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REVIEW ARTICLE

Structure and Function of Cationic and Ionizable Lipids for Nucleic Acid Delivery

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Abstract

Hereditary genetic diseases, cancer, and infectious diseases are affecting global health and become major health issues, but the treatment development remains challenging. Gene therapies using DNA plasmid, RNAi, miRNA, mRNA, and gene editing hold great promise. Lipid nanoparticle (LNP) delivery technology has been a revolutionary development, which has been granted for clinical applications, including mRNA vaccines against SARS-CoV-2 infections. Due to the success of LNP systems, understanding the structure, formulation, and function relationship of the lipid components in LNP systems is crucial for design more effective LNP. Here, we highlight the key considerations for developing an LNP system. The evolution of structure and function of lipids as well as their LNP formulation from the early-stage simple formulations to multi-components LNP and multifunctional ionizable lipids have been discussed. The flexibility and platform nature of LNP enable efficient intracellular delivery of a variety of therapeutic nucleic acids and provide many novel treatment options for the diseases that are previously untreatable.

Keywords cationic lipid · ionizable lipid · lipid nucleic acid nanoparticles · nucleic acid delivery · structural effect

Introduction

Gene therapy corrects malfunctional genes and has brought hope to treat the diseases that are untreatable with conventional approaches [1]. Effective gene therapy requires correction of the mutated genes, replacement of the malfunctional genes with normal genes, or regulation of abnormal gene expression [1]. However, the limiting step for clinical development and application of gene therapy has been the safe and efficient delivery of genetic materials into target cells [2]. Despite of various challenges, significant progress has been made in the development of delivery systems for therapeutic genetic materials [3, 4]. Non-viral gene delivery platforms, which have demonstrated advantages of low immunogenicity, unlimited payload capacity, cost-friendly manufacturing, flexibility, and multi-dosing capability, have received substantial attention [5]. A variety of materials have been used for non-viral gene delivery, including lipid-based nanoparticles, cationic polymers, cell penetrating peptides, and inorganic nanoparticles [6–12].

Lipid nucleic acid nanoparticles (LNP) have emerged as the most promising delivery systems for clinical applications among the various non-viral delivery systems. Various lipids have been reported for intracellular delivery of nucleic acids in the past decades. The lipid structures play the most important role for safe and efficient intracellular delivery of nucleic acids. One key consideration in the design of lipids for nucleic acids delivery is to overcome endosomal barriers for efficient intracellular delivery. The pH-sensitive protonatable or ionizable lipids have emerged as the most promising class of lipids for efficient intracellular nucleic acid delivery. These lipids are neutral at physiological pH for safe systemic nucleic acid delivery. The protonation or ionization of the lipids in acidic endosome presents the lipids with amphiphilicity, which destabilizes endosome membrane for endosomal escape [13, 14]. The structures of the lipids can be fine-tuned to achieve safe and efficient intracellular delivery of nucleic acids.

LNP has been successfully used in the development of gene therapy using small interfering RNA (siRNA) for clinical applications [15] and mRNA vaccine to combat

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SARS-Cov-2 (Covid-19) [16]. Lipids with various structures have been reported in the literature to develop LNP for nucleic acids delivery. In this review, the evolution of lipid structures and functions as well as LNP development, especially the optimization of the essential lipid structure and formulations of LNP, has been discussed with a focus on the relationship of the structure, function, and formulation of cationic and ionizable lipids in LNP for nucleic acid delivery.

The Evolution of LNP for Gene Delivery

Liposomes

The development of LNP for nucleic acid delivery can be dated back to 1970s [17], when liposomes were explored for drug delivery, including enzyme replacement therapy, insulin delivery, cancer chemotherapy [18–20]. One subtype of the liposomes, large unilamellar vesicles (LUV), became the first generation of LNP technology for drug delivery [21]. An early attempt of using liposomes for intracellular DNA delivery showed reduced interaction of DNA containing liposomes with cells caused by those without DNA [22], suggesting that the DNA encapsulation efficiency in liposomes might play a role for gene transfection. In early 1980s, liposomes loaded with DNA were demonstrated with the ability to transfect both eukaryotic and prokaryotic cells [23, 24].

Continuous efforts have been made to optimize DNA encapsulation in formulating liposome based LNP to improve their physicochemical properties and gene transfection efficiency. Reverse-phase evaporation (REV), Ca^{2+}-EDTA chelation, and detergent dialysis were some of the methods tested for DNA encapsulation in liposomes in 1980s. In the REV process, an emulsion containing DNA, lipid, a nonpolar solvent and a buffered aqueous solution was first prepared [25]. The nonpolar solvent was then removed under partial vacuum to form DNA encapsulated liposomes. Spermine or lysozyme was used in some cases to protect DNA from degradation caused by vortex or sonication and to improve the encapsulation efficiency of REV [26, 27]. Ca^{2+}-EDTA could facilitate the loading of DNA in liposomes and was used to improve DNA encapsulation of liposome LNPs [28]. In detergent dialysis, a solution containing DNA, phospholipid, detergent, and aqueous buffer was dialyzed against an excess of buffer to remove the detergent. When the concentration of detergent is lower than critical micelle concentration (CMC), liposomes were formed spontaneously with DNA in the cavity [29]. These methods demonstrated promise in DNA encapsulation and LNP formulations. However, they were greatly limited by low encapsulation efficiency, narrow selection of phospholipids, and uncontrolled sizes [30, 31].

Cationic lipids were later introduced for efficient DNA encapsulation in liposomes with improved transfection efficiency. Quaternary ammonium lipids such as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) were used as the cationic lipids with the ability to mediate efficient intracellular transfection [32]. Cationic liposomes can condense and encapsulate DNA or other genetic materials in LNP formulations for intracellular delivery. Cationic liposome and DNA complexes have affinity to the negatively charged cell membrane and facilitate intracellular delivery of genetic materials.

Cationic Lipid/DNA Complex

Cationic lipids are amphiphilic molecules, which consist of a hydrophilic and a hydrophobic region connected by a linker structure [33]. DOTMA and DOTAP (1,2-dioleoyl-3-trimethylammonium propane) are two most commonly investigated cationic lipids for transfection (Fig. 1). DOTMA directly interacts with plasmid DNA to form lipid-DNA complexes with 100% entrapment [32]. DOTMA facilitates fusion of the complexes with the membrane of cultured cells, resulting in intracellular uptake and expression of the DNA [32]. In order to reduce the cytotoxicity of DOTMA, a series of cationic lipids were synthesized and investigated [34]. DOTAP containing degradable ester bonds between the cationic head group and hydrophobic lipids showed higher transfection efficiency and reduced toxicity compared to DOTMA, when formulated with DOPE (dioleoylphosphatidylethanolamine) at a 1:1 ratio [34, 35].

DOTMA and DOTAP differ only in the linkage bonds and share similar *in vitro* gene transfection efficiency in different cells. However, their efficiency *in vivo* was significantly different at the gene expression level in the lung after intravenous administration of DNA/DOTMA or DOTAP complexes, where DNA/DOTMA with stable ether linkage demonstrated better efficiency than DOTAP with the hydrolyzable ester linkage [36]. The structures of DOTMA

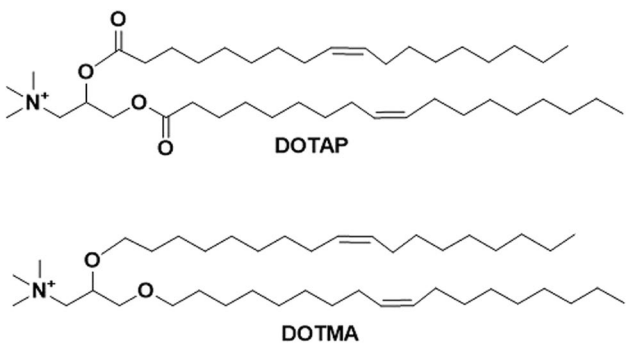


Fig. 1 Chemical structures of DOTAP and DOTMA, which have different linkage bonds (two ester bonds in DOTAP and two ether bonds in DOTMA).

and DOTAP were further altered in order to optimize the transfection efficiency of the cationic lipid and DNA complexes. Ren *et al*. synthesized a small library of cationic lipids to demonstrate the impact of each component of the structure of DOTMA and DOTAP on the transfection efficiency after intravenous administration (Fig. 2) [37]. A decrease in the efficiency in the lung was observed with an increase in the number of carbons between the cationic head group and the branch point of the lipid tails of DOTMA (DOTMA > 1a > 1b) [37]. Cationic lipids 1c and 1d showed similar transfection efficiency as DOTMA, indicating the branching position of the aliphatic chain with respect to the polar head group plays a role in transfection activity [37]. However, such phenomenon was not observed for the DOTAP analogues, which indicated that those effects might be exclusive for cationic lipids with two stable ether bonds [37]. Cationic lipids 2c, 2d with two different acyl chains showed a lower transfection activity than 2b, which has two identical unsaturated lipid chains. Cationic lipids 3b, 3c with different aliphatic lengths and ether/ester linkage, showed lower transfection than 3a, which has two identical hydrocarbon chains. These results demonstrated the importance of the composition of two identical unsaturated lipid chains for efficient transfection [37]. The variation of ester and ether linkages only showed difference under different transfection conditions, indicating other factors such as formulation and transfection conditions may be responsible for transfection efficiency of DOTMA, DOTAP, and their derivatives based DNA complexes *in vivo*.

Helper Lipids for Cationic LNP of DNA

Since both DOTMA and DOTAP are cationic lipids containing a head group with a permanent positive charge [33], they don't respond to pH changes during subcellular trafficking in endosomes. Their gene transfection efficiency could be enhanced by adding helper lipids, such as DOPE and other helper lipids (Fig. 3). DOPE has an amino head group, phosphoethanolamine, and two unsaturated oleoyl lipid chains, which have kinked structure and provide a cone geometry that is favorable for the non-bilayer hexagonal (H_{II}) phase, which is believed crucial for membrane fusion and bilayer disruption during endosomal escape [38–40]. Formulating LNP of plasmid DNA, DOPE and DOTMA or DOTAP demonstrated enhanced efficiency compared with pDNA/DOTMA or pDNA/DOTAP complexes. The formulation processes involve in organic solvents such as chloroform and methanol, in which the lipids are dissolved. Lipid/DNA nanoparticles are formed after a process similar to reverse-phase evaporation [41]. Currently, ethanol is used to dissolve the lipids and to mix with an acidic aqueous solution of genetic materials using a microfluidic system [42, 43]. The cationic lipids and DOPE are often used at the molar ratio of 1:1 in the formulation of LNP [44]. Cholesterol has also been used as a helper lipid in DOTMA or DOTAP based LNP formulations for intravenous administration. It was demonstrated that the presence of at least 25 molar percent of cholesterol in LNP could significantly increase the stability and the retention within the circulation [45]. Massing *et al.* developed a small library of cationic lipids with various lipid chain lengths based on DOTAP (Fig. 4) [41, 46]. All the cationic lipids were formulated with DOPE (1:1 ratio) and cholesterol (2:1 ratio). The presence of DOPE or cholesterol could reduce the minimum temperature required for formulation of temporarily stable liposome formulations containing DOTAP and its analogs (Table I). The presence of DOPE and cholesterol could promote the transition of the LNP into the H_{II} phase. Consequently, the LNP formulated with either DOPE and/or cholesterol could result in enhanced transfection efficiency due to their better performance in the membrane fusion and endosomal escape. No apparent efficacy was observed when cationic lipids directly formed lipid complexes with DNA [41, 46].

The structure of cationic lipids was also modified using fluorinated lipid in order to enhance the efficiency of LNP formulations with helper lipids. For example, Vierling *et al.* synthesized a fluorinated version of DOTMA with one or both of the lipid chains fluorinated [47]. The fluorinated cationic lipids could condense DNA, with or without DOPE, to form fluorinated LNP, which demonstrated little cytotoxicity and high efficiency transfecting lung epithelial cells [47].

A homologous series of dioleyl (Cl8) compounds containing increasing hydroxyalkyl chain lengths on the quaternary amine were synthesized (Fig. 5A), formulated with 50 mol % DOPE [44]. The lipids showed structure-dependent transfection efficacy following an order of (DORIE) ethyl > (DORIE-HP) propyl > (DORIE-HB) butyl > (DORIE-HPe) pentyl > DOTMA [44]. These findings suggest an important role for the hydroxyl moiety in the activity of cationic lipid compounds, where the hydroxyl group may affect the interaction of the lipids with DNA or improve the interaction of the cationic LNP with cellular membranes leading to greater activity [44]. Based on the hydroxylalkyl quaternary amino head group structure, a series of cationic lipids of a hydroxyethyl quaternary ammonium with various alkyl chains were synthesized (Fig. 5B), and formulated with 50 mol % DOPE and plasmid DNA. The resulting LNP showed transfection efficacy in an order of dimyristyl (DMRIE) (di-Cl4) > dioleoyl (DORI) (di-Cl8, unsaturated) > dipalmityl (DPRIE) (di-Cl6) > disteryl (DSRIE) (di-C18). These results demonstrated shorter tails of the cationic lipid and unsaturated double bonds could facilitate better efficiency [44].

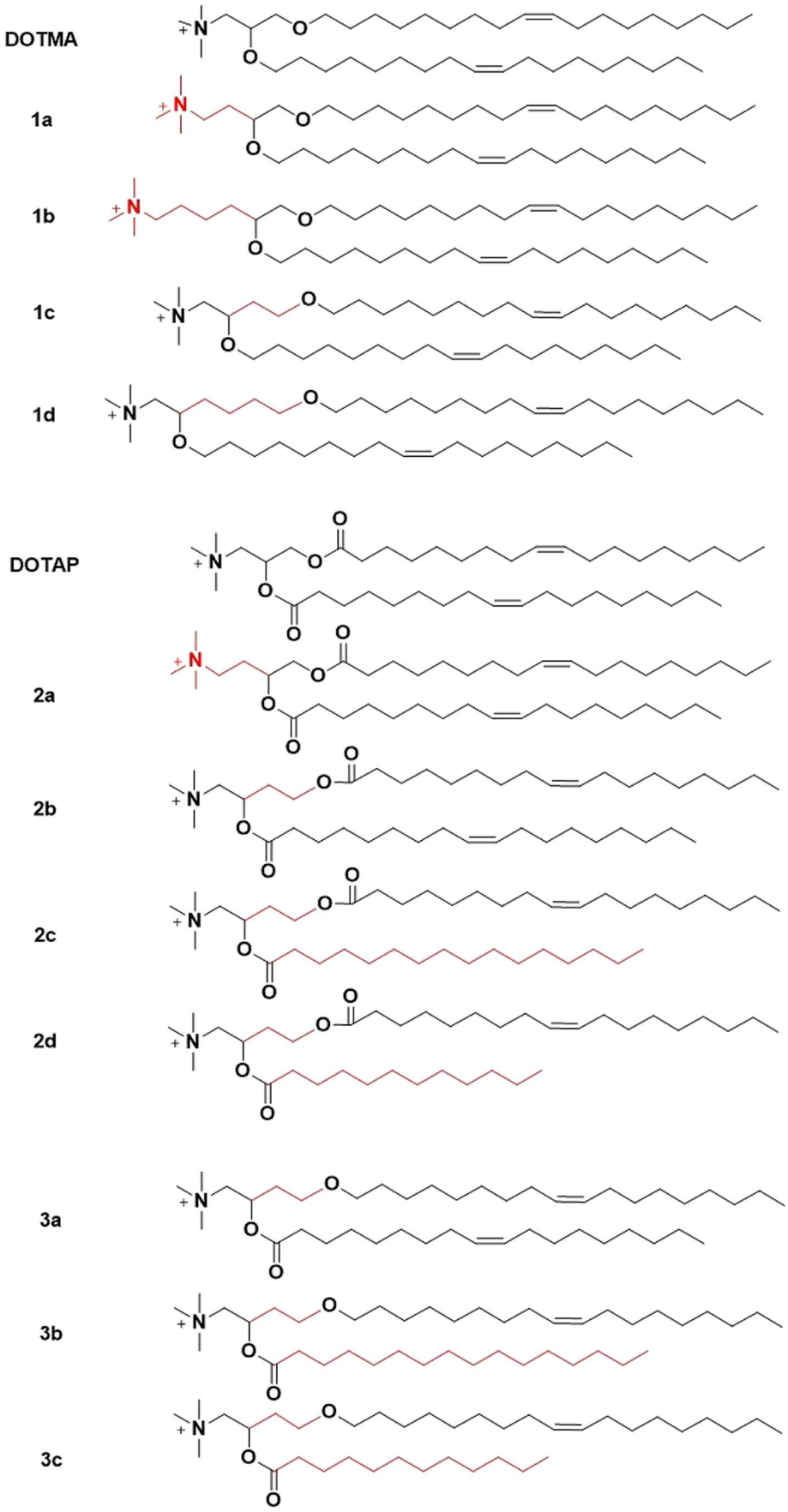


Fig. 2 Structures of the lipid analogues of DOTMA and DOTAP.

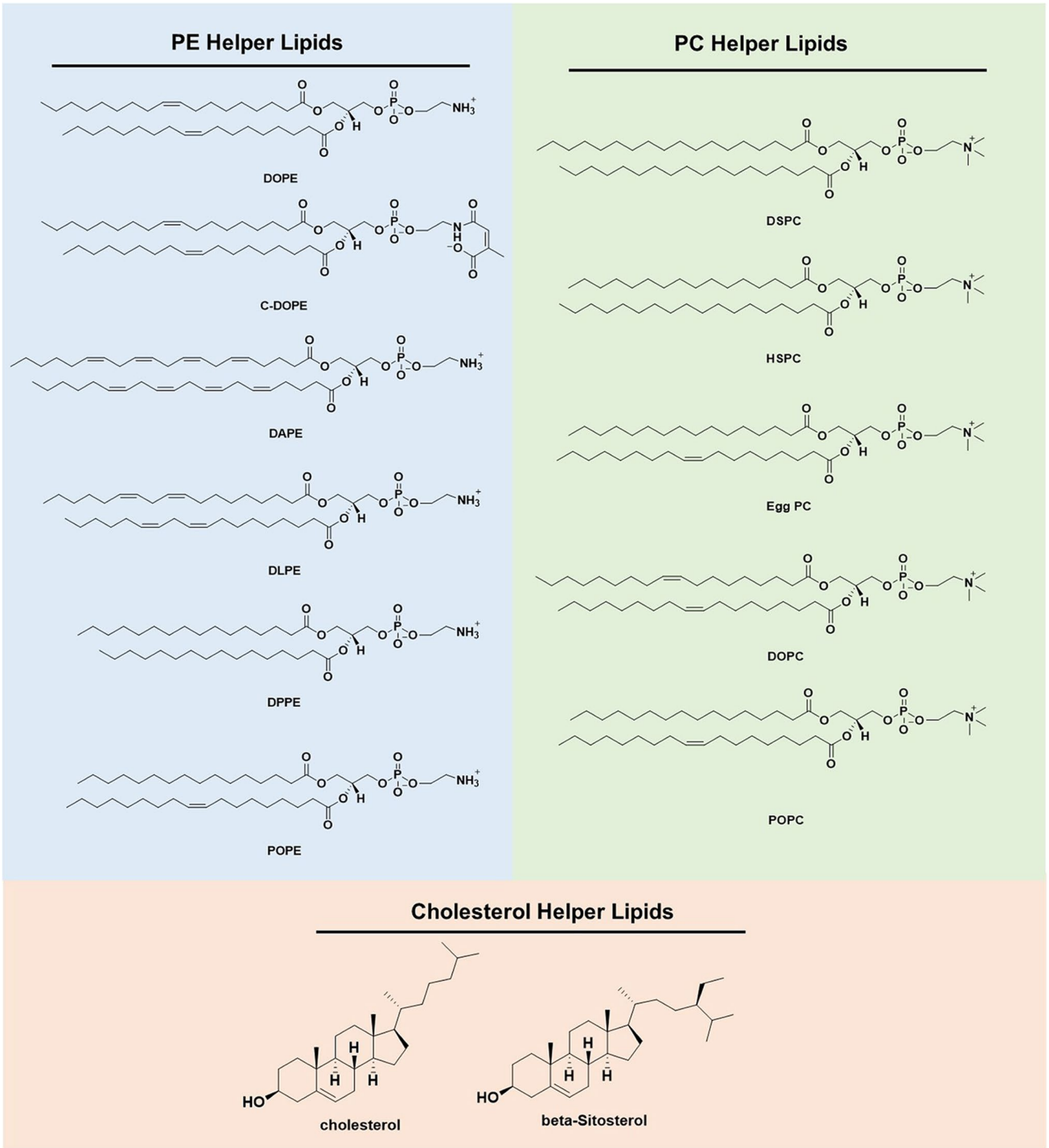


Fig. 3 Structures of commonly used phosphatidylethanolamine (PE), phosphatidylcholine (PC) and cholesterol helper lipids.

Stable Nucleic Acid Lipid Nanoparticles (SNALP)

Stabilized Plasmid-lipid Particles (SPLP)

Modification of cationic lipid plasmid nanoparticles with PEG was shown to improve their stability. A stabilized plasmid-lipid particle (SPLP) formulation was established by incorporating poly(ethylene glycol)-ceramides (PEG-Cer, PEG_{2000} linked to ceramides) with cationic lipid dioleoyldimethylammonium chloride (DODAC), palmitoyloleoylphosphatidylcholine (POPC), or dioleoylphosphatidylethanolamine (DOPE) (Fig. 6) [48]. SPLP was formulated at a DOPE: DODAC: PEG-Cer molar ratio of 84:6:10. The plasmid DNA was first incubated with DODAC in 0.2 M

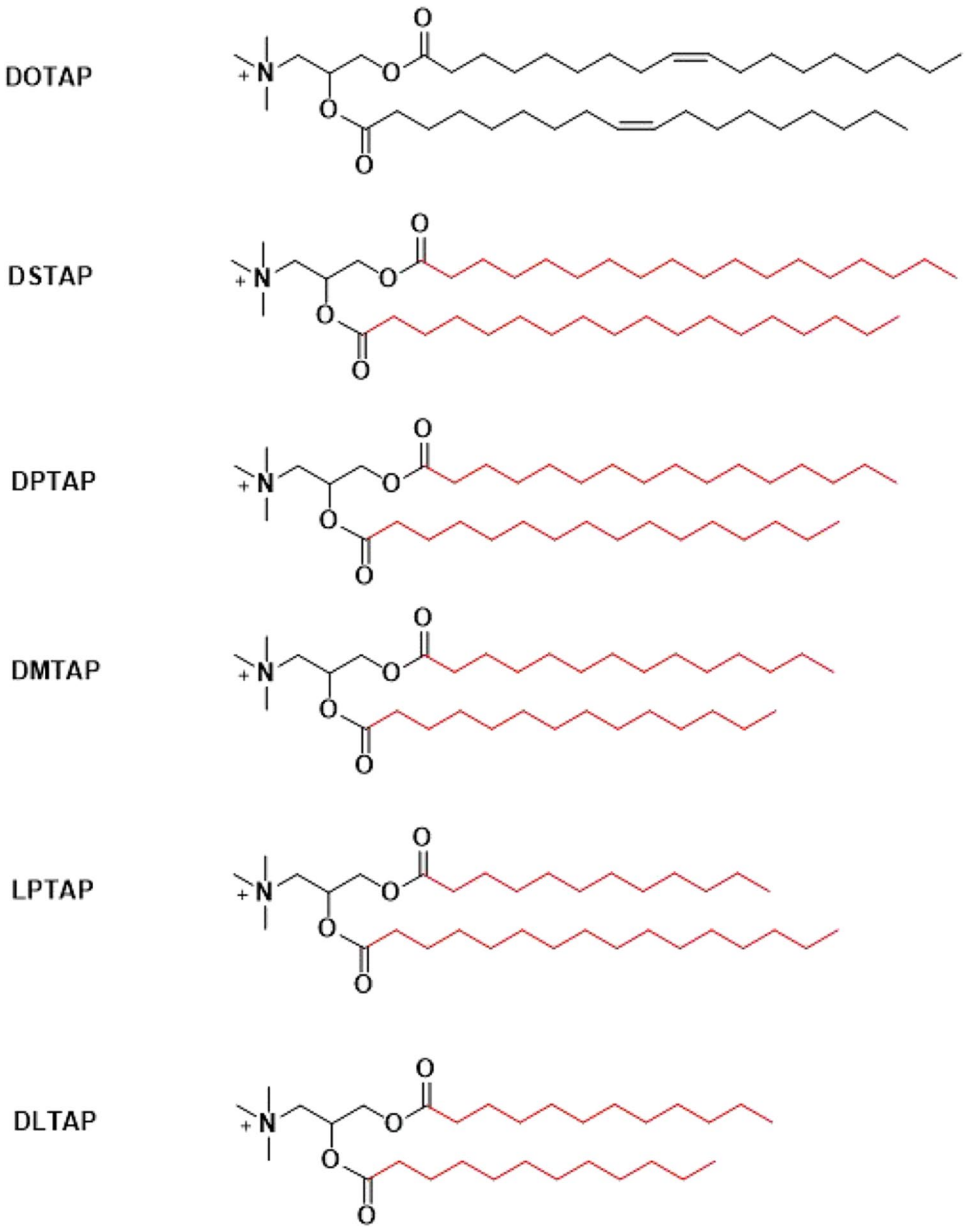


Fig. 4 Chemical structures of a lipid library based on DOTAP with various lipid chain lengths and combination.

Table I Minimum Temperature Required for Formation of Temporarily Stable Liposomes Containing DOTAP or DOTAP Analogs

Lipid	Phase transition temperature (T_c)	Without helper lipid	With Dope (1:1)
DOTAP	< 5°C	RT	RT
DSTAP	62.9°C	55°C	50°C
DPTAP	52.8°C	50°C	35°C
DMTAP	39.1°C	40°C	RT
LPTAP	43.1°C	35°C	RT
DLTAP	24.9°C	25°C	RT

octylglucoside, NaCl (5 mM) HEPES buffer (pH 7.4) and then the plasmid-DODAC mixture was added to DOPE and PEG-CerC_{14} or PEG-CerC_{20} dissolved in 0.2 M octylglucoside, NaCl (5 mM) HEPES buffer (pH 7.4) [48]. SPLP consisted of a unilamellar lipid bi-layer encapsulating a single copy of plasmid DNA with smaller size and better circulation stability. SPLP had an average size of 70 nm, with maximum entrapment observed at DODAC contents of 5 to 10 mol%. Helper lipid POPC demonstrated lower transfection efficiency than DOPE, which had better fusogenic properties. The *in vitro* transfection efficiency of this SPLP was also affected by the length of the acyl chain

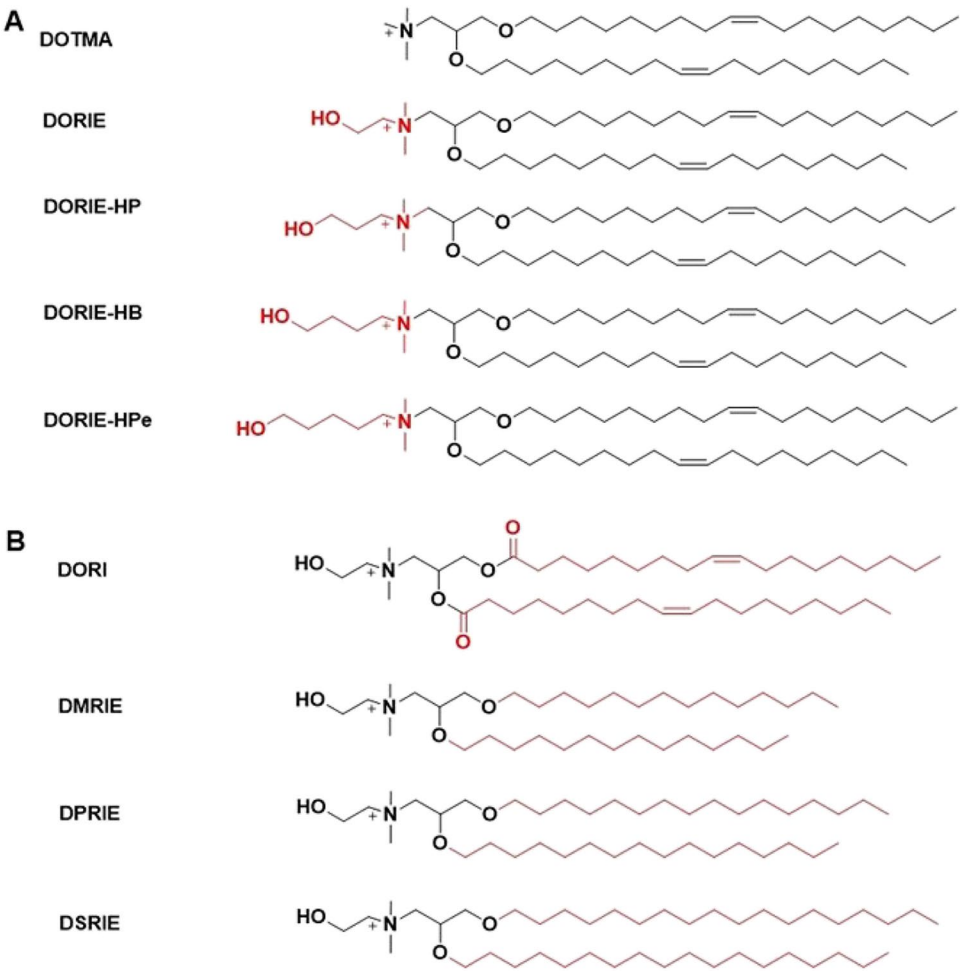


Fig. 5 Structures of a lipid library containing hydroxyalkyl chain lengths on the quaternary amine head group (**A**) and different alkyl chains (**B**).

anchor of the PEG lipid, where shorter acyl chain lengths resulted in better ability of dissociation from the SPLP surface, thereby a better *in vitro* transfection efficiency [48]. A following study characterized the effects of the cationic lipid and PEG-Cer species on SPLP formation and *in vitro* transfection properties [49]. A series of cationic lipids and poly(ethylene glycol)-ceramides (PEG-Cer) were tested for SPLP formulation (Fig. 6). The *in vitro* transfection levels of SPLP were affected by the lipids following the order of DODMA-AN > DOTMA > DODAC > DSDAC > DC-CHOL. PEG molecular weight had modest effect on the transfection efficiency, which demonstrated slight increase in transfection efficiency with lower molecular weight PEG. It appears that the length of the acyl chain had the most dramatic effects, where PEG-Cer with short lipid anchor showed substantially better transfection [49]. SPLP composed of DOPE/DODAC/PEG-CerC20 (83:7:10; mol: mol: mol) were intravenously administered in mice and demonstrated a long circulation life, no evidence of systemic toxicities and significant levels of reporter gene expression were observed [50]. Taking this formulation strategy, lipid 1,2-dioleyloxy-3-dimethylaminopropane (DODMA) and 1,2-dioleoyl-3-dimethylaminopropane (DODAP) (Fig. 7) with ionizable head groups were also used to formulate SPLP using similar methods and formulation composition, and demonstrate excellent *in vivo* efficiency [51–55].

Stabilized Antisense-Lipid Particles (SALP)

PEGylation was also applied to develop stable lipid antisense nanoparticles or stabilized antisense-lipid particles (SALP) [56]. SALP was designed specifically for intravenous administration, which requires an ionizable lipid with pK_a between pH 5.0 and 6.5, a PEGylated lipid, and neutral helper lipids [56]. The encapsulation and particle formulation were achieved through a spontaneous self-assembly process in an aqueous/ethanol solution with pH below the pK_a of the ionizable lipid [56]. The protonated lipid complexed with an oligonucleotide by electrostatic interactions, and PEGylated lipid was used to reduce the particle aggregation. The helper lipids such as (phosphatidylcholine) PC and cholesterol assisted stable bilayer formation. SALP had diameters ranging from 150 to 200 nm. [56] An SALP with

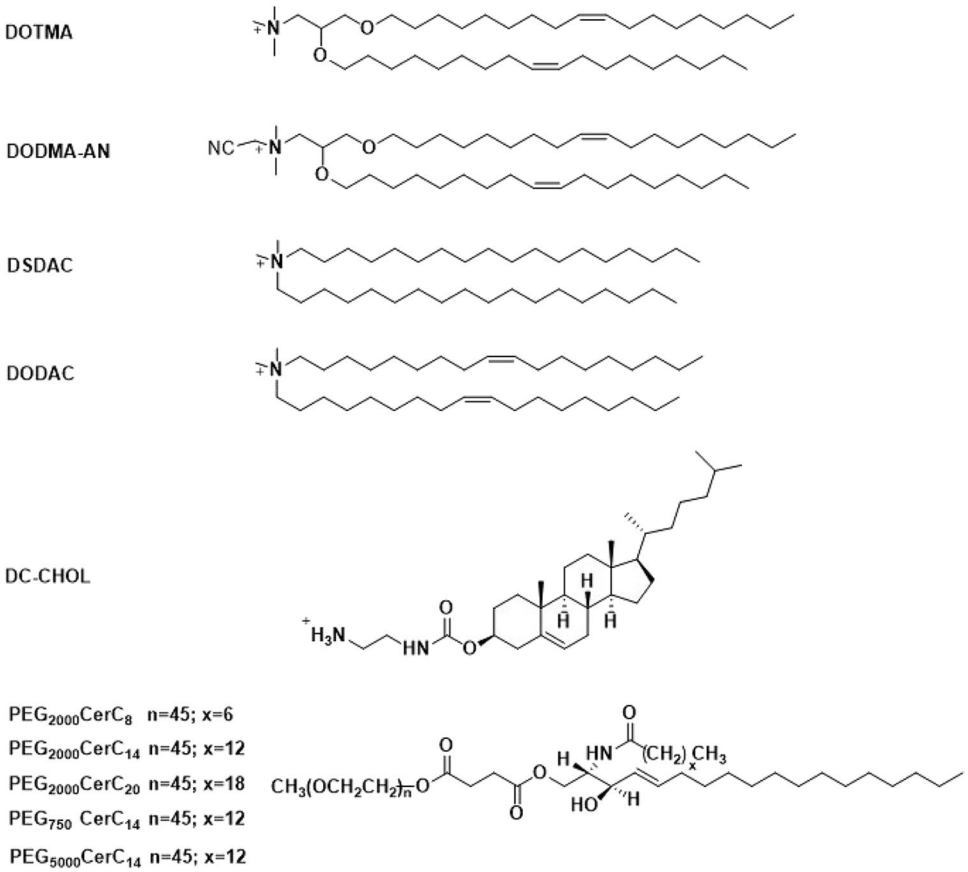


Fig. 6 Structures of the cationic lipids and poly(ethylene glycol)-ceramides (PEG-Cer) for formulation of stabilized plasmid-lipid particles.

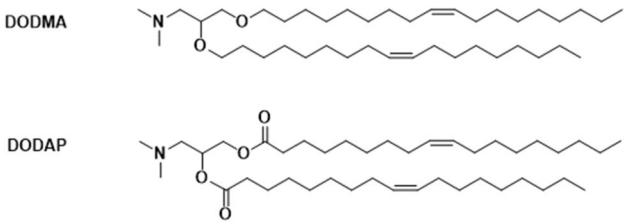


Fig. 7 Chemical structures of 1,2-dioleyloxy-3-dimethylaminopropane (DODMA) and 1,2-dioleoyl-3-dimethylaminopropane (DODAP) lipids.

a composition of DODAP/DSPC/Cholesterol/PEG-C14 (25/20/45/10 mol%) demonstrated efficacy in the liver after systemic delivery [57], as well as other SALP nanoparticle formulations [58, 59].

Stable Nucleic Acid Lipid Nanoparticles (SNALP)

Stable nucleic acid lipid nanoparticles (SNALP) were established specifically for encapsulating siRNA into liposomes with a lipid composition of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, 3-N-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), and 1,2-dilinoleyloxy-3-(N,N-dimethyl)aminopropane (DLinDMA) (20:48:2:30 mol%), Fig. 8 [60]. SNALP was effective at mediating RNAi *in vitro* and *in vivo*, and effective for inhibiting viral replication in a murine model of hepatitis B and silencing gene expression in non-human primates [61–65].

DlinDMA is a linolyl analogue of DODMA [66]. It was shown that the extra double bond in the lipid tails could increase propensity to form the non-bi-layer phase, thereby could increase the transfection efficiency of the LNP [67]. A series of DODMA analogues of 0, 1, 2 or 3 double bonds in the alkyl chains were synthesized and investigated in SNALP, Fig. 9 [66]. The effect of addition of double bonds on bi-layer transition temperature was studied using [31] P-NMR [66]. SNALP containing the saturated lipid DSDMA showed no sign of adopting the H_{II} phase, even at relatively high temperatures. However, DODMA (1 double bond per alkyl chain) containing LNP exhibited a much lower phase transition temperature for H_{II} phase transition. The presence of a 2nd double bond (DLinDMA) further reduced the phase transition temperature, while incorporation of a 3rd double bond (DLenDMA) has little additional effect [66]. Another study also demonstrated similar results, where lipid containing two double bonds in alkyl chains (DLinDMA) to be optimal [68].

The ionizable lipid DLinDMA was further modified by varying the linker structure between the head group and lipid

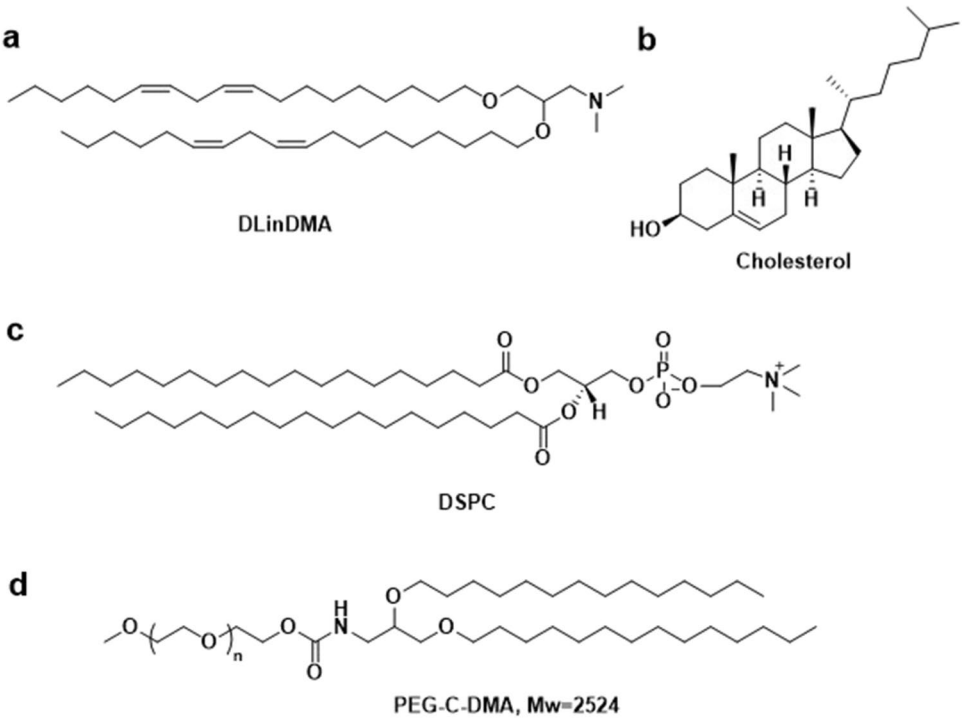


Fig. 8 SNALP Lipids. (a)1,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane (DLinDMA), (b)cholesterol (Chol), (c)1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), (d)3-N-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA).

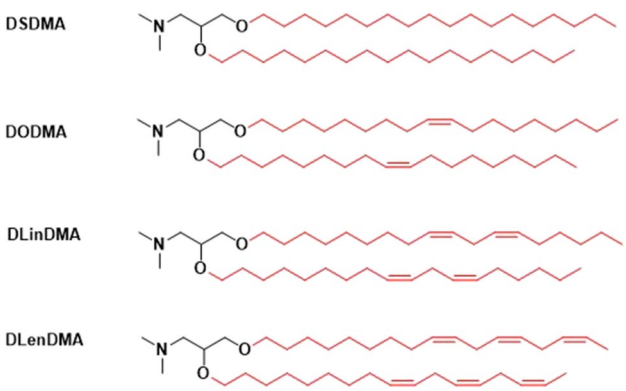


Fig. 9 Structures of ionizable lipids DODMA analogues for SNALP.

tails to introduce degradability, Fig. 10 [69]. Each of the ionizable lipids, distearoylphosphatidylcholine (DSPC), cholesterol, and PEG-lipid (40:10:40:10 mol/mol) were formulated to form SNALP for *in vivo* assessment of their efficacy in a mouse Factor VII model after a single intravenous injection [69]. The introduction of ester, carbamate, or thio-ether linkages to the ionizable lipids resulted in a substantial reduction in *in vivo* activity as compared to the LNP with DLinDMA [69]. DLin-2-DMA with one ether linkage and one ester linkage yielded intermediate activity [69]. Interestingly, the introduction of a ketal ring linker in DLin-K-DMA resulted in LNP with 2.5-fold potency in the same study [69].

Further structural modification was made based on DLin-K-DMA by adding methylene units between the dimethylamino group and the dioxolane linker, Fig. 11 [69]. The modifications affected the pK_a and flexibility of in the lipid bilayer interface [69]. Interestingly, the addition of a methylene unit in DLin-KC2-DMA produced significant increase in potency relative to DLin-K-DMA [69]. As a result, DLin-KC2-DMA became a better ionizable lipid for SNALP formulation than DLinDMA for systemic delivery to the liver.

A library of 56 ionizable lipids were designed and tested by modifying the amino head groups of various pK_as and linkage structures, Table II [70]. LNPs were formulated with the compositions of an ionizable lipid, DSPC, cholesterol and PEG-lipid in the molar ratio 40/10/40/10. (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate (DLin-MC3-DMA) (16 in Table II) demonstrated the best potency in mice [70]. The change of ratio of DLin-MC3-DMA, DSPC, cholesterol and PEG-lipid to 50/10/38.5/1.5 in the LNP composition resulted in a six-fold improvement relative to the 40/10/40/10 molar ratio composition in non-human primate [70]. DLin-MC3-DMA (MC3) is the ionizable lipid for the FDA approved siRNA-based drug ONPATTRO (Patisiran) for patients with hereditary transthyretin-mediated amyloidosis.

Multifunctional Envelope-type Nano Device (MEND)

The concept of multifunctional envelope-type nano device (MEND) was proposed to make a cleavable PEG ligand for LNP formulations to shed the PEG layer during the subcellular trafficking [71–73]. MEND was formulated using a cationic lipid, a phospholipid, cholesterol and a cleavable PEG-DMG or PEG-DSG. The cargo siRNA was complexed with protamine first before formulation [74]. A film of lipid

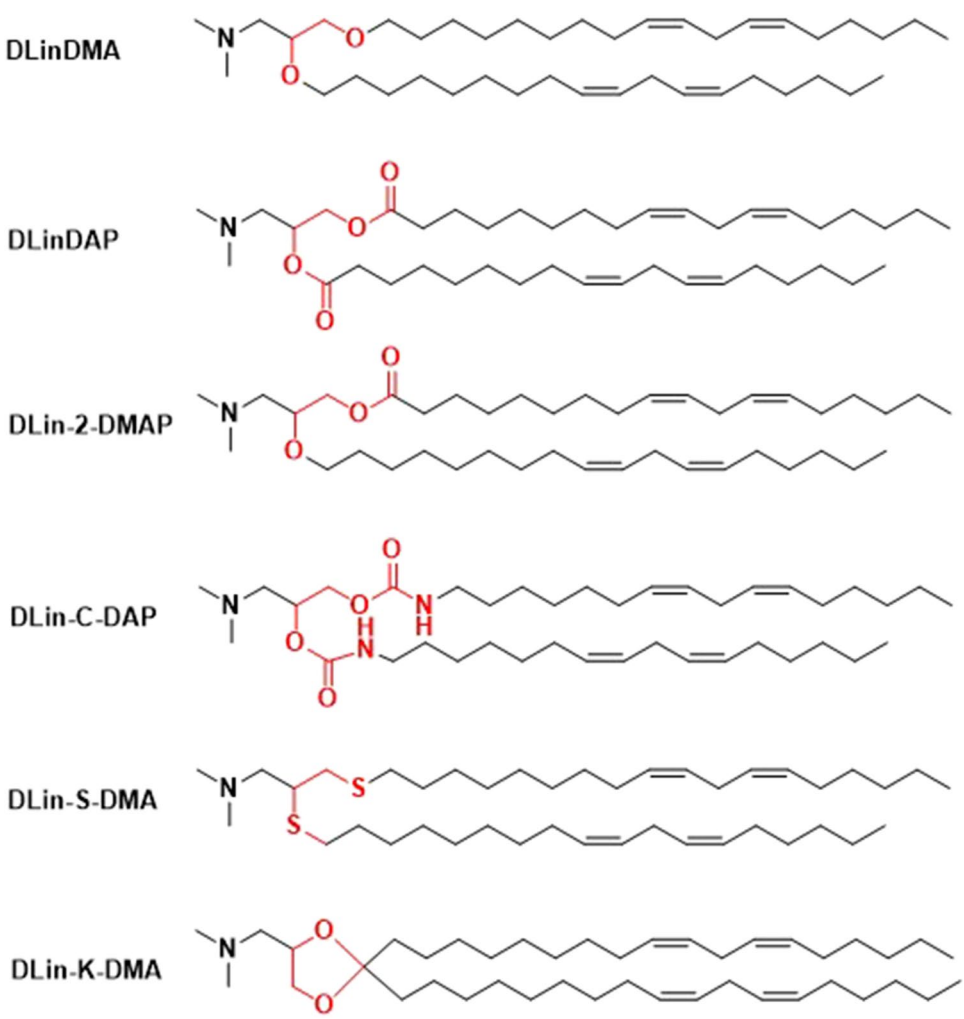


Fig. 10 Structures of DLinDMA analogues.

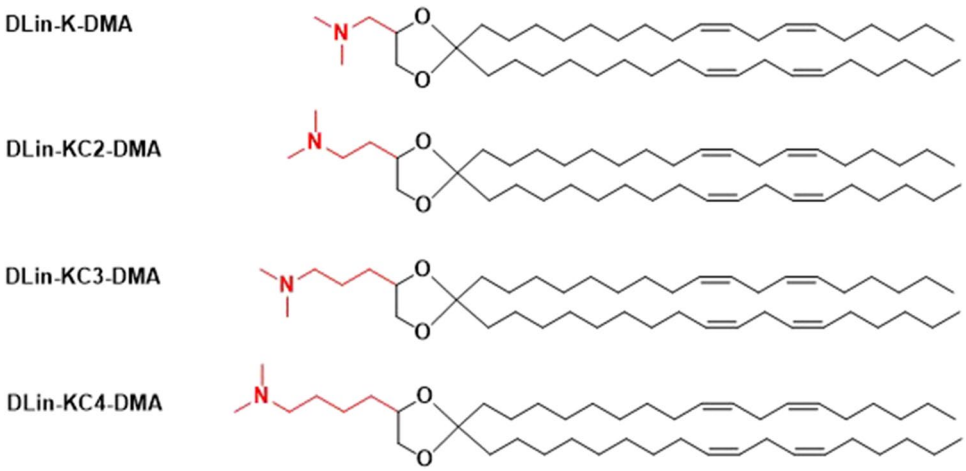
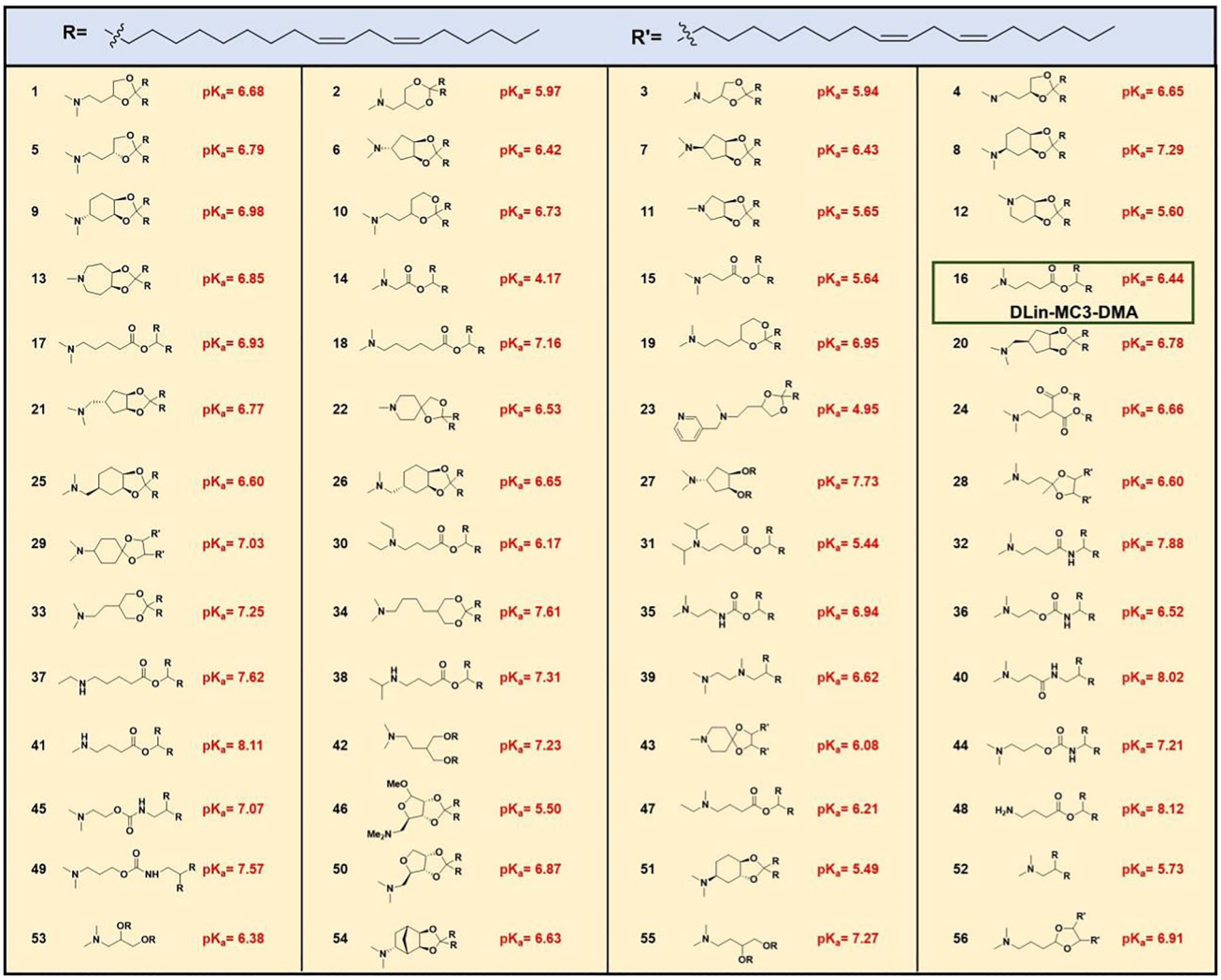


Fig. 11 Structures of DLin-K-DMA analogues.

mixture was formed by the evaporation of the solvent or solvents in a chloroform or chloroform/ethanol solution of the cationic lipid, DOPE, cholesterol and PEG lipids, and then mixed with the siRNA HEPES buffer solution [71–73]. A new ionizable lipid, YSK05, was later designed to improve the intracellular trafficking and gene silencing activity of MEND, Fig. 12 [74]. The initial MEND nanoparticles had a molar ratio of 30/40/30/3 for DOTAP, DODAP, or YSK05/DOPE/cholesterol/PEG-DMG, which was optimized to 50/25/25/3 for YSK05/POPE/cholesterol/PEG-DMG

Table II Structures of the 56 Ionizable Lipids Tested for Gene Silencing Activity *In Vivo*



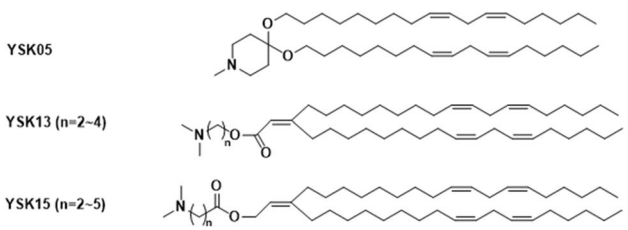


Fig. 12 Structures of YSK13 and YSK15 lipids.

in YSK05-MEND [74]. YSK05-MEND could efficiently escape from endosomes and the addition of PEG-DSG (5 mol% of total lipid) in YSK05-MEND could facilitate excellent *in vivo* gene silencing activity [74]. YSK05-MEND was also effective to deliver nucleic acid to various organs *in vivo* [75–78].

Two additional series of ionizable lipids, referred as YSK13 and YSK15, were developed for MEND formulations to enhance membrane destabilization, (Fig. 12) [79]. A carbon–carbon double bond was introduced in the acyl chains for enhanced fusogenic activity, and an ester bond for improved biodegradability. Both YSK13 and YSK15 series were formulated in MEND (YSK13 or YSK15/Cholesterol/PEG-DMG: 70/30/3) and the effect of the ester bond on siRNA delivery was examined [79]. The gene-silencing activity in hepatocytes dramatically reduced with the decrease in the pK_a value of YSK13 and YSK15 lipids [79]. YSK15-C4-MEND ($pK_a = 7.10$) demonstrated the strongest gene-silencing activity in the liver [79].

YSK12-MEND also demonstrated promising results as a delivery system of siRNA for dendritic cell-based therapy [80]. MEND formulated with YSK12-C4 loading siRNA (YSK12-MEND) (YSK12-C4/cholesterol/PEG-DMG: 85/15/1) was effective for endosomal escape in dendritic cells [80]. Based on the structure of YSK12-C4, a library of ionizable lipids have been established with various head groups, linkages and tail structures for liver siRNA delivery (Fig. 13) [81]. LNP formulated with a lipid CL4H6

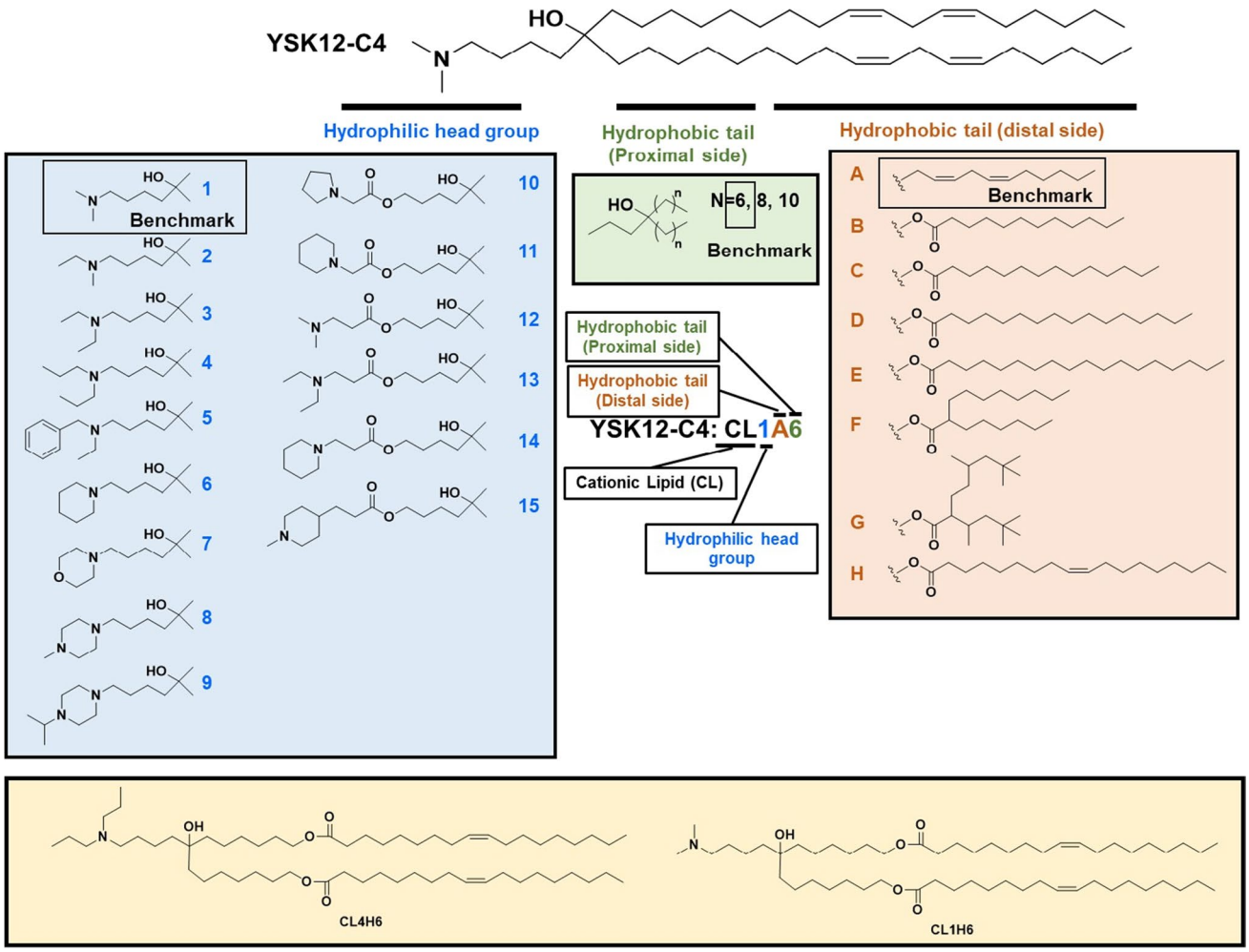


Fig. 13 Systematic derivatization of YSK12-C4. The YSK12-C4 is divided into 3 sections, an ionizable head group, distal, and proximal side of hydrophobic tails. Each part of the YSK12-C4 was derivatized to assess structure–activity relationship. The notation for the ionizable lipids used in this study are abbreviated as cationic lipid (CL), followed by the order of the number of carbons in the head group (1 to 15), distal (A to H) and proximal side (6 to 10) of the hydrophobic tails.

(CL4H6-LNP) (Fig. 13) showed efficient gene silencing activity, biodegradability, and was tolerated. The *in vivo* experiments demonstrated that the CL4H6-LNP had a superior efficiency for endosomal escape, cytosolic release, and the RNA-induced silencing [81]. For human natural killer (NK) cells, LNP formulated with CL1H6 (CL1H6-LNP) (Fig. 13) demonstrated an increased gene silencing and cell viability as compared to YSK12-LNP due to the ability to avoid endosomal disruption, resulting in a decreased level of cytotoxicity [82].

Multifunctional pH-Sensitive Amino Lipids

Multifunctional pH-sensitive amino lipids are a series of pH-sensitive protonatable or ionizable lipids that are designed to address the challenges of cytosolic delivery of nucleic acids [14, 83, 84]. Unlike the cationic or ionizable lipids discussed in the previous sections, these pH-sensitive amino lipids can form stable LNP by self-assembly with nucleic acids without helper lipids and mediate efficient cytosolic delivery of nucleic acids, including RNA and DNA of any sizes for various applications. The multifunctional pH-sensitive amino lipids were designed based on the concept of pH-sensitive amphiphilic endosomal escape and reductive cytosolic delivery, the PERC effect [14, 85]. Multiple functionalities were introduced in the structures of the pH-sensitive amino lipids to form stable LNP and to facilitate pH-sensitive amphiphilic endosomal escape and environment-sensitive release of nucleic acid cargo in cytosol.

The core structure of the multifunctional amino lipids is shown in Fig. 14 [14, 84]. It is composed of three key components in its structure, including a protonatable or ionizable amino head group, dual cysteinyl residues, and dual lipid tails. The amino head group was designed to complex with the gene cargo and to enable pH-sensitive amphiphilicity of the lipids due to their ionization in acidic endosome during

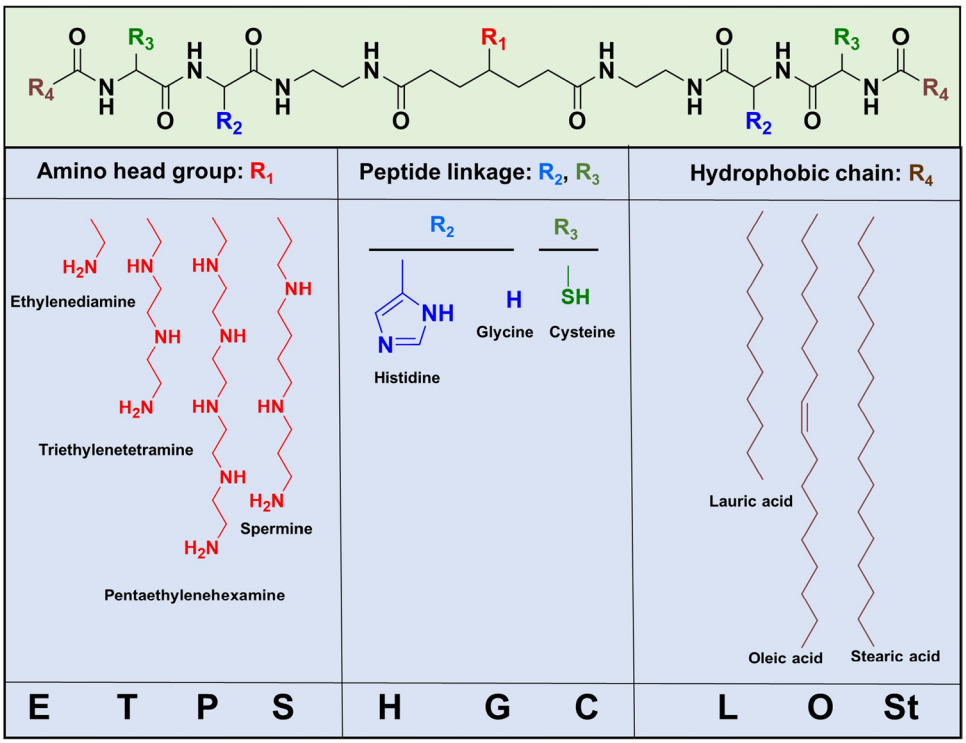


Fig. 14 The design of the multifunctional pH-sensitive amino lipids.

subcellular trafficking and to facilitate endosomal escape by amphiphilic interactions of the ionized lipids with endosomal membrane. Primary, secondary, tertiary, and aromatic amino groups have been tested as the protonatable amino head group. Lipophilic tails are designed to provide hydrophobic interactions to stabilize the LNP formulations and to induce lipophilicity to interact with cellular membrane during endosomal escape. Cysteinyl residues are introduced to further stabilize LNP by forming disulfide bonds, which are relatively stable in the plasma during the delivery process and can be reduced in the reductive cytosolic environment to facilitate the gene cargo release [13, 14]. The thiol groups can also be used for functionalization of the LNP with PEG or targeting agents. The pH-sensitive amphiphilicity can be tuned by varying the composition of amino head groups, including ethylenediamine, triethylenetetraamine, pentaethylenehexamine, spermine, and histidine, in combination with different lipid tails. Fatty acids of various chain lengths and saturation conditions were used, including lauric acid, stearic acid, and unsaturated oleic acid. The pH-sensitive amphiphilic cell membrane disruption, siRNA transfection, and silencing efficiency of the amino lipids were affected by the structures of both the head group and lipid tails. Among these amino lipids, EHCO with a combination of a ethylenediamine head group and unsaturated lipid tails exhibited no hemolytic activity at pH 7.4 and 6.5, and high hemolytic activity at the endosomal-lysosomal pH (5.4), indicating pH-sensitive membrane destabilization in acidic endosomes for efficient endosome escape [14]. The amino lipids with head group of more amino groups and/or saturated lipid did not show good pH sensitivity in this pH range. The siRNA delivery into U87 cells demonstrated that the lipid EHCO with an ethylenediamine head group, histidylcysteinyl linkers, and oleoyl tails had the best *in vitro* transfection and silencing efficiency [14]. More multifunctional amino lipids were also synthesized and investigated for plasmid DNA delivery [84]. The plasmid DNA transfection experiment demonstrated amino lipid SKACO showed high efficacy for LNP formulation with plasmid DNA [84].

Unlike other LNP formulations where a lipid anchor is required for PEGylation, the multifunctional amino lipid-based LNP can be readily modified with PEG by conjugating a maleimido-PEG to a small portion (ca. 2.5 mol-%) of the free thiol groups of the amino lipids [86, 87]. A targeting ligand could be used at the other end of PEG. Bombesin peptide was used in EHCO/siRNA nanoparticles to target CHO-d1EGFP cells *in vitro*, which had specific overexpression of bombesin receptors, resulting in specific targeting and enhanced delivery and gene silencing efficiency [86]. RGD peptide on PEG was also used for EHCO based LNP formulation, which resulted in receptor-mediated cellular uptake and high gene silencing efficiency in U87 cells *in vitro* and *in vivo* [87].

The structure of multifunctional amino lipids was modified by altering the head groups and lipid tails based on the core structure of EHCO, Fig. 15 [83]. The impact of the unsaturation degree in oleoyl or linoleoyl tails and the role of the protonatable amino groups and histidine residues on

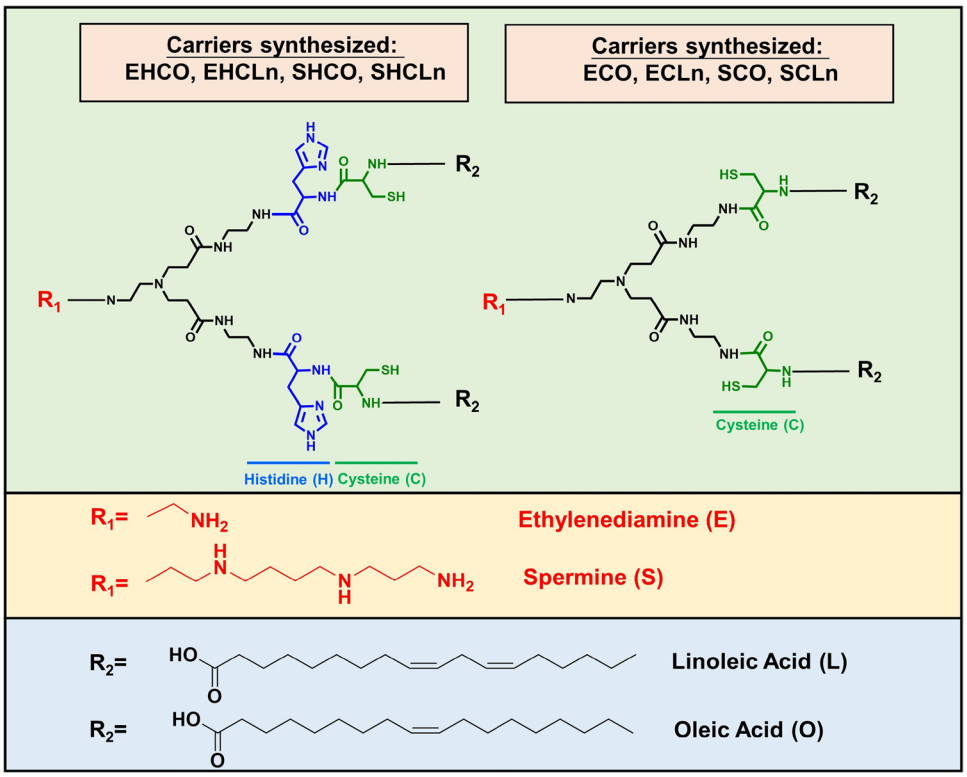


Fig. 15 Structural modification of the multifunctional pH-sensitive amino lipids based on EHCO.

siRNA LNP formulation, pH sensitivity, gene silencing was further investigated. The presence of additional protonatable amines in the head group helped the encapsulation of siRNA at low N/P ratios, but show less sensitivity to pH changes. ECO and ECLn without the histidyl residues mediated the best gene silencing efficiency among these new amino lipids. The number of double bonds in the lipid tails did not affect gene silencing efficiency of the lipids with the same head group [83].

ECO with an ethylenediamine head group, two cysteine-based linker groups, and two oleoyl tails has been extensively explored for *in vivo* delivery of nucleic acids for various applications, including siRNA gene regulation for cancer therapy, CRISPR/Cas9 for gene editing, and gene replacement therapy with plasmid DNA for eye genetic disorders [88–101]. ECO readily self-assembles with nucleic acids of any sizes to form stable LNPs without helper lipids. The cysteinyl residues stabilize the LNP formulation by disulfide bonds formation, and can also be used for PEGylation with targeting functions. ECO is not amphiphilic and does not induce cell membrane destabilization at physiological pH 7.4. It can be protonated or ionized at the endosomal pH (5.4–6.5) to become amphiphilic to facilitate endosomal membrane destabilization and to promote efficient endosomal escape [85, 90]. In the reductive cytoplasm, the disulfide crosslinks in ECO LNP can undergo reductive cleavage, further aid on the release of the gene cargo [97], which is defined as the PERC effect [85].

In the formulation of multifunctional amino lipid LNP, the amino lipids are dissolved in ethanol, which accounts for 5% of total final volume, and mixed with nuclease free aqueous solution of nucleic acids to form LNP by self-assembly with uniformed distribution and size in the range of 80–200 nm with simple agitation. The presence of 5% ethanol in the final volume does not affect stability and transfection efficiency of the LNP. No dialysis process is needed after the formulations. Addition of sucrose in a range of 5 -10% significantly improve the stability of the LNP formulation. The frozen formulation of RGD-PEG-ECO/siRNA LNP with as low as 5% sucrose retained nanoparticle integrity (90% siRNA encapsulation), size distribution (polydispersity index [PDI] 20%), and functionality (at least 75% silencing efficiency) at -80 ºC for at least 1 year [97]. The frozen LNP formulation also exhibited excellent biocompatibility, with no adverse effects on hemocompatibility and minimal immunogenicity [97].

RGD peptide with a PEG spacer was used to modify siRNA ECO LNP for targeted delivery in cancer via systemic injection, which has demonstrated excellent *in vivo* efficacy in mouse tumor models [91, 96, 97, 101]. Retinoid analogues with a PEG spacer were also used to target the retinal pigmented epithelium for retinal gene therapy using ECO plasmid LNP [94, 99]. ECO was also able to deliver a large plasmid DNA encoding *ABCA4* gene for treating Stargardt disease. ECO is a versatile and highly efficient multifunctional amino lipid for delivery of nucleic acid for

treating disease with abnormal genetic functions [94, 98, 99, 102, 103]. A pH-sensitive hydrazone linker was also incorporated in the PEG spacer to develop dual pH-sensitive ECO LNP, enabling the shed of PEG layer at acidic endosomal pH, resulting in increased endosomal escape properties than previous targeted ECO LNP both for cancer and retinal gene therapies [89, 99].

Ionizable Lipids for mRNA LNP

Amino lipids have also been used to form mRNA LNP as vaccines to combat viral infections. The FDA approved two LNP based Covid-19 vaccines that contain mRNA encoding the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [104, 105], which are the first mRNA vaccines approved for human use. The mRNA is encapsulated in LNPs containing an ionizable lipid, PEG-lipid, the phospholipid 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol. Ionizable lipid ALC-0315 ((4-hydroxybutyl) azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) is used in the mRNA vaccine developed by Pfizer, and SM-102 (heptadecan-9-yl 8-(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl] amino octanoate) is used in Moderna's vaccine, Fig. 14. It was shown that SM-102 in Moderna's mRNA vaccine outperformed MC3 [106, 107]. Interestingly, both ACL-315 and SM-102 share some common structure features, including a tertiary amine with a hydroxyalkyl group, branched tails, and ester linkers. ALC-0315 has four very short tails, while SM-102 has only three and one of them is along lipid [108]. As compared MC3, the unique structural feature of ALC-0315 and SM-102 is the branched lipid tails, which has also been seen from YSK based ionizable lipids development [81]. Ionizable lipid CL4F6 has demonstrated as excellent properties as CL4H6 (Fig. 13) [81]. It seems that multiple branched short tails could achieve similar property as unsaturated long tails, which largely affect the lipid phase transition and thereby endosomal escape properties. Molecular dynamics simulations demonstrated differences in the stability of SM-102 and ALC-0315 due to the tail structure difference, which SM-102 demonstrated better stability using a three-tailed design whereas ALC-0315 used a four-short-tail design [108]. The mRNA-loaded LNPs for Covid-19 vaccine are formulated through microfluidic mixing, where the pH of the solution is kept low to protonate the ionizable lipid's amino groups for electrostatic complexation of negatively charged mRNA. Dialysis or ultrafiltration is used to neutralize the pH, resulting in uncharged, solid-core LNPs that densely package the mRNA. PEGylation stabilizes the mRNA LNP by preventing aggregations during manufacturing and storage. Due to a short anchor and the biodegradability of the PEG lipid, it can quickly dissociate from the LNP following injection and allow efficient intracellular delivery (Fig. 16.).

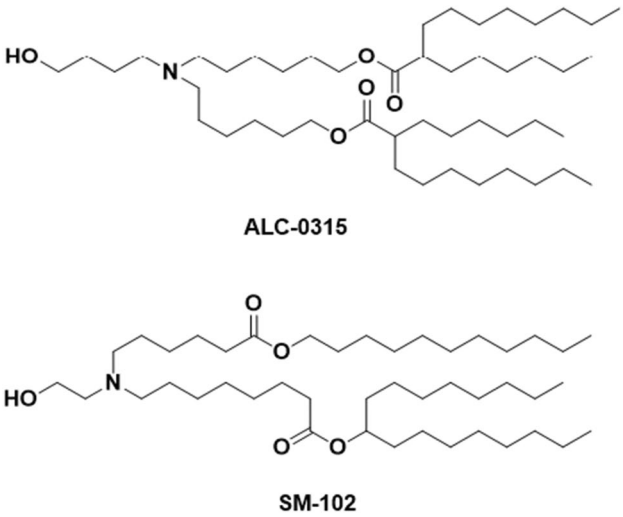


Fig. 16 Chemical structures of ionizable lipids in mRNA LNP.

The Effects of Lipid Structure on Formulation and Intracellular Nucleic Acid Delivery Of LNP

Safe and efficient delivery of nucleic acids into cytoplasm of target cells is the key consideration in the design and development of LNP for biomedical applications. The structure of lipids plays the essential role in cytosolic delivery of nucleic acids with LNP. The main route of entry of LNP into cells is by endocytosis, including clathrin-mediated, caveolae-mediated endocytosis, receptor-mediated endocytosis, macropinocytosis, or phagocytosis, based on its size and properties. Once LNP is internalized by cells, it is transported through endosomes and lysosomes, where the internalized materials are digested or exocytosed. In order to allow nucleic acid to perform the intended function, the gene cargo has to escape the endocytic pathway and redistributes in intact form in cytoplasm or nucleus of target cells. Thus, endosomal escape is a crucial step for effective gene delivery with LNP. The ability of the lipids to promote endosomal escape is a critical factor in the design and development of LNP for nucleic acid delivery.

Cationic lipids, which have permanent positive charges, can facilitate better electrostatic interactions with negatively charged cell membrane, but are cytotoxic due to their permanent positive charges and amphiphilicity, which causes cytotoxicity by dissolving lipid bilayer cell membrane [109]. Neutral amino lipids such as DODMA and DODAP were then developed to reduce the cytotoxicity of the cationic lipids with permanent changes. The concept of pH-sensitive protonatable or ionizable amino lipids for pH-sensitive amphiphilic endosomal escape was first introduced in the design of the multifunctional pH-sensitive protonatable or ionizable lipids [14, 90] and systemically demonstrated

with these lipids. The pH-sensitive protonatable or ionizable lipids are neural at physiological pH and are protonated or ionized at acidic endosomal pH to become amphiphilic to destabilize the endosomal membrane for endosome escape. It is shown that the structures of both amino head group and lipid tails are essential to control the pH sensitivity and pH-dependent cell membrane destabilization [14]. The amino lipids EHCO and ECO with a small amino head group, ethylenediamine, and unsaturated oleoyl tails exhibited the best pH sensitivity in the pH range of 5.4–7.4 [14, 83]. ECO shows effective pH-sensitive cell membrane destabilization and mediates efficient endosomal escape for cytosolic delivery of nucleic acids [90]. The concept has now been broadly adopted in the design and development of ionizable lipids to promote membrane destabilization and facilitate endosomal escape of LNP [70, 107, 108, 110–115].

Since the pH-sensitivity of ionizable lipid plays a crucial role in gene delivery using LNPs, the pK_a of the head group has significant impact on the pH-sensitivity [111]. It has been shown that the most effective amino lipids have a pKa around 6.5 [111] and the lipids with pK_a values less than or equal to 5.4 demonstrates significantly lower efficiency [113]. For systemic delivery for the liver targeting, it was reported that the pK_a between 6.2 and 6.5 of amino lipids are optimal for systemic siRNA delivery to the liver [70]. If the criterion for a proper pK_a value is not met, LNP may have with low efficiency. Proper pK_a values can facilitate a non-bilayer (hexagonal H_{II}) phase structure when mixed with anionic lipids, which serves as a measure of their bilayer-destabilizing capacity and relative endosomolytic potential [110, 115–117].

Saturation status of the lipid tails also affects the overall property of the cationic or ionizable lipids that, in turn, determines the endosomal escape properties [118]. Cationic/ionizable lipids can have various dynamic structural phases, including the micellar, lamellar, cubic and inverted hexagonal phase [119]. The type of structure can be predicted by the packing parameter (P), which represents the ratio of the area occupied by the hydrophobic tails *versus* the hydrophilic head [120–122]. When P value exceeds 1, the area occupied by the hydrocarbon lipid tails is much larger than the head group, the lipid tends to adopt the inverted hexagonal phase, which is in favor of endosomal membrane destabilization, which is crucial for LNP gene delivery [112, 123]. The study utilized lipids of the same alkyl chain length (C18) modified by a systematic addition of double bonds and the effect of double bonds addition against bi-layer transition temperature demonstrated that the lipid tails containing 1 or 2 double bonds had much lower phase transition temperature for H_{II} phase transition to get the 'cone' shape character [66]. Lower phase transition temperature and the 'cone' shape structure can facilitate efficient membrane fusion and thereby endosomal escape.

Helper lipid structure can also affect the endosomal escape properties of LNP formulations [124–127]. As one of the most commonly used helper lipid used for LNP formulations, DOPE has a small head group, phosphoethanolamine, and two unsaturated oleoyl lipid chains, providing kinked structure and creating a cone geometry that is favorable for the non-bilayer hexagonal (H_{II}) phase crucial for membrane fusion and bilayer disruption process during endosomal escape [38–40]. Therefore, DOPE aids endosomal escape of some of the cationic and ionizable lipids, especially when they have low pH-sensitivity. For example, combination of DOPE with a cationic lipid that originally did not have effective transfection resulted in efficient gene transfection [114]. On the other hand, saturated phosphatidylcholines, such as distearoylphosphatidylcholine (DSPC) and hydrogenated soybean phosphatidylcholines (HSPC), have high phase transition temperatures [128–131]. They have been used to formulate LNP such as stabilized nucleic acid lipid particles (SNALP) to improve its stability [117, 132]. However, LNPs formulated with saturated phosphatidylcholine helper lipids potentially have lower endosomal release properties compared with unsaturated helper lipids, which may affect the gene delivery efficiency. Cholesterol is another commonly used component in LNP formulations, which stabilizes lipid bilayers by filling in the gaps between phospholipids and give better circulation stability to LNPs [133]. It can facilitate membrane fusion, thereby promoting the endosomal release [134]. The lower solubility of cholesterol in the LNP core may lead to its enrichment on the LNP surface, promoting its crystallization and potentially contributing to endosomal fusion [135]. As a result, cholesterol can enhance the activity of LNP especially at a high concentration [136]. Derivatives of CHOL has also demonstrated ability enhancing the efficiency of LNP, such as phytosterols and the derivatives of β-sitosterol [137, 138]. These derivatives with longer alkyl chains can improve the fusogenic properties and endosomal escape property [135].

Cationic or ionizable lipid can mediate electrostatic interaction between LNPs and the cellular or endosomal membranes, and facilitate cellular uptake and endosomal release of gene cargos [139]. At the same time, the interactions of LNP may cause cytotoxicity and related toxic side effects *in vivo* if the amphiphilic properties of lipids and surface property of the LNP are not precisely controlled in the range physiological pH (7.4) and endosomal pH. The pH sensitive protonation or ionization of amino lipids in this pH range is essential in the design of safe and effective LNP for clinical translation, especially for systemic administration. The pH sensitive ionizable lipids with little to no amphiphilicity at physiological pH and high amphiphilicity at endosomal pH can have better control of cytotoxicity and side effects of LNP for *in vivo* use. In addition, pH-sensitive amphiphilic cell membrane destabilization of LNP also depends on the

concentrations of the lipids [83]. The pH-sensitive ionizable lipids with the ability to form stable LNP and mediate efficient cytosolic delivery of nucleic acids at low N/P ratios are preferred to minimize the potential toxic side effects for *in vivo* applications. Surface modification of LNP with biocompatible polymers or biopolymers could also minimize the toxic side effects of LNP [140, 141]. Nevertheless, the breakthrough of LNP based mRNA vaccines and other therapies has demonstrated the potential of LNP systems, especially the pH-sensitive ionizable LNP systems, for the delivery of therapeutic nucleic acids, including DNA, mRNA, miRNA, and siRNA. As such, it has ignited a new wave of the development of ionizable lipid based LNP systems for delivering various therapeutic nucleic acids for preventing and treating human diseases that are not treatable with conventional therapeutic regimens. It is expected that more LNP based gene therapies or vaccines will be approved for clinical use.

Summary

Lipid nanoparticle systems hold great promise in treating untreatable diseases including inherited genetic diseases, cancer, and infectious diseases. Through the modifications in lipid structures, formulations with helper components, and preparation processes, LNP systems have shown significantly improved delivery efficiency and therapeutic efficacy in the targeted tissue, from simple lipid/DNA complexes to the mRNA vaccine to combat SARS-CoV-2. Understanding the structure/formulation/function relationship allows tunable designs of LNP systems addressing different applications, which will facilitate more innovative design and development of LNP systems for broader applications in future clinical practice.

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Declarations

Conflicts of Interests/Competing Interests There is no conflict of interests/competing interests for the authors in this work.

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