Development of Lipid Nanoparticles for the Delivery of Macromolecules Based on the Molecular Design of pH-Sensitive Cationic Lipids

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Considerable efforts have been made on the development of lipid nanoparticles (LNPs) for delivering of nucleic acids in LNP-based medicines, including a first-ever short interfering RNA (siRNA) medicine, Onpattro, and the mRNA vaccines against the coronavirus disease 2019 (COVID-19), which have been approved and are currently in use worldwide. The successful rational design of ionizable cationic lipids was a major breakthrough that dramatically increased delivery efficiency in this field. The LNPs would be expected to be useful as a platform technology for the delivery of various therapeutic modalities for genome editing and even for undiscovered therapeutic mechanisms. In this review, the current progress of my research, including the molecular design of pH-sensitive cationic lipids, their applications for various tissues and cell types, and for delivering various macromolecules, including siRNA, antisense oligonucleotide, mRNA, and the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) system will be described. Mechanistic studies regarding relationships between the physicochemical properties of LNPs, drug delivery, and biosafety are also summarized. Furthermore, current issues that need to be addressed for next generation drug delivery systems are discussed.

Key words lipid nanoparticle; pH-sensitive cationic lipid; endosomal escape; macromolecule delivery; nucleic acid; ribonucleoprotein

1. Introduction

Abnormal genetic status, including mutations and deletions in genomes and the mis regulation of gene expressions, causes a variety of diseases.1–4 These therapeutic targets are sometimes difficult to treat using conventional small molecule drugs due to limitations regarding their selectivity, affinity, and untranslated RNAs.5 Therefore, alternative approaches using (modified) DNAs/RNAs, including short interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), aptamers, decoys, and mRNAs, and genome editing, including clustered regularly palindromic repeat (CRISPR)/Cas, have been considered for use as novel therapeutics against various refractory diseases. Among these, some nucleic acid therapeutics have already been approved by the Ministry of Health, Labour and Welfare (MHLW) in Japan, including pegaptanib (Macugen®; aptamer), nusinersen (Spinraza®; ASO), patisiran (Onpattro®; siRNA), givosiran (Givlaari®; siRNA), viltolarsen (Viltepso®; ASO), BNT162b2 (Comirnaty®; mRNA), and mRNA-1273 (Spikervax®; mRNA) as of the writing of this review. In particular, Onpattro® is a first-ever RNA interference (RNAi) medicine that can be used in the treatment of familial amyloid polyneuropathy caused by mutations in the transthyretin gene.6 In addition, both Comirnaty® (Pfizer/BionTech) and Spikervax® (Moderna) are the first mRNA vaccines against the coronavirus disease 2019 (COVID-19) that were developed for treatment of infections caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).7 Both siRNA medicines and mRNA vaccines are lipid nanoparticle (LNP) preparations. The LNP can encapsulate RNAs in its core thus protecting the cargo from enzymatic degradation by ribonucleases (RNases) present in biological environments and to efficiently deliver the cargo to the cytosol of target cells (Fig. 1A). The LNPs are typically composed of 4 types of lipids, including a cationic lipid (CL), a phospholipid (PL), cholesterol, and a pegylated (PEG)-lipid (Fig. 1A). Among them, CL is the major component (approx. 50 mol% of the total lipid) and is essential for successfully encapsulating negatively charged nucleic acids through membrane fusion-mediated endosomal escape. Therefore, as of this writing, numerous attempts have been made to develop original potent CLs. Quaternary ammonium-based permanently charged polymeric structures, such as polyethylene glycol (PEG)-lipid (Fig. 1A), have been reported to be the major component (approx. 50 mol% of the total lipid) and is essential for successfully encapsulating negatively charged nucleic acids through electrostatic interactions and to achieve the efficient cytosolic delivery of nucleic acids through membrane fusion-mediated endosomal escape. Therefore, as of this writing, numerous attempts have been made to develop original potent CLs. Quaternary ammonium-based permanently charged polymeric structures, such as polyethylene glycol (PEG)-lipid (Fig. 1A), have been reported to be
the positive charge greatly hinders the control of the blood circulation, biodistribution, and activation of complement systems due to non-specific interactions with biocomponents (Fig. 1A). Therefore, tertiary amine-based pH-sensitive CLs, which are near neutral at physiological pH (e.g. blood stream) but develop cationic properties in a weakly acidic environment (e.g. endosomes/lysosomes), were first reported by Bailey and Cullis in 1994 (Fig. 1A). After reports on the efficient encapsulation of ASOs in LNPs composed of the pH-sensitive CLs, these pH-sensitive CLs have now become the current gold standard. Hayes et al. clearly demonstrated that unsaturation in the scaffolds in CLs is critical for inducing membrane fusion for endosomal escape, and found that DLinDMA, which is composed of 2 scaffolds derived from linoleic acid (C18:2), showed the best endosomal escape and cytosolic delivery of siRNA (Fig. 1B). In 2006, Zimmermann et al. first demonstrated successful induction of hepatic apolipoprotein B (ApoB) gene silencing in mice and in non-human primates after the intravenous injection of siApoB-loaded DLinDMA-LNPs (referred to as a stable nucleic acid lipid particle; SNALP) at a clinically relevant dose (50% effective dose; ED<sub>50</sub> of approx. 1 mg siRNA/kg). In 2010, Semple et al. reported that the linker structure between the tertiary amino group and the scaffolds was critical for maximizing fusogenic activity. Specifically, the efficacy of a ketal ring linker was found to be superior to a 1,2-diol-derived linker, which was adopted in DLinDMA. The best performing CL, DLin-KC2-DMA, showed an ED<sub>50</sub> of approx. 0.02 mg siRNA/kg in the mouse factor VII (FVII) model, which was a 50-fold improvement in gene silencing efficiency in hepatocytes (Fig. 1C). Further structural optimizations resulted in the identification of the optimal CL, DLin-MC3-DMA (MC3) (Fig. 1D), which exhibited an ED<sub>50</sub> of approx. 0.005 mg siRNA/kg in the FVII model and is used as a main ingredient in Onpattro<sup>®</sup>. Based on this structure, numerous potent CLs, including ALC-0315 and SM-102 used for COVID-19 mRNA vaccines, have been developed so far (Figs. 1E, F). Based on the above findings, the development of potent CLs is one of the major breakthroughs in the field of LNPs.

I began work on developing LNPs for the in vivo delivery...
of macromolecules based on the molecular design of original pH-sensitive cationic lipids in 2010 in the laboratory for the molecular design of pharmaceutics organized by Prof. Hideyoshi Harashima. In this review, current progress of my research, including the molecular design of pH-sensitive cationic lipids, their applications for liver, extra-hepatic tissues and cell types, and for the delivery of various macromolecules, including siRNA, antisense oligonucleotides, mRNA, and clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) system are described. Mechanistic studies regarding the relationship between the physicochemical properties of LNPs, drug delivery, and biosafety will be also summarized. Current limitations that should be addressed for next generation drug delivery systems are also discussed.

2. Development of pH-Sensitive CLs and Their Applications for Liver

In 2012, a pH-sensitive CL, YSK05, was first synthesized as the 1st generation CL (Fig. 2A). YSK05 can be synthesized in a one-step condensation reaction of N-methyl-4-piperidone and linoleyl alcohols. The structure that two linoleyl alcohol-derived scaffolds that are attached to one carbon atom leads to an increase in the angle between the two scaffolds (109.5°) (Fig. 2A). Based on theory of critical packing parameter (CPP), a framework for determining the type of aggregates formed by surfactants, this design emphasizes the formation of a CL with a cone shape. The cone shape of the CLs is important for inducing membrane fusion-mediated endosomal escape. Membrane fusion can be achieved through the phase transition of the lipid bilayer of biological membranes from a lamellar (L) to an inverted hexagonal (HII) phase. The pH-sensitive CLs acquire a cationic charge in weakly acidified endosomes, bind to anionic lipids in the endosomal membranes and form ion pairs (Fig. 2B). When the ion pairs adopt a cone shape that is sufficient to destabilize lipid bilayers and to induce a phase transition into non-bilayer structures (e.g. HII phase), efficient endosomal escape can be achieved. 31 Phosphorus (31P) NMR can be utilized to understand lipid polymorphism of lipid dispersion. Due to the anisotropy of the chemical shift of the 31P of PLs that is derived from different limitations in molecular motion between lipid polymorphism, it is known that L and HII phases show a discriminative broad signal with a high-field peak and a low-field peak, respectively (Fig. 2B). A 31P-NMR analysis using lipid dispersions composed of an equimolar amounts of a pH-sensitive CL, zwitterionic 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and negatively charged 1,2-distearoyl-sn-glycero-3-phosphatidylserine (DSPS) suspended in an acidic buffer revealed that the phase transition temperature from L to HII phase (TH) for YSK05 was significantly lower (21–25 °C) than that for a conventional pH-sensitive CL, 1,2-dioleoyloxy-3-dimethylaminopropane (DODAP) (35–40 °C) (Fig. 2C). This result indicates that YSK05 is more fusogenic than DODAP. A hemolysis assay which can be used to examine the fusogenicity of LNPs using red blood cells as model biomembranes also revealed that YSK05-containing LNPs (YSK05-LNPs) are more fusogenic. Indeed, the siRNA-loaded YSK05-LNPs showed much higher gene silencing activity compared to DODAP or 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP)-LNPs in HeLa cells despite the fact that the cellular uptake of the YSK05-LNPs was consistent or lower than the counterparts. Treatment with chloroquine or ammonium chloride, endosome buffering reagents, revealed that the acidification of endosomes is an essential process for the successful...
The YSK05-LNPs were applied to liver tissue. It is known that size-controlled LNPs with diameters of 100 nm or less can reach hepatocytes due to the structural features of the liver, including the presence of fenestrae which are pores with diameters in the range of 100 to 150 nm in liver sinusoidal endothelial cells (LSECs) and a lack of basement membranes between LSECs and hepatocytes.\(^{41,42}\) In addition to this, the presence of the apolipoprotein E (ApoE)-low density lipoprotein receptor (LDLR) pathway as an endogenous uptake mechanism in hepatocytes greatly contributes to the development of liver-targeting LNPs.\(^{43,44}\)

It was found that the bare YSK05-LNPs were cleared from blood stream very quickly (\(t_{1/2}\) of approx. 1.8 min) after intravenous administration through the ApoE-LDLR pathway\(^{45}\) (Fig. 3A). This rate was over 10-fold faster than that for \(N\)-acetyl-D-galactosamine (GalNAc)-modified YSK05-LNPs which were cleared through the GalNAc-asialoglycoprotein receptor (ASGPR) pathway (Fig. 3A). It is well known that GalNAc functions as a ligand of ASGPRs, which are highly and specifically expressed on hepatocytes, and are used for active targeting to hepatocytes.\(^{46-50}\) Intrahepatic observations revealed that bare YSK05-LNPs are adsorbed to microvillar surface of hepatocytes in wild-type mice but not in ApoE-deficient mice, indicating that the presence of the ApoE protein is essential for adsorption to occur. This adsorption was competitively inhibited by treatment with heparin or heparinases, suggesting that involvement of heparan sulfate proteoglycans (HSPGs),\(^{51,52}\) which are abundantly expressed on the surface of hepatocytes and are known to be a receptor of the ApoE protein.\(^{53}\) These findings that liver accumulation is a complex process in which the bare LNPs rapidly adsorb to the microvillar surface of hepatocytes through ApoE-HSPG interactions following the binding of ApoE proteins to the LNPs in the blood circulation after intravenous administration (Fig. 3B).

The YSK05-LNPs showed \(ED_{50}\) values of 0.06 mg siRNA/kg and a durable gene silencing for up to 2 weeks in a mouse FVII model through optimizing the lipid composition.\(^{54}\) To apply this to the treatment of hepatitis C virus (HCV), mice with chronic HCV infections were produced by the inoculation of the HCV genotype 1b in chimeric mice with humanized liver cells and the siHCV-loaded YSK05-LNPs were then intravenously administered. HCV genomic RNA levels in serum were suppressed up to 90% for periods of up to 2 weeks (Fig. 3C). A significant decrease in the levels of HCV core proteins in liver tissue was also observed (Fig. 3D).

The optimized YSK05-LNPs were also applied to the in vivo validation of candidate genes as therapeutic targets.\(^{55}\) A comparative transcriptome analysis in liver between diabetic and normal mice identified an elevated expression of monoglyceride \(O\)-acyltransferase 1 (Mogat-1), an enzyme that is involved in triglyceride synthesis and its storage.\(^{56-58}\) Long term Mogat-1 gene silencing by the repeated administration of siMogat-1-loaded YSK05-LNPs in diabetic mice resulted in preventive effects of type 2 diabetes, including reduced blood glucose levels, triglycerides and cholesterol, and increased levels of adiponectin. Related to this study, the durable silencing of Lipin1, which regulates fatty acid utilization in the triglyceride biosynthesis pathway,\(^{59}\) by YSK05-LNPs successfully revealed that Lipin1 silencing in liver tissue led to a marked decrease in adipose tissue mass and adipocyte diameters.\(^{60}\) These results suggest that the established LNPs could be applied not only for the treatment of a specific disease but also for the validation and investigation of in vivo functions of genes of interest.

PEGylation of the LNP surface is a widely used strategy for inhibiting non-specific interactions with biocomponents and to maximize the efficiency of active targeting.\(^{61-63}\) However, the PEGylation is known to severely inhibit the endosomal escape
process, a phenomenon known as the ‘PEG dilemma.’\(^{64,65}\) Although pH-sensitive CLs with an acid dissociation constant (pK\(_a\)) around 6.4 can be used to reduce the concentration of PEG on the surface of LNPs needed for long blood circulation compared to permanently CLs, even such a lower concentration of surface PEG can inhibit both cellular uptake and endosomal escape.\(^{32}\) PEGylation of a LNP surface via a pH-labile linkage is one of the more useful strategies for overcoming this dilemma.\(^{66–70}\) Maleic anhydride reacts with amino groups and forms a labile amide bond in an acidic environment, and the pH-sensitive degradability of the amide bond can be modulated by the substituents on the cis-double bond of the maleic anhydride\(^{71–74}\) (Fig. 4A). The siRNA-loaded GalNAc-modified YSK05-LNPs were PEGylated though carboxylated dimethylmaleic anhydride (CDM) or carboxylated ethylmaleic anhydride (CEM), a maleic anhydride derivative, linker.\(^{73}\) The use of a maleic anhydride linker promoted the detachment of the modified PEG and the re-activation of the fusogenisity of the LNPs under endosomal pH mimetic conditions. The efficiency of hepatocyte-targeting and gene silencing activity were observed to be enhanced in vivo. Taken together, the pH-labile PEGylation of GalNAc-modified LNPs through a maleic anhydride linkage successfully shielded the LNPs, enhanced active targeting to hepatocytes, and re-activated the LNPs by the detachment of PEG in response to the acidic environment in endosomes (Fig. 4B).

MicroRNAs (miRNAs) are a class of small non-coding RNAs that are related to homeostasis and diseases and function by regulating gene expression by binding to the 3'-untranslated region (3'-UTR) of target mRNAs, thus triggering the degradation of the mRNAs or the suppression of translation.\(^{76}\) miRNA-122 (miR-122) is a liver-specific miRNA and is known to play important roles in lipid metabolism and to facilitate the replication of HCV RNA.\(^{77–79}\) Therefore, the inhibition of miR-122 by the anti-miRNA oligonucleotide (AMO) is a promising strategy for the treatment of liver diseases.\(^{80}\) Consistent with siRNAs, LNPs can increase the bioavailability of AMOs. Three separate intravenous administrations of AMO122-loaded YSK05-LNPs at doses of 1 mg AMO/kg in mice resulted in a nearly 90% inhibition of miR-122, a value that was significantly higher than free AMO122 (<40% inhibition).\(^{81}\) This resulted in an elevated expression of miR-122 target genes (AldoA, Bckdk, and Ndrg3) in liver tissue and a reduced plasma cholesterol concentration. The YSK05-LNPs also succeeded in inhibiting miR-122 by delivering 2'-OMe-4'-thioribonucleoside-modified AMO122.\(^{82}\) Taken together, these results demonstrate that the use of drug delivery technologies presents a practical and valuable alternative to anti-miRNA therapeutics.

In 2016, the 2nd generation pH-sensitive CLs, YSK13 and YSK15, were designed with the objective of overcoming the limitations of YSK05, including insufficient fusogenisity and potential chemical instability under acidic conditions due to

![Fig. 4. Improvement of Active Targeting Using pH-Labile PEGylation](attachment:image)

![Fig. 5. Development of the 2nd Generation pH-Sensitive CLs, YSK13 and YSK15](attachment:image)
an acid-labile ketal linker. The YSK13 and YSK15 contain \(\alpha,\beta\)-unsaturated ester and allyl ester linkers, respectively, and two linoleic acid-derived scaffolds are attached to one \(sp^2\) carbon atom of the linker (Fig. 5A). This design makes the angle between the two scaffolds be 120°, which is higher than that of YSK05, and therefore would be expected to emphasize a cone shape and to increase fusogenicity based on CPP theory.

A \(^{31}\)P-NMR analysis demonstrated that YSK13 show clearly a lower \(T_H\) compared to that of YSK05, suggesting a higher fusogenicity. Both the YSK13 and YSK15 contain some derivatives with different spacer lengths between an ester bond and a tertiary amino group. A longer spacer length resulted in LNPs with an increased apparent \(pK_a\) value, which can be determined by a 6-(\(p\)-toluidino)-2-naphthalenesulfonic acid (TNS) assay. It is known that the optimal \(pK_a\) range for delivering siRNA to hepatocytes is 6.2–6.5.

A YSK13 derivative, YSK13-C3, with a \(pK_a\) of 6.45 and \(ED_{50}\) of 0.015 mg/kg in a mouse FVII model, was selected as the optimal CL for hepatocytes. The impact of the apparent \(pK_a\) value on cellspecificity is described below. The YSK13-C3 was used in the treatment of chronic hepatitis B virus (HBV) infections. Mice that are persistently infected with HBV were intravenously administered with the siHBV-loaded YSK13-C3-LNPs at a dose of 5 mg siRNA/kg. An approximately 80–90% suppression of HBV antigens (HBsAg and HBeAg) and HBV genomic DNA was observed for periods of up to 10 d (Fig. 5B). The oral administration of a clinically used nucleoside analogue of the reverse transcription (RT) inhibitor, entecavir, strongly suppressed HBV DNA in sera but failed to reduce the levels of HBV antigens in the sera. The elimination or suppression of HBV antigens is important for the treatment of hepatitis because several HBV antigens downregulate the adaptive immune response to HBV in patients. Therefore, the above findings indicate that a gene silencing approach could be the basis for a novel class of anti-HBV drugs which would be superior to RT inhibitors.

More recently, a pH-sensitive CL library was developed from a benchmark CL, YSK12-C4. The YSK12-C4, which shows a high fusogenicity and a high \(pK_a\) (approx. 8.2), has been used for treating hard-to-transfect immune cells.

Fig. 6. Development of an Original pH-Sensitive CL Library

(A) Overall picture of the pH-sensitive CL library. (B) A plot of calculated \(pK_a\) by ChemDraw versus the apparent \(pK_a\) measured by a TNS assay. (C) Structure of one of the best pH-sensitive CLs, CL4H6, for the delivery of siRNAs to hepatocytes. (D) Dose-dependent FVII silencing activity of the siFVII-loaded CL4H6-LNPs. (E) Cytosolic delivery of siRNAs by the CL4H6-LNPs. The siRNAs are observed as diffused patterns in the cytosol of hepatocytes. These figures are reproduced, in part, with permission from Elsevier. (Color figure can be accessed in the online version.)
LNP technology was current COVID-19 mRNA vaccines. The pH-sensitive CLs with different structures around tertiary amine showed various apparent pK_a values. A plot of the cLogp value of the hydrophilic region versus the experimentally determined apparent pK_a value revealed that the apparent pK_a value decreased with increasing hydrophobicity of the hydrophilic region. On the other hand, the pK_a values estimated by the ChemDraw software were significantly lower than the apparent pK_a values and predicting the precise apparent pK_a value clearly failed (Fig. 6B). This could be explained by the fact that a tertiary amino group, a proton donor, is present on the interface of the LNPs where the proton is relatively hard to access and an increased cLogp value leads to the preferential localization of the tertiary amine in the hydrophobic layer of scaffolds, resulting in resistance to protonation. Moreover, extremely high concentrations of the pH-sensitive CLs (approx. 50 mol% of total lipid) in the LNPs results in reduced pK_a values due to an increased charge repulsion between the CLs that are in close proximity with each other. In contrast to the hydrophilic region, the structures of hydrophobic scaffolds had relatively minor effects on apparent pK_a values and unexpected interactions between the hydrophobic tails and the hydrophilic head group on apparent pK_a values was limited. After several examinations regarding structure–activity relationships, a novel pH-sensitive CL, CL4H6, was identified as one of the best CLs for delivering siRNA into hepatocytes and was determined to be a 3rd generation CL (Fig. 6C). The lipid composition-optimized CL4H6-LNPs delivered a dose of 0.0025 mg siRNA/kg in a mouse FVII model, which was a 6- and 2-fold higher efficiency compared to 2nd generation CLs that are in close proximity with each other. In contrast to the hydrophilic region, the structures of hydrophobic scaffolds had relatively minor effects on apparent pK_a values and unexpected interactions between the hydrophobic tails and the hydrophilic head group on apparent pK_a values was limited. After several examinations regarding structure–activity relationships, a novel pH-sensitive CL, CL4H6, was identified as one of the best CLs for delivering siRNA into hepatocytes and was determined to be a 3rd generation CL (Fig. 6C). The lipid composition-optimized CL4H6-LNPs delivered a dose of 0.0025 mg siRNA/kg in a mouse FVII model, which was a 6- and 2-fold higher efficiency compared to 2nd generation YSK13-C3-LNPs and MC3-LNPs, respectively (Fig. 6D). Fluorescent microscopic observations in liver tissue revealed that AlexaFluor647-labeled siRNAs had escaped from endosomes and diffused into the cytosol (Fig. 6E). A quantitative PCR analysis revealed that 4.2% of the siRNAs that had accumulated in liver tissue was successfully loaded in RNA-induced silencing complex (RISC), which indicates a higher bioavailability of siRNAs compared to previous reports regarding MC3 and its biodegradable analogue, L319. Introducing exogenous mRNAs into target cells is a promising strategy for expressing therapeutic proteins of interest. For vaccinations, the introduction of antigen-encoding mRNAs leads to the expression of antigen proteins and the activation of antigen presenting cells, resulting in the activation of both innate and adaptive immunity in attempts to eliminate pathogens or cancer cells. Current progress in mRNA optimization (including cap, codon, UTRs, poly(A) tail, chemically modified nucleosides, purification) have substantially improved the translatability of the RNAs with reduced immunogenicity, resulted in realization of and successful development of the current COVID-19 mRNA vaccines. LNP technology was also naturally critical for this realization. However, the composition of the optimal formulation and the physicochemical properties of the LNPs for mRNA remain unclear. The design of experiment (DOE), an experimental design method that rationally allocates experiments by using mathematical statistics and reaches reliable conclusions with a considerably reduced number of experiments, is a useful methodology for determining the optimal formulation from a large number of manufacturing factors, including the choice of lipids, liposome composition, nitrogen per phosphate (N/P) ratio, and appropriate conditions for synthesis (e.g. buffer pH, flow rate ratio and total flow rate during microfluidic synthesis). However, the conventional DOE used for formulation optimization involves the analysis of only one response such as gene expression in a target tissue. Therefore, a detailed understanding of the relationship between a main response and physicochemical properties was very limited. Therefore, DOE with multiple responses were conducted to optimize liver-targeting mRNA-loaded LNPs and to identify the important properties (e.g. physicochemical properties) needed for gene expression. In addition to the factors examined by DOE (e.g. a type of lipid and lipid composition), it was found that LNP size and PEG-to-PL ratio were other key factors for liver-specific gene expression. The optimized mRNA-loaded CL4H6-LNPs delivered over 8000 ng/mL of human erythropoietin protein in serum at 6h after the intravenous administration of a 0.5mg mRNA/kg dose. The optimized mRNA-loaded CL4H6-LNPs also showed 2.5-fold higher nanocleavage expression in liver tissue compared to MC3-LNPs with no observable toxicity. Since the discovery of the CRISPR/Cas system, targeted genome editing through engineered nucleases has become a major focus of attention in both biological research and therapeutics. Intended genome editing can be achieved through several approaches, including gene disruption by non-homologous end joining and the insertion of a target sequence by homology-directed repair. Moreover, the rapid generation of re-engineered Cas nucleases, including high-fidelity Cas9, protospacer adjacent motif (PAM)-modified Cas9, base editor, transcription activator or repressor (CRISPRa/CRISPRi), a programmable epigenetic memory writer (CRISPRoff), a prime editor, and type I Cas system, has dramatically amplified application diversity and the significance of the CRISPR/Cas system for therapeutics. The introduction of CRISPR/Cas machinery as a ribonucleoprotein (RNP; a complex of Cas nuclease and guide RNA) is known to be preferable for both the efficiency of genome editing and for achieving a lower off-target effect. However, the technology for formulating RNP delivery has been not established yet. To overcome this limitation, RNP-loaded LNPs were synthesized by means of a clinically relevant manufacturing process using a micromixer-equipped microfluidic mixer, iLiNP (Fig. 7A). A detailed examination of manufacturing conditions revealed that both a higher pH (5.5 or over) and a higher flow rate ratio (7 or over) compared to standard conditions for the synthesis of nucleic acid-loaded LNPs were required to avoid aggregation and the loss of RNA cleavage activity of the RNPs. Severe aggregation and the loss of DNA cleavage activity of RNPs were observed under standard conditions used for the synthesis of nucleic acid-loaded LNPs. Furthermore, by using an iLiNP device with 3 inlets, the introduction of a buffer solution from a center inlet between two inlets used for RNP and lipid solutions resulted in the aggregation of RNPs at the junction to be completely inhibited by preventing the RNPs from coming into direct contact with high concentrations of ethanol (Fig. 7A). These optimizations in manufacturing parameters and the structure of the microfluidic device enabled us to synthesize RNP-loaded LNPs...
The optimization of *Streptococcus pyogenes* Cas9 (spCas9)-loaded LNPs was conducted through 2 cycles of DOE using a flowcytometric analysis of enhanced green fluorescent protein (EGFP) knockout (KO) in HeLa stably expressing EGFP (HeLa-GFP) cells. The optimized CL4H6-LNPs showed a IC$_{50}$ of approx. 0.2 nM RNP, which was a 10-to 100-fold lower concentration compared to previous reports regarding non-viral spCas9 RNP delivery systems. To expand the applicability of the optimized LNPs, *Acidaminococcus* BV3L6 Cas12a (Cpf1) RNP delivery was examined. The Cpf1 has a unique PAM sequence that is different from that of spCas9, exhibits robust genome editing activity in mammalian cells and potentially shows lower off-target effects compared to spCas9.117) The Cpf1 utilizes a shorter single crispr RNA (crRNA) compared to the guide RNA (approx. 100nt length) of spCas9, making the loading of this cargo in LNPs difficult due to the poor electrostatic interactions.118) Therefore, both the complexation of single strand oligonucleotide (ssON) with RNPs and the elongation of crRNA were examined in attempts to increase the negative charge density in the Cpf1 RNPs. Interestingly, while the original Cpf1 RNP-loaded LNPs failed to induce a significant EGFP KO (<10% at 2nM RNP), the elongation of crRNA or the complexation of ssON dramatically increased EGFP KO activity (Fig. 7B). A combination of the two approaches further increased the EGFP KO, providing an IC$_{50}$ less than 0.5 nM RNP (Fig. 7B). The total RNA length (crRNA + ssON) was well correlated with the KO activity (Fig. 7C), indicating the significance of the density of negative charges of RNPs on functional delivery. Although an increase in EGFP KO activity was also observed for spCas9 RNP, the impact of ssON complexation was much less than that for Cpf1 RNP due to the longer guide RNA for spCas9 RNP. To confirm that the optimized RNP-loaded LNPs have therapeutic potential, the inhibitory effect of HBV in HBV-infected cells was examined. HBV-infected HepG2-hNTCP-30 cells were consecutively treated with spCas9 RNP-loaded LNPs for 12d, resulting in 60 and 80% inhibition of HBV DNA and covalently closed circular DNA, respectively, a significantly higher inhibitory effect than the adeno-associated virus 2 (AAV2) vectors used for comparison (Fig. 7D). These data suggest that using RNP-loaded LNPs for gene editing would be a novel strategy for the treatment of chronic HBV infections. Optimization of RNP-loaded LNPs targeting liver tissue for gene KO and knock-in is currently an on-going effort.

![Image of Figure 7](https://example.com/figure7.png)
3. Application of LNPs to Extra-Hepatic Tissues and Cell Types

Macrophages are one of the most important contributors to homeostasis and various inflammatory diseases.119) Macrophages in different tissues play various roles in inflammatory diseases and are distinguished by different names, including peritoneal macrophages (PEMs), Kupffer cells, microglia, and tumor-associated macrophages (TAMs) in the peritoneal cavity, liver, brain, and tumor tissue, respectively.120–123) Manipulation of macrophages at the genetic level represents a promising approach for the treatment of a number of diseases in which macrophages are involved.

Gene silencing in PEMs was examined by focusing on the size and route of administration used for the YSK05-LNPs.124) The diameter of the YSK05-LNPs was modulated from 75 to 460 nm by changing the amount of PEG-lipid. Cellular uptake in PEMs (defined as F4/80+ cells in the peritoneal cavity) was clearly size-dependent and reached a maximum at a diameter of 340 nm after intravenous administration. An intravenous administration of siCD45-loaded YSK05-LNPs in mice at a dose of 2 mg siRNA/kg resulted in approx. 80% CD45 silencing at the protein level for particles with diameters of 200 and 340 nm, which was significantly higher than that for 75 nm (approx. 40% silencing). On the other hand, no significant difference in gene silencing activity in PEMs was observed after an intraperitoneal administration of the same LNPs in mice at a dose of 0.003 mg siRNA/kg, suggesting that impact of size on intravenous administration involves translocation from the blood circulation to the peritoneal cavity. The YSK05-LNPs showed an ED50 value of 6 µg siRNA/kg for CD45 gene silencing in PEMs after intraperitoneal administration. This single digit µg/kg range enabled us to easily analyze multiple genes simultaneously without any obvious adverse effect.

The tumor-microenvironment is known to contain large numbers of macrophages, which are referred to as TAMs and are mostly the pro-tumorous (M2) phenotype.125–128) Modifying the functions of TAMs or the manipulation of TAMs from M2 to the anti-tumorous M1 phenotype by siRNAs could be a novel immunotherapy for the treatment of cancer. To target TAMs, the CL4H6-LNPs were modified with distearoylglycerol PEG, which has longer scaffolds (C18:0), dissociates more slowly from the LNPs, and contributes to longer blood circulation.129) The CL4H6-LNPs accumulated in subcutaneously inoculated human renal cell carcinoma (OS-RC-2) tumor tissues. Flowcytometric analysis revealed that high levels of the CL4H6-LNPs were taken up by TAMs (defined as CD45+CD11b+CD4/80+ cells) compared to other cell populations, including neutrophils (defined as CD45+CD11b+CD4/80− cells), other leukocytes (defined as CD45+CD11b−CD4/80− cells), and non-leukocytes (defined as CD45− cells, which include tumor cells, tumor-associated fibroblasts, and endothelial cells). A significant (approx. 70%) gene silencing of CD45 in TAMs was obtained after the intravenous administrations of the CL4H6-LNPs. Motivated by these findings, we examined the manipulation of TAMs from M2 to M1 phenotype by the gene silencing of M2-related genes, signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF-1α), which are elevated in TAMs and involved in various pro-tumorous functions, including immunosuppression, the promotion of tumor growth, and angiogenesis.130–132) The repeated administration of the siSTAT3/siHIF1α-loaded CL4H6-LNPs in OS-RC-2 tumor-bearing mice induced a significant anti-tumor effect. An analysis of the gene expression in tumor tissues revealed the elevation of CD11b (a macrophage mark-
er), CD169 (an M1 macrophage marker), and tumor necrosis factor-α (TNF-α) and the suppression of CD31 (an endothelial cell marker) and transforming growth factor-β (TGF-β) in addition to target genes (STAT3 and HIF-1α). These findings suggest that the use of CL4H6-LNPs leads to the infiltration of M1 macrophages in the tumor microenvironment, the suppression of angiogenesis, and the release of immunosuppression. Importantly, these reactions were derived from gene silencing in TAMs but not in human tumor cells because the siRNAs utilized only target murine genes.

The delivery of siRNAs to dendritic cells (DCs) is a promising strategy for improving the efficacy of DC-based cancer immunotherapy. However, the efficient delivery of siRNAs to DCs is somewhat challenging.133–135 A fusogenic CL, YSK12-C4, was originally designed to overcome this hurdle.136 YSK12-C4 contains two linoleic acid-derived scaffolds that are attached to one carbon atom, which emphasizes the adoption of a cone shape and enhances fusogenicity (Fig. 8A). A hydrophilic tertiary hydroxy group that is present between a tertiary amine and the scaffolds contributes to the higher apparent pKₐ (approx. 8.0) (Fig. 8A), which leads to strong cationic properties in the cell culture medium and promotes cellular uptake in DCs. The optimized YSK12-C4-LNPs showed IC₅₀ values of 1.5nM siRNA and achieved up to 90% gene silencing at higher concentrations in mouse bone marrow-derived DCs (BMDCs), while a commercially available transfection reagent, Lipofectamine RNAiMAX, achieved less than 60% gene silencing at a maximum and showed an IC₅₀ of 25nM, which was much higher than that for YSK12-C4-LNPs (Fig. 8B). The efficient gene silencing was expected to enhance the efficacy of DC-based immunotherapy by targeting the suppressor of cytokine signaling 1 (SOCS1), which blocks the Janus kinase (JAK)–STAT signaling pathways.137 The siSOCS1-loaded YSK12-C4-LNPs achieved approx. 80% silencing of the SOCS1 gene, resulting in an enhanced production of TNF-α, including interleukin-6 (IL-6) in response to interferon (IFN)-γ stimulation. Immunization of the SOCS1-silenced DCs by the YSK12-C4-LNPs in mice bearing the EL4 murine lymphoma cell line expressing chicken ovalbumin (E.G7-OVA) resulted in a significant antitumor effect, while the SOCS1-silenced DCs by Lipofectamine RNAiMAX failed (Fig. 8C). These results clearly suggest that the YSK12-C4-LNPs are a promising technology for use in enhancing DC-based immunotherapy. The silencing of the indoleamine 2,3-dioxygenase 1 gene by the YSK12-C4-LNPs in BMDCs also led to an enhanced antitumor effect against E.G7-OVA tumors.138 Motivated by these results, the gene silencing activity of the YSK12-C4-LNPs in several hard-to-transfect human immune cell lines, including Jurkat, THP-1, KG-1, and NK92, was examined.139 Although substantial cytototoxicity was observed at higher concentrations in the cases of KG-1 and NK92 cells, the YSK12-C4-LNPs showed an efficient gene silencing activity of up to 80 to 90% (Fig. 8D). It was suggested that small-size and colloidal stability in the cell culture medium of the YSK12-C4-LNPs contributed to the enhanced cellular uptake followed by efficient gene silencing in these cells.

The stimulator of the interferon gene (STING) pathway recognizes cytosolic DNA and cyclic dinucleotides (CDNs) to induce type I IFN and inflammatory cytokine responses and appears to be essential for the innate sensing of tumor cells.149 STING agonists such as CDNs are expected to function as potent cancer adjuvants. However, the delivery of the CDNs into cytosol where the STING pathway is located is somewhat challenging due to hydrophilic nature of the CDNs.140 A CDN, cyclic di-GMP (c-di-GMP)-loaded YSK05-LNPs were developed for cancer immunotherapy.141 The optimized c-di-GMP-loaded YSK05-LNPs induced a significantly higher level of IFN-β production in a murine Raw264.7 macrophage cell line compared to Lipofectamine 2000 and DOTAP-based LNPs. The subcutaneous administration of the c-di-GMP-loaded YSK05-LNPs enhanced in vivo antigen-specific cytotoxic T cell (CTL) activities and showed preventive antitumor effects in major histocompatibility complex class I (MHC-I)-expressing E.G7-OVA-bearing mice, suggesting that the induction of MHC-I restricted antitumor immunity. The loss or down regulation of the MHC-I in tumors is a well-known mechanism for escaping from immunosurveillance by CTL, which is observed in malignant melanomas. In this situation, the activation of natural killer (NK) cells would be desirable for inducing an antitumor effect against malignant melanomas.142,143 The intravenous administration of c-di-GMP-loaded YSK05-LNPs resulted in the production of TNF-α and IFN-γ, which are secreted from activated NK cells.144 The activation of NK cells was confirmed by the observation of a significant increase in the population of NKG2D- and CD69-positive NK cells. Significant antitumor effects in a lung metastatic mouse model with a B16-F10 melanoma was observed after the intravenous administration of c-di-GMP-loaded YSK05-LNPs and was cancelled by the depletion of NK cells, thus confirming that the c-di-GMP-loaded YSK05-LNPs are capable of activating NK cells and inducing antitumor effects against tumors with the loss or downregulation of MHC-I. It was recently demonstrated that c-di-GMP-loaded YSK12-C4-LNPs overcome anti-programmed cell death 1 (PD-1) resistance and show a synergistic antitumor effect by activating NK cells followed by inducing the formation of PD ligand 1 on tumor cells in the same melanoma model.145

A technology for the treatment of brain disorders, including traumatic brain injury, Parkinson's disease, and Alzheimer's disease, is still an unmet medical need. Nucleic acid and gene therapy is a promising strategy for realizing efficacious therapeutics for the hard-to-treat brain disorders, as demonstrated by the clinically approved zolgensma (wild-type AAV9-based gene therapy) and spinraza (aso for exon inclusion) for the treatment of spinal muscular atrophy, intractable genetic diseases caused by the deletion of a responsible gene, the survival motor neuron 1.146 A disorder of the brain endothelium is known to be associated with the pathophysiology of brain disorders.147 Therefore, targeting brain endothelial cells (BECs) would be a useful approach for the treatment of brain disorders. The gene silencing activity of siRNA-loaded YSK05-LNPs in BECs was examined as a possible approach. YSK05-LNPs were modified with recombinant ApoE proteins as ligands against LDLR family-expressing BECs.148 An ApoE-dependent increase in both cellular uptake and gene silencing activity was clearly obtained. Motivated by the impact of ApoE modification on delivery efficiency, ApoE-modified YSK05-LNPs containing pDNA were intracerebroventricularly administered to mice for transgene expression in brain.149 A significant increase in transgene expression was observed after the ApoE modification in vivo. Immunostaining analysis
revealed transgene (mCherry) expression in neural stem cells and/or neural progenitor cells, suggesting usefulness of this system for the treatment of neurodegenerative diseases.

Targeting the lung endothelium is promising approach for the treatment of various diseases, including sepsis, pulmonary hypertension, and lung cancer.150 Previously, our laboratory coincidentally found that a pH-sensitive fusogenic GALA peptide, functions as a specific ligand for the lung, and, using it, we were able to successfully induce gene silencing of an endothelial cell marker, CD31, in lungs with an ED$_{50}$ of 0.21–0.4 mg siRNA/kg.151,152 Lung-targeting LNPs composed of YSK05 were recently finely optimized, and an ED$_{50}$ of 0.01 mg siRNA/kg was achieved, a value that was 20- to 40-fold lower than that for previous formulations.153 A clinically relevant dose (0.5 mg siRNA/kg) was sufficient to eradicate a metastatic lung cancer model without any signs of toxicity. Development of novel lung targeting formulations for mRNA delivery is currently an on-going project.

4. Elucidation of Structure–Activity Relationships

The particle diameter of LNPs is a critical factor that affects their biodistribution and infiltration into deep tissue. It is possible for small-sized nanoparticles to be translocated from the lung to lymph nodes where they then drain into the lymphatic system and penetrate deeply into stromal-rich tumor tissues.154–159 It is known that subcutaneously administered small-sized LNPs show an excellent transitivity to and distribution within the lymph nodes.159 Furthermore, the subcutaneous administration of small-sized siRNA-loaded LNPs modified with GalNac successfully reached hepatocytes and induced substantial gene silencing while a larger counterpart, resulting in poor gene silencing activity in hepatocytes.163 However, the detailed mechanism responsible for this poor delivery that is associated with downsizing remains unknown. Therefore, siRNA-loaded LNPs with different diameters, approx. 35 and approx. 65 nm, were synthesized by changing amount of PEG-lipid to 3% and 1%, respectively, to examine the impact of particle size (Fig. 9A). The 3%PEG-LNPs showed a 4-fold lower FVII gene silencing activity compared to the 1%PEG-LNPs. Fluorescent microscopic observations of intrahepatic siRNA distribution revealed that most of the siRNAs showed a punctate pattern in the case of the 3%PEG-LNPs, indicating failure of endosomal escape, while substantial amounts of siRNAs showed a diffused pattern in the case of 1%PEG-LNPs, indicating the successful cytosolic delivery of siRNAs. The quantification of siRNAs also permitted the amount of siRNAs delivered by 3%PEG-LNPs to be measured and the findings indicated that the amount was significantly lower than that delivered by 1%PEG-LNPs, suggesting the premature release of siRNAs from the 3%PEG-LNPs in the blood stream. An in vitro study confirmed that the siRNAs released from the 3%PEG-LNPs showed a lower gene silencing activity only in the presence of fetal bovine serum (FBS) but not in the absence of FBS (Fig. 9B), suggesting that serum components promote the destabilization and inactivation of small-sized LNPs. Measurement of the relative surface area using a TNS probe revealed that the actual relative surface area of 3%PEG-LNPs was 2.99-fold higher than that of the 1%PEG-LNPs (Fig. 9C), which was significantly higher than the theoretically calculated relative surface area (1.72-fold) (Fig. 9D), indicating that lower lipid packing on the interface may have occurred, possible due to...
packing stress derived from strain between the actual positive curvature on the interface of the LNPs and the spontaneous negative curvature of the fusogenic CLs (Fig. 9E). Measurement of the hydration level of the interface of the LNPs using laurdan supported the above finding (Fig. 9F). The interface of the small-sized LNPs can cause instability, thus facilitating the dissociation of lipid components and the premature release of siRNAs, and the adsorption of large quantities of serum proteins on the interface, resulting in the inhibition of fusogenesity, a process that was confirmed by a hemolysis assay and an siRNA release assay established by the Merck research group.163) Cholesterol is known to contribute to the stability of LNPs.166,167) The premature release of siRNAs from small-sized LNPs was successfully inhibited by increasing the molar ratio of cholesterol in the lipid composition. However, the gene silencing activity of the small-sized LNPs was still significantly lower than that of the larger counterparts, suggesting that reduced endosomal escape needs to be addressed if small-sized LNPs with efficient nucleic acid delivery characteristics are to be developed.

The apparent pKa value is another key factor that can affect the biodistribution and endosomal escape of LNPs. As described above, the optimal pKa range for hepatic siRNA delivery is known to be from 6.0 to 6.5.20) Therefore, the original pH-sensitive CLs, YSK05, YSK13-C3, and CL4H6, that can deliver nucleic acids into hepatocytes efficiently also show pKa values in the range from 6.35 to 6.45, which are within the desired optimal range.32,54,83,84,89) However, the impact of the apparent pKa on intrahepatic distribution has not yet been examined. The effect of the apparent pKa on the intrahepatic distribution of the LNPs was examined using the 2nd generation pH-sensitive CLs, YSK13 and YSK15, with varied apparent pKa values ranging from 5.70 to 7.2583) (Fig. 5A). The intrahepatic distribution of the LNPs clearly changed from hepatocytes to LSECs. The LNPs with lower pKa values were optimal from the viewpoint of hepatocyte-specificity. The LNPs with optimal pKa (approx. 6.4) values for gene silencing activity in hepatocytes naturally accumulated in hepatocytes but also were substantially co-localized with LSECs. The LNPs with pKa values of 6.8 specifically accumulated in LSECs. Gene silencing activity in LSECs was examined using siRNA targeting CD31, and a plot of CD31 expression in the liver versus the apparent pKa value of the LNPs clearly showed that gene silencing activity in LSECs was clearly pKa-dependent and became maximum at pKa values in excess of 7.0 (Fig. 10A). A similar experiment was conducted using a pH-sensitive CL library with different hydrophilic regions, and it was revealed that the optimal pKa range for gene silencing activity in LSECs was from 7.0 to 7.5 (Fig. 10B), which was just 1 unit higher than that in hepatocytes. Although the apparent pKa value of the LNPs can be controlled by the design of chemical structure of the CL as examples of the 2nd generation CLs and CL library,83,89) precise adjustment of apparent pKa value is somewhat challenging due to time-consuming and labor-intensive processes and is sometimes impossible due to limitations in the permissibility of chemical structure. Therefore, the manipulation of the apparent pKa value of the LNPs using a combination of two pH-sensitive CLs with different apparent pKa values would be an alternative strategy for precisely adjusting apparent pKa to the target values. LNPs composed of both YSK05 and YSK12-C4 (YSK05/12-LNPs) were developed in attempts to achieve new pKa values.168) Interestingly, a plot of pKa of the final LNP versus the YSK12-C4 ratio in total CLs indicated that contribution of YSK12-C4 was higher than that of YSK05 for the final pKa of the LNPs (Fig. 10C). For example, the actual final pKa of the LNPs composed of equal molar amount of YSK05 and YSK12-C4 was clearly higher than the pKa value that was simply calculated as an average of the apparent pKa value of each lipid (Fig. 10C). Similar results were obtained when mixtures of YSK13,
YSK13-C2 and YSK13-C4 lipids were used (Fig. 10D). Importantly, the contribution of the pH of LNPs composed of two CLs with different pKₐ values was superimposed on the theoretical curve for the new pKₐ but not on the averaged curve of original two pKₐ (Fig. 10E), strongly suggesting that the new pKₐ value was contributed by the mixing of two CLs.

Neutral zwitterionic lipids with a bulky phosphocholine, phosphatidylethanolamine (PC) and sphingomyelin (SM) are known to stabilize the L phase and inhibit the phase transition to an H II phase due to their cylindrical structure. As described above, a phase transition is essential for achieving membrane fusion-mediated endosomal escape. Therefore, a high amount of PCs or SMs in siRNA-loaded LNPs greatly inhibits endosomal escape followed by the cytosolic release of siRNAs. Indeed, the YSK05-LNPs showed robust gene silencing activity, but escape followed by the cytosolic release of siRNAs. Indeed, the YSK05-LNPs showed robust gene silencing activity, but only when phosphatidylethanolamines and not PCs were used as a helper lipid. Interestingly, siRNA-loaded LNPs composed of CL15H6, which has much longer scaffolds (C24 + O1) compared to the conventional linoleic acid-derived scaffolds (C18), and 50 mol% egg-derived SM showed excellent gene silencing in HeLa-dluc cells, while that with CL15A6, a counterpart with conventional scaffolds, failed. Gene silencing activity was also clearly observed when any PCs with C16 or C18 scaffolds were used but not when brain- and milk-derived SMs which contain longer scaffolds (C22 to C24) were used. It is known that lipids with different scaffold lengths have a tendency to be segregated from each other due to the higher Gibbs free energy for mixing. The inhibitory effect of PCs and SMs on the L-to-H II phase transition should be affected only when fusogenic CLs are miscible with the PCs and SMs during the phase transition. Taking the above into consideration, CLs with much longer scaffolds would be immiscible with typical PCs and SMs consisting of C16 to C18 scaffolds and would be resistant to the inhibitory effect of PCs and SMs on membrane fusion (Fig. 11A). CL4H6, which is made up of scaffolds with CL15H6, formed well-dispersed lipid suspensions composed of equal molar ratios of CL4H6, distearoyl PC (DSPC), and DSPS in an acidic buffer even at higher temperatures (50°C), and therefore, a ⁴¹P-NMR analysis failed to detect a phase transition because the anisotropic ⁴¹P spectra from the L or H II phases were averaged as the result of the rapid rotation of relatively small-sized dispersed particles (Fig. 11B, insertion). A lipid suspension consisting of CLs with conventional scaffolds, including YSK05, adopts a completely aggregated form at temperatures higher than T_H due to the highly hydrophobic nature of the H II phase. These findings lead to the hypothesis that PCs (L phase) are segregated from the H II structures formed by CH4H6-DSPS ion pairs and cover the H II structure, which leads to a dispersion of small vesicles that are well-suspended, even at high temperatures (Fig. 11A). A small-angle X-ray scattering (SAXS) analysis of the same lipid dispersion revealed the existence of a mixture of L and H II phases at higher temperature (Fig. 11B), supporting the hypothesis that CL4H6 is immiscible with PCs when in the H II structure. The immiscible property of the CLs would greatly contribute to membrane fusion-mediated endosomal escape because the main lipid component of endosomal membranes is PCs that can inhibit phase transition. This hypothesis is also supported by the fact that mRNA-loaded CL4H6-LNPs with high amounts (25 mol%) of DSPC showed consistent gene expression in liver tissue compared to a lower amount (5 mol%) in liver tissue.

5. Improving the Biosafety of LNPs

While the administration of nucleic acid-loaded LNPs sometimes causes toxicity, our knowledge regarding LNP-associated inflammatory toxicities remains limited and is somewhat controversial. Two reports demonstrated that a co-treatment of dexamethasone (Dex), a glucocorticoid receptor agonist, and a JAK 2 inhibitor with siRNA-loaded LNPs dramatically inhibited LNP-associated toxicities including elevated serum chemistry parameters and inflammatory cytokines. Another study reported on the controversial finding that a pro-inflammatory cytokine response was observed after the administration of LNPs even with pre-medication with Dex and other drugs. Differences in the components, characteristics, and biodistribution of LNPs, and in the animal model being used likely explain the difficulties associated with the interpretation of these findings. Elucidation of the mechanism regarding LNP-associated toxicity would contribute to the development of new strategies for improving the safety of LNPs.

To elucidate mechanism responsible for LNP-associated hepatotoxicity, elevated doses of siRNA-loaded YSK13-C3-LNPs were intravenously administered to mice. Interestingly, dose-dependent hepatotoxicity was observed with changes in the intrahepatic distribution of the LNPs from hepatocytes to LSECs (Figs. 12A, B). Hepatotoxicity was also observed at all dose range regardless of the siRNA-loading, indicating that the toxicity is derived from the LNP itself and appears

Fig. 11. Impact of Scaffold Length of CLs on Immiscibility with Neutral PLs

(A) Schematic illustration of the impact of scaffold length of CLs on the segregation of neutral PLs from H II structures of CL-anionic PL complexes. (B) SAXS analysis of CL4H6/DSPC/DSPS dispersion that the inserted isotropic ⁴¹P-NMR spectra were obtained. These figures are reproduced, in part, with permission from Elsevier. (Color figure can be accessed in the online version.)
to be associated with changes in intrahepatic distribution. In toxic conditions, adhesion molecules, including the vascular cell adhesion molecule-1, E-selectin, and intercellular adhesion molecule-1, are upregulated in liver tissue. A multiplex suspension assay revealed the production of neutrophil-related cytokines, including keratinocyte-derived cytokines and granulocyte colony stimulating factor, in addition to some inflammatory cytokines, the IL-6, IFN-\(\gamma\)-induced protein 10. Neutrophil depletion resulted in the significant suppression of LNP-associated toxicity, suggesting the involvement of neutrophilic inflammation on hepatotoxicity.

Fig. 12. Strategies for Improving the Safety of the LNPs

(A) Dose-dependency of intrahepatic distribution of the LNPs. The LNPs accumulated to LSECs at a higher dose. (B) Dose-dependency of the hepatotoxicity of the LNPs. The hepatotoxicity was correlated with the change in intrahepatic distribution. (C) Schematic illustration of the estimated mechanism of LNP-associated hepatotoxicity. The LNPs partially accumulate in LSECs, leading to the expression of adhesion molecules and cytokines followed by neutrophilic inflammation that aggravates hepatotox性和 systemic toxicity. (D) GalNAc modification significantly decreased the accumulation of the LNPs in LSECs. (E) Impact of surface modification of the LNPs on hepatotoxicity. GalNAc modification significantly decreased the LNP-associated hepatotoxicity. Further PEG modification completely suppressed the hepatotoxicity. (F) Schematic illustration of the development of the LLC-NPs. (G) Weight of each component. The amount of lipids for the LLC-NPs is significantly lower than that for conventional HL-NP formulations. The amount of protamine is much lower than that for lipids or siRNAs. (H) Estimated cleavage of the CL4H6 by endogenous lipases. (I) Biodegradation of the CL4H6 in both the liver and spleen. These figures are reproduced, in part, with permission from Elsevier. (Color figure can be accessed in the online version.)
cytokines including neutrophil-related cytokines followed by the induction of neutrophil inflammation (Fig. 12C). Based on this supposition, the LNPs were modified with GalNAc to improve hepatocyte-specific accumulation and to avoid the unintended accumulation to LSECs. Hepatocyte-specificity was substantially improved by the GalNAc modification (Fig. 12D). Surprisingly, LNP-associated hepatotoxicity was dramatically suppressed by GalNAc modification (Fig. 12F).

Further modification of GalNAc-LNPs with PEG to minimize the ApoE-mediated unintended accumulation to LSECs completely inhibited hepatotoxicity (Fig. 12E). Importantly, gene silencing activity in hepatocytes was fully maintained (ED$_{50}$ of 0.015 mg siRNA/kg in a mouse FVII model), even after surface modification. A single intravenous administration of the modified YSK13-C3-LNPs in mice persistently infected with HBV resulted in the suppression of HBV antigens by approx. 90% without any signs of toxicity.

It is known that high doses or high concentrations of CLs can cause undesired toxicity through multiple mechanisms, including the production of reactive oxygen species, the induction of apoptosis and pro-inflammatory cytokines. Therefore, the reduction of CLs for functional delivery would be a useful and straightforward strategy for improving safety. However, the reduction of CLs by reducing the molar ratio or reducing the total lipids against nucleic acids typically causes a dramatic decrease in delivery efficiency because the CLs are critical components for endosomal escape, which is a limiting step in nucleic acid delivery (Fig. 12F).

To address this dilemma, the negative charges of siRNAs were neutralized by protamines, cationic proteins, in an effort to reduce the consumption of CLs for siRNA-loading and to reduce the net dose of CLs. The siRNA/protamine core-loaded LNPs with low lipid/siRNA charge ratios are referred to as low lipid core-nanoparticles (LLC-NPs) (Fig. 12F). The LLC-NPs showed consistent gene silencing activity (IC$_{50}$ of approx. 1 nM siRNA) when conventional siRNA-loaded LNPs with high lipid/siRNA charge ratios (high lipid-nanoparticles; HL-NPs) were used in HeLa cells stably expressing dual-luciferase (HeLa-dluc). On the other hand, the gene silencing activity of the siRNA-loaded LNPs with low lipid/siRNA charge ratios (low lipid-nanoparticles; LL-NPs) were dramatically reduced (approx. 10% gene silencing at 30 nM siRNA). These data suggest that the neutralization of the negative charges of the siRNAs by protamines significantly restored the gene silencing activity of the LNPs with low lipid/siRNA ratios. A liposome fusion assay revealed that the neutralization restored fusogenic activity, indicating that the amount of CLs available for endosomal escape was increased by the reduced consumption of CLs for siRNA loading by protamines. Importantly, while the total amount of lipid was significantly decreased from 152.3 µg (HL-NPs) to 38.1 µg (LLC-NPs), only 7.9 µg of protamines were added against 10 µg siRNA (Fig. 12G).

Moreover, protamines are biodegradable and were eliminated from liver tissue with a half time (t$_{1/2}$) of less than 4h. The restoration of gene silencing activity in liver tissue and the reduced hepatotoxicity for LLC-NPs was also confirmed. Furthermore, a similar strategy was also successful in the case of human immune cells.

Because CLs cause toxicity, the addition of biodegradability into CLs would be also promising strategy in addition to the above strategy for reducing the amount of CLs used. The 3rd generation CL, CL4H6, contains biodegradable ester bonds in its hydrophobic scaffolds. Cleavage of the ester bonds produces a water-soluble alkanol amine and two oleic acids (Fig. 12H). The amount of CL4H6 in both the liver and spleen tissues rapidly dropped with time (Fig. 12I). The expected alkanol amine was detected at 30 min in both tissues, indicating that the CL4H6 was rapidly hydrolyzed after the delivery of siRNAs, as expected. The incubation of CL4H6-LNPs in 90% mouse plasma revealed that the extent of degradation of CL4H6 was negligible for periods of up to 2h, suggesting that CL4H6, when contained within the LNPs is protected from enzymatic degradation and is rapidly degraded once the integrity of the LNPs is lost by membrane fusion with endosomal membranes. A single dose toxicity test revealed that the CL4H6-LNPs were tolerated for at least up to levels of 5 mg siRNA/kg while the undegradable MC3-LNPs showed a dose-dependent elevation in both alanine transaminase and aspartate transaminase and a decrease in body weight. Furthermore, repeated dose toxicity also confirmed that the CL4H6-LNPs were well tolerated.

These strategies for improving the safety of LNPs, including maximizing hepatocyte-specificity, reduction of the CL dose, and the addition of biodegradability to CLs, are not exclusive and could be combined with each other. It is expected that these findings will contribute to the further development of LNPs with excellent safety profiles.

6. Perspectives

LNPs technology has been enjoyed dramatic progress in the last 10 years, which in part, was driven by a breakthrough of development of potential CLs. However, the limitations that yet need to be addressed to realize ideal drug delivery systems and to overcome widespread refractory diseases still remain. There is still much room for the development of the efficient extra-hepatic targeting of therapeutically important tissues, including heart, muscles, and brain, and cell types, including specific subpopulations of immune cells. In addition, payloads that can be loaded and delivered by LNPs are still limited. Developing general loading/delivery strategies for various classes of payloads (e.g. proteins, peptides, and others) remains an important issue. It is also important to understand the fundamental reasons for in vivo fate of the LNPs (e.g. biodistribution, stability, endosomal escape, toxicity, and so on) at the molecular and particle levels. Therefore, the importance of related technologies such as synthetic devices (e.g. sophisticated and flexible microfluidic devices) and analytical methodologies (e.g. NMR, SAXS, small-angle neutron scattering, thermal analyses, transmission electron microscopy, robotics, machine learning and so on) promise to become more and more important.

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