA was formed by the addition of each nanoparticle or liposome to siRNA with gentle shaking and left at room temperature for 15 min. The charge ratio (+/−) of nanoparticles/siRNA or liposomes/siRNA is expressed as the molar ratio of cationic lipid to siRNA phosphate.

Size and ζ-Potential of Nanoparticles and Nanoplexes The particle size distributions of nanoparticles, nanoplexes, liposomes and lipoplexes were measured by the cumulant method using a light-scattering photometer (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan), at 25°C after diluting the dispersion to an appropriate volume with water. The ζ-potentials were measured using ELS-Z2 at 25°C after diluting the dispersion to an appropriate volume with water.

Cell Culture Human breast cancer MCF-7-Luc (TamR-Luc#1) cells stably expressing *firefly* luciferase (pGL3) were donated by Dr. Kazuhiro Ikeda (Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/mL kanamycin and 0.5 mg/mL G418 at 37°C in a 5% CO_2 humidified atmosphere.

Luciferase Activity MCF-7-Luc cells were plated into 6-well culture dishes at a density of 3×10^5 cells per well. For transfection, each lipoplex and nanoplex of 100 pmol Luc siRNA or Cont siRNA formed at various charge ratios (+/−) was diluted in 2 mL of medium supplemented with 10% FBS and then the mixture was added to the cells. Forty-eight hours after the transfection, luciferase activity was measured as counts per second (cps)/μg protein using the luciferase assay system (Pica Gene, Toyo Ink Mfg. Co., Ltd., Tokyo, Japan) and BCA reagent (Pierce, Rockford, IL, U.S.A.), as previously reported.[14] Luciferase activity (%) was calculated as relative to the luciferase activity (cps/μg protein) of untransfected cells.

Flow Cytometric Analysis MCF-7-Luc cells were prepared by plating on 6-well culture dishes at a density of 3×10^5 cells per well 24 h prior to each experiment. Each nanoparticle and liposome was mixed with 50 pmol AF-siRNA at a charge ratio (+/−) of 7/1. The nanoplexes and lipoplexes were then diluted in 1 mL of medium containing 10% FBS and the mixture was added to the cell. After 3-h incubation, the dish was washed 2 times with 1 mL of PBS to remove any unbound nanoplex. The amount of AF-siRNA in the cells was determined by examining fluorescence intensity on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.), as previously described.[13]

Biodistribution of siRNA after Intravenous Injection of Cationic Nanoplex and Lipoplex to Mice All animal experiments were performed with approval from the Institutional Animal Care and Use Committee of Hoshi University. Cationic nanoplexes or lipoplexes of 50 μg of Cy5.5-siRNA were intravenously administered *via* lateral tail veins into female BALB/c mice (8 weeks of age; Sankyo Lab Service Corp., Tokyo, Japan). One hour after injection, the mice were sacrificed and the tissues were frozen on dry ice and sliced at 16 μm. The localization of Cy5.5-siRNA was examined using an Eclipse TS100-F microscope (Nikon, Tokyo, Japan).

Statistical Analysis Data were compared using analysis of variance and evaluated by Student's *t*-test. A p value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Synthesis of Cationic Cholesterol Derivatives For efficient gene silencing in tumor cells, the intracellular trafficking of siRNA after endocytosis must be controlled. Previously, we reported that cationic nanoparticles (NP) composed of diamine-type cholesteryl-3-carboxamide, OH-Chol (Fig. 1) and Tween 80 could deliver siRNA with high transfection efficiency into tumor cells.[8,9] Furthermore, to facilitate the endosomal escape of siRNA transfected by cationic nanoparticles, we synthesized another cationic cholesterol derivative, triamine-type cholesteryl-3-carboxamide (OH-N-Chol, *N*-(2-(2-(2-hydroxyethylamino)ethylamino)ethyl)cholesteryl-3-carboxamide) (Supplemental Fig. S1), which has 2 units of aminoethylene between the amino head group and the cholesterol skeleton, and then prepared NP-N composed of OH-N-Chol and Tween 80. In the transfection for tumor cells, NP-N nanoplex with a small size exhibited high gene knockdown by siRNA.[13] In NP-N, we expected that OH-N-Chol may show single protonation at neutral pH due to the strong electrostatic repulsion between two protonated amines in OH-N-Chol, while exhibiting double protonations at an endosomal pH of 5.5 (proton sponge effect). However, the carboxamide-type cholesterol derivatives such as OH-Chol and OH-N-Chol had a main drawback in their synthesis, especially for large-scale synthesis. Thus, OH-Chol and OH-N-Chol were prepared from cholesteryl carboxylic acid as the starting material, which was obtained by CO_2 treatment of Grignard reagent (cholesteryl magnesium bromide) derived from cholesteryl bromide.[13] This step is very sensitive to moisture and air, which decreases the reproducibility of the reaction. Formation of the minor isomer in this step is also problematic. We expected that, if the cholesteryl carboxamide moiety (cholesteryl-CONHR) can be replaced by a cholesterol-type structure (cholesteryl-OR), the synthesis costs would be considerably reduced. Thus, we designed cholesteryl carbamates, (2-((2-hydroxyethyl)amino)-ethyl)carbamate (OH-C-Chol) and (2-((2-((2-hydroxyethyl)-amino)ethyl)amino)ethyl)carbamate (OH-NC-Chol) (Fig. 1). These carbamates of diamine and triamine can be easily prepared from cholesteryl chloroformate (Figs. 2, 3), which is easily available on the order of one hundred grams from several companies.

Preparation of Nanoplexes and Lipoplexes First, we examined formulation of cationic cholesterol-based liposomes and nanoparticles for siRNA delivery. Here, we used OH-Chol, OH-C-Chol and OH-NC-Chol as cationic cholesterol derivatives for the preparation of cationic liposomes and nanoparticles. Cationic cholesterol derivatives can self-assemble into cationic nanoparticles without any helper lipids. Previously, we reported that cationic nanoparticles composed of cationic cholesterol derivative and Tween 80 could efficiently deliver siRNA into tumor cells.[9,13] Therefore, as cationic cholesterol derivative-based nanoparticles, we prepared NP, NP-C and NP-NC composed of OH-Chol/Tween 80, OH-C-

Table 1. Particle Size and ζ-Potential of Cationic Cholesterol-Based Nanoplexes and Lipoplexes at Various Charge Ratios (+/−)

Nanoparticle	Formulation (molar ratio)	Charge ratio (+/−)	Size[a] (nm)	ζ-Potential[a] (mV)	Liposome	Formulation (molar ratio)	Charge ratio (+/−)	Size[a] (nm)	ζ-Potential[a] (mV)
NP	OH-Chol/ Tween 80 =95/5	Without siRNA	111.7±1.6	45.7±5.7	LP	OH-Chol/ DOPE =60/40	Without siRNA	98.8±4.3	49.6±6.4
		1	Aggregation	N.D.			1	173.9±11.0	−37.4±4.4
		3	237.5±20.9	40.0±3.8			3	237.5±21.0	43.5±1.2
		4	182.5±11.4	47.5±1.7			4	283.2±32.2	40.5±2.1
		7	165.5±3.1	46.9±3.2			7	237.6±9.0	43.3±1.8
		10	190.5±9.9	47.9±1.4			10	221.5±12.0	44.7±1.5
NP-C	OH-C-Chol/ Tween 80 =95/5	Without siRNA	115.5±1.1	51.0±1.7	LP-C	OH-C-Chol/ DOPE =60/40	Without siRNA	70.6±0.1	53.0±0.8
		1	184.3±8.8	−30.2±0.6			1	168.9±0.6	−47.5±0.6
		3	226.7±2.1	47.1±3.9			3	Aggregation	N.D.
		4	208.3±14.1	48.7±1.0			4	Aggregation	N.D.
		7	186.4±8.0	47.4±1.1			7	174.0±14.0	43.0±0.2
		10	174.4±3.8	48.8±1.3			10	162.3±11.0	46.1±1.1
NP-NC	OH-NC-Chol/ Tween 80 =95/5	Without siRNA	116.3±2.7	49.3±6.3	LP-NC	OH-NC-Chol/ DOPE =60/40	Without siRNA	109.0±1.6	38.7±5.1
		1	271.6±7.8	4.6±0.3			1	181.2±18.3	−18.6±9.7
		3	211.0±6.4	35.3±0.4			3	Aggregation	N.D.
		4	185.2±3.1	37.2±2.9			4	207.2±6.7	33.4±0.7
		7	162.2±5.6	42.5±1.0			7	247.0±24.3	38.6±1.1
		10	172.4±15.5	44.9±6.4			10	217.4±10.5	40.0±2.0

a) In water. N.D.: not determined. Values represent mean±S.D. (n=3).

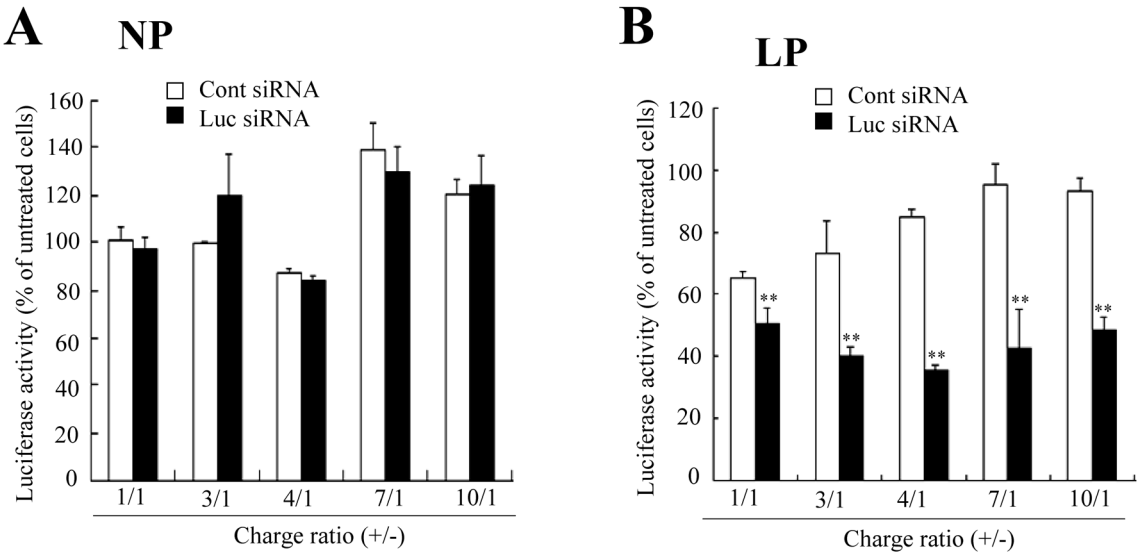

Fig. 4. Effect of Charge Ratio (+/−) in the Formation of NP Nanoplex and LP Lipoplex of siRNA on Gene Silencing Activity in MCF-7-Luc Cells

NP nanoplexes and LP lipoplexes were formed at charge ratios (+/−) of 1/1, 3/1, 4/1, 7/1 and 10/1. They were added to MCF-7-Luc cells at 50 nM siRNA, and the luciferase assay was carried out 48 h after incubation of the nanoplexes and lipoplexes. Each column represents the mean±S.D. (n=3). **p<0.01, compared with Cont siRNA.

Chol/Tween 80 and OH-NC-Chol/Tween 80 at a molar ratio of 95/5, respectively. In liposomal formulation, cationic liposomes composed OH-Chol and DOPE were previously used for gene delivery.[15,16] Therefore, as cationic cholesterol derivative-based liposomes, LP, LP-C and LP-NC were prepared from OH-Chol/DOPE, OH-C-Chol/DOPE and OH-NC-Chol/DOPE at a molar ratio of 3/2, respectively.

The sizes of NP, NP-C and NP-NC were about 110–120 nm, and their ζ-potentials were 46, 51 and 49 mV, respectively (Table 1). In contrast, the sizes of LP, LP-C and LP-NC were about 100, 70 and 110 nm, and their ζ-potentials were about +50, 53 and 39 mV, respectively (Table 1). When the nanoplexes and lipoplexes were prepared with siRNA at various charge ratios (+/−), large-sized aggregates were found at around neutral ζ-potential in NP, LP-C and LP-NC. However, beyond a charge ratio (+/−) of 7/1, their sizes were about 200 nm and their ζ-potentials were about 39−47 mV.

Effect of Charge Ratio (+/−) for Formation of Nanoplex and Lipoplex on Gene Knockdown Efficacy We examined the effect of charge ratio (+/−) on the gene knockdown effect by the nanoplex or the lipoplex at a concentration of 50 nM siRNA using a luciferase assay system with MCF-7-Luc cells.

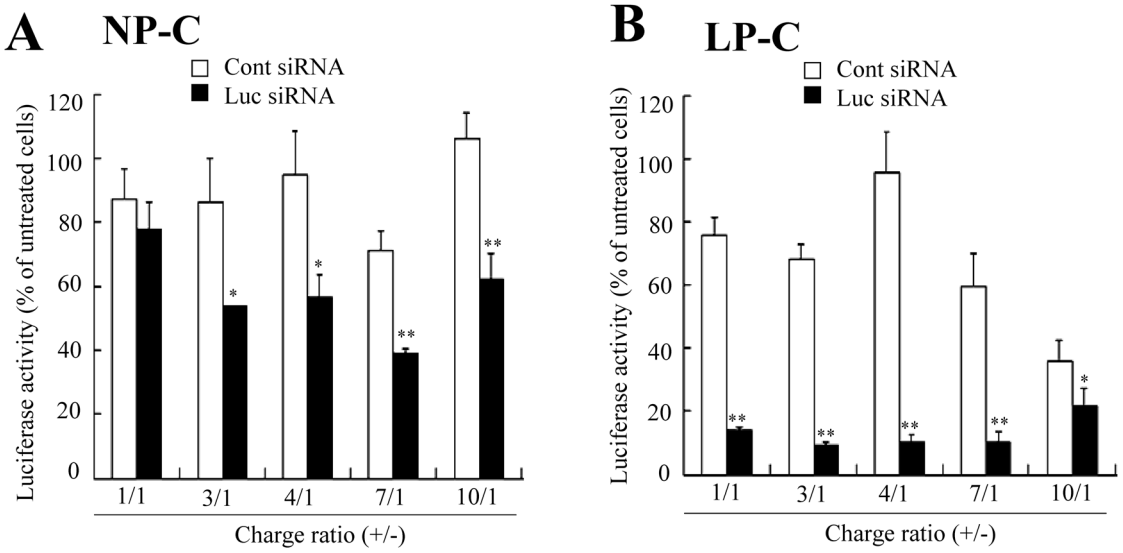

Fig. 5. Effect of Charge Ratio (+/−) in the Formation of NP-C Nanoplex and LP-C Lipoplex of siRNA on Gene Silencing Activity in MCF-7-Luc Cells

NP-C nanoplexes and LP-C lipoplexes were formed at charge ratios (+/−) of 1/1, 3/1, 4/1, 7/1 and 10/1. They were added to MCF-7-Luc cells at 50 nM siRNA, and the luciferase assay was carried out 48 h after incubation of the nanoplexes and lipoplexes. Each column represents the mean±S.D. (n=3). *p<0.05 and **p<0.01, compared with Cont siRNA.

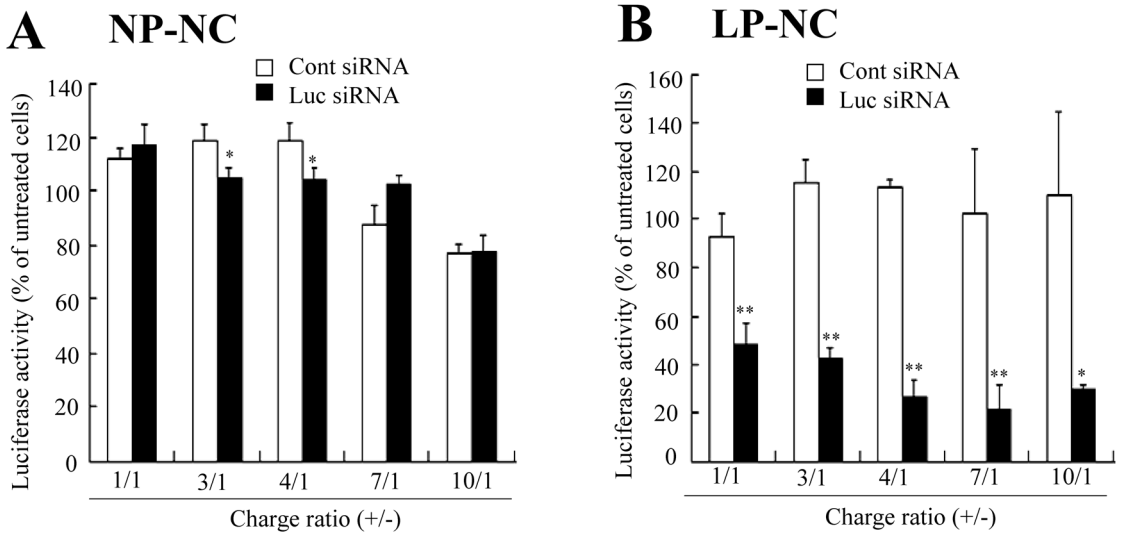

Fig. 6. Effect of Charge Ratio (+/−) in the Formation of NP-NC Nanoplex and LP-NC Lipoplex of siRNA on Gene Silencing Activity in MCF-7-Luc Cells

NP-NC nanoplexes and LP-NC lipoplexes were formed at charge ratios (+/−) of 1/1, 3/1, 4/1, 7/1 and 10/1, respectively. They were added to MCF-7-Luc cells at 50 nM siRNA, and the luciferase assay was carried out 48 h after incubation of the nanoplexes and lipoplexes. Each column represents the mean±S.D. (n=3). *p<0.05 and **p<0.01, compared with Cont siRNA.

For OH-Chol-based formulae, NP nanoplex of Luc siRNA did not exhibit suppression of luciferase activity at any charge ratio (+/−) (Fig. 4A). However, for LP lipoplex, moderate suppression of luciferase activity was observed beyond a charge ratio (+/−) of 3/1 (Fig. 4B). For OH-C-Chol-based formulae, NP-C nanoplex of Luc siRNA moderately suppressed luciferase activity (Fig. 5A), and LP-C lipoplex could strongly suppress luciferase activity at charge ratios (+/−) of 1/1, 3/1, 4/1 and 7/1, compared with those of Cont siRNA (Fig. 5B). For OH-NC-Chol-based formulae, NP-NC nanoplex of Luc siRNA exhibited slight suppression of luciferase activities at charge ratios (+/−) of 3/1 and 4/1 (Fig. 6A). In contrast, LP-NC lipoplex could strongly suppress luciferase activity at all charge ratios (+/−) (Fig. 6B). However, the slight reductions of luciferase activity were observed in transfection of Cont siRNA by LP, NP-NC or LP-C compared with untreatment, indicating that their lipoplexes or nanoplexes might induce changes in expression of luciferase mRNA by a target-independent fashion (off-target effect). The liposomes or nanoparticles without siRNA did not significantly affect the luciferase activities with the exception of the same amount of NP and LP with NP nanoplex and LP lipoplex at charge ratios (+/−) of 10/1 and 1/1, respectively (Supplemental Fig. S2). From the results of lipoplex size and gene silencing effects, LP-C and LP-NC lipoplex formed at a charge ratio (+/−) of 7/1 exhibited high gene knockdown by siRNA without aggregation. Therefore, in subsequent experiments, we used 7/1 as an optimal charge ratio (+/−) for siRNA transfection.

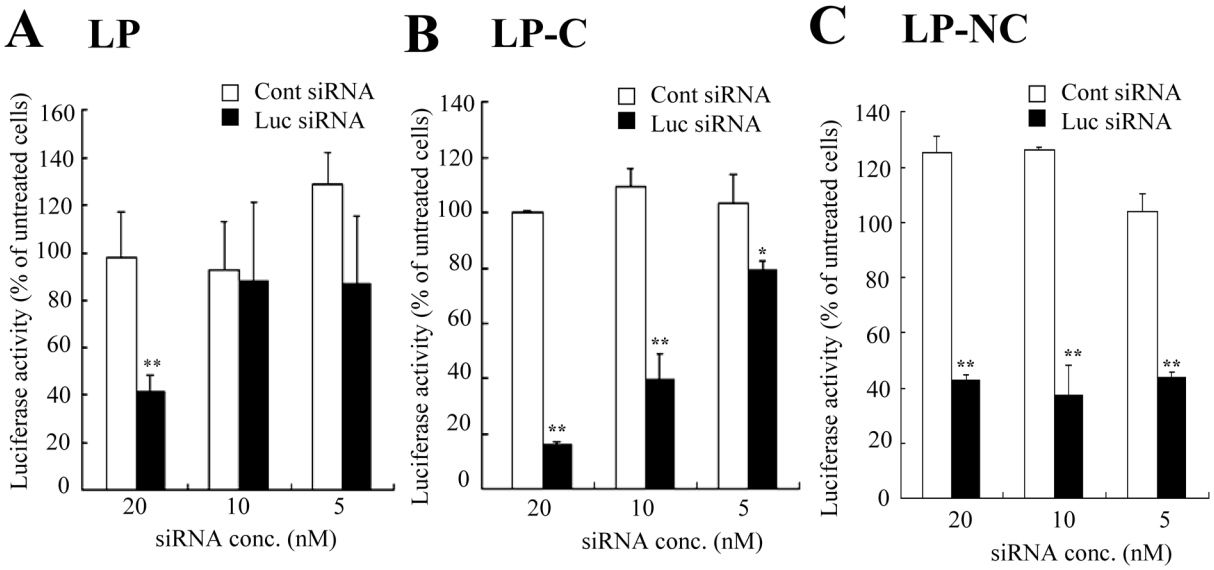

Fig. 7. Dose Dependence of siRNA-Mediated Inhibition of Luciferase Activity 48 h after Incubation with LP, LP-C and LP-NC Lipoplexes Prepared at a Charge Ratio (+/−) of 7/1

MCF-7-Luc cells were treated with each lipoplex at a final concentration of 20, 10 and 5 nM siRNA. Each value represents the mean±S.D. (n=3). *p<0.05 and **p<0.01, compared with Cont siRNA.

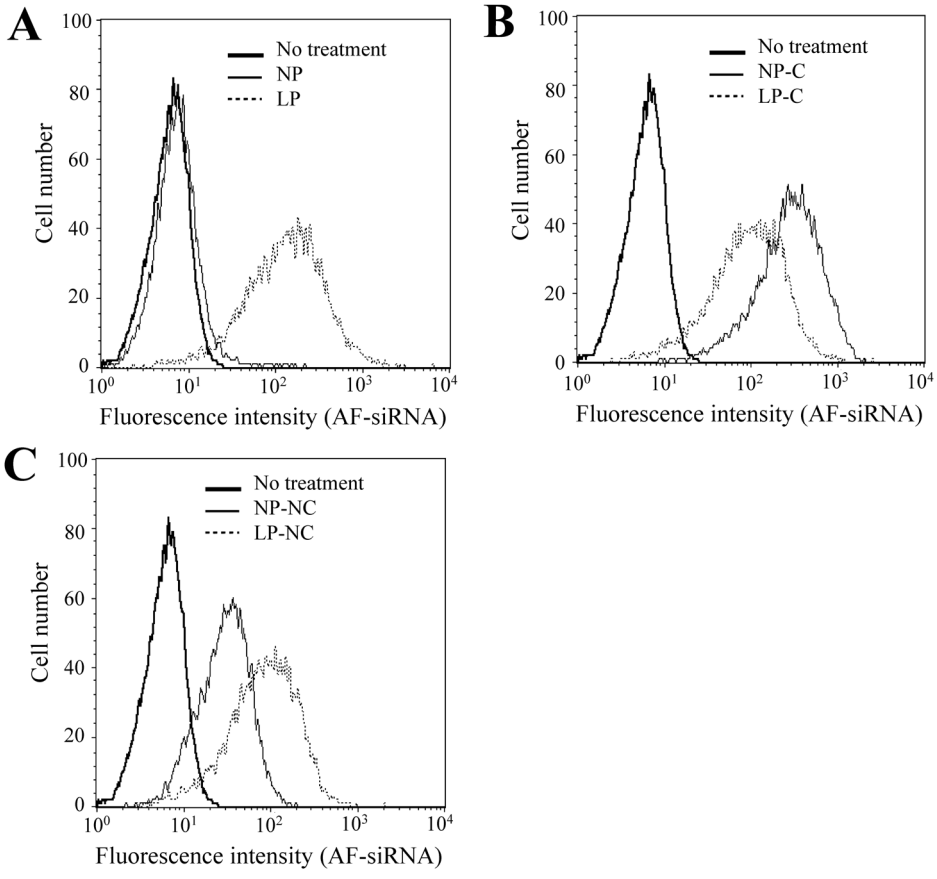

Fig. 8. Cellular Association at 3 h after Transfection of Nanoplex and Lipoplex

NP nanoplex and LP lipoplex (A), NP-C nanoplex and LP-C lipoplex (B), NP-NC nanoplex and LP-NC lipoplex (C), were formed by mixing with AF-siRNA at a charge ratio (+/−) of 7/1. The association of nanoplex or lipoplex with MCF-7 cells was determined on the basis of Alexa Fluor®488-fluorescence by flow cytometry.

The persistence of gene silencing is a key factor when considering the therapeutic uses of siRNA. We thus studied the dose dependence of inhibition produced by LP, LP-C and LP-NC lipoplexes (Figs. 7A–C). In terms of the concentration of siRNA, LP-NC lipoplex could significantly suppress luciferase activity (39% of untreated cells) even at 5 nM siRNA, compared with that of Cont siRNA (Fig. 7C). Furthermore, Cont siRNA transfected by LP, LP-C or LP-NC at low concen-

trations did not induce changes in luciferase activity.

Association of Nanoplexes with the Cells To clarify the relationship between gene silencing effect and cellular uptake in the transfection of nanoplexes and lipoplexes, we examined the cellular association by flow cytometric analysis. Cellular associations with LP and LP-NC lipoplexes were higher than with NP and NP-NC nanoplexes, respectively (Figs. 8A, C). This result corresponds to those of gene knockdown efficacies (Figs. 4, 6). In contrast, NP-C nanoplex exhibited higher cellular association than LP-C lipoplex (Fig. 8B), although the gene silencing effect by NP-C nanoplex was lower than that by LP-C lipoplex (Figs. 5A, B). This might indicate that the cellular association could be increased by the inclusion of OH-C-Chol into the nanoparticles, but siRNA transduced by NP-C could not be efficiently released from the nanoplex. From the results, the LP-NC composed of OH-NC-Chol and DOPE could deliver siRNA *via* efficient cellular association, and induce a large gene silencing effect by siRNA.

Previously, we reported that NP nanoplex formed in NaCl solution exhibited marked RNAi activity in human prostate tumor PC-3 cells.[8,9] *In vitro* transfection activity increased in parallel with ζ-potential and the size of the lipoplex.[17,18] In plasmid DNA transfection, large lipoplexes over 700 nm in mean diameter induced efficient transfection; in contrast, lipoplexes of less than 250 nm were inefficient.[19] The presence of NaCl during the formation of NP nanoplex increased the size of the nanoplex (*ca.* μm in size) and affected the gene silencing effect by siRNA.[8,9] However, in this study, the NP nanoplex was formed in water to prepare small nanoplexes (Table 1); therefore, the NP nanoplex might not be able to induce a gene knockdown effect (Fig. 4A). In contrast, LP lipoplex could induce the gene silencing effect in the cells (Fig. 4B). To overcome poor endosomal escape and subsequent degradation of siRNA in the late lysosomes, pH-sensitive lipids such as DOPE have been used as a component of liposomal siRNA vectors.[20,21] The role of DOPE is not fully understood, but it might affect the structural transition of cationic nanoparticles at the acidic pH of late endosomes in the cells.[22] Although the sizes and ζ-potentials of LP lipoplexes were similar with those of NP nanoplex (Table 1), LP lipoplex exhibited gene silencing effect in the cells (Fig. 4). These findings indicate that LP lipoplex might increase the gene silencing effect by the improvement of intracellular trafficking.

Upon comparing the transfection efficiency between OH-Chol- and OH-C-Chol-based liposomes, LP-C lipoplex exhibited a larger gene silencing effect than LP lipoplex. In plasmid DNA transfection, it has been reported that the linker of cationic cholesterol derivatives affected transfection efficiency by cationic lipoplex.[9,23] However, it was not clear why the OH-C-Chol with a carbamate-type linker could induce higher gene suppression by siRNA than OH-Chol with a carboxamide-type linker.

To facilitate the endosomal escape of siRNA transfected by cationic nanoparticles, we prepared NP-NC that had aminoethylene units on the surface of the nanoparticles; however, NP-NC nanoplex could not induce a silencing effect in the cells (Fig. 6A). Previously, we reported that NP-N composed of OH-N-Chol and Tween 80 could efficiently deliver siRNA into cytoplasm *via* efficient endosomal escape and induce a large gene silencing effect.[13] The only difference between OH-N-Chol and OH-NC-Chol is in the structure of the linker, indicating that a carbamate-type linker might affect protonation of aminoethylene units of OH-NC-Chol in the endosome. However, for liposomal formulae, LP-NC lipoplex could induce a larger gene silencing effect than LP-C lipoplex at low concentrations of siRNA (Figs. 7B, C). These findings suggested that liposomal formulation might improve the double protonation of the aminoethylene units of OH-NC-Chol and increase gene silencing effect in the cells by LP-NC lipoplex. However, in this study, we have no evidence that LP-NC lipoplex could facilitate endosomal escape after internalization into the cells; therefore, further study must be performed to investigate the mechanism to improve the gene silencing effect.

In a preliminary study, we intravenously injected the lipoplexes or nanoplexes of Cy5.5-siRNA into mice and observed the biodistribution of siRNA 1 h after injection by fluorescent microscopy (Supplemental Fig. S3). Injections of NP and NP-C nanoplex and LP-C lipoplex produced the accumulation of siRNA both in lung and in liver, and those of LP and LP-NC lipoplexes, and NP-NC nanoplex in the liver. These findings indicate that their nanoparticles and liposomes might be useful for siRNA delivery to lung or liver tumor metastasis. Further *in vivo* application should be performed to examine the gene knockdown effect in tumors by intravenous injection of the nanoplex or lipoplex.

In this study, we synthesized new cholesteryl derivatives with a carbamate-linked spacer as cationic lipids for lipid-based siRNA vectors. LP-NC composed of OH-NC-Chol and DOPE could form siRNA lipoplexes of 200 nm in size, and could induce a high level of gene knockdown in the cells. These findings suggest that OH-NC-Chol may have potential as a cationic lipid to transfect siRNA efficiently into tumor cells.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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