新規脂質ナノ粒子を用いた

全身投与型 siRNA 送達システムの開発

Development of systemic siRNA delivery system
using novel lipid-based nanoparticles

本論文は静岡県立大学大学院薬学研究科博士論文である

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Abbreviations

AF750-siRNA: AlexaFluor750-conjugated siRNA
ALNP: anionic lipid nanoparticles
ANOVA: analysis of variance
APRPG: polyethylene glycol-grafted Ala-Pro-Arg-Pro-Gly
APRPG-TL: TL decorated with APRPG
CL: cationic liposomes
C-siRNA: small interfering RNA conjugated with cholesterol
DCP: dicetyl phosphate
DCP-TEPA: dicetylphosphate-tetraethylenepentamine conjugate
DDS: drug delivery system
DL: dioleylphosphate-diethylenetriamine conjugate -based liposomes
DL/siRNA: DL carrying siRNA
DOPC: dioleoylphosphatidylcholine
DOP-DETA: dioleylphosphate-diethylenetriamine conjugate
DOPE: dioleoylphosphatidyl-ethanolamine
DOPG: dioleoylphosphatidylglycerol
DPPC: dipalmitoylphosphatidylcholine
DSG: distearoylglycerol
DSPE: distearoylphosphatidylethanolamine
EDTA: ethylenediaminetetraacetic acid
EE: encapsulation efficacy
EPR: enhanced permeability and retention
FBS: fetal bovine serum
GFP: green fluorescent protein
HT1080-EGFP: HT1080 human fibrosarcoma cells constitutively expressing green fluorescent protein
IVIS: in vivo imaging system
LL2-luc-m38: Lewis lung adenocarcinoma cells constitutively expressing luciferase protein
miRNA: microRNA
O/W: ethanol in aqueous buffer
PCL: polycationic liposomes
PdI: polydispersity index
PEG: polyethylene glycol
PEG-DL: PEGylated DL
PLK1: polo-like kinase 1

PMSF: phenylmethylsulfonyl fluoride
RGD: polyethylene glycol-grafted Arg-Gly-Asp
RGD-TL: TL decorated with RGD
RISC: RNA-induced silencing complex
RNAi: RNA interference
SDS: sodium dodecyl sulfate
siCont: control siRNA
siGFP: siRNA against green fluorescent protein
siLuc: siRNA against luciferase 2
siPLK1: siRNA against mouse polo-like kinase 1
TEM: transmission electron microscope
TL: dicetylphosphate-tetraethylenepentamine conjugate-based liposomes

Contents

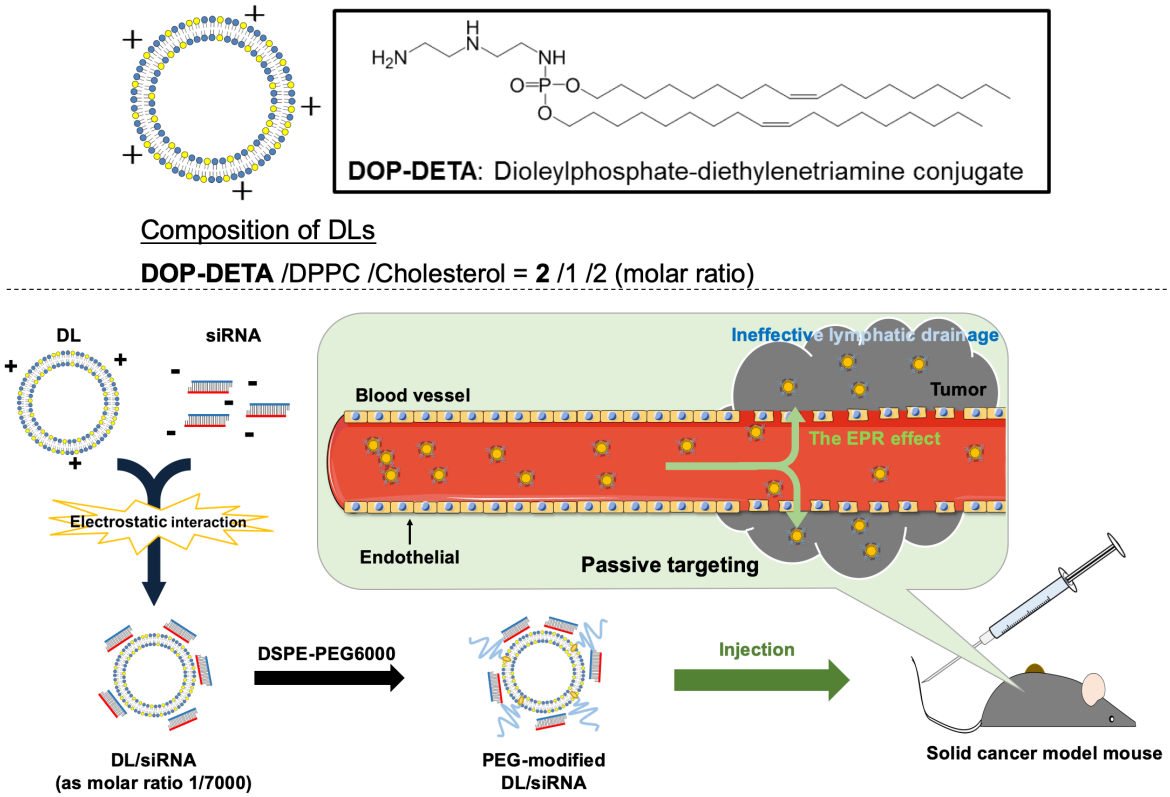
Background

RNA interference (RNAi) is an endogenous biological process that occurs in eukaryotic cells.[1] When double-stranded RNA is introduced into the cytoplasm, it is dissociated into single-strand RNA, which then forms an RNA-induced silencing complex (RISC). RISC specifically cleaves target mRNA in a sequence dependent manner.[2] Small RNAs such as small interfering RNA (siRNA) and microRNA (miRNA) are 19-23 base-pair double-strand RNAs. They are easy to synthesize and have strong potential for gene suppression. Because they are theoretically capable of targeting any gene, they are considered attractive drug candidates for the treatment of intractable diseases such as cancer.[3–5] However, RNAs are immediately degraded by RNases in the body and penetrate the cell membrane poorly because of their negative charge, high molecular weight and hydrophilicity.[6] Therefore, an effective drug delivery system (DDS) is indispensable for the development of RNAi-based drugs. Recently, many non-viral vectors such as liposomes, micelles, and lipid nanoparticles have been studied for small RNA delivery.[7–9] Among these, liposomes in particular have been receiving considerable attention as a small RNA delivery system, as they can enhance the stability of small RNA in serum and increase siRNA cellular delivery. Additionally, liposomes can be easily formulated and modified their surfaces with various molecules.

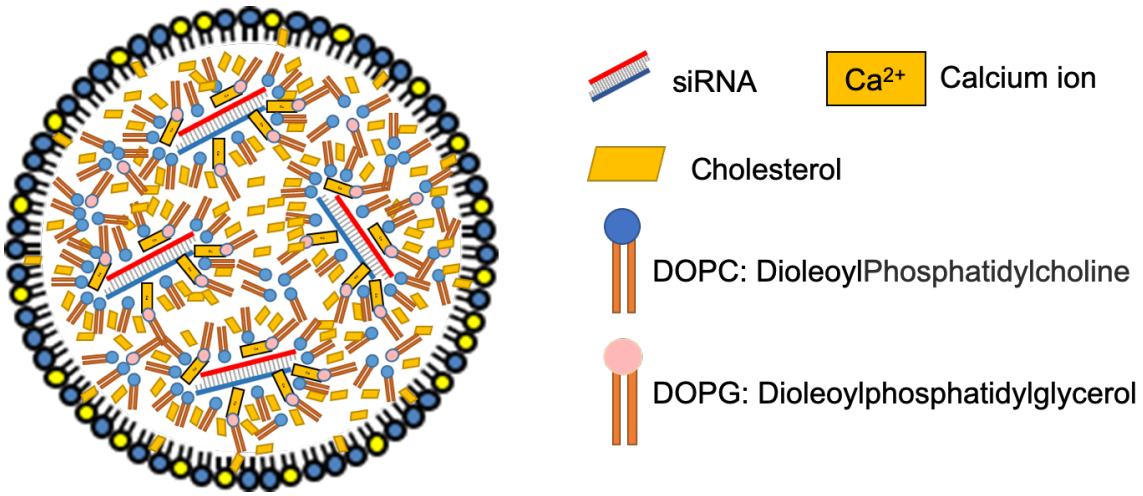
The World Health Organization reported that malignant tumor is now the second cause of death in the world.[10] Although numerous chemotherapies have been developed for the treatment of malignant tumors, most of the anticancer drugs cause problems such as serious side effects.[11] Cancer treatments by gene silencing with small RNAs including miRNA and siRNA have been receiving a great attention because of their specific therapeutic effects. However, the development of safe and efficient delivery systems to achieve effective cancer therapy is still a challenging task.

Cationic liposomes (CL) have been developed as one of non-viral vectors for siRNA

delivery. Especially, pH-responsive CL carrying siRNA are expected as a drug candidate because standard CL have some disadvantages such as cytotoxicity and low transfection efficiency due to siRNA degradation in the lysosomes. It was previously reported that pH-responsive polycationic lipid in lipoplexes, which are DNA-liposome complexes, promotes the interaction of lipoplex with cellular membranes and endosomal escape of the lipoplex by the proton sponge effect.[12] Based on these benefits, a series of polycationic lipids such as cetyl-polyethylenimine[1800], dicetyl phosphate (DCP)-spermidine, and DCP-spermine were synthesized for preparation of pH-responsive polycationic liposomes (PCL).[13,14] siRNA formulated in these PCL shows high gene-transfer activity without severe cytotoxicity *in vitro*.[15] Recently, dicetylphosphate-tetraethylenepentamine conjugate (DCP-TEPA) was synthesized as a pH-responsive polycationic lipid, and PCL containing DCP-TEPA (TL) were prepared for efficient siRNA delivery.[16] In addition, TL were decorated with polyethylene glycol (PEG)-grafted Arg-Gly-Asp (RGD-TL) or Ala-Pro-Arg-Pro-Gly (APRPG-TL) for active targeting to tumors via systemic administration.[17] It is known that the RGD peptide specifically binds to integrin αvβ3.[18] The APRPG peptide, a peptide originally identified by *in vivo* bio-panning, binds to vascular endothelial growth factor receptor-1.[19] Intravenous injection of siRNA against mammalian rapamycin target complexed with APRPG-TL significantly inhibits the growth of pulmonary tumors in the tumor-bearing mice.[20] Moreover, intravenous injection of APRPG-TL carrying miR-499 suppresses target gene expression and the growth of subcutaneous tumors in the tumor-bearing mice.[21] On the other hand, for systemic administration of siRNA, TL needed to be improved because of the requirement of a large amount of additional helper lipids such as dioleoylphosphatidyl-ethanolamine (DOPE) to promote their cellular uptake and endosomal escape, thus resulting in the need for an increased dose of total lipids. In this study, pH-responsive liposomes containing a novel polyamine lipid derivative were newly designed and studied to establish more advanced vector for systemic delivery of siRNA.



Scheme 1. Schematic image of the novel systemic siRNA delivery system for cancer therapy with DOP-DETA-based CL



Scheme 2. Schematic image of anionic lipid nanoparticle (ALNP)

Part 1. Development of novel pH-responsive lipid-based liposomes for siRNA delivery

Chapter 1. Design and *in vitro* evaluation of novel pH-responsive lipid-based liposomes for siRNA delivery

1-1-1. Introduction

In this chapter, a novel dual-function polyamine lipid derivative, dioleylphosphate-diethylenetriamine conjugate (DOP-DETA, **Fig. 1**), was used siRNA delivery. It is expected that the positively charged diethylenetriamine residue in DOP-DETA would contribute to the capture of siRNA, to the interaction between the lipid and the cell membrane, and to the interaction between the lipid and the endosomal membrane, and that the unsaturated carbon chain in DOP-DETA would contribute to higher membrane fluidity and induce membrane fusion. A variety of siRNA formulation using DOP-DETA-based liposomes (DL) was prepared and characterized its properties. The effectiveness on RNAi inducing of DL carrying siRNA (DL/siRNA) was evaluated in gene silencing experiments on (HT1080-EGFP) cell line with low doses of siRNA *in vitro*.

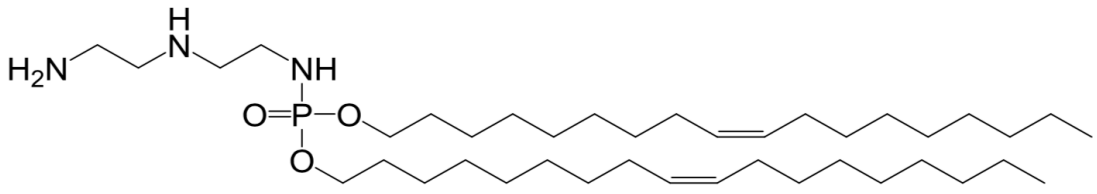


Fig. 1. Chemical structure of DOP-DETA.

1-1-2. Materials and methods

1-1-2-1. Materials

DOP-DETA was synthesized and kindly donated by Dr. Takehisa Dewa (Nagoya Institute of Technology, Nagoya, Japan). Dipalmitoylphosphatidylcholine (DPPC) and cholesterol were kindly donated by 名前　Nippon Fine Chemical Co. (Takasago, Hyogo, Japan). All siRNAs including cholesterol-conjugated siRNA were purchased from Hokkaido System Science Co. (Hokkaido, Japan). The nucleotide sequences of siRNA against green fluorescent protein (siGFP) with a 2-nucleotide overhang (underline) were 5'-GGC UAC GUC CAG GAG CGC ACC-3' (sense) and 5'-UGC GCU CCU GGA CGU AGC CUU-3' (antisense); those of siRNA against mouse polo-like kinase 1 (siPLK1) with a 2-nucleotide overhang (underline) were 5'-CCA AAG GAA UUC CGA GAA ATT-3' (sense) and 5'- UUU CUC GGA AUU CCU UUG GTT-3' (antisense); and those of siRNA against luciferase 2 (siLuc2) with a 2-nucleotide overhang (underline) were 5'-GCU AUG GGC UGA AUA CAA ATT-3' (sense) and 5'-UUU GUA UUC AGC CCA UAG CTT-3' (antisense). Phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin A, n-Octyl-β-D-glucoside, and bovine serum albumin (BSA) were purchased from SIGMA-Aldrich (St Louis, MO, USA). Sodium dodecyl sulfate (SDS) and GelRed were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

1-1-2-2. Preparation of DL carrying siRNA

For the preparation of liposomes, DOP-DETA, DPPC, and cholesterol were dissolved in *tert*-butyl alcohol and lyophilized. The liposomes were produced by hydration of the lipid mixture with UltraPure™ DNase/RNase-Free Distilled Water (RNase-free water; Invitrogen, Carlsbad, CA, USA). The liposomes were extruded 10 times through a polycarbonate membrane filter having a pore size of 100 nm (Nuclepore, Maidstone, UK). Particle size,

polydispersity index (PdI), and ζ-potential of DL were measured using a Zetasizer Nano ZS (Malvern, Worcs, UK) after dilution of liposomes with RNase-free water. DL and siRNA were mixed gently and incubated for 20 min at room temperature to obtain DL complexed with siRNA (DL/siRNA).

1-1-2-3. Electrophoretic assay

siRNAs that were unattached to DL were separated using 15% polyacrylamide gel electrophoresis, where the siRNA in stable complexes did not enter the gel. After electrophoresis, free siRNA was stained 30 min with GelRed, and fluorescence was observed using a LAS-3000 mini imaging system under UV irradiation (Fuji Film, Tokyo, Japan).

1-1-2-4. Transmission electron microscope (TEM) imaging

DL/siRNA (total lipid concentration; 5 mM) in a volume of 5 μL was placed on a grid (Nisshin EM, Tokyo, Japan) and dehydrated with warm air. After this step was performed for 3 cycles, each sample was negatively stained with 10 μL of 1 % ammonium molybdate for 1 min and air dried for 1 h. Samples were imaged with an HT7700 TEM System (Hitachi High-Technologies, Tokyo, Japan).

1-1-2-5. Cell cultures

HT1080 human fibrosarcoma cells constitutively expressing enhanced green fluorescent protein (HT1080-EGFP), previously established,[22] and colon26 NL-17 murine colon carcinoma cells were established and kindly provied by Dr. Yamori *et.al.*.[23] They were cultured in D-MEM/Ham's F-12 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Both cell lines were cultured in medium supplemented with 10% inactivated fetal bovine serum (FBS; AusGeneX, Oxenford, Australia), 100 units/mL penicillin, and 100 μg/mL streptomycin (MP Biomedicals) in a CO_2 incubator.

1-1-2-6. Gene silencing effect assay

Gene silencing effect was evaluated with GFP assay. HT1080-EGFP cells were seeded onto 24-well plates and precultured overnight. The medium was replaced with antibiotic-free medium before transfection, then each sample was added to the cells. Forty eight hours after the sample addition, the medium was exchanged for the medium containing 10% FBS and antiboitics and further cultured for 24 h. These cells were then lysed with 1% reduced *n*-Octyl-β-D-glucoside containing protease inhibitors (1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 2 μM pepstatin A). After cell lysis, the fluorescence intensity of EGFP was measured using a Tecan Infinite M200 microplate reader (Tecan, Salzburg, Austria) and corrected for protein amount using a BCA Protein Assay Reagent Kit (PIERCE Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Water was used as a control free of silencing effect.

1-1-2-7. Real-time RT-PCR

Colon26 NL-17 cells (1.5×10^5 cells/well on 6-well plate) were transfected with DL/siPLK1. Total RNA was extracted with TRIZOL LS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction at 24 h after the transfection. The cDNA was generated from the total RNA samples (2 μg) by using a first-strand cDNA synthesis kit (GE Healthcare, Buckinghamshire, England). In the presence of PLK1 (Takara Bio, Shiga, Japan) or β-actin primers (Takara Bio) and SYBR Premix Ex Taq II (Takara Bio), real-time RT-PCR was performed with a Thermal Cycler Dice Real Time System (Takara Bio). The nucleotide sequences of the primers of PLK1 were 5'-CTT CGC CCA AAT GCT TCG AGA T-3' (forward) and 5'-TAG GCT GCG GTG AAT TGA AGA T-3' (reverse); and those of β-Actin, 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' (forward) and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' (reverse). The conditions for real-time RT-PCR were as

follow: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s.

1-1-2-8. Western blotting

Anti-PLK1 rabbit polyclonal antibody (Cell Signaling Technology Japan, Tokyo, Japan) and anti-β-actin rabbit polyclonal antibody (SIGMA-Aldrich, St Louis, MO, USA) were diluted according to the manufacturer's instructions. Colon26 NL-17 cells (1.5×10^5 cells/well on 6-well plates) were transfected with DL/siPLK1. The culture medium was replaced with a fresh one at 24 h after the transfection. At 48 h after the transfection, the cells were lysed with lysis buffer (10 mM Tris-HCl buffer pH 7.4 containing protease inhibitors and 0.1% SDS). Total protein content was measured with a BCA Protein Assay Reagent Kit (PIERCE Biotechnology, Rockford, IL, USA). The cell extracts were loaded into 10% SDS-PAGE gels and separated by electrophoresis, and then transferred electrophoretically onto an Immobilon R-P Transfer Membrane (Merck Millipore, Billerica, MA, USA). After having been blocked for 1 h at room temperature with 5% BSA in Tris-HCl-buffered saline, pH 7.4, containing 0.1% Tween 20 (TTBS; Bio-Rad, Hercules, CA, USA), the membrane was incubated with a primary antibody (against PLK1 or β-actin) overnight at 4°C. Then, it was incubated with an horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. After the membrane had been washed with TTBS, each sample was developed with a chemiluminescent substrate (GE Healthcare, Buckinghamshire, UK), and each protein was detected with a LAS-3000 mini imaging system.

1-1-2-9. Evaluation of anti-proliferation effect of DL/siPLK1 on colon26 NL-17 cells

Colon26 NL-17 cells (1.0×10^3 cells/well in a 96-well plate) were transfected with DL/siPLK1 (n=6). The culture medium was replaced to the fresh one at 24 h after the

transfection. The cell growth was evaluated at 0, 24, 48, 72, 96 h after the transfection by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. The absorbance was measured by use of a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) operated at a test wavelength of 450 nm and a reference wavelength of 630 nm.

1-1-2-10. Statistical analysis

Differences between groups were evaluated by analysis of variance (ANOVA) with the Tukey *post-hoc* test. P values of < 0.05 were considered to be significant. Data are presented as the mean ± SD.

1-1-3. Results and discussion

1-1-3-1. Design of DL carrying siRNA for efficient gene silencing

The lipid composition of DL was first examined to optimize the formulation of the DL/siRNA. Summaries of lipid composition, particle size, PdI, and ζ-potential of each DL are shown in **Table 1**. Here, the ratio of DOP-DETA to cholesterol in the liposomes was fixed at 1:1, and the amount of DPPC varied from 0 to 30% (as molar ratio). The liposomes containing DPPC showed a uniform particle size smaller than 200 nm. The particle size of DL not containing DPPC was larger than that containing DPPC despite the preparation procedure being the same for each DL, suggesting that DPPC affects the membrane stability of DL.

Table 1. Characteristics of DL with various amounts of DPPC

	Size (d. nm)	PdI	ζ-potential (mV)
DPPC 0%	214.5	0.105	+ 43.0
DPPC 10%	145.2	0.069	+ 46.2
DPPC 20%	151.4	0.075	+ 48.5
DPPC 30%	148.4	0.101	+ 52.3

Particle size and ζ-potential of samples diluted with RNase-free water were measured by Zetasizer Nano ZS.

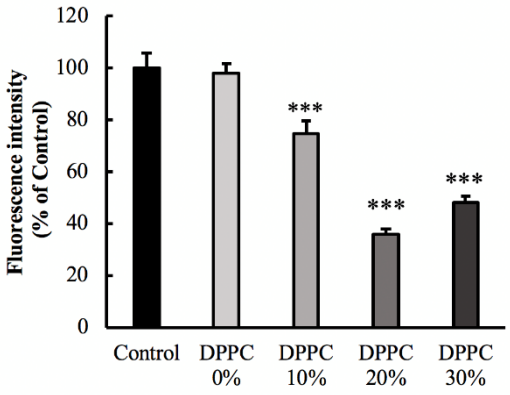
For the evaluation of RNAi inducing efficiency of DL with different lipid composition, HT1080-EGFP cells were transfected with DL/siGFP (DL/siRNA ratio = 840/1) at an siRNA concentration of 50 nM. As the result, the highest knockdown efficiency was obtained when the lipid composition of DL was DOP-DETA/DPPC/cholesterol = 40%/20%/40%（as a molar ratio **(Fig. 2A)**. This DL composition was used for the subsequent

experiments.

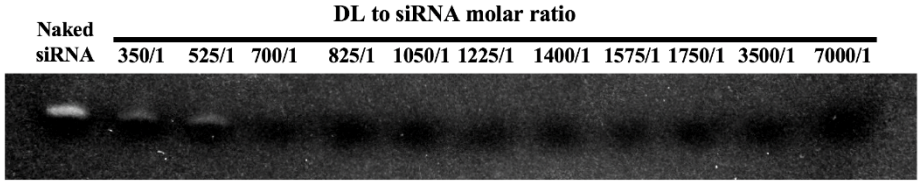
Reports on the development of lipid-based siRNA carriers have affirmed the importance of appropriate lipid/siRNA molar ratio for avoiding degradation, instability, immune reaction, and cytotoxicity.[15,24–26] In order to confirm the retention of siRNA in DL, an electrophoretic assay was performed. DL/siLuc2 was prepared by varying the DL/siRNA ratio between 350/1 and 7000/1. The results showed that the siRNA band was detected when the DL/siRNA ratio was lower than 525/1, but not when it was higher than 700/1 (**Fig. 2B**). These results indicate that all of the siRNA was attached to DL when the DL/siRNA ratio was higher than 700/1.

Next, the knockdown efficiency of DL/siGFP was evaluated using HT1080-EGFP cells. The results showed that knockdown effect of DL/siGFP was dependent on DL/siRNA ratio (**Fig. 2C, D**). The knockdown efficiency of siRNA was enhanced by a higher concentration of DL. However, cytotoxicity was observed when the DL/siRNA ratio was 14000/1 (**Fig. 2E**), suggesting that a DL/siRNA ratio of 7000/1 is suitable for obtaining high knockdown efficiency with minimal cytotoxicity. These results indicate that this novel siRNA delivery system using DL can induce RNAi even at a very low siRNA concentration. DL/siRNA ratio was fixed at 7000/1 for the subsequent experiments.

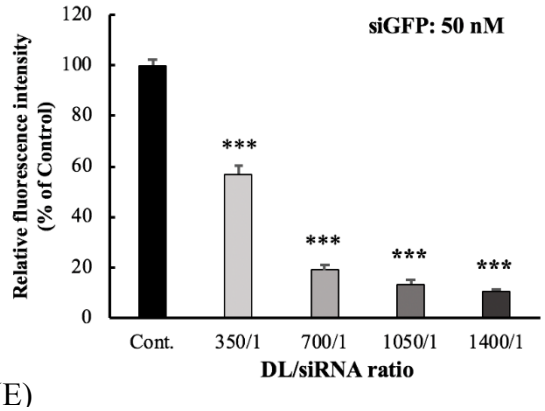
(A)


Fluorescence intensity (% of Control)
120
100
80
60
40
20
0
***
***
***
Control
DPPC 0%
DPPC 10%
DPPC 20%
DPPC 30%

(B)


DL to siRNA molar ratio
Naked siRNA
350/1
525/1
700/1
825/1
1050/1
1225/1
1400/1
1575/1
1750/1
3500/1
7000/1

(C)


siGFP: 50 nM
Relative fluorescence intensity (% of Control)
120
100
80
60
40
20
0
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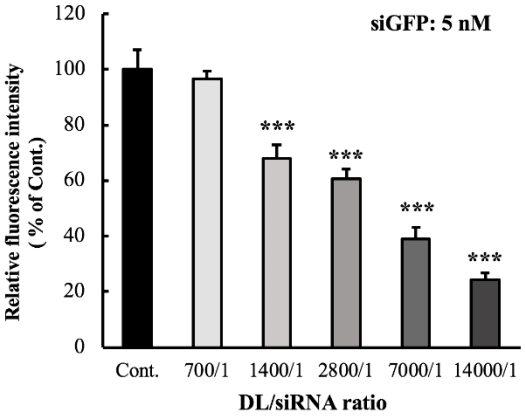
Cont.
350/1
700/1
1050/1
1400/1
DL/siRNA ratio

(D)


siGFP: 5 nM
Relative fluorescence intensity (% of Cont.)
120
100
80
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40
20
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Cont.
700/1
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2800/1
7000/1
14000/1
DL/siRNA ratio

(E)

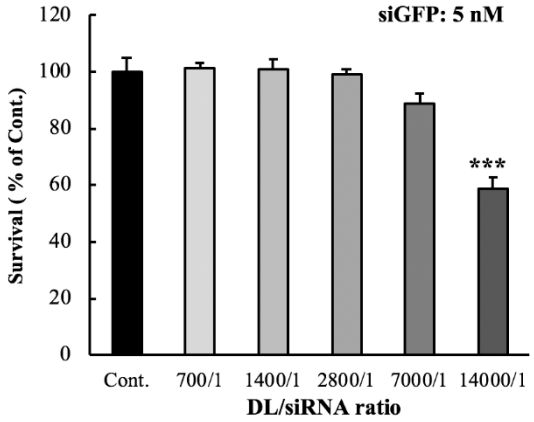

siGFP: 5 nM
Survival (% of Cont.)
120
100
80
60
40
20
0
***
Cont.
700/1
1400/1
2800/1
7000/1
14000/1
DL/siRNA ratio

Fig. 2. Gene Silencing with DOP-DETA liposomes (DL).

(A) Evaluation of the influence of varying amounts of DPPC in DL/siGFP (siRNA concentration = 50 nM, lipid siRNA molar ratio = 840) on gene silencing effect. (B) Small interfering RNA was mixed with DL at the indicated molar ratio. Various amounts of DL were complexed with siGFP at siRNA concentrations of (C) 50 nM or (D) 5 nM, with DL/siRNA ratios between 350/1 and 1400/1 (C) and 700/1 and 14000/1 (D). HT1080-EGFP cells (1.5×10^4 cells/well) were transfected with DL/siGFP complexes for (C) 72 or (D) 48 h. (E) Cytotoxicity of DL/siRNA with DL/siRNA ratios from 700/1 to 14000/1. HT1080-EGFP cells were seeded and incubated with DL/siGFP for 48 h. After incubation, cell survival was determined by LDH assay. Significant differences are indicated ***$P <$ 0.001 vs. Control. Mean ± SD (n = 6).

1-1-3-2. Gene silencing effects of DL carrying siRNA

The particle size and ξ-potential of DL and DL/siRNA (DL/siRNA ratio of 7000/1) were not significantly different (**Table 2)**. The average size of these particles was approximately 150-160 nm, with a narrow size distribution (PdI = <0.100); and their ξ-potential was approximately +50 mV.

Table 2. Characteristics of DL and DL/siRNA

	Size (d. nm)	PdI	ζ-Potential (mV)
DL	152 ± 2	0.09 ± 0.04	+50 ± 5
DL/siRNA	161 ± 12	0.07 ± 0.04	+48 ± 5

DOP-DETA/DPPC/cholesterol = 40%/20%/40% DL/siRNA ratio = 7000/1. Mean ± SD (n = 3).

Representative images obtained by transmission electron microscopy (TEM) showed that DL were round in shape and that their size distribution was almost uniform (**Fig. 3**).

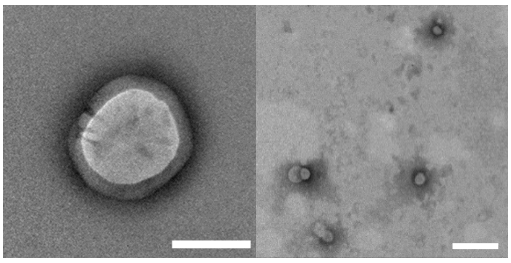
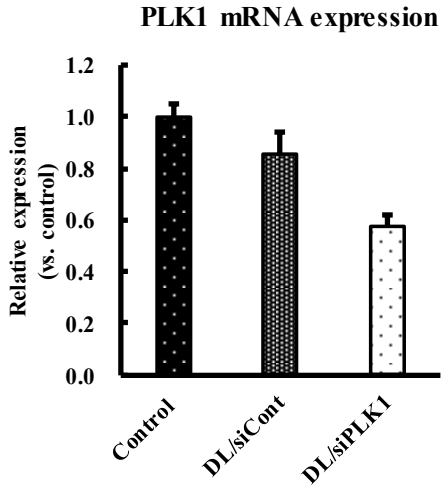


Fig. 3. Transmission electron microscopy images of DL.

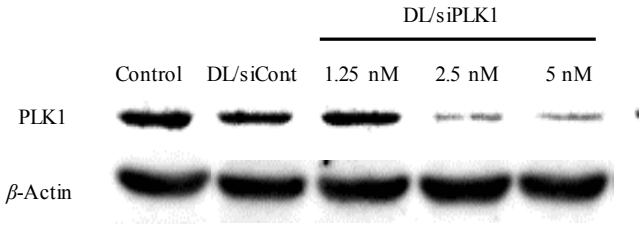
The images were obtained after negative staining. Scale bars indicate 200 nm (left) or 500 nm (right).

DL carrying siRNA for PLK1, a serine/threonine protein kinase, induced the knockdown of its target mRNA and protein in colon26 NL-17 murine colon carcinoma cells in a sequence-dependent manner (**Fig. 4A, B**). Inhibition of the PLK1 protein expression was observed even at a low siRNA concentration (2.5 nM). Although TL, previously developed pH-responsive liposomes, require a large amount of DOPE to induce gene silencing efficiently, our newly developed DL induced gene silencing at low doses of siRNA even without DOPE. Because DOP-DETA contained unsaturated lipids in their structure, these liposomes might have facilitated membrane fusion between DL/siRNA and biomembranes. Our recent study also showed that DL induced a sufficient change in surface charge in response to a change in pH, resulting in efficient endosomal escape and gene silencing.[27] DL/siPLK1 (1.0 nM siRNA) significantly suppressed the growth of colon26 NL-17 cells (**Fig. 4C**). Since PLK1 is a cell-cycle protein overexpressed in certain cancer cells,[28] delivery of siPLK1 to cancer cells is a promising approach for cancer treatment.[29,30]

(A)



(B)



(C)

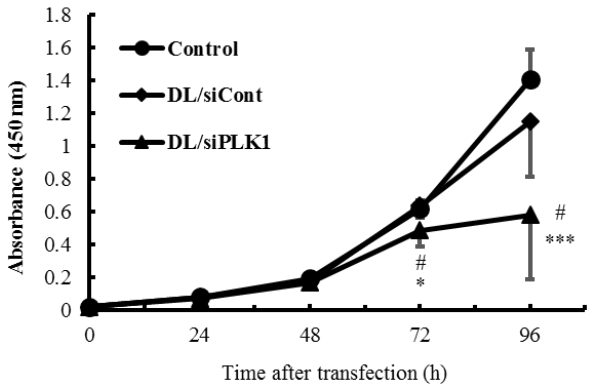


Fig. 4. Knockdown effects of DL/siRNA *in vitro*

Colon26 NL-17 cells were incubated with DL/siCont or DL/siPLK1. The amount of PLK1 mRNA and the expression of PLK1 protein were evaluated by RT-PCR (A, siRNA: 5 nM, Mean ± SD) and Western blotting (B, siRNA: 1.25, 2.5 or 5 nM), respectively. Data are presented as a relative expression of PLK1 against the control (water). (C) Colon26 NL-17 cells were incubated with DL/siCont or DL/siPLK1 (siRNA: 1 nM). Significant differences: $^{*}P<0.05$ vs. control, $^{***}P<0.001$ vs. control, $^{\#}P<0.05$ vs. siCont. Mean ± SD ($n = 6$).

Chapter 2. PEGylation of DL for systemic delivery of siRNA

1-2-1. Introduction

Since DOP-DETA has both 2 unsaturated carbon chains for membrane fusion and a relatively shorter than polycationic hydrophilic head for endosomal escape, DL can induce significant gene silencing without the inclusion of DOPE as shown in the chapter 1 Fig.1. DL have been shown to be a promising carrier capable of inducing gene silencing with low doses of siRNA *in vitro*. Here, in this chapter a new formulation for systemic injection of siRNA by modifying DL with PEG (PEG-DL) was designed and studied a key determinant to obtain *in vivo* effects of PEGylation of DL. Two types of PEG-lipid conjugates, distearoylphosphatidylethanolamine (DSPE-PEG) and distearoylglycerol (DSG-PEG), were used for PEGylation of DL; and their effects on the biodistribution of PEG-DL and siRNA in mice were compared. In addition, the gene-silencing effects of PEGylated DL carrying siRNA (PEG-DL/siRNA) in tumors were examined in mice to evaluate the potential of PEG-DL for systemic siRNA delivery.

1-2-2. Materials and methods

1-2-2-1. Materials

DSPE-PEG6000 were kindly donated by Nippon Fine Chemical Co. (Takasago, Hyogo, Japan). DSPE-PEG5000 and DSG-PEG5000 were purchased from NOF Corporation (Tokyo, Japan). Other materials are the same as those described in the "1-2-1. Materials".

1-2-2-2. Experimental animals

Five-week-old BALB/c male mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal experiments were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

1-2-2-3. Preparation of DL/siRNA complexes

DOP-DETA, DPPC, cholesterol (2:1:2 as a molar ratio), and a trace amount of [^{3}H] cholesteryl hexadecyl ether (GE Healthcare UK Ltd., Buckinghamshire, England) were dissolved in *tert*-butyl alcohol and freeze–dried for the preparation of radiolabeled DL. DL were produced by the hydration of the freeze–dried lipids with UltraPure^{TM} DNase/RNase-Free Distilled Water (RNase-free water; Invitrogen, Carlsbad, CA, USA). Sizing was performed by extrusion for 10 times through a polycarbonate membrane filter having 100-nm pores (Nucleopore, Maidstone, UK). The DL and siRNA were mixed and incubated for 20 min at room temperature to form DL/siRNA complexes. The molar ratio of DL (total lipid amount) to siRNA was 7000/1. The PEG modification was performed by mixing DL/siRNA with DSG- or DSPE-PEG5000 at 40°C for 10 min.

1-2-2-4. *In vivo* biodistribution of PEG-DL/siRNA

In order to label DL with a radioisotope, we added a trace amount of [^{3}H] cholesteryl hexadecyl ether (GE Healthcare UK Ltd., Buckinghamshire, England) to the initial t-butyl alcohol solution and freeze-dried it with the other lipids of the DL preparation. Five-week-old BALB/c male mice (n=6) were injected with [^{3}H]-labeled PEG-DL *via* a tail vein (74 kBq/mouse). Three hours after the injection, the mice were sacrificed under deep anesthesia with 2.0% isoflurane for collection of the blood. The post-heparin plasma was obtained by centrifugation (4°C, 700 × *g*, 10 min). Then, the heart, lungs, liver, spleen, and kidneys were removed, washed with saline, and weighed. Each tissue was minced and solubilized in 1 mL of Solvable (PerkinElmer, Kanagawa, Japan) overnight at 50°C. After treatment with hydrogen peroxide, a Hionic-Fluor (PerkinElmer) scintillation cocktail was added and the radioactivity in each organ and plasma was determined with a liquid scintillation counter (LSC-7400, Hitachi Aloka Medical, Tokyo, Japan). The total amount in the plasma was calculated based on the body weight of the mice, where the plasma volume was assumed to be 4.27% of the body weight based on the data for total blood volume.

1-2-2-5. Statistical analysis

Differences between groups were evaluated by ANOVA with the Tukey *post-hoc* test. *P* values of < 0.05 were considered to be significant. Data are presented as the mean ± SD.

1-2-3. Results and discussion

For the delivery of siRNA to a target tissue by intravenous injection, DL/siRNA was PEGylated to obtain stability in the blood and long-circulation property. In this chapter, 2 types of PEG-lipid conjugates, DSPE-PEG and DSG-PEG, were used to investigate the importance of the lipid structure of PEG-lipid derivatives for biodistribution. The structural difference between DSPE-PEG5000 and DSG-PEG5000 is shown in **Fig. 5A**. DSPE-PEG has a negatively charged phosphate group, while DSG-PEG does not have. The biodistribution of PEG-DL/siRNA modified with DSPE-PEG or DSG-PEG was examined 3 h after intravenous injection into mice (**Fig. 5B**). The result showed that DL/siRNA modified with DSPE-PEG showed excellent blood retention compared with non-PEGylated DL/siRNA. However, DL/siRNA modified with DSG-PEG did not show such improvement of distribution. This result suggests that the electrostatic interaction between the phosphate group of DSPE-PEG and the amine group of DOP-DETA is important for PEG-lipid to remain stably on the surface of PEG-DL/siRNA. Since DL contained 40% molar ratio of DOP-DETA (unsaturated cationic lipids), it is considered that DSG-PEG was easily eliminated from the surface of DL in the blood. Thus, the most important conclusion is that the lipophilic interaction between DSG-PEG and lipids of nanoparticles was not sufficient for stable PEGylation to obtain a long-circulation property.

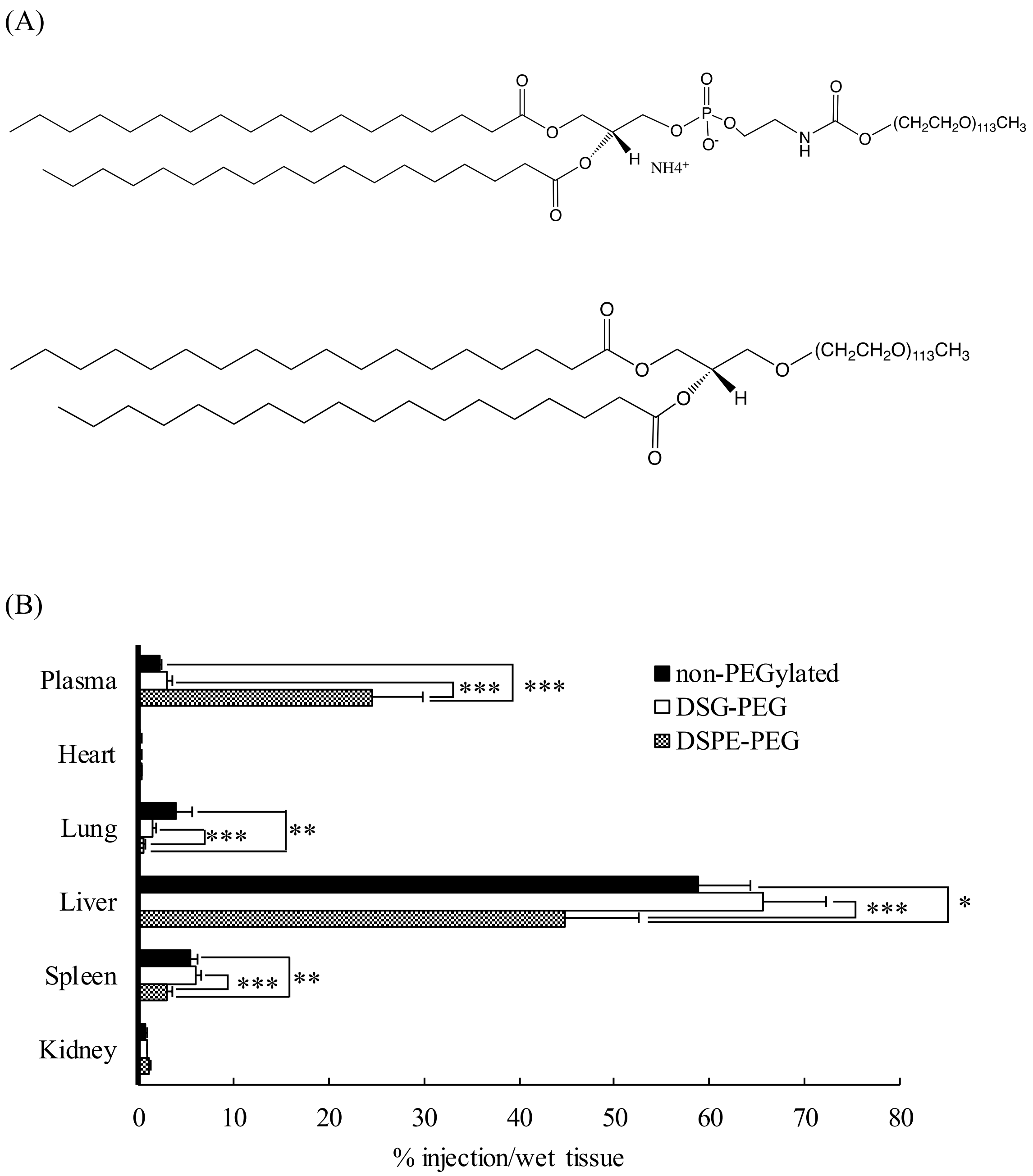


Fig. 5. Comparison between DSPE-PEG- and DSG-PEG-modified DL/siRNA

(A) Structures of DSPE-PEG5000 (upper) and DSG-PEG5000 (lower). (B) Biodistribution of PEG-DL/siRNA modified with DSPE-PEG or DSG-PEG. [^{3}H]-labeled PEG-DL/siRNA was intravenously administered to mice. The radioactivity in each organ was determined at 3 h after the injection. Data are represented as a percent of the injected dose per tissue. Significant differences are indicated **P*<0.05, ***P*<0.01; ****P*<0.001. Mean ± SD (*n* = 6).

Chapter 3. Protein knockdown in tumors after systemic injection of PEG-DL/siRNA

1-3-1. Introduction

In this chapter, *in vivo* gene silencing with PEG-DL/siRNA was examined in mice bearing subcutaneous tumors. Based on the results in the chapter 2, DSPE-PEG was used for PEGylation of DL/siRNA. For *in vivo* gene silencing experiments, DL/siRNA were modified with DSPE-PEG6000 instead of DSPE-PEG5000 because DL/siRNA modified with DSPE-PEG6000 showed longer circulation than that modified with DSPE-PEG5000 (data not shown). In the chapter 2, DSPE-PEG5000 was compared with DSG-PEG5000 since DSG-PEG6000 is not commercially available. In this chapter, various formulations of PEG-DL/siRNA were prepared with DSPE-PEG6000 and evaluated their potentials to achieve *in vivo* gene silencing in the tumors after systemic administration.

1-3-2. Materials and methods

1-3-2-1. Materials

Cholesterol-conjugated siRNA (C-siRNA) was purchased from Hokkaido System Science Co. Other materials are the same as those described in the "1-1-2-1. Materials" and "1-2-2-1. Materials".

1-3-2-3. Physicochemical characteristics of PEG-DL/siRNA in the presence of serum

The PEG modification was performed by mixing DL/siRNA with DSPE-PEG 6000 at 40°C for 10 min. The molar ratios, as a percentage, of PEGylation to total lipids were 2.5, 5 and10%. Then, these samples were incubated in 50% FBS at 37°C for 1 h. After the incubation, the absorbance was measured by using the Tecan Infinite M200 microplate reader at 600 nm. Particle size of the complexes diluted with RNase-free water was measured by using a Zetasizer

Nano ZS.

1-3-2-4. Cell cultures

LL/2-luc-M38 cells (Bioware cell line, Caliper LifeScience, Waltham, MA, USA, 1×10^6 cells/mouse) were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a CO_2 incubator. Colon26 NL-17 cells were cultured as described in the "1-1-2-5. Cell cultures".

1-3-2-5. Experimental animals

Five-week-old BALB/c or C57BL/6 male mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal experiments were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

1-3-2-6. *In vivo* biodistribution of PEG-DL/siRNA

The method for *in vivo* biodistribution of PEG-DL/siRNA is described in the "1-2-2-4. *In vivo* biodistribution of PEG-DL/siRNA "

1-3-2-7. Evaluation of the stability of siRNA in the presence of serum

Naked siRNA or siRNA formulated in DL or PEG-DL was incubated in 90% FBS at 37°C for 24 h. The siRNA was extracted from the serum by using TRIZOL LS Reagent and subjected to 15% polyacrylamide gel electrophoresis. Un-degraded siRNA was stained with GelRed, and the fluorescence was observed with a LAS-3000 mini imaging system under UV irradiation.

1-3-2-8. *Ex vivo* biodistribution of fluorescence-labeled siRNA after administration with DL or PEG-DL

Colon26 NL-17 cells (1.0×10^6 cells/mouse) were implanted subcutaneously into the right flank of 5-week-old BALB/c male mice. The mice were fed an alfalfa-free feed (Oriental Yeast Co. Ltd., Tokyo, Japan) to reduce the influence of background fluorescence. Ten days after the tumor implantation, the tumor-bearing mice were anesthetized continuously *via* inhalation of 2% isoflurane (Mylan Pharmaceuticals, Morgantown, WV, USA) and intravenously injected *via* a tail vein with AF750-siRNA or AF750-siRNA formulated in DL or PEG-DL (15 μg). The mice were subsequently sacrificed under anesthesia at 24 h post injection. Then, the organs (heart, lungs, liver, spleen, kidneys) and tumor were collected and imaged *ex vivo* with a Xenogen IVIS Lumina System coupled with Living Image software for data acquisition (Xenogen, Corp, Alameda, CA, USA).

1-3-2-9. Electrophoretic assay

siRNAs detached from DL during PEGylation were separated by performing 15% polyacrylamide gel electrophoresis. After electrophoresis, free siRNA was stained with GelRed; and fluorescence was observed by using the LAS-3000 mini imaging system under UV irradiation.

1-3-2-10. Evaluation of RNAi effect of C-siRNA by Gene silencing effect assay

HT1080-EGFP cells (2.0×10^4 cells/well in a 24-well plate) were transfected with DL/C-siGFP. The method for Gene silencing effect assay is described in the "1-1-2-6. Gene silencing effect assay "

1-3-2-11. Knockdown of luciferase expression in vivo using PEG-DL/C-siRNA

LL/2-luc-M38 cells were implanted subcutaneously into the back of 5-week-old C57BL/6 male mice (n=5 or 6). At day 9 after the tumor implantation, the mice were given an i.v. injection of PBS (control), PEG-DL/C-siCont or PEG-DL/C-siLuc (0.1 mg/kg as siRNA). For the assessment of luciferase gene expression, on day 0, 1 or 3 after the injection, luciferin (3 mg/mouse, Promega KK, Tokyo, Japan) was injected *i.p.* and the luminescence intensity from the mice was measured 10 min post luciferin injection by the IVIS. Data are presented as the luminescence intensity relative to that at day 0 after the sample injection.

1-3-2-12. Statistical analysis

Differences among groups were evaluated by ANOVA with the Tukey *post-hoc* test. P values of < 0.05 were considered to be significant. Data are presented as the mean ± SD.

1-3-3. Results and discussion

DL/siRNA were modified with 2.5, 5, or 10% DSPE-PEG6000. The particle size of PEG-DL/siRNA was not altered in the presence of serum, whereas that of non-PEGylated DL/siRNA was dramatically increased by it (**Table 3**).

Table 3. Size distribution of each PEG-DL

	FBS (-)	FBS (+)
PEG 0%	146 ±5	4750 ±730
PEG 2.5%	156 ±4	137 ±0.8
PEG 5%	146 ±1	122 ±15
PEG 10%	96 ±9	73 ±0.7

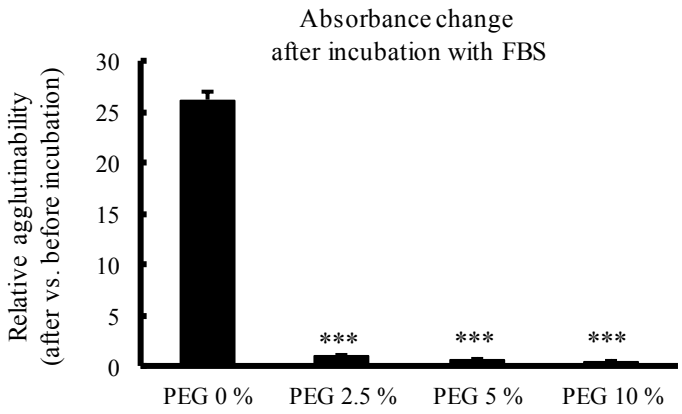
Particle size (d. nm) of liposomes were measured by use of a Zetasizer Nano ZS. Mean ± SD (n = 3).

Similarly, although the turbidity of DL/siPLK1 (without PEGylation) was approximately 25-fold increased by incubation with 50% FBS, that of the PEG-DL/siPLK1 was not increased (**Fig. 6A**). These data indicate that PEGylation prevented aggregation of DL/siPLK1 in serum. In addition, the particle size of PEG-DL/siRNA seemed to be more suitable for long circulation and passive targeting to tumors via the enhanced permeability and retention (EPR) effect. The biodistribution of PEG-DL/siPLK1 at 24 h was evaluated after intravenous injection (**Fig. 6B**). The circulation of DL/siPLK1 was significantly improved by PEGylation in a PEG amount-dependent manner. About 20% of the injected PEG-DL/siRNA remained in the blood when DL/siRNA was modified with 10% DSPE-PEG. In addition, the accumulation of PEG-DL/siRNA in the liver and spleen was decreased by the 10% modification.

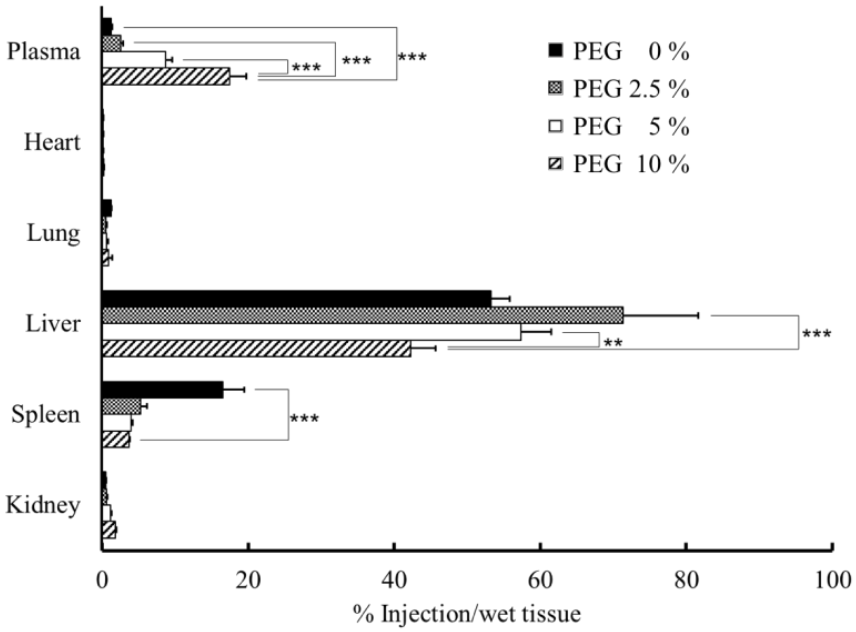
In general, 10% PEG would seem to be a high amount for the modification of liposomes, but our previous study also indicated that a relatively high amount and long PEG chain are effective to prolong the circulation time of lipoplexes. Masking of the surface property of lipoplexes with an adequate amount and length of PEG would be a key determinant to control their biodistribution. For these reasons, DL/siRNA modified with 10% DSPE-PEG was used for subsequent experiments.

PEG-DL/siRNA was incubated with 90% serum at 37°C for 24 h to test the protective effect of PEG-DL on siRNA degradation **(Fig. 6C**). Degradation of siRNA was prevented by use of PEG-DL/siRNA, whereas almost all naked siRNA and most of siRNA formulated in DL was degraded in the presence of serum. To explore siRNA biodistribution in mice bearing a subcutaneous tumor, we prepared AlexaFluor750-conjugated siRNA (AF750-siRNA) with PEG-DL and intravenously injected them into mice. Fluorescence *ex vivo* imaging at 24 h post injection showed that AF750-siRNA formulated in PEG-DL accumulated in the colon26 NL-17 tumor to a greater extent compared with that in DL or naked siRNA (**Fig. 6D**). AF750-siRNA formulated in non-PEGylated DL accumulated in the liver and spleen more than that in PEG-DL, which is consistent with the data shown in **Fig. 6B**. These data suggest that systemically injected PEG-DL/siRNA could accumulate in solid tumors via the EPR effect.

(A)


Absorbance change
after incubation with FBS
Relative agglutinability
(after vs. before incubation)
30
25
20
15
10
5
0
***
***
***
PEG 0 %
PEG 2.5 %
PEG 5 %
PEG 10 %

(B)


Plasma
Heart
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Liver
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PEG 0 %
PEG 2.5 %
PEG 5 %
PEG 10 %
0
20
40
60
80
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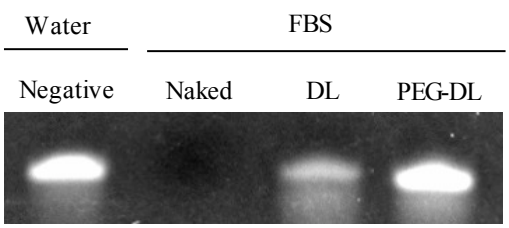
% Injection/wet tissue

(C)


Water
FBS
Negative
Naked
DL
PEG-DL

(D)

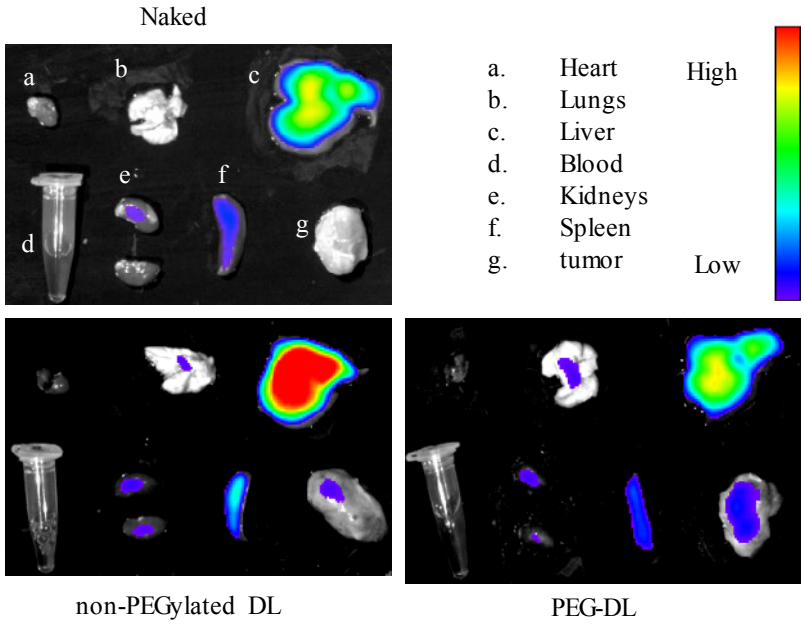


Fig. 6. Biodistribution of PEG-DL/siRNA in mice after intravenous injection

(A) PEG-DL/siRNA (0, 2.5, 5, 10 % PEGylation to total lipid) were incubated with or without 50% serum at 37°C for 1 h. The agglutination ability of each sample was evaluated (600 nm). Data are presented as the agglutination ability relative to that of samples incubated with water (control). Significant differences: ***P<0.001 vs. non-PEGylated DL/siRNA. Mean ± SD (n = 3). (B) Biodistribution of PEG-DL/siRNA. [^{3}H]-labeled PEG-DL/siRNA were intravenously administered to mice. Then, the radioactivity in each organ was determined at 24 h after the injection. Data are presented as a percent of the injected dose per tissue. Significant differences are shown (**P<0.01, ***P<0.001; Mean ± SD (n = 6)) (C) Evaluation of the stability of siRNA in serum. Naked siRNA, DL/siRNA or PEG-DL/siRNA were incubated in 90 % FBS at 37 °C for 24 h. After the incubation, intact siRNA was separated by electrophoresis and stained with GelRed. (D) *Ex vivo* imaging of AF750-siRNA in tumor-bearing mice. AF750-siRNA, DL/AF750-siRNA or PEG-DL/AF750-siRNA was intravenously injected into tumor-bearing mice. The fluorescence of AF750 in each organ (heart, lungs, liver, spleen, kidneys) and tumor was acquired *ex vivo* at 24 h after the injection.

It was previously reported that siRNA can be detached from liposomes during PEGylation. The phosphate group in DSPE-PEG may interfere with the electrostatic interaction

between siRNA and DL. Therefore, the detachment of siRNA in DL after PEGylation was examined by performing an electrophoretic assay. The results showed that a small amount of siRNA was detached from DL after modification with 10% DSPE-PEG **(Fig. 7A)**. But it seems that most of the siRNA was not detached from PEG-DL and accumulated in the tumors from the data of **Fig. 6D**. This detachment of siRNA from DL was prevented by conjugation of siRNA with cholesterol at the 3'-prime of the sense strand, possibly through hydrophobic interaction between the cholesterol of C-siRNA and lipids of DL **(Fig. 7B)**. The knockdown effect of C-siRNA formulated in DL was examined by performing a GFP silencing assay before the *in vivo* experiment. C-siRNA for GFP (siRNA: 2.5 nM) formulated in DL suppressed the expression of GFP in a sequence-dependent manner **(Fig. 7C)**. The *in vivo* gene silencing effect of PEG-DL/C-siRNA was examined in mice bearing a subcutaneous tumor of LL/2-luc-M38 firefly luciferase-transduced mouse lung carcinoma. C-siRNA for luciferase (C-siLuc, 0.1 mg/kg) formulated in PEG-DL was intravenously injected into the tumor-bearing mice at day 9 after the tumor implantation. The luminescence intensities in the tumors were increased over the whole monitoring period in the control groups **(Fig. 7D)**. In contrast, PEG-DL/C-siLuc treatment significantly suppressed the increase in luminescence intensity in the tumors. We hypothesized that DSPE-PEG was gradually detached from DL/siRNA in the body, which triggered the interaction between DL/siRNA and the tumor cells. However, precise mechanism of gene silencing *in vivo* with PEG-DL/siRNA is unclear at the present. In previous studies, the siRNA dosage was often set at around 1.0-10 mg/kg to achieve *in vivo* knockdown by systemic administration.[31] However, in our present study significant knockdown of target protein was observed at only one-tenth to -hundredth siRNA concentration (0.1 mg/kg) by use of PEG-DL. These results suggest that PEG-DL could be a useful systemic siRNA vector to induce gene silencing in solid tumors.

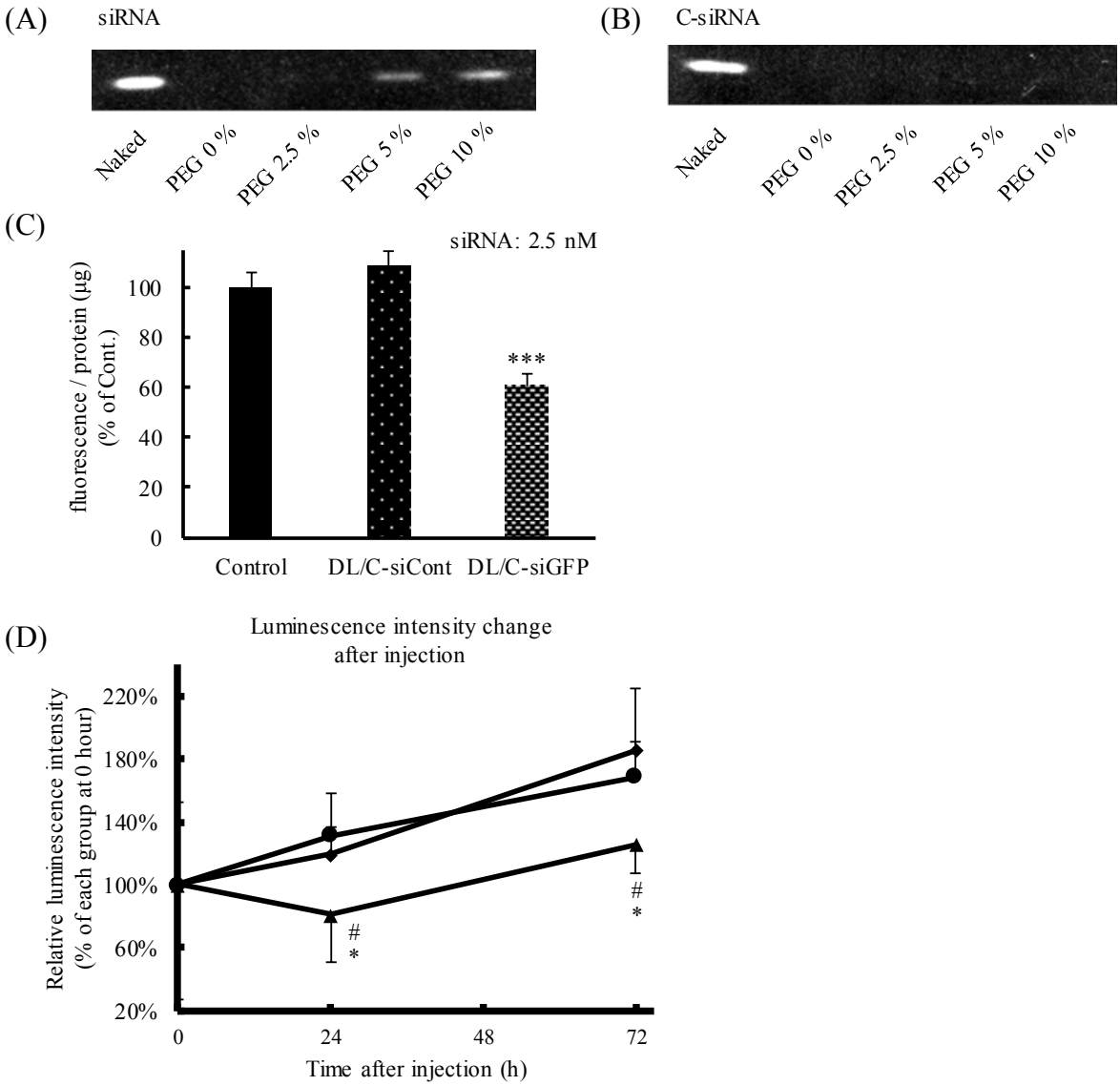


Fig. 7. Gene silencing in the tumors after injection of PEG-DL/C-siRNA

(A, B) siRNAs detached from DL were detected by use of an electrophoretic assay. (C) Gene silencing efficiency of cholesterol-conjugated siRNA formulated in DL. A significant difference is indicated ***P<0.001 vs. DL/C-siCont (D) *In vivo* gene silencing effect in tumors after intravenous injection of PEG-DL/siRNA. PBS (circle), PEG-DL/C-siCont (diamond) or PEG-DL/C-siLuc (triangle) were administered to tumor-bearing mice via a tail vein at day 9 after the tumor implantation (siRNA: 0.1 mg/kg). Significant differences are indicated *P<0.05 vs. Control, $^{\#}P$<0.05 vs. siCont. (n=5-6)

Part 2. Development of anionic lipid nanoparticle

Chapter 1. Design of anionic lipid nanoparticles

2-1-1. Introduction

Cationic vector have been widely studied because of their strong electrostatic affinity with nucleic acids and the cytoplasm gene delivery ability in target cell. Various cationic agent have been developed for gene delivery. However, after cationic vectors entered into a cell, the cytotoxicity through non-specific adsorption with the protein and affecting the normal activity of cell.[32] It has been reported that gene is introduced into cell with cationic vector, the intrinsic normal gene expression is also affected.[33] Furthermore, cationic vector must be modified with PEG before application *in vivo* to avoid non-specifically adsorbed on body tissues and trapped by immune cells after intravenous injection.[34] In addition, most of the cationic delivery systems are derived from chemical synthesis, their biocompatibility and biodegradability *in vivo* are also concern by researchers. Therefore, to develope a nucleic acid delivery system which does not use cationic materials has been received a lot of attention.

Non-cationic material siRNA delivery systems, which are considered less toxic are used in various applications.[35–37] However, it is an important challenge to design a non-cationic vector with a higher siRNA loading effect because it cannot bind to the nucleic acid through electrostatic interaction like a cationic vector. In this study, I attempted to develop a new siRNA delivery anionic lipid nanoparticle (ALNP) which consist of neutral lipid dioleoylphosphatidylcholine (DOPC), negatively charged lipid dioleoylphosphatidylglycerol (DOPG), cholesterol, calcium chloride and siRNA. Each component of the formulation was determined with the physical properties and the siRNA loading effect of siRNA in ALNP. GFP assay was used for the evaluation of RNA interference induction ability of siRNA encapsulated ALNP.

2-1-2. Materials and Methods

2-1-2-1. Materials

DOPC, DOPG were donated by the Nippon Fine Chemical Co. Triton X-100 was purchased from SIGMA-Aldrich. Ethylenediaminetetraacetic acid disodium salt, dihydrate (EDTA) was purchased from Wako Pure Chemical Industries, Ltd. Other materials were described in the 1-1-2-1.

2-1-2-2. Preparation of ALNP

Ethanol containing DOPC, DOPG, cholesterol and $CaCl_2$ and 10 mM Tris-HCl buffer (pH 7.0) containing siRNA were heated at 40°C, respectively. The ethanol (50 μL) was added to the buffer (siRNA concentration: 1 μM, lipid/siRNA molar ratio: 500/1) and incubated with a continuous vortex for 30 s at room temperature. Then the mixture was incubated for 2 min at 40°C. After the incubation, Tris-HCl buffer (pH 7.0) was added to the mixture up to 1 mL. Ethanol was removed by dialysis with Tris-HCl buffer (pH 7.0) overnight. Particle size, PdI), and ζ-potential of ALNP diluted with RNase-free water were measured using a Zetasizer Nano ZS.

2-1-2-3. Electrophoretic assay

Method was described in the 1-1-2-3. Materials.

2-1-2-4. Detection of siRNA in ALNP after the ultracentrifugation

ALNP was precipitated by ultracentrifugation (453,000 x *g*, 4°C, 10 min). After the supernatant was removed, the precipitation was treated with 2.5 mM EDTA, 2.5 mM EDTA and 0.5% (v/v) Triton X-100, or 2.5 mM EDTA, 0.5% (v/v) Triton X-100 and sodium acetate buffer (pH 4.0). siRNA in ALNP was detected by the electrophoresis assay.

2-1-2-5. Quantification of siRNA amount in ALNP

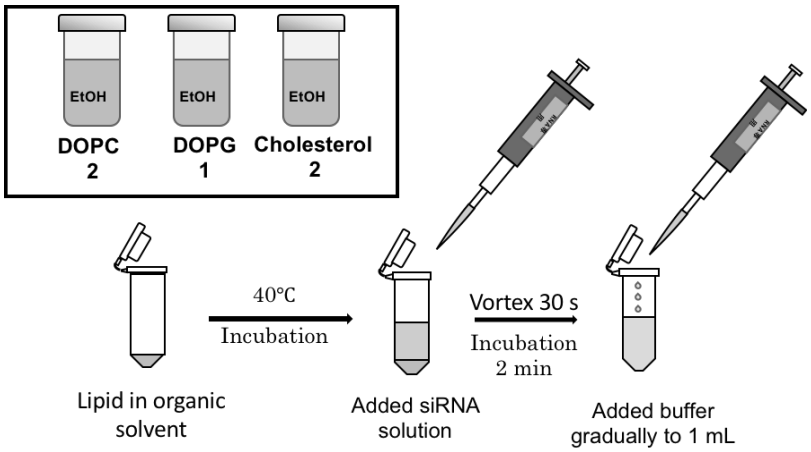
After ALNP had been precipitated by ultracentrifugation (453,000 x *g*, 4°C, 10 min), the amount of siRNA in the precipitation or supernatant was measured by the Quant-iT RiboGreen RNA Reagent (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturer's instructions. Encapsulation efficacy (EE) was calculated as follows: EE (%) = (F_{ALNP}) / (F_{total}) × 100, where F_{ALNP} is the siRNA fluorescence intensity in the precipitation (after the incubation with 2.5 mM EDTA, 0.5% (v/v) Triton-X100 and sodium acetate buffer (pH 4.0)), F_{total} is the fluorescence intensity of total (feed) siRNA dissolved in 2.5 mM EDTA, 0.5% (v/v) Triton-X100 and sodium acetate buffer (pH 4.0) .

2-1-2-6. Transmission electron microscope (TEM) image of ALNP

ALNP (5 mM total lipid concentration) in a volume of 5 μL was placed on a grid (Nisshin EM, Tokyo, Japan) and dehydrated with warm air. After this step was repeated for 3 cycles, each sample was negatively stained with 10 μL of 1 % ammonium molybdate for 1 min and air dried for 1 h. Samples were imaged with HT7700 TEM System (Hitachi High-Technologies, Tokyo, Japan).

2-1-3. Results and discussion

It was previously reported that the volume ratio of ethanol and aqueous buffer (O/W ratio) is the most critical factor to form siRNA-loaded LNP.[38] The organic solvent increases the fluidity of lipids and contributes to make a complex of calcium ion and the phosphate group of siRNA or lipids. It seems that siRNA and calcium will be precipitated and lipids will be completely dissolved in the presence of excessive organic solvent. On the other hand, excessive aqueous solution attenuates complex formation. Consequently, optimization of O/W ratio is important to obtain DOPG (or DOPC)-Ca-siRNA complexes. To determine the effect of O/W ratio on the properties of ALNP, various ALNP were prepared by changing the O/W ratio from 4:1 to 1:3 (calcium concentration: 5 mM, lipid/siRNA molar ratio = 500/1). An illustration of ALNP is shown in **Scheme 2.** A graphical method for ALNP preparation is shown in **Scheme 3**. Particle size, PdI and ζ-potential of ALNP were summarized in **Table 4**. These data indicated that the O/W ratio affected the particle size and ζ-potentials of ALNP. Detachment siRNA from ALNP was lowest when the O/W ratio was 1:1, suggesting that this ratio is suitable to obtain high encapsulation efficiency (**Fig. 8**). The O/W ratio was fixed at 1:1 for the subsequent experiments.



Scheme 3. A preparation method of ALNP

Table 4. Particle size, PdI and zeta-potential of siRNA-encapsulated ALNP.

O/W ratio	Size (d. nm)	PdI	ζ-potential (mV)
4:1	144.8	0.12	-25.2
3:1	130.8	0.132	-24.9
2:1	109.4	0.15	-23.8
1:1	224.1	0.11	-35.2
1:1.5	112.5	0.232	-41.1
1:2	104	0.132	-38.9
1:3	102	0.185	-36

Particle size and ζ-potential of samples diluted with RNase-free water were measured by Zetasizer Nano ZS.

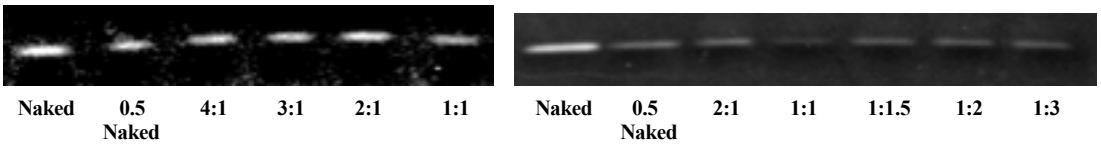


Fig. 8. Effect of O/W ratio on siRNA encapsulation into ALNP.

Encapsulation ratio was evaluated with the O/W ratio from 4:1 to 1:1 and from 2:1 to 1:3, respectively. siRNA not encapsulated in ALNP was separated by electrophoresis. The bands "Naked" and "0.5 Naked" represented 100% and 50% of total amount of free siRNA, respectively.

The effects of the amount of DOPG on the properties of ALNP were examined. The molar ratio of DOPC and cholesterol was fixed at 1:1. The particle sizes of ALNP were not significantly changed by changing DOPG percentage (Table 5). Despite DOPG is a negatively charged lipid, ζ-potential of ALNP was not correlated with the DOPG amount. ALNP containing 2.5% DOPG showed the highest encapsulation efficiency of siRNA (Fig. 9A). Increase in the DOPG amount more than 20% decreased the encapsulation efficiency and increased the particle size and PdI. Excess DOPG may cause 1) structural instability of ALNP, 2) electrostatic repulsion between DOPG and siRNA, and/or 3) failure of linkage with Ca2+.

Importantly, siRNA was encapsulated in ALNP without DOPG, suggesting that Ca2+ interact with the phosphate group of DOPC.39 Based on the data of encapsulation efficiency, ALNP containing 2.5% DOPG were used for the subsequent experiments.

Next, various ALNPs were prepared by changing lipid/siRNA molar ratio from 250/1 to 2000/1 and calcium concentration. The particle size of ALNP had a tendency to reduce with increase in increase in lipid/siRNA molar ratio (**Table 6**) and in calcium concentration (**Table 7**). On the other hand, ζ-potential of ALNP was not significantly changed. Electrophoresis data suggest that the high encapsulation of sRNA into ALNP was obtained when the lipid/siRNA ratio was 500/1 and the calcium concentration was 5 mM (**Fig. 9B**). From these results, it seems that 2.5% DOPG, lipid/siRNA ratio = 500/1 and calcium concentration 5 mM were the best condition for the preparation of ALNP. TEM images of ALNP prepared under optimized condition showed that they had a uniform size and multiple layers (**Fig. 9C**). In addition, these data indicate that siRNA encapsulation in ALNP involves a complicate interaction among the lipid, Ca^{2+} and siRNA. Thus, lipid concentration, lipid/siRNA molar ratio and Ca^{2+} concentration are very sensitive parameters for the preparation of ALNP.

Table 5. Characteristics of ALNP with different DOPG amounts

	Size (d. nm)	PdI	ζ-potential (mV)
0% DOPG	267.2	0.566	-2.17
2.5% DOPG	219.9	0.211	-13.8
5% DOPG	283.1	0.276	-22.6
10% DOPG	242.8	0.116	-33.6
20% DOPG	252.1	0.129	-64.7
40% DOPG	299.7	0.38	-38.8

Particle size, PdI, and ζ-potential of ALNP diluted with RNase-free water were measured by Zetasizer Nano ZS.

Table 6. Characteristics of ALNP with different lipid/siRNA ratio

Lipid/siRNA ratio	Size (d. nm)	PdI	ζ-potential (mV)
250/1	302.6	0.337	-25.2
500/1	199.5	0.126	-27.4
1000/1	182.6	0.126	-27
2000/1	184.4	0.136	-32.1

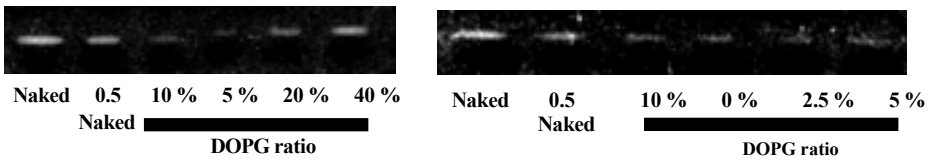
Particle size and ζ-potential of ALNP diluted with RNase-free water were measured by Zetasizer Nano ZS.

Table 7. Characteristics of ALNP with different calcium concentration

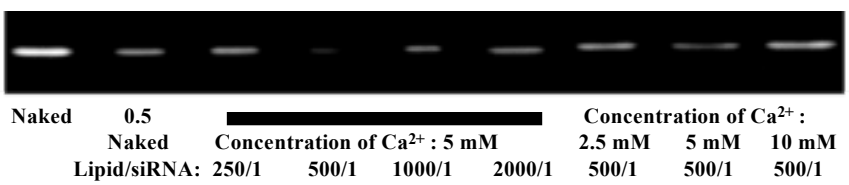
Calcium ion concentration	Size (d. nm)	PdI	ζ-potential (mV)
2.5 mM	186.3	0.105	-30.3
5 mM	188.6	0.126	-31.2
10 mM	168.0	0.124	-27.6

Particle size and ζ-potential of ALNP diluted with RNase-free water were measured by Zetasizer Nano ZS.

(A) DOPG amount



(B) Lipid/siRNA ratio and the concentration of calcium



(C)

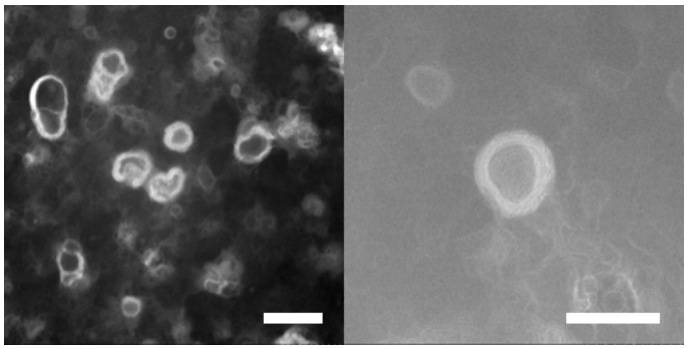
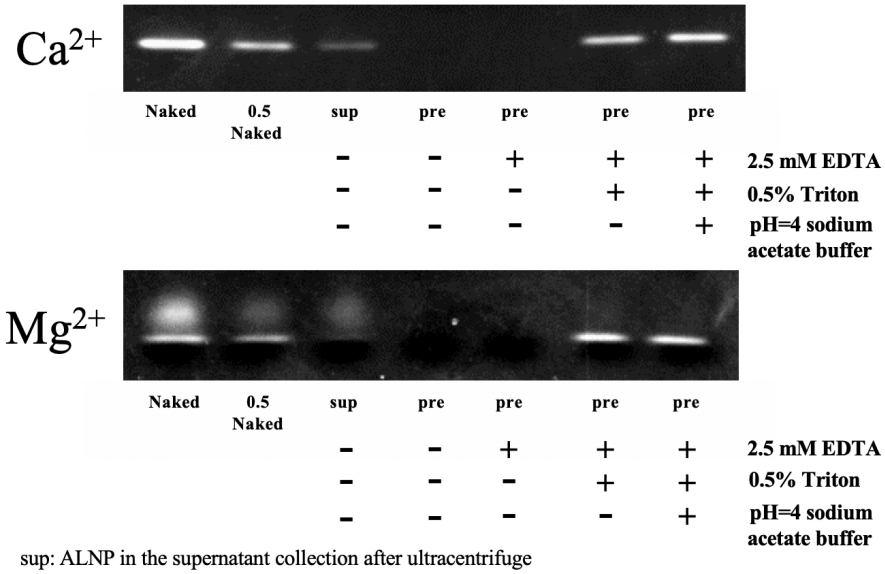


Fig. 9. Effects of preparation condition on the properties of ALNP

siRNA not encapsulated in ALNP was separated by electrophoresis. After staining of siRNA with GelRed^{TM}, free siRNA was detected under UV. Naked and 0.5 Naked represent 100% and 50% of a total amount of siRNA, respectively used for ALNP preparation. (A) siRNA was formulated in ALNP with different DOPG amounts (B) ALNP were prepared by changing the lipid/siRNA ratio and calcium concentration. (C) Transmission electron microscopy (TEM) image of ALNP negatively stained with ammonium molybdate. Scale bars indicate 500 nm (left) and 200 nm (right).

To detect siRNA encapsulated in the ALNP, ALNP were ultracentrifuged and solubilized with 2.5 mM EDTA and 1% Triton-X100 at pH 4. Results showed that most of siRNA was detected in the ALNP (precipitate) fraction. A small amount of siRNA was detected in the free siRNA (supernatant) fraction (**Fig. 10A upper**). This experiment also indicates that only 2.5 mM EDTA or 2.5 mM EDTA and 1% Triton-X100 were not enough to solubilize ALNP. In addition, similar results were obtained with the use of magnesium instead of calcium (**Fig. 10A bottom**). Encapsulation efficacy of siRNA into ALNP determined by a RiboGreen assay was approximately 80% (**Fig. 10B**). These results suggest that siRNA was efficiently encapsulated inside ALNP.

(A)



sup: ALNP in the supernatant collection after ultracentrifuge
pre: ALNP in the precipitates collection after ultracentrifuge

(B)

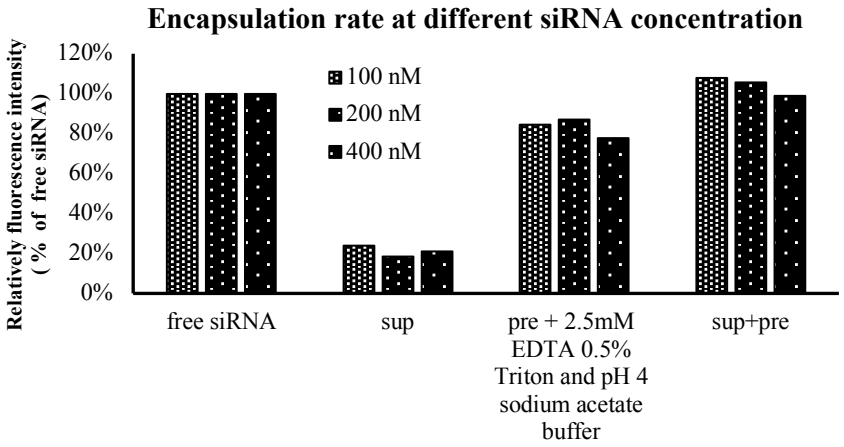


Fig. 10. Encapsulation efficacy of siRNA into ALNP

(A) ALNP were precipitated by ultracentrifugation and solubilized with 2.5 mM EDTA, 0.5% (v/v) Triton X-100 (Triton) or sodium acetate buffer (pH 4.0). siRNA in the precipitates or supernatant was stained with GelRed and detected under UV. Naked and 0.5 Naked represent 100% and 50% of a total amount of siRNA, respectively used for ALNP preparation. sup: free siRNA, pre: siRNA in ALNP. (B) Encapsulation efficacy of siRNA into ALNP at indicated concentrations was determined by the RiboGreen assay.

Chapter 2. RNA interference induced by ALNP treatment

2-2-1. Introduction

Non-cationic vectors are expected as a safe vector for nucleic acid delivery but in general, their transfection efficiency is relatively low compared with cationic vectors. In the chapter 1, although ALNP were successfully prepared and characterized, their potential for gene silencing was not yet determined. Therefore, in this chapter, the potential of ALNP for siRNA delivery was evaluated by a gene silencing experiment. For this purpose, HT1080-EGFP cells were transfected with siGFP encapsulated in ALNP.

2-2-2. Materials and method

2-2-2-1. Materials

All material information is described in the chapter 1.

2-2-2-2. Preparation of ALNP carrying

ALNP were prepared under optimized condition determined in the chapter 1. The preparation method is described in the chapter 1.

2-2-2-3. Cell cultures

The method for culturing HT1080-EGFP cells is described in the "1-1-2-5. Cell cultures"

2-2-2-4. Gene silencing effect assay

The method for Gene silencing effect assay is described in the "1-1-2-6. Gene silencing effect assay "

2-2-2-5. Statistical analysis

Differences between groups were evaluated by ANOVA with the Tukey *post-hoc* test. *P* values of < 0.05 were considered to be significant. Data are presented as the mean ± SD.

2-2-3. Results and discussion

HT1080-EGFP cells were transfected with siGFP formulated in ALNP at the siRNA concentration of 50 nM. The expression of GFP was significantly inhibited by ALNP carrying siGFP in a sequence dependent manner (**Fig. 11**). Since ALNP induced the knockdown of GFP in HT1080-EGFP cells, they could be taken up into cells and release siRNA into the cytosol. It was reported that calcium-phosphate complexes decomposes in the endosome in response to low pH. Subsequently, endosome destruction could be triggered by the increase in osmotic pressure, which promote the transfer of siRNA to the cytosol.[40] However, the potential of ALNP to deliver siRNA into the cytosol is not so high at the present stage because the knockdown efficiency of ALNP was relatively low.

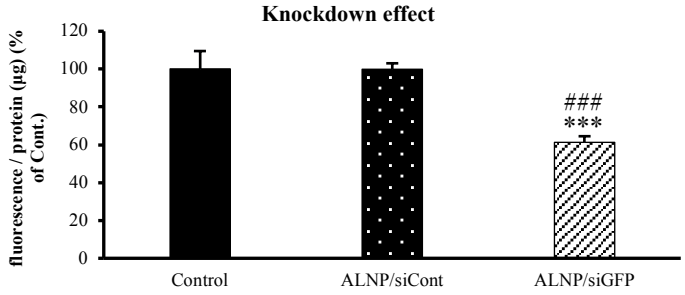


Fig. 11. Gene silencing effect of ALNP.

ALNP carrying siGFP (final siRNA concentration = 50 nM) was added to HT1080-EGFP cells and removed after 48 h incubation. After additional 24 h incubation, the fluorescent intensity and protein amount were measured. Data are indicated as the percentage of relative fluorescence intensity. ($^{###}P<0.001$ vs. Control, $***P<0.001$ vs. siCont; Mean ± SD ($n = 4$).)

Conclusion

In the Part 1, DL carrying siRNA capable of inducing efficient gene silencing with low doses of siRNA were modified with PEG for systemic injection of siRNA. The biodistribution of DL and siRNA in the PEG-DL/siRNA was studied by using radiolabeled DL and fluorescence-labeled siRNA, respectively. DL in the PEG-DL/siRNA showed a high retention in the plasma, accumulation in the tumor, and low accumulation in the liver and spleen after intravenous injection. The *in vivo* effects of PEGylation were observed only when DSPE-PEG but not DSG-PEG were used. This result suggests that the electrostatic interaction between lipid molecules on the surface of PEG-DL/siRNA was a critical determinant for the in vivo effect of PEGylation. When PEG-DL/siRNA (0.1 mg/kg siRNA) was intravenously injected into tumor-bearing mice, *in vivo* gene silencing was observed in subcutaneous tumors. These results indicate that PEG-DL/siRNA designed in this study is a promising formulation for systemic use of siRNA.

In the Part 2, ALNP, a non-cationic siRNA vector, were newly designed and successfully prepared. ALNP composed of only biodegradable and biocompatible components were expected to deliver siRNA to target cells without severe side effects. In this study, a preparation method of ALNP was studied in detail, which allow to encapsulate siRNA into ALNP efficiently. In addition, ALNP induced the knockdown of a target protein in a sequence dependent manner. ALNP might become an alternative to conventional cationic carriers although further study is required to improve their transfection efficiency.

In this thesis, two types of siRNA delivery systems were newly developed for RNA interference-based therapy. I strongly believe that my study provides new insights for the development of siRNA drugs.

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