Neutralization of negative charges of siRNA results in improved safety and efficient gene silencing activity of lipid nanoparticles loaded with high levels of siRNA

Yusuke Sato†, Hideki Matsui†, Risa Sato†, Hideyoshi Harashima★

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12 Nishi 6, Kita-ku, Sapporo 060-0812, Japan

†These authors are contributed equally to this work.

★Corresponding author
Hideyoshi Harashima, PhD
Faculty of Pharmaceutical Sciences
Hokkaido University
Kita-12 Nishi-6, Kita-ku, Sapporo, Hokkaido, 050-0812, Japan.
Tel.: +81 11 706 3919
Fax: +81 11 706 3734
E-mail: harasima@pharm.hokudai.ac.jp
Abstract

Lipid nanoparticles (LNPs) are one of the leading technologies for the in vivo delivery of short interfering RNA (siRNA). Cationic lipids are an important component for efficient endosomal escape via membrane fusion followed by release of siRNAs in cytosol where the site of action is located. A high cationic lipid/siRNA charge ratio is usually necessary for maximizing the gene silencing activity of the siRNA-loaded LNPs. However, high levels of cationic lipids are known to cause cytotoxicity through interactions with negatively charged biocomponents. A strategy for solving this dilemma is important, in terms of producing clinically applicable LNPs with a wide therapeutic window. We herein report on the development of LNPs with a high gene silencing activity and a low cationic lipid/siRNA charge ratio, which we refer to as low lipid core-nanoparticles (LLC-NPs). The negative charges of the siRNAs were neutralized by protamines, cationic proteins, to reduce the net dose of cationic lipid, YSK05, which was developed in our laboratory, for endosomal escape, resulting in preserved fusogenic activity and gene silencing activity, both in vitro and in vivo factor VII mouse model. In addition, the LLC-NPs showed an improved hepatotoxicity compared to conventional LNPs, which have a relatively higher cationic lipid/siRNA charge ratio. The concept of the LLC-NPs helps to realize clinically applicable LNPs with a wide therapeutic window and has the potential for use in various applications and for the delivery of different classes of nucleic acid.

Key words
Lipid nanoparticles, short interfering RNA, charge ratio, therapeutic window
1. Introduction

The use of short interfering RNA (siRNA) in the treatment of various refractory diseases is a promising strategy because of its specific and highly potent gene silencing via RNA interference mechanism [1]. The physicochemical properties of siRNA, which include a high molecular weight, hydrophilic properties and a polyanionic charge, represent a challenge for use in *in vivo* applications as well as clinical applications. Use of lipid nanoparticles (LNPs) is one of the most extensively investigated technologies for the delivery of siRNA *in vivo* [2]. Many of the studies of siRNA-loaded LNPs have focused on the efficiency of siRNA delivery. The recent development of sophisticated cationic lipids through rational design or combinatorial screening approaches has led to dramatic improvements in the efficiency of the LNP-mediated siRNA delivery [3-8].

The safety of the siRNA delivery system, as well as the efficiency of delivery are both important for any applications from basic research to clinical use. While cationic lipids are important for the efficient cytosolic delivery of siRNAs through their ability to enhance cellular uptake and endosomal escape, it is known that the exposure of high concentrations of cationic lipids can cause undesired side effects through multiple mechanisms, including the production of reactive oxygen species, the induction of pro-inflammatory cytokine production and apoptosis, both *in vitro* and *in vivo* [9-13]. Attempts have been made to avoid or minimize such cationic lipid-mediated cytotoxicity. These include the introduction of biodegradable linkages, including an ester bond, into the structure of the cationic lipids, which leads to their rapid elimination after delivery of the cargo [14-16]. Maier *et al.* reported that biodegradable cationic lipids are rapidly degraded and eliminated from the animal body, and showed a higher degree of safety compared to a counterpart with low biodegradability. Premedication with anti-inflammatory drugs is also a useful strategy for avoiding an elevation in the levels of pro-inflammatory cytokines which are induced by exposure to the cationic lipid [17, 18]. Abrams *et al.* reported that an intravenous injection of siRNA-loaded LNPs resulted in an elevation in serum chemistry parameters and the production of various inflammatory cytokines, and these reactions were significantly decreased by a co-treatment with dexamethasone, a glucocorticoid receptor agonist [17] and with a Janus kinase 2 inhibitor [18]. On the other hand, an elevation in pro-inflammatory cytokine levels was reported in a phase I clinical trial of ALN-VSP in spite of such a pretreatment, indicating that the efficacy of the pretreatment continues to be a controversial subject [19]. We recently found that the accumulation of the LNPs in liver sinusoidal endothelial cells causes their activation, cytokine production and subsequent
neutrophilic inflammation followed by elevated blood chemistry levels including alanine transaminase (ALT) and aspartate transaminase (AST) [20]. It is also noteworthy that this toxicity can be completely avoided by the actively targeted delivery of LNPs to hepatocytes through the strategic modification of their surface with both polyethyleneglycol (PEG) and a hepatocyte-specific ligand, N-acetyl-D-galactosamine (GalNAc) [20].

In addition to the above-mentioned strategies for reducing cationic lipid-mediated cytotoxicity, decreasing the cationic lipid/siRNA charge ratio, which is consistent with reducing cationic lipid mass per siRNA or increasing the drug/lipid ratio, would be an alternative and straightforward strategy. However, it has been reported that reducing the cationic lipid/siRNA charge ratio of LNPs results in a decrease in the efficiency of siRNA delivery [21]. This issue can be attributed to a decrease in the fusogenicity of the LNPs with endosomal membranes due to a significant consumption of active cationic lipids for membrane fusion [22]. Because negatively charged siRNAs are encapsulated by electrostatic interactions with cationic lipids, most cationic lipids are consumed in binding with siRNAs and are not available for interacting with endosomal membranes in cases of a low cationic lipid/siRNA charge ratio. Therefore, excess molar ratio of cationic lipid against siRNA is typically used in most formulations.

We herein report on the use of the protein protamine, which carries a net positive charge at physiological pH, to reduce the net dose of cationic lipids, YSK05, which was developed in our laboratory, for endosomal escape, resulting in preserved fusogenic activity and gene silencing activity, both in vitro and in vivo factor VII mouse model. Protamine sulfate is one of the widely used and well characterized cationic proteins in the field of drug delivery system [23, 24] and is an FDA-approved compound which can be injected intravenously with a documented safety profile. Therefore, we decided to use protamines for neutralizing the negative charges of the siRNA as a proof of concept. In addition, the newly developed LNPs showed superior safety properties compared to the conventional LNPs with relatively higher cationic lipid/siRNA charge ratios. The current concept of the design for potent and safe LNPs advances our efforts to create clinically applicable LNPs with a wide therapeutic window and has the potential for use in a wide variety of applications as well as for the delivery of different classes of nucleic acids.

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2. Materials and methods

2.1 Materials

Cholesterol (chol), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE), 1,2-dimyristoyl-sn-glycerol methoxypolyethylene glycol 2000 (mPEG2k-DMG), 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) were purchased from the NOF Corporation (Tokyo, Japan). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoazadiazol-4-yl) (NBD-DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-DOPE) were obtained from Avanti Polar Lipids (alabaster, AL). YSK05 was synthesized in our laboratory as described previously [25] (please refer to Fig. 6C for the structure). EverFluor TMR-labeled YSK05 was synthesized in our laboratory. Protamine was purchased from Calbiochem (San Diego, CA, USA). EverFluor TMR-X was purchased from Setareh Biotech (Eugene, OR, USA). 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS) was obtained from Wako Chemicals (Osaka, Japan). Ribogreen, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD) were obtained from Molecular Probes (Eugene, OR, USA). TRIzol reagent was obtained from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase Reporter Assay Reagent was purchased from Promega Corporation (Madison, WI, USA). FITC-conjugated Isolectin B4 was purchased from Vector Laboratories (Burlingame, CA). HeLa human cervical carcinoma cells was purchased from RIKEN Cell Bank (Tsukuba, Japan). HCT116 human colorectal carcinoma cells was purchased from ATCC (Manassas, VA, USA). All siRNAs ware obtained from Hokkaido System Science Co. Ltd. (Sapporo, Japan). The sequences for the sense and antisense strands of the siRNAs used in this study are listed in Supplementary Table.

2.2 Animals

Female ICR mice, 4 weeks of age, were purchased from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals.

2.3 Preparation of LNPs

A 400 µL of 90% t-BuOH solution containing YSK05/POPE/chol/mPEG2k-DMG at a molar ratio of 50/25/25/3 (for in vitro experiments) or 50/0/50/1.5 (for in vivo
experiments) were prepared. Dii or DiiD from 0.1 to 1 mol% of total lipid was added to the above solution for labeling. These lipid solutions were mixed with 300 μL of an siRNA in 1 mM citrate buffer (pH4.5) to give the indicated lipid/siRNA charge ratio. Concerning the LLC-NPs, a negatively charged siRNA core with the indicated nitrogen/phosphate (N/P) ratio was prepared by adding 138.5 μL of a protamine in 1 mM citrate buffer (pH4.5) to 171.5 μL of an siRNA in 1 mM citrate buffer (pH4.5) under vigorous mixing, and the siRNA core solution was diluted to become 400 μL by 1 mM citrate buffer (pH4.5) and then added to the above lipid solutions under vigorous mixing. LNPs were prepared by rapidly diluting this mixture with 1 mM citrate buffer (pH4.5). The resulting LNP solution was diluted with Dulbecco’s PBS(-) (D-PBS(-)) and subjected to two ultrafiltrations to remove the i-BuOH, replacing external buffer with D-PBS(-) and concentrating the LNPs using amicon ultra centrifugal filters. The size of the LNP in D-PBS(-) was measured by means of dynamic light scattering using a Zetasizer Nano ZS ZEN3600 instrument (Malvern Instruments, Worcestershire, UK). Number-weighted average sizes are expressed. The encapsulation efficiency and total concentration of siRNA were measured by a Ribogreen assay as described previously [25]. Apparent acid dissociation constant (pKa) of the LNPs was measured by TNS assay as described previously [25].

2.4 Cell culture and transfection

HeLa cells stably expressing Firefly and Renilla luciferase (HeLa-dluc) were cultured in growth medium, DMEM supplemented with 10% fetal bovine albumin, penicillin (100 U/mL), streptomycin (100 mg/mL) and G418 (0.4 mg/mL) at 37 °C in 5% CO₂. For luciferase gene silencing, HeLa-dluc cells were seeded at a density of 5×10³ cells per well in 96-well plates in growth medium 24 hrs prior to transfection and incubated overnight at 37°C in an atmosphere of 5% CO₂. For transfection, growth media containing LNPs at the indicated concentration of siGL4 were added to cells after aspiration of the spent media. The LNPs were allowed to incubate with cells for 24 hrs prior to analysis for luciferase expression.

HCT116 cells were cultured in growth media, DMEM supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in 5% CO₂. For polo-like kinase 1 (PLK1) silencing, HCT116 cells were seeded at a density of 1.5×10⁵ cells per well in 6-well plates in growth media 24 hrs prior to transfection and incubated overnight at 37°C in atmosphere of 5% CO₂. For transfection, growth media containing LNPs at the indicated concentration of siPLK1 were added to cells after aspiration of the spent media. The LNPs were allowed to incubate with cells for 24 hrs prior to
analysis for PLK1 mRNA expression.

2.5 Measurement of gene silencing activity *in vitro*

HeLa-dluc cells were lysed with 1× Passive Lysis Buffer 24 hrs after transfection. Firefly and Renilla luciferase activities were determined using the dual-luciferase reporter assay system according to the manufacturer’s protocol. Luminescence was measured using a luminometer (Luminescencer-PSN, ATTO, Japan). For data analysis, Firefly luciferase activity was normalized by Renilla luciferase activity and treated samples were compared to untreated samples to determine degree of luciferase silencing.

HCT116 cells were lysed with the TRIzol reagent 24 hrs after transfection. Purified total RNA was acquired according to manufacturer’s protocol. The total RNA (0.25 µg) was reverse transcribed using a ReverTra Ace qPCR RT Kit (TOYOBO, Tokyo, Japan) according to manufacturer’s protocol. A quantitative PCR analysis was performed using Thunderbird SYBR qPCR Mix (TOYOBO) and LightCycler 480 system II (Roche). All reactions were performed at a volume of 5 µL. The primers for human PLK1 were (forward) 5’-AAT AAA GGC TTG GAG AAC CC-3’ and (reverse) 5’-ACC TCA CCT GTC TCT CGA AC-3’ and for human GAPDH were (forward) 5’-CCT CTG ACT TCA ACA GCG AC-3’ and (reverse) 5’-CGT TGT CAT ACC AGG AAA TGA G-3’.

2.6 Cellular uptake

Twenty-four hours prior to transfection, HeLa-dluc cells were seeded at a density of 1×10^5 cells per well in 6-well plates in growth media and incubated overnight at 37°C in atmosphere of 5% CO₂. For transfection, media containing DiD-labeled LNPs at a concentration of 30 nM siRNA were transferred to the cells after aspiration of spent media. The LNPs were allowed to incubate with the cells for 2 hr. The cells were washed twice with PBS(-) and lysed with 1% TritonX-100 solution. DiD-derived fluorescent intensity was measured by Enspire 2300 Multilabel Reader (PerkinElmer). Cell number was normalized based on the protein concentration, as calculated by a BCA protein assay. The amount of cellular uptake is represented as the percentage of total transfected DiD fluorescence.

2.7 Measurement of fusogenic activity by fluorescence resonance energy transfer (FRET)

Anionic liposomes containing DOPS/DOPC/NBD-DOPE/Rho-DOPE at a molar ratio of 50/50/1.0/0.5 were prepared by simple hydration method in MES buffer (20 mM
MES and 130 mM NaCl). Three hundred microliter of anionic liposome (0.25 mM lipid) was diluted with 600 µL of MES buffer (20 mM MES and 130 mM NaCl) and 1800 µL of saline. Three hundred microliters of LNPs (0.25 mM lipid) was added to the above solution under stirring and fluorescence derived from Rho-DOPE was monitored for 30 min at 37°C using a spectrofluorometer (FP-6300, JASCO, Japan) with settings of λ_{ex} = 470 nm, λ_{em} = 530 nm. A 300 µL aliquot of 10 w/v% TritonX-100 was added instead of the LNP solution and was used as a positive control.

2.8 Measurement of plasma coagulation factor VII (FVII) activity

ICR mice were intravenously injected with the LNPs at the indicated siRNA doses at a volume of 10 mL/kg of body weight. At 24 hours after injection, the mice were euthanized and blood was collected by cardiac puncture and processed to plasma using heparin. Plasma FVII activity was measured using a Biophen FVII chromogenic assay kit (Aniara Corporation, West Chester, OH, USA) according to manufacturer’s protocol.

2.9 Evaluation of biodistribution and elimination from liver

As for evaluation of biodistribution, ICR mice were intravenously injected with Cy5-siRNAs formulated in DiI (0.5 mol% of total lipid) labeled LNPs at a dose of 1 mg siRNA/kg at a volume of 10 mL/kg of body weight. At 30 minutes after injection, tissues were collected, weighed and homogenized using a MicroSmash MS-100R (TOMY SEIKO Co., LTD., Tokyo, Japan) in 1% sodium dodecyl sulfate (SDS) solution. The resulting homogenates were transferred to black 96-well plates and the fluorescence was measured using an Infinite M200 Microplate reader (Tecan Group Ltd., Männedorf, Switzerland) with λ_{ex} = 650 nm, λ_{em} = 680 nm and with λ_{ex} = 550 nm, λ_{em} = 580 nm for Cy5 and DiI, respectively.

Regarding the evaluation of the elimination of LNP components from liver tissue, ICR mice were intravenously injected with LLC-NPs labeled with EverFluor TMR-labeled YSK05 (FL-YSK05) or EverFluor TMR-labeled protamine (FL-protamine) at a dose of 0.5 mg siRNA/kg at a volume of 10 mL/kg weight. At the indicated time points, liver tissues were collected, weighed and homogenized using a MicroSmash MS-100R in 1% SDS solution. The resulting homogenates were transferred to black 96-well plates and the fluorescence was measured using an Enspire 2300 Multilabel Reader with λ_{ex} = 544 nm, λ_{em} = 570 nm.

2.10 Observations of intrahepatic distribution

ICR mice were injected with Cy5-siRNA-loaded LNPs at a dose of 1 mg siRNA/kg.
At 30 minutes after injection, liver tissues were collected and stained with 50 µg/mL FITC-conjugated Isolectin B4 to visualize blood vessels. Intrahepatic distribution of the LNPs was observed by confocal laser-scanning microscopy, Nikon A1 (Nikon Co. Ltd. Tokyo, Japan). Images were captured by ×60 objective.

2.11 Measurement of blood chemistry level
ICR mice were injected with the LNPs at a dose of 7 mg siRNA/kg. Blood samples were collected 24 hr after injection and processed to plasma using heparin. Plasma alanine transaminase (ALT) and aspartate transaminase (AST) activities were determined using a commercially available kit (Wako Chemicals, Osaka, Japan).

2.12 Separation of sub-population of LLC-NPs by ultracentrifugation
Fifty microliter of the LLC-NPs (2 mM lipid) labeled with FITC-labeled protamine (5 mol% of total protamine), Cy5-siRNA (10 mol% of total siRNA) and DiI for lipid (0.5 mol% of total lipid) was added to 950 µL of a 10% sucrose containing D-PBS(-) in a polycarbonate tube. The LLC-NP suspension was centrifuged at 74,500g for 1 hr. Two hundred microliter of the solution were carefully collected from upper side. The collected samples were mixed with same volume of 2 w/v% SDS solution to lyse the LLC-NPs in black 96-well plates and the fluorescence was measured using an Infinite M200 Microplate reader with $\lambda_{ex} = 488$ nm, $\lambda_{em} = 530$ nm, with $\lambda_{ex} = 550$ nm, $\lambda_{em} = 580$ nm and with $\lambda_{ex} = 650$ nm, $\lambda_{em} = 680$ nm for FITC, DiI and Cy5, respectively.

2.13 Statistical analysis
Results are expressed as the mean±SD. Statistical comparisons between two groups were evaluated by the Student’s $t$-test and corrected by ANOVA for multiple comparisons.
3. Results and discussion

3.1 Encapsulation of siRNA core restores gene silencing activity in vitro

As mentioned above, it is known that the encapsulation of high amounts of siRNAs in LNPs results in a reduced gene silencing activity through reduced fusogenic activity, which results in lower endosomal escape [21, 22]. This disadvantageous phenomenon is thought to be due to the fact that most of the cationic lipids are consumed during the siRNA encapsulation process, which then resulted in a relocation of cationic lipids, which are available for endosomal escape via membrane fusion, from the surface to the core of the LNPs. We hypothesized that the fundamental cause of the issue is the high amount of negative charges on the siRNA molecules, and therefore, that neutralization of the siRNA-derived negative charges by cationic peptides, such as protamines, would permit the reduced gene silencing activity to be avoided. In the current study, we prepared 3 types of LNPs (Fig. 1). The first type, high lipid (HL)-nanoparticles (NPs), are the LNPs with a high cationic lipid/siRNA charge ratio, which are a conventional formulation, show a high gene silencing activity and are relatively cytotoxic due to the high content of cationic lipid. The second, low lipid (LL)-NPs, are LNPs with a low cationic lipid/siRNA charge ratio, which show a reduced gene silencing activity due to the above-mentioned mechanism and with improved safety due to the reduced amount of cationic lipids in the formulation. The third type, low lipid core (LLC)-NPs, are the currently developed LNPs, in which a siRNA/protamine core with a low cationic lipid/siRNA charge ratio is encapsulated and are predicted to show both high gene silencing activity and to be more safe for use.
Fig. 1 Schematic illustration of the 3 types of LNPs used in this study. The HL-NPs, formed by free siRNAs and a high amount of lipids, shows a high activity but lower safety due to the high amount of cytotoxic lipids that are present. The LL-NPs, formed by free siRNAs and a low amount of lipids, shows a high safety but lower activity due to its reduced fusogenic activity because the cationic lipids are consumed by the negatively charged siRNAs. The LLC-NPs are expected to show an improved activity due to the neutralization of the negative charges of the siRNAs by cationic protamines, and a high safety level, as the result of a lower amount of lipid.

We first attempted to examine the effect of the lipid/siRNA ratio on gene silencing activity in HeLa-dluc cells. In an in vitro study, the LNPs composed of YSK05, a cationic lipid that was synthesized in our laboratory, POPE, chol and \( n \text{PEG}_{2k} \)-DMG at a molar ratio of 50/25/25/3, which is the optimized lipid composition determined in a previous study [25]. The gene silencing activity of the LNPs with lipid/siRNA charge ratios from 1 to 4 was examined. The LNPs with a lipid/siRNA charge ratio of 4 showed dose-dependent gene silencing and the activity was significantly higher than that of counterparts with a lower lipid/siRNA charge ratio (Fig. 2A). We next attempted to confirm the positive contribution caused by the neutralization of siRNAs by protamines on LNP-mediated gene silencing activity. The lipid/siRNA charge ratio was fixed at 1, and siRNA/protamine core was formed with an N/P ratio within a range from 0 to 1.3. All siRNA/protamine cores were negatively charged (their \( \zeta \)-potentials were -17 to -30 mV) and their size was reproducible (diameters in the range of 54 to 70 nm) (Supplementary Fig. S1A). We confirmed that approximately 80% of the siRNAs were covered with protamines at an N/P ratio of 1.2 by a Ribogreen exclusion assay (Supplementary Fig. S1B). The gene silencing activity of the LNPs with a lipid/siRNA charge ratio of 1 were greatly restored and this restoration increased with increasing N/P ratio of the siRNA/protamine core (Fig. 2B). The 50% inhibitory concentration (IC\(_{50}\)) of the LNPs with a lipid/siRNA charge ratio of 4 was 0.75 nM siRNA and that of the counterpart with a lipid/siRNA charge ratio of 1 was above 30 nM siRNA (Fig. 2C). The IC\(_{50}\) value decreased significantly with increasing N/P ratio, and the IC\(_{50}\) value for the LNPs with N/P ratios of 1.0 and 1.3 was comparable to that for LNPs with a lipid/siRNA ratio of 4. For all in vitro experiments, LNPs with a siRNA/lipid ratio of 4 and 1 without protamines were defined as HL-NPs and LL-NPs, respectively. In addition, the LNP with a siRNA/lipid ratio of 1 and with a siRNA/protamine core at an N/P ratio of 1.3 was defined as LLC-NPs. These 3 types of LNPs had diameters in the
range from 58 to 88 nm, were monodisperse (polydispersity <0.2), near neutral, and showed a high siRNA encapsulation efficiency (approximately 90%) and had a consistent acid dissociation constant (pKa) value of 6.8 (Table 1). Physicochemical properties of the LNPs used in in vitro study (except for HL, LL and LLC-NPs) are listed in Supplementary Table S2. It can theoretically be calculated that the LLC-LNPs contain only approximately 7.9 µg of protamines against 10 µg siRNAs, whereas the difference in the amount of lipid between the HL-NPs and LLC-NPs was 114.2 µg against 10 µg siRNAs (The HL-NPs and LLC-NPs contain 152.3 µg and 38.1 µg of lipid against 10 µg siRNA, respectively) (Supplementary Fig. S2A). To confirm the generality and impact of both the lipid/siRNA ratio and the formation of an siRNA/protamine core, the gene silencing activity of the 3 types of LNPs were evaluated in a different cell line (HCT116) and a different target gene (PLK1). The physicochemical properties of the LNPs used in this study are listed in Supplementary Table S3. Both the HL-NPs and the LLC-NPs showed comparable activity (IC₅₀: 10 nM siRNA) and the activity was significantly higher than that of the LL-NPs (Fig. 2D). These results clearly suggest that the neutralization of the negatively charged siRNA with protamines restores the gene silencing activity of the LNPs.

<table>
<thead>
<tr>
<th>LNP</th>
<th>Lipid/siRNA charge ratio</th>
<th>siRNA/protamine NP ratio</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>ζ-potential (mV)</th>
<th>siGL4 encapsulation (%)</th>
<th>Apparent pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL</td>
<td>4.0</td>
<td>-</td>
<td>72.8±5.5</td>
<td>0.13±0.01</td>
<td>+2.4±2.7</td>
<td>93.5±1.3</td>
<td>6.8</td>
</tr>
<tr>
<td>LL</td>
<td>1.0</td>
<td>-</td>
<td>58.8±1.8</td>
<td>0.12±0.04</td>
<td>-4.5±1.8</td>
<td>84.3±2.7</td>
<td>6.8</td>
</tr>
<tr>
<td>LLC</td>
<td>1.0</td>
<td>1.3</td>
<td>88.4±5.7</td>
<td>0.16±0.02</td>
<td>+1.0±3.5</td>
<td>93.7±1.2</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Particle size, polydispersity (PDI) and ζ-potential were measured using a Malvern Zetasizer. n=3. Percentage of siGL4 encapsulation was determined by Ribogreen fluorescent assay. n=3. The apparent pKa value was determined by a TNS fluorescent assay. n=2.
Fig. 2 Gene silencing activities of the LNPs in vitro. (A) Effect of lipid/siRNA charge ratio on gene silencing activity in HeLa-dluc cells. Data are expressed as relative luc protein expression against untreated cells. n=3. **P<0.01. (B) Effect of N/P ratio of siRNA/protamine core on gene silencing activity in HeLa-dluc cells. The lipid/siRNA charge ratio was fixed at 1. Data are expressed as relative luc protein expression against untreated cells. n=3. **P<0.01. (C) IC<sub>50</sub> values of the LNPs in HeLa-dluc cells. n=3. **P<0.01. N.S.: not significant. (vs. LNP with lipid/siRNA ratio of 4). (D) Comparison of the 3 types of LNPs in HCT116 cells. Data are expressed as relative PLK1 mRNA expression against untreated cells. n=3. **P<0.01.

To clarify the mechanism responsible for the restoration of the gene silencing activity in the LLC-NPs, the cellular uptake of the 3 types of LNPs was measured. As shown in Fig. 3A, the cellular uptakes of the 3 types of LNPs were similar, suggesting that the amount of cellular uptake is not a factor in the difference in gene silencing activity between the 3 types of LNPs. As mentioned above, a high siRNA-loading results in the consumption of a large fraction of the cationic lipids for membrane fusion. Therefore,
the fusogenic activity of the 3 types of LNPs was examined by a membrane fusion assay using negatively charged bilayer lipid vesicles composed of DOPS and DOPC that contained the FRET pair, NBD-DOPE and Rho-DOPE. Each of the 3 types of LNPs with fixed amounts of lipid were added to the negatively charged vesicles at pH 5.5, which imitates the endosomal pH. Because the pKa value of the 3 types of LNPs is 6.8 (Table 1), more than 90% of the YSK05 would be in a net positively charged state under this condition, which would, in turn, promote membrane fusion with the negatively charged vesicles. In this experimental setting, LNPs with high fusogenic activity fuse with anionic liposomes, resulting in the cancellation of FRET efficiency due to the dilution of the FRET pair. On the other hand, it is expected that the mixing of LNPs with low fusogenic activities with anionic liposomes would fail to fuse, resulting in a decrease in the cancellation of FRET efficiency. As shown in Fig. 3B, approximately a 10% FRET cancellation was observed in the HL-NPs, which was used as a positive control. In this condition, the LL-NPs showed an obviously lower fusogenic activity (approximately 2% FRET cancellation), which is consistent with a previously reported finding [22]. Importantly, the fusogenic activity of the LLC-NPs was similar to that for the HL-NPs, even though the amount of cationic lipid against siRNA in the LLC-NPs was 4-fold lower than that in the HL-NPs, which is consistent with gene silencing activity (Fig. 2). The relatively lower value of FRET cancellation in this experiment (only ~10% on the HL-NPs) would result from the use of an excess amount of TritonX-100 (as a positive control), resulting in the drastic dilution of the FRET pair. On the other hand, an equimolar amount of the LNPs and negatively charged vesicles were mixed to compare fusogenic activity. Therefore, FRET pair lipids are diluted only by half, even if complete lipid mixing had occurred. This has been demonstrated in a previous study [22]. According to the literature, the %FRET cancellation can be increased by decreasing the molar amount of fluorescent lipids that are incorporated in anionic liposomes, but this minor modification should not influence the interpretation of this experimental data. Taken together with these in vitro experiments, it can be concluded that the neutralization of the negative charges of the siRNAs is a useful strategy to producing LNPs with low lipid/siRNA ratios and high gene silencing activity. Cytotoxicity was negligible, as evidenced by cell viability in HeLa-dluc cells, even at the highest concentration (Supplementary Fig. S3). This result provides support that the gene silencing observed in HeLa-dluc cells did not result from undesired side effects. The safety of the 3 types of LNPs was examined in vivo (see below).
Fig. 3 Cellular uptake and fusogenic activity of the 3 types of LNPs. (A) Cellular uptake of the 3 types of LNPs in HeLa-dluc cells after a 2 hr incubation. Y-axis indicates the percentage of added lipid. n=3. N.S.: not significant. (B) Fusogenic activity of the 3 types of LNPs were evaluated by monitoring FRET cancellation after mixing with anionic liposomes at pH 5.5. n=2.

3.2 The LLC-NPs show gene silencing activity and safety in vivo

For in vivo experiments, the lipid composition of the 3 types of LNPs was changed to a molar ratio of YSK05/chol/mPEG2k-DMG=50/50/1.5. POPE was removed from the lipid composition for in vivo experiments, because the silencing activity of the LNPs with the POPE-containing lipid composition used in the in vitro study was negligible in the in vivo model probably due to complement-mediated rapid clearance by the reticuloendothelial system [28]. Both the molar ratio between YSK05 and cholesterol, and the molar amount of mPEG2k-DMG were determined by factor VII (FVII) gene silencing activity of the HL-NPs by varying the lipid molar ratio of YSK05/chol/mPEG2k-DMG=X/100-X/1.5-3.0 (X=35-70). The lipid/siRNA charge ratio was fixed at 8.0 and 1.5 for the HL-NPs and both LL-NPs and LLC-NPs, respectively, and the N/P ratio of the siRNA/protamine core was fixed at 1.0 through optimizing the formulation based on physicochemical properties. Similar to the in vitro formulation, based on theoretical calculations, a reduction in lipid weight (219 µg against 10 µg siRNA) is much higher than the increase in protamine weight (6.6 µg against 10 µg siRNA) through changing from the HL-NPs to the LLC-NPs (Supplementary Fig. S2B). The properties of the 3 types of LNPs were consistent with the properties for the previous particles, including a diameter of approximately 90 nm, monodisperse (polydispersity < 0.1), near neutral and high siRNA encapsulation efficiency (approximately 90%) (Table 2). To determine if neutralizing the negative charges of the siRNAs restores the gene silencing activity mediated by the LNPs with a low
lipid/siRNA ratio *in vivo*, the gene silencing activity of the 3 types of LNPs was examined in a mouse FVII model. As shown in Fig. 4A, the median effective dose (ED$_{50}$) of the HL-NPs, LL-NPs and LLC-NPs was 0.015, 0.15 and 0.05 mg siRNA/kg, respectively, indicating that the ED$_{50}$ for the LLC-NPs was approximately 3-fold higher than that of the HL-NPs. To examine the extent of accumulation of siRNAs in liver tissue, the Cy5 fluorescence in liver homogenates was quantified after the injection of Cy5-labeled siRNAs formulated in the 3 types of LNPs. As shown in Fig. 4B, both the HL-NPs and LL-NPs delivered siRNAs efficiently whereas the LLC-NPs delivered only one third of the siRNAs compared to the other formulations. Taken together with these *in vivo* results, the efficiency of the intracellular process, especially endosomal escape, of the LLC-NPs appears to be consistent with that of the HL-NPs, suggesting that our current strategy, which restores the fusogenic activity of the LNPs with a low lipid/siRNA charge ratio by neutralization of the negative charges of the siRNAs with protamines, and is functional *in vivo*. It should be noted that the concept of the LLC-NPs is not restricted in a specific lipid composition based on both *in vitro* and *in vivo* results.

**Table 2 Physicochemical properties of the 3 types of LNPs with lipid composition for *in vivo* experiments.**

<table>
<thead>
<tr>
<th>LNP</th>
<th>Lipid/siRNA charge ratio</th>
<th>siRNA/protamine NP ratio</th>
<th>Size (nm)</th>
<th>Pdi</th>
<th>ζ-potential (mV)</th>
<th>siFVII encapsulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL</td>
<td>8.0</td>
<td>-</td>
<td>82.9±8.1</td>
<td>0.07±0.07</td>
<td>+0.4±1.1</td>
<td>93.9±3.5</td>
</tr>
<tr>
<td>LL</td>
<td>1.5</td>
<td>-</td>
<td>97.3±20.4</td>
<td>0.06±0.01</td>
<td>-4.4±6.6</td>
<td>88.1±2.3</td>
</tr>
<tr>
<td>LLC</td>
<td>1.5</td>
<td>1.0</td>
<td>93.2±4.0</td>
<td>0.08±0.06</td>
<td>-1.5±2.0</td>
<td>87.5±4.3</td>
</tr>
</tbody>
</table>

Particle size, polydispersity (Pdi) and ζ-potential were measured using a Malvern Zetasizer. Percentage of siFVII encapsulation was determined by a Ribogreen fluorescent assay. n=3-5.
Fig. 4 FVII gene silencing activity and accumulation of the 3 types of LNPs in the liver. (A) Plasma FVII activity was measured 24 hr after injection of siFVII at the indicated dose. Data are expressed as the percentage of FVII protein expression against the PBS treated group. n=3. *P<0.05 (vs. HL-NPs), **P<0.01 (vs. HL-NPs), ##P<0.01 (vs. LLC-NPs). (B) Liver accumulation of the Cy5-labeled siRNAs 30 minutes after injection. n=3. **P<0.01.

3.3 Analysis of heterogeneity of the LLC-NPs

Based on the in vivo results, a lower hepatic uptake of the siRNAs, when formulated in the LLC-NPs, limited their in vivo FVII gene silencing activity (Fig. 4B). To examine the biodistribution of the LNPs in more detail, both siRNAs and lipids of the HL-NPs and LLC-NPs were labeled with different fluorophores, Cy5 and DiI, respectively. Regarding liver tissue, the siRNA accumulation of the LLC-NPs was approximately 3-fold lower than that of the HL-NPs (Fig. 5A) whereas the lipid accumulation of both LNPs remained essentially the same (Fig. 5B), suggesting the possibility that siRNA leaks from the LLC-NPs into the blood circulation. Microscopic observations also supported the lower hepatic accumulation of the siRNA formulated in the LLC-NPs compared to that of the HL-NPs (Fig. 5C). The assumption of siRNA leakage in the LLC-NPs is supported by the fact that the LLC-NPs-mediated siRNA accumulation in kidneys was significantly higher than that for the HL-NPs (Fig. 5A). Although the LLC-NPs protected the siRNAs from degradation in mouse serum, a fraction of the siRNAs was degraded during the incubation of up to 24 hr (Supplementary Fig. S4). This result supports the conclusion that a part of siRNAs leak from the LLC-NPs upon intravenous injection. The accumulation of the LLC-NPs to the spleen was higher than that of the HL-NPs (Fig. 5B). To clarify the heterogeneity of the LLC-NPs, the lipids,
siRNAs and protamines in the LLC-NPs were labeled with Dil, Cy5 and FITC, respectively. Before the following experiment, an electrophoretic mobility shift assay was performed to confirm that the Cy5-labeled siRNAs were correctly loaded in the LNPs (Supplementary Fig. S5). The result indicated that most Cy5-labeled siRNAs were associated with the LNPs. The sub-population of the LLC-NP mass was removed by ultracentrifugation. It can be estimated that LNPs that contain no or lower amounts of siRNA/protamine core would be lighter whereas particles that contain high amounts of the core would be heavier. After ultracentrifugation of the LLC-NPs suspended in 10% sucrose containing D-PBS(-), 5 fractions were collected from the upper side. High amounts of all components were detected in both fraction 1 and 5, suggesting that the LLC-NP mass consisted of both light and heavy sub-populations (Fig. 5D). Negligible amounts of the HL-NPs were detected in fraction 5 under this condition (data not shown). The relative amount of siRNAs and protamines against lipids in fractions 1 and 5 was calculated. The siRNA/lipid ratio in fraction 5 was determined to be higher than that in fraction 1 (Fig. 5E). This suggests that the LLC-NP mass consists of two sub-populations, one of which included LNPs with a lower amount of siRNA (light sub-population) and the other with a higher amount of siRNA (heavy sub-population). The LLC-NPs with lipid composition for *in vitro* experiments (containing POPE) showed similar patterns with that for *in vivo* experiments (Supplementary Fig. S6), suggesting that the lipid composition had a negligible effect on the heterogeneity of the LLC-NPs.

The heterogeneity would likely be the result of the deformation of the siRNA/protamine core during particle formulation. In this study, LLC-NPs were prepared by dilution of a mixture of the siRNA/protamine cores and lipids with an acidic buffer. Before the dilution, it is possible that the positively-charged lipids remove some of the siRNAs from the siRNA/protamine cores competitively. A one-step, rapid and homogenous mixing of all components and formation of LLC-NPs using a microfluidic device equipped with micromixer structure would be an efficient strategy for addressing the heterogeneity [8, 27] and this aspect is currently under investigation.
Fig. 5 Heterogeneity of the LLC-NPs. (A, B) Biodistribution of the HL-NPs and LLC-NPs 30 min after injection. The LNPs were labeled with Cy5-siRNA (A) and DiD for lipid (B). n=3. *P<0.05. **P<0.01. N.S.: not significant. (C) Intrahepatic distribution of the Cy5-siRNAs 30 min after injection. The siRNAs and blood vessels are visualized as red and green, respectively. Bars represent 50 μm. (D, E) The LLC-NP population was separated by ultracentrifugation in 10% sucrose containing D-PBS(-). (D) Five fractions were collected from upper side (fraction 1) to the bottom (fraction 5). (E) The siRNA/lipid ratio and the protamine/lipid ratio in fraction 1 and 5 were calculated. n=3. **P<0.01. N.S.: not significant.
3.4 Toxicity of the LNPs *in vivo*.

Finally, the toxicity of the 3 types of the LNPs was evaluated *in vivo*. Since the LNPs are hepatotropic, we measured blood ALT and AST levels as an indicator of hepatotoxicity following a single administration of the LNPs at 7 mg siRNA/kg. It is known that chemically unmodified siRNAs induce the production of proinflammatory cytokines through a response to type I interferon, which can potentially result in hepatotoxicity. Therefore, in this experiment, a chemically modified siRNA targeting human polo-like kinase 1 (siPLK1), which was previously reported to not induce immune stimulation [28, 29]. Administration of the HL-NPs resulted in a significant elevation in both ALT and AST levels in this experimental setting (Fig. 6A). On the other hand, both the LL-NPs and LLC-NPs caused no hepatotoxicity or loss of body weight (Fig. 6A, B). One possible reason for why the LLC-NPs showed improved safety compared to the HL-NPs is the dosed lipid mass was significantly reduced (from 183.3 to 34.4 mg/kg in the case of 7 mg siRNA/kg). In addition, several inflammatory cytokines and chemokine levels were measured by means of a multiplex suspension array at 3 hr after the intravenous injection of the LNPs at a dose of 7 mg siRNA/kg (Supplementary Fig. S7). Few or no elevation in the levels of inflammatory cytokines including TNFα, IFNγ and IL-6 was observed in any of the LNPs. Both HL-NPs and LLC-NPs but not LL-NPs induced an elevation of keratinocyte-derived cytokine (KC), IFNγ-induced protein 10 (IP-10) and granulocyte-derived colony stimulating factor (G-CSF). This cytokine pattern is consistent with findings reported in our previous study [20]. The accumulation of the LNPs resulted in the activation of liver endothelial cells and an elevation in the latter 3 types of cytokines but not inflammatory cytokines. A drastic elevation of neutrophil-related cytokines including KC and G-CSF potentially causes neutrophilic inflammation which aggravates hepatotoxicity [20]. The result suggests that the reduction in the amount of lipid clearly resulted in a reduced immune response (HL vs. LL). On the other hand, the incorporation of protamines in the LNPs contributed to the induction of the latter 3 types of cytokines. Protamines have been reported to bind to the negatively charged surface of endothelial cells and, at high doses, can induce vasodilation. The leaked protamines from the LLC-NPs upon intravenous injection would be involved in the elevated cytokine levels. Further optimization of the LLC-NPs in the light of *in vivo* stability would improve the safety profile as well as gene silencing activity. It should be noted that
the elevated cytokines did not contribute to hepatotoxicity and body weight. Protamine is a small naturally occurring protein and therefore would be expected to be enzymatically metabolized and rapidly eliminated from liver tissue. To confirm this assumption, EverFluor TMR-X, a fluorescent probe, was covalently conjugated to protamine and YSK05 (denoted as FL-protamine and FL-YSK05, respectively) in order to trace these compounds (Fig. 6C). The amount of the remaining FL-protamine or FL-YSK05 in liver tissue was quantified for times of up to 24 hrs after the administration of the LLC-NPs labeled with trace amounts of FL-protamine or FL-YSK05. Negligible elimination of the FL-YSK05 from liver tissue was observed for at least up to 24 hrs (Fig. 6D), indicating that the YSK05 is relatively resistant to degradation and is eliminated from liver tissue within one day, potentially causing some toxicity in the target tissue. On the other hand, the FL-protamine was rapidly eliminated from liver tissue ($t_{1/2}$: < 4 hrs) (Fig. 6D), indicating that the biodegradability of the protamine would contribute to the improved safety of the LLC-NPs in vivo, in addition to the lower amount of the dosed lipids.
Fig. 6 Toxicity of the LNPs. (A, B) Plasma ALT and AST levels (A) and body weights (B) were measured 24 hr after the injection of the 3 types of LNPs at a dose of 7 mg siRNA/kg. n=3. *P<0.05 (vs. PBS), #P<0.05 (vs. HL-NPs). N.S.: not significance. (C) Chemical structure of EverFluor TMR-labeled YSK05 (FL-YSK05). (D) Time-dependent elimination of FL-YSK05 (solid) and FL-protamine (open). Mice were injected with the LLC-NPs labeled with trace amounts of FL-YSK05 or FL-protamine and liver tissues were then collected at the indicated time points. n=3. **P<0.01 (vs. FL-YSK05 at the same time point).
Conclusions

We report herein on the development and formulation of LLC-NPs that show consistent gene silencing activity and in which the safety is improved, compared to HL-NPs, a conventional formulation. The neutralization of the negative charges of the siRNAs by protamines significantly restored the fusogenic activity and gene silencing activity of the LNPs with low lipid/siRNA ratio both in vitro and in vivo. A dramatic reduction in lipid mass, and biodegradability and a low amount of protamines contributed to this improved safety. Although the issue of the heterogeneity of the LLC-NPs remains, we believe that the concept behind the LLC-NP would help to realize the production and use of clinically applicable LNPs with a wide therapeutic window.
Acknowledgement
This work was supported in parts by Research Program on Hepatitis from Japanese Agency for Medical Research and Development (AMED) (Grant Number PJ44280010), JSPS KAKENHI Grant Numbers JP15K20831 and JP17H0505207. The authors also wish to thank Dr. Milton S. Feather for his helpful advice in writing the English manuscript.

Conflict of interest
The authors who have taken part in this study declared that they have nothing to disclose regarding funding or conflict of interest with respect to this manuscript.
References


Supplementary Information

Neutralization of negative charges of siRNA results in improved safety and efficient gene silencing activity of high siRNA-loaded lipid nanoparticles

Yusuke Sato†, Hideki Matsui†, Risa Sato†, Hideyoshi Harashima*

Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12 Nishi 6, Kita-ku, Sapporo 060-0812, Japan

†These authors are contributed equally to this work.
2'-OMe modified nucleotides are indicated in lower case letters, 2'-fluoro modified nucleotides are in bold lower case letters, and phosphorothioate linkages are indicated by an s. Only T denotes deoxyribonucleotides and the other bases (A, U, G and C) denote ribonucleotides.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>sense strand (5'-3')</th>
<th>antisense strand (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>siGL4</td>
<td>CCG UCG UAU UCG UGA GCA ATT</td>
<td>UUG CUC ACG AAU ACG ACG GTT</td>
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<tr>
<td>siFVII</td>
<td>GGA ucA ucu cAA Guc uuA CTsT</td>
<td>GuA AGA cuu GAG AuG Auc cTsT</td>
</tr>
<tr>
<td>siPLK1</td>
<td>AGA uCA CCC uCC UUA AAu AUU</td>
<td>UAU UUA AGG AGG GUG AuC UUU</td>
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<tr>
<td>Cy5-siRNA</td>
<td>ACA UGA AGC AGC ACG Acu UTsT</td>
<td>AAG UCG UGC UGC UUC AUG UTsT-Cy5</td>
</tr>
</tbody>
</table>

Table S1. List of siRNA sequences used in this study.
Table S2. Physicochemical properties of the LNPs used for HeLa-dluc cells.

<table>
<thead>
<tr>
<th>Lipid/siRNA charge ratio</th>
<th>siRNA/protamine N/P ratio</th>
<th>Size (nm)</th>
<th>PdI</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
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<td>53.3±3.2</td>
<td>0.11±0.05</td>
<td>-5.9±7.2</td>
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<td>1.5</td>
<td>-</td>
<td>57.9±6.7</td>
<td>0.09±0.03</td>
<td>-5.5±4.3</td>
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<tr>
<td>1.0</td>
<td>0.6</td>
<td>49.1±1.0</td>
<td>0.15±0.03</td>
<td>-6.6±2.4</td>
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<tr>
<td>1.0</td>
<td>1.0</td>
<td>52.8±3.1</td>
<td>0.20±0.02</td>
<td>+1.3±0.6</td>
</tr>
</tbody>
</table>

Particle size, polydispersity (PdI) and ζ-potential were measured using a Malvern Zetasizer. n=3.
Table S3. Physicochemical properties of the LNPs used for HCT116 cells.

<table>
<thead>
<tr>
<th>LNP</th>
<th>Size (nm)</th>
<th>Pdl</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL</td>
<td>43.2</td>
<td>0.26</td>
<td>+0.6</td>
</tr>
<tr>
<td>LL</td>
<td>52.4</td>
<td>0.19</td>
<td>-5.4</td>
</tr>
<tr>
<td>LLC</td>
<td>71.4</td>
<td>0.13</td>
<td>+7.3</td>
</tr>
</tbody>
</table>

Particle size, polydispersity (Pdl) and ζ-potential were measured using a Malvern Zetasizer. n=3.
Figure S1. Effect of N/P ratio on the properties of the siRNA/protamine core. (A) Size (open) and $\zeta$-potential (solid) of siRNA/protamine core with different N/P ratio. $n=3$. (B) Effect of N/P ratio on coverage of siRNA. The accessible siRNA was detected by Ribogreen. $n=3$. 
Figure S2. Weight of the components in each LNP. The values are expressed as relative amounts (μg) of each component against 10 μg siRNA. The LNPs with lipid composition for *in vitro* (A) and *in vivo* (B) experiments are shown.
Figure S3. Cytotoxicity of the 3 types of LNPs \textit{in vitro}. HeLa-dluc cells were transfected with each of the LNPs at the indicated siRNA concentration for 24 hr. Cell viability was measured by WST-8 assay using a Cell Counting Kit-8 ((Dojindo, Kumamoto, Japan) according to the manufacturer’s recommended protocol. Non-treated cells were used as positive control. n=3.
Figure S4. Evaluation of the stability of the LLC-NPs in mouse serum. The LLC-NPs encapsulating siGL4 was incubated in 80% mouse serum at 37°C for the indicated times. After a phenol-chloroform-isoamyl alcohol extraction, polyacrylamide gel electrophoresis was performed, the siGL4 was stained by ethidium bromide and visualized by means of a transilluminator.
Figure S5. Confirming siRNA loading by a electrophoretic mobility shift assay. The LNPs encapsulating Cy5-labeled siRNAs were run on a 2% agarose gel at 100 V for 20 min. The siRNAs were stained with ethidium bromide and visualized by means of a transilluminator.
Figure S6. Separation of a sub-population of the LLC-NPs with lipid composition for *in vitro* experiments. Five fractions of the suspension of the LLC-NPs were collected from the upper side after ultracentrifugation. (A) Relative amount of each component in each fraction was measured. n=3. (B) The siRNA/lipid ratio and the protamine/siRNA ratio in fraction 1 and 5 was calculated. n=3. *P<0.05, **P<0.01.
Figure S7. Measurement of cytokines in plasma. Plasma concentration of TNFα (A), IFNγ (B), IL-6 (C), KC (D), IP-10 (E) and G-CSF (F) were measured at 3 hr after intravenous injection of the 3 types of LNPs at a dose of 7 mg siRNA/kg. n=3-4.