Next-Generation Lipids in RNA Interference Therapeutics

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ABSTRACT: RNA is emerging as a potential therapeutic modality for the treatment of incurable diseases. Despite intense research, the advent to clinical utility remains compromised by numerous biological barriers, hence, there is a need for sophisticated delivery vehicles. In this aspect, lipid nanoparticles (LNPs) are the most advanced platform among nonviral vectors for gene delivery. In this review, we critically review the literature and the reasons for ineffective delivery beyond the liver. We discuss the toxicity issues associated with permanently charged cationic lipids and then turn our attention to next-generation ionizable cationic lipids. These lipids exhibit reduced toxicity and immunogenicity and undergo ionization under the acidic environment of the endosome to release the encapsulated payload to their site of action in the cytosol. Finally, we summarize recent achievements in therapeutic nucleic acid delivery and report on the current status of clinical trials using LNP and the obstacles to clinical translation.

KEYWORDS: RNA interference, RNAi therapeutics, cationic lipids, ionizable amino lipids, lipidoids, lipid nanoparticles, siRNA, delivery, toxicity, gene silencing

In recent years, lipid nanoparticles (LNPs) containing permanent aminated cationic lipids have garnered considerable attention as delivery vehicles for nucleic acids to target cells. The discovery of the RNA interference (RNAi) phenomena in 1998 and subsequently small interfering RNAs (siRNAs) that mediate gene silencing has led to interest in exploiting this endogenous process to address incurable diseases, as now potentially any target could become druggable.

siRNA is a double-stranded oligonucleotide consisting of 19−25 base pairs with 3 in. overhangs that guide RNAi. It binds to RNA-induced silencing complex (RISC) in the cytoplasm of cells and results in cleavage of the mRNA sequence to suppress the expression of specific genes. Such selective gene silencing has potential for the treatment of a variety of diseases including viral infections, cancer, and autoimmune diseases that cannot be treated using existing drug classes. It is anticipated that RNA-based therapeutics will enable the realization of personalized medicine via sequencing of the patient’s transcriptome.

Although promising, the progress of nucleic-acid-based medicines has been hindered by challenges to their delivery as well as a lack of understanding of the link between physicochemical properties of delivery vehicles, biological barriers, and silencing activity. Nucleic acids are notoriously difficult to deliver intracellularly due to their physicochemical properties, including their relatively large molecular weight of ~13.5 kDa, anionic charge due to >40 phosphate groups, and hydrophilic nature.

Chemical modifications to the backbone or ribose to increase stability have been sufficient for delivery to the brain and lung, and local administration has successfully delivered siRNA to the eye, skin, mucous membranes, and solid tumors. However, systemic delivery of nucleic acids for far-reaching applications suffer from barriers including half-life of only minutes due to degradation by plasma and cellular nucleases, rapid plasma clearance, activation of the immune system, and poor cellular uptake. Nevertheless, there has been success in intravascular delivery to the liver by siRNA conjugated to cholesterol, which led to 60% knockdown of ApoB mRNA in a mouse model. For broader applications, for example, to metastasis, the aforementioned issues must be overcome, and successful nanocarriers need to be efficient, safe, and specific.

Lipid formulations are regarded as one of the leading strategies for nonviral gene delivery. The incorporation of cationic lipids in LNP formulations provides an effective means to promote efficient encapsulation or neutralization of siRNAs,
cytoplasmic delivery, and endosomal escape, with some formulations having progressed to clinical trials. The advantages of LNPs over viral-based carriers include low immunogenicity, ease of manufacturing, and higher loading capacity with the potential to incorporate targeting moieties. However, LNPs exhibit lower transfection efficiency and colloidal instability, and cationic lipids can induce toxicity.

Here, we will focus on the use of the next-generation ionizable cationic lipids in lipid formulations for nucleic acid delivery, advances in clinical trials, and the key limitations to translation of these LNPs into the clinic.

**LIPID SELF-ASSEMBLY**

The self-assembly of amphiphilic lipid molecules, such as cationic lipids, in excess water is determined by the nature of the hydrophilic and hydrophobic regions of the lipid. The resulting mesophases are thermodynamically stable, and the type of structure produced is dictated by packing constraints of the individual lipid molecules to minimize exposure of the hydrophobic chains to water (Figure 1), as well as lipid concentration, temperature, pH, pressure, and additives. The geometry of the molecules has been defined using the packing parameter $P = V/a$, where $V$ is the surfactant tail volume, $a$ is

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**Figure 1.** Sequence of phases as a function of critical packing parameter, $P = V/a$, and amphiphile concentration (b). Names and abbreviations for the different mesophases are micelles ($L_1$), micellar cubic ($I_1$), hexagonal ($H_1$), bicontinuous cubic ($V_1$), lamellar ($L_\alpha$), reversed bicontinuous cubic ($V_2$), reversed hexagonal ($H_2$), reversed micellar cubic ($I_2$), and reversed micelles ($L_2$), where subscripts 1 and 2 refer to “normal” and “reversed” phases, respectively. Adapted with permission from ref 10. Copyright 2006 The Royal Society of Chemistry.

**Figure 2.** Chemical structure of cationic lipid subtypes. Reproduced with permission from ref 8. Copyright 2008 Elsevier.
the effective area per molecule at the interface, and \( l \) is the surfactant tail length.\(^7\)

In the case of \( P = 1 \), the lamellar liquid-crystalline structure \( L_\alpha \) is formed with zero mean curvature. The membrane of cells, which is composed of phospholipids arranged in a bilayer or lamellar fashion, is a classic example of lipid self-assembly exhibited \textit{in vivo}.\(^{11}\) The resulting self-assembled structures of amphiphilic lipids with a large polar head relative to the hydrophobic chain result \( (P > 1) \) form type I structures such as normal micelles \( (L_\text{G}) \) and normal hexagonal phase \( (H_\text{II}) \). They possess positive mean curvature and curve toward the lipid region to form oil in water structures. In the event that \( P < 1 \), type II or inverse structures (water in oil) are adopted by inverted or wedge-shaped amphiphiles that have a small polar head and large chain. The reversed hexagonal \( (H_\text{I}) \) is a bilayer destabilizing structure critical for delivery of nucleic acid into the cytosol following internalization of the cationic lipid–nucleic acid complex.\(^{12}\)

### PERMANENTLY CHARGED CATIONIC LIPIDS

In 1987, Felgner \textit{et al.} first reported on the formation of complexes between the cationic lipid \( \text{N}-[1-(2,3\text{-dioloyloxy})-propyl]-\text{NNN}-\text{trimethylammonium chloride} \) (DOTMA) and plasmid DNA, which when formulated with dioleyl phosphatidylethanolamine (DOPE) to make liposomes resulted in successful transfection of cells \textit{in vitro}.\(^{13}\) The synthesis of cationic lipids for the purpose of gene delivery for \textit{in vitro}, \textit{in vivo}, and clinical applications followed, with the basic structure of a positively charged headgroup (monocation or polycation, linear or heterocyclic), attached to a hydrophobic group (cholesterol or aliphatic) \textit{via} a linker bond.\(^{14}\) Subtypes include monovalent aliphatic lipids, multivalent aliphatic lipids, and cationic cholesterol derivatives (Figure 2).

The lipid structure is an important determinant of the cellular toxicity and transfection efficiency. Cellular toxicity is mainly dictated by the structure of the headgroup, which commonly are a primary, secondary, tertiary amine, or quaternary ammonium salt, although phosphorus or arsenic have also been employed.\(^{15}\) Transfection efficiency is linked to the cationic lipids, and their ability to complex to negatively charged nucleic acid induces cellular uptake and endosomal escape.

Farhood \textit{et al.} reported that cationic derivatives of cholesterol (Chol) with quaternary headgroups are more toxic than tertiary derivatives. When formulated with DOPE to form liposomes and mixed with plasmid containing the \textit{Escherichia coli} CAT gene, they also have little to no transfection ability in mouse \textit{L929} cells and inhibit protein kinase C (PKC).\(^{16,17}\) Cholesterol-derived cationic lipids with tertiary and quaternary nitrogen headgroups are reported to interact with PKC and induce cytotoxicity.\(^{18}\) Heterocyclic cationic lipids have also been investigated, using imidazolium, pyridinium, and guanidine heads. They display higher gene knockdown and reduced cytotoxicity, which is believed to be due to delocalization of the cationic charge into the ring.\(^{19-22}\)

The hydrophobic anchor is based on aliphatic chains or cholesterol derivatives, and the chain length and degree of saturation influence transfection and cytotoxicity. In a homologous series that ranged from \( \text{C}_{14} \) to \( \text{C}_{18} \) lipids, a shorter alkyl chain was revealed to provide better transfection, deemed to be a result of a more fluid bilayer that allows for better intermembrane mixing.\(^{23-25}\) Unsaturated chains are shown to be the most effective, with oleyl-based lipids being most prevalent in studies. Double-chained lipids are predominantly investigated and have the advantage of forming lamellar phases in the absence of helper lipids, although single-tailed aliphatic lipids are traditionally more efficient but toxic due to their tendency to form micelles.\(^{26,27}\) Steroid-based (\textit{e.g.}, cholesterol) cationic lipids are biodegradable, rigid, and fusogenic; however, they can be potent PKC inhibitors, rendering them more toxic than their straight-chain analogues.\(^{18}\)

Amide, ester, and ether bonds are typically used as the linker group between the hydrophobic and hydrophilic regions. Although the ether bond is stable and yields higher transfection efficiency, this occurs at the expense of toxicity. In contrast, less stable ester bonds are biodegradable and hence display reduced cytotoxicity. Reduced potency in ester-linked lipids has been attributed to degradation of the carrier by lipases, resulting in premature release of cargo in endosomes and degradation by nucleases in lysosomes.\(^{28}\) On the other hand, carbamate bonds which remain stable during circulation but undergo hydrolysis in the acidic endosomal environment are also associated with less toxicity.\(^{29,30}\)

Cationic liposomes have been formulated to protect oligonucleotides from nucleases, mediate cellular delivery, and target specific cell types.\(^{16}\) Lipofectin, comprising DOTMA/DOPE, was the first cationic liposomal formulation to mediate delivery of DNA into mammalian cells.\(^{13}\) On the other hand, liposomes without cationic lipid such as lipofectin or DOTMA displayed no activity.\(^{31,32}\) DC-Chol/DOPE unilamellar liposomes have no PKC inhibition activity and were demonstrated to transfect cell lines, primary cells, and \textit{in vivo} in mice.\(^{16,33}\)

The pH-sensitive liposomes mimicking the fusing behavior of envelope viruses have been designed to avoid lysosome degradation. DOPE/oleic acid/Chol pH-sensitive was shown to deliver 90% of encapsulated oligonucleotides in 90 min at acidic pH, compared to only 15% by the DOPC/OA/Chol formulation.\(^{34}\)

Major drawbacks are associated with the use of cationic lipids, which is linked to their high net positive charge, cellular toxicity, aggregation with erythrocytes, recruitment of the immune response (interaction with Toll-like receptor or other intracellular proteins), and rapid plasma clearance.\(^{35-37}\) Kedmi \textit{et al.} reported immune toxicity in mice following systemic administration of positively charged lipid nanoparticles composed of HSPC/Chol/DOTAP, likely due to activation of Toll-like receptor 4 on leukocytes.\(^{38}\) Furthermore, it is well-known that cationic particles undergo aggregation and accumulation in the lung, liver, and spleen.\(^{39}\) In addition, cationic lipid–nucleic acid complexes, called lipoplexes, formed from the cationic lipids DDAB/DOPE, DMRIE, DOTMA, and DOTAP were found to result in inflammatory toxicity following systemic administration, activation of immune cells, cell shrinkage, cytoplasmic vacuolization, and inhibition of PKC.\(^{16,39,41}\) The majority of lipoplexes are also taken up by Kupffer cells\(^{42}\) and less by the spleen.\(^{43}\)

For these reasons, attention has been directed to the rational design of less toxic lipids that are known as ionizable lipids. These lipids are charged at mildly acidic pH and can be complexed with small RNAs to generate a stable nanoparticle. Under physiological pH conditions, the net charge of the particles formed is predominantly neutral to mildly cationic surface charge, and thus they do not induce a robust immune response. In addition, these lipids are only destabilized in the
acidic environment of the endosome to release their RNA cargo.

IONIZABLE CATIONIC LIPIDS

Structure and Characterization. Ionizable cationic lipids typically consist of three parts: a hydrophobic tail, a linker group, and an amine headgroup that is capable of undergoing protonation (Figure 3). A pKₐ < pH 7 of the ionizable amino lipid enables complexation with negatively charged nucleic acid at low pH, but a neutral or slightly cationic surface charge is maintained at physiological pH to prevent nonspecific interactions with serum proteins and improve circulation time. The chemical structure of the ionizable cationic lipid is an important determinant of the transfection potency and fusogenic behavior. Increased unsaturation of acyl chains is reported to lead to decreased Lₐ–H₂ transition temperature and enhanced silencing capacity. This is attributed to increased flexibility due to a fluid lipid state, allowing for membrane fusion between anionic and cationic lipids via ion pairing, inducing a stable Lₐ to unstable H₂ phase and promoting nucleic acid release into the cytoplasm.

DODAP with a pKₐ value of 6.6–7 was the first ionizable amino lipid used in a lipid formulation, encapsulating up to 70% oligodeoxynucleic acid in uni- and multilamellar vesicles under acidic conditions. Its structure, containing two oleyl chains, has been used as the basis for the development of other ionizable lipids (Figure 4). In general, unsaturated ionizable lipids are capable of gene silencing, with two double bonds being more effective than those with three, and saturated acyl chains exhibit no silencing ability.

More recently, a combinatorial approach has been employed in the design and synthesis of amino lipids in order to elucidate the structure–activity relationship (SAR). The linoleyl hydrocarbon chains in the first generation of ionizable cationic lipid 1,2-dilinoleylxoy-N,N-dimethyl-3-aminopropane (DLinDMA) were found to be optimal, thus multiple hydrophobic tails of varying lengths and unsaturation, lengths of linker group, and multiamine headgroups were systematically investigated. A mouse factor VII model (a blood clotting factor) was employed to assess in vivo delivery to hepatocytes, where mice receive a tail vein injection of a dose of mouse factor VII siRNA formulated in lipid nanoparticle (composed of ionizable cationic lipid/DSPC/Chol/PEG-lipid), and serum factor VII protein level was analyzed after 24 h. LNP composed of DLinDAP, an ionizable lipid with ester linker groups, displayed >40-fold reduction in in vivo activity compared to alkoxy-linked

Figure 3. Ionizable lipids consist of an amine headgroup, linker group, and hydrophobic tails (a). Lipids with a small polar head and unsaturated hydrophobic tails form conical structures compared to lipids with a large headgroup and saturated hydrophobic tails that form cylindrical structures (b). The interaction of cationic liposomal membrane with anionic endosomal membranes results in a lamellar to reversed hexagonal phase transition (c). Adapted with permission from ref 47. Copyright 2013 Macmillan Publishers Ltd.

Figure 4. Chemical structures of ionizable cationic lipids.
DLinDMA, which has an ED$_{50}$ of $\sim$1 mg/kg. It has been postulated that esters are rapidly hydrolyzed and cannot protect the siRNA from degradation by endogenous lipases, leading to low potency. This trend is also observed in carbamate and thioester containing lipids.

A ketal ring linker and the addition of methylene groups gave rise to the potent lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), with an ED$_{50}$ of 0.1 mg/kg in non-human primates and 0.01 mg/kg in rodents. Other systematic studies have shown that $pK_a$ values of the LNP between 6 and 7 display in vitro luciferase silencing in HeLa cells and in vivo mouse factor VII silencing (after formulation into LNP with DSPC/Chol/PEG-lipid), and those below 5.5 did not, highlighting the importance of ionization behavior on potency. $pK_a$ was measured using the 2-(p-toluidino)-6-naphthalene sulfonic acid assay and titrating LNPs from pH 2.5 to pH 8.5. Aibi et al. reported that electron-withdrawing groups near the amine head and aromatic and bulky headgroups led to low $pK_a$ values and poor gene knockdown; headgroups with five- or six-membered ring were better than six- or seven-membered rings, and $pK_a$ was more important for gene silencing than LNP size and siRNA entrapment. Liposomal $pK_a$ is a more crucial determinant of efficacy than the $pK_a$ of the individual lipid components.

Heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA) is currently the most potent ionizable lipid used in LNPs. It was found to have an optimal $pK_a$ value at 6.44 and displays in vivo hepatic activity in doses of 0.005 mg/kg in rodents and 0.03 mg/kg in non-human primates. Jayaraman et al. showed that for amino lipids with similar structure, the $pK_a$ was more critical for activity than headgroup structure or linker length.

Next-generation biodegradable ionizable lipids containing ester linkages have been synthesized to boost the safety profile. They have been found to improve biocompatibility and tolerability, as well as maintain high potency (ED$_{50}$ < 0.01 mg/kg in mouse factor VII model) when administered in LNP composed of ionizable lipid/DSPC/Chol/PEG-DMG in rodents and non-human primates. Shifting the placement of the ester group toward the headgroup decreased potency, which was attributed to an increase in $pK_a$. The hydrolysis products were rapidly eliminated from plasma and tissues following potent in vivo delivery of siRNA to a mouse model. The proprietary lipid-enabled and unlocked nucleic-acid-modified RNA (LUNAR) delivery platform by Arcturus Therapeutics contains an ionizable lipid with ester bonds in the lipid backbone, rendering it biodegradable.

The asymmetric ionizable lipid L101 synthesized by Suzuki et al. contained a biodegradable ester group in the middle of the lipid tail and a non-biodegradable cyclic tertiary amine headgroup. LNP encapsulating L101/DSPC/Chol/PEG-DMG showed potent gene silencing in mouse hepatocytes, with ED$_{50}$ < 0.02 mg/kg in a mouse factor VII model. The particles were well-tolerated at single siRNA doses up to 16

Figure 5. Schematic illustrating the endosomal escape hypothesis. The endosomal membrane is destabilized by electrostatic interaction between cationic lipids and endosomal anionic lipids. The cationic and anionic lipids form a neutral ion pair, which causes dissociation of nucleic acid into the cytoplasm. Adapted from ref 27. Copyright 2013 American Chemical Society.
mg/kg in a rat model, with no adverse clinical signs or alterations in body weight and serum chemistry parameters. A single dose of LNP containing 0.1 mg/kg siPCSK9 in cynomolgus monkeys led to 90% protein silencing and 50% decrease in plasma low-density lipoprotein cholesterol, which persisted for 2 months. In addition, the lipids were degradable in the liver and underwent rapid hepatic clearance in mice and non-human primates.

**Interaction of Ionizable Lipids with Cell Membranes and Intracellular Delivery of Nucleic Acid.** To initiate transfection, LNP must first interact with cell membranes and undergo internalization. The accepted mechanism for the transfection process is binding and endocytosis of LNP to target cell membrane, encapsulation of LNP in early endosomes, fusion of LNP with endosomal membrane, and perturbation and dissociation of nucleic acid into the cytoplasm. Endosomal escape is considered to be necessary to avoid trafficking and subsequent degradation in lysosomes and for efficient transfection; however, interestingly lipid fusion has not been correlated with transfection efficiency. This is perhaps due to incomplete release of the payload into the cytoplasm.

The ionization state of lipids is important for escape from early endosomes, and there is a correlation between pH of the endosomal environment and the narrow pKₐ range of successful ionizable lipids. In the acidic endosomal environment (pH 6), the amino group undergoes protonation, allowing for fusion with anionic endosomal phospholipids via ion pairing. This leads to formation of nonlamellar phases, preferentially the reversed hexagonal phase (Hₓ) and the subsequent destabilization of endosomal membranes.

The nature of the lipids constituting the LNP influences the dissociation of the nucleic from the ionizable cationic lipids; for example, chain saturation imparts rigidity and limits membrane fusion. In addition to ionizable cationic lipids, fusogenic lipids (DOPE, cholesterol) and PEGylated lipids are included in the formulation to optimize formation of nonlamellar structures and internalization into target cells and enable long circulation.

Techniques such as small-angle X-ray scattering and differential scanning calorimetry have been employed by Zhang et al. to investigate the phase behavior upon interaction between ionizable amino lipids and biomembranes. Bilayer disruption in the form of lamellar to inverted hexagonal phase transition was correlated with increasing ionizable amino lipid concentration, cholesterol-conjugated amino lipids, increasing aliphatic chain length from C₁₄–C₁₅, and longer tails with a cis double bond.

Red blood cell membrane lysis at pH 5.5 has been employed as a model for endosomal escape from LNP, where <10% was an indication of poor in *vivo* transfection and potent LNP (>50% lysis) had pKₐ values between 6 and 7. In a separate study, *in vitro* silencing data were shown to correlate well with membrane lysis in a model using membrane mimicking liposomes (45:20:20:15 DOPC/DOPE/DOPG/Chol) conducted at pH 5.5 and 7.4. DLinDMA liposomes exhibited pH-dependent lysis, with 86% lysis at pH 5.5 compared to 16% at pH 7.4.

**ADVANCES IN PRODUCTION OF LNP CONTAINING IONIZABLE CATIONIC LIPIDS**

**Microfluidic Mixing for Production of LNPs.** LNPs produced by microfluidic mixing are the most advanced LNP for systemic siRNA delivery. They are approximately 90 nm in diameter, and contrary to the aqueous core characteristic of liposomes, LNP possess an electron-dense core of inverted micelles with high encapsulation efficiency (Figure 6). This is the result of spontaneous self-assembly driven by electrostatic interaction of negatively charged nucleic acid with ionizable cationic lipids. Such complexes are termed lipoplexes, and their structure has been shown by NMR measurements, membrane fusion assays, density measurements, cryo-transmission electron microscopy, and computer simulations. LNPs are produced by microfluidic mixing, where siRNA in acetate buffer pH 4.0 is rapidly mixed with a mixture of lipids dissolved in ethanol to achieve 100% encapsulation efficiency. LNPs are well-tolerated and have been demonstrated to efficiently induce gene silencing in the mouse and non-human primate hepatocytes.
Lipidoid-Based LNPs. Lipidoids are synthetic, lipid-like molecules that can form liposomes and can be complexed with siRNA or miRNA to mediate gene silencing. Akinc et al. produced a combinatorial library of amino alkyl acrylate and -acrylamide-based lipidoids to assess the impact of alkyl chain length, linker and R group on the amine on luciferase and factor VII silencing. They reported that lipidoids with two amine headgroups and more than two alkyl chains (C₈−C₁₂) and an amide linker were shown to effectively deliver siRNA against luciferase in vitro to carcinoma cells.

The top-performing lipidoid compounds were formulated with cholesterol and PEG-lipid for in vivo delivery of siRNA. Decreased mRNA levels in the liver after systemic delivery of lipidoid-siFVII and lipidoid-siApoB formulations indicated specific and dose-dependent silencing occurred in mouse, rat, and non-human primate hepatocyte model. A decrease in FVII and ApoB expression in hepatocytes by 75−90% was observed in non-human primates and mice, with 50% silencing apparent for 14 days. In addition, silencing was observed following local in vivo delivery to lung and peritoneal macrophages, demonstrating utility in nonhepatic applications.

In another combinatorial study, epoxide-based lipidoid formulations effective in doses below 0.01 mg/kg were produced. These formulations contained cholesterol, DSPC, and PEG-DMG. A single IV injection of siRNA sequences against factor VII, ApoB, PCSK9, XBP1, and SORT1 formulated in C12-200 lipidoid formulation was shown to simultaneously inhibit gene expression of five hepatic genes in an in vivo mouse model. Greater than 65% silencing of all five genes was observed at a dose of 0.2 mg/kg per siRNA (1 mg/kg total siRNA dose) with no adverse side effects. The C12-200 lipidoid formulation with siRNA against transthyretin also exhibited specific gene knockdown in a non-human primate model at doses as low as 0.03 mg/kg, reflecting potential treatment for transthyretin amyloidosis.

More recently, ester-containing biodegradable lipidoid-based formulations comprising cholesterol, DSPC PEG-DMG, and 10 nM dose of siGAPDH were shown to induce >80% gene silencing 24−60 h post-transfection in an in vitro model of intestinal epithelium. The effects lasted for a week and did not induce cytotoxicity or alter intestinal barrier function.

The latest combinatorial studies by Knapp et al. and Whitehead et al. further shed light on structure−function relationship of lipidoid materials formulated with DSPC, cholesterol, and PEG-DMG. Over 1400 degradable lipidoids were synthesized and tested by Whitehead et al. The presence of tertiary and secondary amines, alcohols, branched or linear chains, and piperazine rings reduced firefly luciferase in HeLa cells. Finally, four necessary criteria were established for robust prediction of in vivo efficacy in gene silencing in mouse hepatocytes, monocytes, macrophages, and dendritic cells. These were an O₁₃ tail, ≥3 tails, alkylamine-
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"ApoB, apolipoprotein B; KSP, kinesin spindle protein; LNP, lipid nanoparticle; PCSK9, proprotein convertase subtilisin/kexin type 9; PLK1, polo-like kinase 1; PKN3, protein kinase N3; TTR, transthyretin; VEGF, vascular endothelial growth factor; VP24, Ebola viral protein 24; VP35, Ebola viral protein 35."
based with at least one tertiary amine, surface $pK_a$ of LNP $\geq 5.5$. Methacrylate and ether-containing tails length were found by Knapp et al. to be less efficient, postulated to be a result of unfavorable change in lipid tail alignment and subsequent nanoparticle packing by the methyl groups. The branched tail structure of isodecyl acrylate lipidoids were capable of inducing $>90\%$ in vitro and in vivo mouse FVII gene silencing at 40 nM and 0.1 mg/kg. Tail chemistry influenced surface $pK_a$ values of LNP, and tails that conferred higher $pK_a$ facilitate higher degree of gene knockdown. The same group also demonstrated transfection of B cells, which are notoriously difficult to transfect, in a study that is relevant for treatment of mantle cell lymphoma. Their potent 306O13 lipidoid, DSPC, cholesterol, PEG-DMG formulation containing siMcl-1 displayed dose-dependent gene downregulation. A dose of 100 mM reduced the gene expression by 80 and 50% 3 days post-transfection in JeKo-1 and MAVER-1 cell lines, respectively, and induced 30% apoptosis in both.

**Therapeutic Nucleic Acid Delivery.** Despite high in vitro silencing, recent therapeutically relevant studies have provided insight into the mechanism of siRNA uptake by cells and rate-limiting steps in delivery (Figure 7). Sahay et al. utilized confocal microscopy and tracked cellular uptake and intracellular transport of lipidoid-based LNP, formulated with cholesterol, DSPC, PEG-DMG, and labeled siRNA in HeLa cells. The particles were found to internalize by microinocytosis; however, 70% of the siRNA underwent endocytic recycling via late endosomes and lysosomes. This may account for a lack of correlation between the level of siRNA uptake and degree of silencing.

Using quantitative fluorescence imaging and electron microscopy, Gilleron et al. have shown that uptake of LNP in vitro occurs via micropinocytosis and clathrin-mediated endocytosis. In the same study, mice injected with LNP composed of ionizable lipid, cholesterol, DSPC, and PEG-DMG with siRNA conjugated with gold particles revealed that only 1–2% of internalized siRNA was released from moderately acidic, early endosomes. It is a testament to the reusability of the siRNA transcript by the machinery that even a low amount of siRNA can lead to significant knockdown. Together, these studies highlight areas in the design of LNP, which need further optimization to achieve efficient intracellular delivery. One strategy that has been explored is inhibition of Niemann-Pick type C1, a late endosomal/lysosomal membrane protein involved in endosomal recycling. During in vitro experiments, the compound NP3.47 was found to enhance intracellular uptake and colocalization of LNP-siRNA in cell lines. Nevertheless, efforts toward therapies for inflammatory disorders such as cardiovascular disease and cancer have demonstrated success in animal models due to uptake of LNP by macrophages. Systemic administration of LNP (C12-200/DSPC/Chol/PEG-DMG) silenced CCR2 mRNA in mice to inhibit recruitment of inflammatory monocytes. The treatment led to diminished size of myocardial infarction, reduced inflammatory atherosclerosis, and reduced tumor-associated macrophages and tumor size. Majmuda et al. found that these nanoparticles also improved healing after myocardial infarction and attenuated left ventricular remodeling. In another study with applications for the treatment of cardiovascular disease, a siRNA formulation (7C1 compound/C14PEG2000) simultaneously silenced five key adhesion molecules, Icam1, Icam2, Vcam1, Sele, and Selp, in atherosclerotic mice. Suppressed
leukocyte recruitment to atherosclerotic plaques and reduced inflammation after myocardial infarction were observed. In another study, mice treated with lipoid/Chol/PEG-lipid LNP encapsulated siRF5 resulted in dampened inflammatory effects of cardiac macrophages, downregulated expression of cytokines, improved infarct healing, and more rapid skin wound healing.\(^\text{81}\)

Novobrantseva \textit{et al.} demonstrated the therapeutic efficacy of siTNFα in an ionizable lipid/DSPC/Chol/PEG-DMG formulation by reduction in inflammatory cell infiltration, synovial joint inflammation, and edema in a murine model of rheumatoid arthritis.\(^\text{82}\) Furthermore, efficient and durable CD45 knockdown in macrophages of mouse and non-human primates was observed after administration of LNP containing siRNA.

In attempts to promote bone formation and treat progressive bone diseases, siRNA targeting the scleroste gene was delivered in DLin-MC3-DMA/DSPC/chol/PEG-DMG LNP \textit{in vitro} and IV to mice.\(^\text{83}\) The particles were effectively delivered to at least 50% of osteocytes, by confocal microscopy and knocked down serum sclerostin for 1 week. However, the potency of this system to silence genes in extrahepatic tissue and knocked down serum sclerostin for 1 week. However, the potency of this system to silence genes in extrahepatic tissue was much lower, and 15 mg siRNA/kg body weight was required, compared to the 0.005 mg/kg required in hepatocytes.\(^\text{84}\)

These studies have indicated that delivery of siRNA to immune cells such as monocytes and macrophages can alter disease progression, and a number of LNP formulations containing siRNA have already entered clinical trials for the treatment of hypercholesterolemia, solid tumors, transthyretin-meditated amyloidosis, and Ebola virus infection.\(^\text{85,86,84}\) (Table 1). However, the trial for ApoB was terminated as subjects developed flu-like symptoms consistent with activation of immune system, and the trials for Ebola virus terminated due to adverse Toll-like receptor-mediated immune response in one and lack of effectiveness in the phase II trial. ALN-TTR02 by Ahylam Pharmaceuticals is currently undergoing phase III testing to evaluate safety and efficacy and is set to be the first FDA-approved siRNA-based therapeutic.

**Active Cellular Targeting.** Targeting moieties such as antibodies, aptamers, proteins, and natural ligands can be attached to nanocarriers to enhance delivery to a specific cell population \textit{in vivo}. Active targeting provides the advantages of minimizing toxicity to neighboring cells and reducing the nucleic acid payload required for therapeutic benefit.\(^\text{85}\) There are reports in literature to support that active targeting does not change the overall pharmacokinetic profiles or biodistribution and others that suggest otherwise.\(^\text{86-90}\) Nevertheless, the improved therapeutic response is attributed to enhanced receptor-mediated uptake by the specific target cells to induce gene knockdown.

Antibody-mediated targeting has demonstrated success in gene silencing; for example, liposomes with targeted antibodies for T cell receptor molecule CD3 containing antisense TNA inhibited replication of HIV-1 \textit{in vitro}.\(^\text{91}\) Similarly, stabilized liposomes with lymphocyte function-associated antigen-1 antibody were revealed to deliver CCR5 siRNA to T cells and macrophages and protect from infection in a HIV mouse model.\(^\text{92}\)

Leukocytes are known to be difficult to transfect; however, recent studies with LNP coated with the targeting moiety leading to therapeutic gene silencing have been reported. Rameshetti \textit{et al.} used anti-CD4 monoclonal antibody as a targeting moiety on LNP and showed specific binding, uptake, and CD45 silencing in murine T lymphocytes following IV administration.\(^\text{93}\) In the study, a dose of 1 mg/kg, which is lower than other nontargeted systems to leukocytes, was effective in silencing T cells in the blood, spleen, bone marrow, and inguinal lymph nodes. Interestingly, it was also demonstrated that two CD4+ T cell populations exist, whereby internalization of the targeted LNP was observed by the CD4\textsuperscript{low} population, leading to CD45 knockdown, and localization of particles on the surface of the CD4\textsuperscript{high} population did not alter CD45 expression.

For B cell malignancy, anti-CD38 monoclonal antibody-coated LNP specifically delivered encapsulated siRNA against cyclin D1 to induce gene silencing in mantle cell lymphoma cells.\(^\text{94}\) The study showed that treatment suppressed tumor cell growth and prolonged survival of mice.

Hyaluronan (HA) is a natural ligand of the CD44 receptor, which is overexpressed on the surface of many forms of cancer cells. Local delivery of HA-coated LNPs containing siPLK1 were shown to specifically target CD44 cells in a murine glioma model.\(^\text{95}\) The treatment induced internalization of the LNP, robust PLK1 silencing, and cell death of glioma cells to prolong survival of animals. In a different study, HA-stabilized liposomes decorated with monoclonal antibody against γ7 integrin have been used as a carrier to deliver cyclin D1 siRNA into leukocytes to treat colitis in mice with efficacy in doses as low as 2.5 mg/kg.\(^\text{96}\)

Other examples of ligands used for targeting are GalNAc, which binds with high affinity to the asialoglycoprotein receptor on hepatocytes,\(^\text{97}\) lipoprotein ApoE, which absorbs to the surface of liposomes to facilitate receptor-mediated uptake into hepatocytes\(^\text{97}\) and folate to target cancer cells.\(^\text{98,99}\)

**Barriers to Clinical Translation.** Despite intense research, no nucleic-acid-based products exist on the market. The development of therapeutic products is reliant on establishing levels of specifications and controls to ensure product quality, stability, safety, and \textit{in vivo} performance. Analytical techniques are required to understand and characterize physicochemical properties of the particles, their biological interactions, and degradation. These include chemical composition, morphology, particle size, surface properties, phase behavior, polydispersity, and serum stability.\(^\text{100}\) Furthermore, control of formulation, processing, and storage conditions is critical to the robust and reproducible manufacturing of stable products.

Scale up of LNP formulations poses problems for translation due to alterations in mass transport processes and production conditions, resulting in particles with polydisperse size, morphology, and structure. Advancements in mixing technologies and production of microfluidic devices have been significant in nanoparticle manufacturing and \textit{in vitro} evaluation and screening, as previously reviewed.\(^\text{101}\) LNP encapsulating siRNA has been produced mixing preformed vesicles with siRNA in ethanol–water solutions\(^\text{102}\) and by mixing lipids dissolved in ethanol with an aqueous component containing siRNA in a T tube mixer.\(^\text{103,104}\) More recently, the staggered herringbone micromixer has enabled precise controlled production of nanoparticles in a reproducible manner, with potential for scale up by using mixing devices in parallel.\(^\text{105}\)

The flow rate controls the particle size, where small monodisperse particles are produced with faster mixing of the aqueous and ethanol solutions. During mixing, the increase in polarity leads to rapid nucleation of nanoparticles and conversion of the intermediate disk-like planar fragments to vesicles, preventing growth in particle size. Microfluidic mixing
is a versatile technique; it has been used to encapsulate a variety of macromolecules, such as mRNA, plasmid DNA, and gold nanoparticles into LNP. Furthermore, siRNA encapsulation efficiencies approaching 100% have been achieved, with potent in vivo gene silencing.

Another major issue precluding clinical use of cationic vectors and lipoplexes is toxicity and activation of the innate immune system. Positively and negatively charged liposomes incubated in sera activate the complement system and prolong clotting times. Cationic LNPs can invoke an immune response by activating Toll-like receptor 4 and stimulate production of inflammatory cytokines including interleukin-6 and tumor necrosis factor-α. The cytotoxicity and immunostimulation associated with cationic lipids has been largely addressed with the development of ionizable lipids and PEGylated lipids which shield the positive surface charge. The use of neutral lipids decreases toxicity and increases in vivo transfection efficiency. LNPs containing ionizable lipids have been shown to be well-tolerated in rodents and non-human primates, with no dose-dependent clinically significant changes in key serum chemistry or hematology parameters.

Animal models of human diseases are essential for the development and assessment of therapeutics. Rodents remain the most popular models due to their low cost, fast reproduction rate, and genetic accessibility; however, care must be taken when extrapolating to humans. Their small size makes it difficult to scale the dose from preclinical animal studies to humans, and systems that clear through kidney may scale with body weight, and those that clear through the mononuclear phagocyte system (MPS) may scale with body surface area. Preclinical studies for RNAi therapeutics in clinical trials used rodent and non-human primate as toxico logical species to determine the maximum tolerated dose.

CONCLUSION AND FUTURE PERSPECTIVE

Harnessing RNA interference as a therapeutic modality has merits to suppress expression of disease-initiating genes to combat currently untreatable human diseases, with less off-target effects than conventional small molecule agents. Lipid-based delivery systems have emerged as one of the most mature approaches to attain clinical translation. Advancements in the field such as synthesis of more potent and biocompatible ionizable lipids by combinatorial and screening approaches, and microfluidic engineering for controlled, rapid, and scalable nanoparticle production have been major stakeholders in the progression of a number of RNA therapeutics into clinical testing.

Currently, LNP formulations containing ionizable lipids have proven effective for liver-associated diseases, transthyretin-mediated amyloidosis and lipid metabolism diseases due to inherent accumulation via discontinuous vasculature. For extra-hepatic delivery, better receptor-specific targeting ligands that will internalize into target cells and release payload into the cytosol are critical. Optimization to enhance endosomal escape is also necessary for functional delivery. This could be achieved via use of incorporated endosomal escape enhancers such as pH-sensitive polymers or peptides or design of more potent and safe ionizable lipids. Elucidation of receptor–ligand interactions and underlying mechanisms of cellular uptake, endosomal trafficking, and recycling will undoubtedly play a role in future success in the field and aid in the rational design of safe, specific, and efficient lipid-based carriers for RNAi therapeutics.

REFERENCES


