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Lipid Nanoparticle Technology for Clinical Translation of siRNA Therapeutics

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Jayesh A. Kulkarni,[†,‡,#] Dominik Witzigmann,[†,#] Sam Chen,[†,§] Pieter R. Cullis,[*,†] and Roy van der Meel[†,¶,⊥]

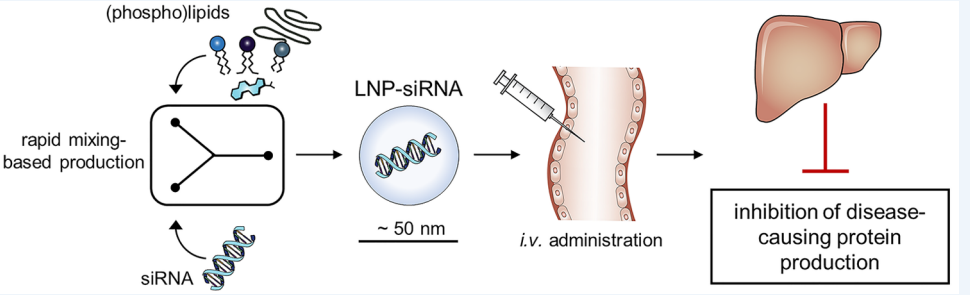
[†]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

[‡]Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, BC Children's Hospital Research Institute, University of British Columbia, Vancouver, BC V5Z 4H4, Canada

[§]Integrated Nanotherapeutics, Vancouver, BC V6T 1Z3, Canada

[¶]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht 3584 CX, The Netherlands

[⊥]Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex Molecular Systems, Eindhoven University of Technology, Eindhoven 5612 AE, The Netherlands



CONSPECTUS: Delivering nucleic acid-based therapeutics to cells is an attractive approach to target the genetic cause of various diseases. In contrast to conventional small molecule drugs that target gene products (i.e., proteins), genetic drugs induce therapeutic effects by modulating gene expression. Gene silencing, the process whereby protein production is prevented by neutralizing its mRNA template, is a potent strategy to induce therapeutic effects in a highly precise manner. Importantly, gene silencing has broad potential as theoretically any disease-causing gene can be targeted. It was demonstrated two decades ago that introducing synthetic small interfering RNAs (siRNAs) into the cytoplasm results in specific degradation of complementary mRNA via a process called RNA interference (RNAi). Since then, significant efforts and investments have been made to exploit RNAi therapeutically and advance siRNA drugs to the clinic.

Utilizing (unmodified) siRNA as a therapeutic, however, is challenging due to its limited bioavailability following systemic administration. Nuclease activity and renal filtration result in siRNA's rapid clearance from the circulation and its administration induces (innate) immune responses. Furthermore, siRNA's unfavorable physicochemical characteristics largely prevent its diffusion across cellular membranes, impeding its ability to reach the cytoplasm where it can engage the RNAi machinery. The clinical translation of siRNA therapeutics has therefore been dependent on chemical modifications and developing sophisticated delivery platforms to improve their stability, limit immune activation, facilitate internalization, and increase target affinity.

These developments have resulted in last year's approval of the first siRNA therapeutic, called Onpattro (patisiran), for treatment of hereditary amyloidogenic transthyretin (TTR) amyloidosis. This disease is characterized by a mutation in the gene encoding TTR, a serum protein that transports retinol in circulation following secretion by the liver. The mutation leads to production of misfolded proteins that deposit as amyloid fibrils in multiple organs, resulting in progressive neurodegeneration. Patisiran's therapeutic effect relies on siRNA-mediated TTR gene silencing, preventing mutant protein production and halting or even reversing disease progression. For efficient therapeutic siRNA delivery to hepatocytes, patisiran is critically dependent on lipid nanoparticle (LNP) technology.

In this Account, we provide an overview of key advances that have been crucial for developing LNP delivery technology, and we explain how these developments have contributed to the clinical translation of siRNA therapeutics for parenteral administration. We discuss optimization of the LNP formulation, particularly focusing on the rational design of ionizable cationic lipids and

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poly(ethylene glycol) lipids. These components have proven to be instrumental for highly efficient siRNA encapsulation, favorable LNP pharmacokinetic parameters, and hepatocyte internalization. Additionally, we pay attention to the development of rapid mixing-based methods that provide robust and scalable LNP production procedures. Finally, we highlight patisiran's clinical translation and LNP delivery technology's potential to enable the development of genetic drugs beyond the current state-of-the-art, such as mRNA and gene editing therapeutics.

1. INTRODUCTION

Introducing exogenous nucleic acids into cells to modulate gene expression, such as gene silencing, is an attractive approach to achieve highly specific and potent therapeutic effects. Following the discovery of RNA interference (RNAi)[1] and the subsequent demonstration that small interfering RNA (siRNA)[2] can transiently induce specific mRNA (mRNA) degradation, tremendous efforts have been made in the last two decades to exploit gene silencing therapeutically.

These efforts have resulted in last year's approval of the first-ever siRNA drug, named Onpattro (patisiran), for treatment of hereditary amyloidogenic transthyretin (ATTRv) amyloidosis. This disease is characterized by hepatocytic production of mutant TTR proteins, which deposit as amyloid fibrils in multiple organs resulting in progressive neurodegeneration.[3] Patisiran is critically dependent on lipid nanoparticle (LNP) technology for delivering TTR siRNA to hepatocytes in the liver and inhibit protein production following systemic infusion. LNPs protect the siRNA from degradation and ensure their stability in the circulation, reduce immune activation, enable localization to the target tissue, and facilitate intracellular delivery. LNP delivery systems are typically around 50 nm in diameter and composed of ionizable cationic lipids, cholesterol, phospholipids, and poly(ethylene glycol) (PEG)-lipids (Figure 1). All of these components have been optimized to efficiently deliver siRNA into the cytoplasm of hepatocytes where it can engage the RNAi machinery and subsequently inhibit specific mRNA translation.

In this Account, we provide an overview of factors that have been crucial for successfully developing LNP technology and its clinical application that resulted in approval of the first siRNA therapeutic. These include the rational design of ionizable cationic lipids[4,5] and understanding their structure−function relationship, application of diffusible PEG-lipids, and development of a robust, scalable self-assembly manufacturing process.[6,7] We also discuss our current understanding of the self-assembling LNP formation process and provide functionalization strategy examples. Finally, we highlight important features of patisiran's clinical success and the potential to exploit LNP technology for developing mRNA (mRNA) or gene editing therapeutics. Of note, for other important developments, such as chemical RNA modifications, and alternative gene silencing approaches, including GalNAc−siRNA conjugates and antisense oligonucleotides (ASO), the reader is referred to several excellent recent review articles.[8,9]

2. LIPID NANOPARTICLE COMPOSITION

LNP systems have evolved substantially over the past 25 years from initial formulations composed primarily of phospholipids and cholesterol. Building on the LNP design parameters established while developing lipid-based carrier systems for small molecule therapeutics, nucleic acid delivery requires additional functionalities. These include components for entrapping nucleic acids efficiently, maintaining a neutral surface charge in circulation, and evading immune clearance for successful delivery in vivo. The synthesis of ionizable cationic lipids, modifications to the lipid composition, and development of diffusible PEG-lipids are discussed in this section.

2.1. Ionizable Cationic Lipid Development and Lipid Nanoparticle Composition

Ionizable cationic lipids play several important roles in LNP-based siRNA delivery. First, under acidic conditions, the lipid is positively charged, as required to entrap the negatively charged nucleic acid polymers within the nanoparticle. Second, the lipid has to produce LNPs with an apparent acid-dissociation constant (pK_a) such that at physiological pH values the overall LNP surface charge is close to neutral. Third, the lipid must exhibit a positive charge in an acidified endosome (pH ≈ 5−6) in order to interact with endogenous anionic lipids. Finally, to efficiently destabilize the endosomal membrane and deliver the nucleic acid payload, the lipid must exhibit a physical shape that promotes the formation of the hexagonal (H_{II}) lipid phase.[10] As such, rational lipid design was combined with an iterative screening process to determine the optimal combination of acyl chains, linkers, and ionizable head groups.

An initial study comparing analogues of 1,2-dioleyloxy-*N*,*N*-dimethyl-3-aminopropane (DODMA) with varying degrees of unsaturation in the acyl chains determined that linoleyl ($C_{18:2}$) chains offered an optimal combination of particle uptake, intracellular delivery, and apparent pK_a.[11] The linoleyl chains were hypothesized to produce a lipid with an inverted cone geometry with a higher propensity for adopting nonbilayer phases such as the H_{II} phase. The first LNPs that resulted in significant hepatocyte gene silencing *in vivo* contained 1,2-dilinoleyl-*N*,*N*-dimethyl-3-aminopropane (DLinDMA, Figure 2A),[12] but the formulations displayed relatively low potency requiring high doses of siRNA.

A murine factor VII (FVII) model[13] was employed to screen and select progressively more potent ionizable lipids with a focus on headgroup and linker region chemistry. FVII is a blood clotting protein produced by hepatocytes and secreted into the circulation (as occurs with TTR) with a half-life of 5−6 h.[14] Therefore, the LNP−siRNA formulations' potency could be determined by measuring FVII levels in serum 24 h after systemic administration with optimized formulations requiring lower siRNA doses to achieve 50% gene silencing (ED_{50}). In an effort to vary the linker region to exhibit differential chemical and enzymatic stability, a lipid based on ester linkages (1,2-dilinoleoyl-3-dimethylaminopropane; DLinDAP) was synthesized that displayed an even lower gene silencing potency. It was later suggested that endogenous lipase digestion of DLinDAP resulted in transfection-incompetent LNPs.[15] The introduction of a ketal ring into the linker region of the lipid to form 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (KC2, Figure 2A) resulted in improved lipid stability and gene silencing potency (ED_{50} 0.02 mg/kg). This indicated that ionizable cationic lipids with pK_a values in the range of 6.2−6.7 resulted in optimal gene silencing.[4]

Subsequent screening of a large number of ionizable cationic lipids with varied headgroup and linker structure resulted in

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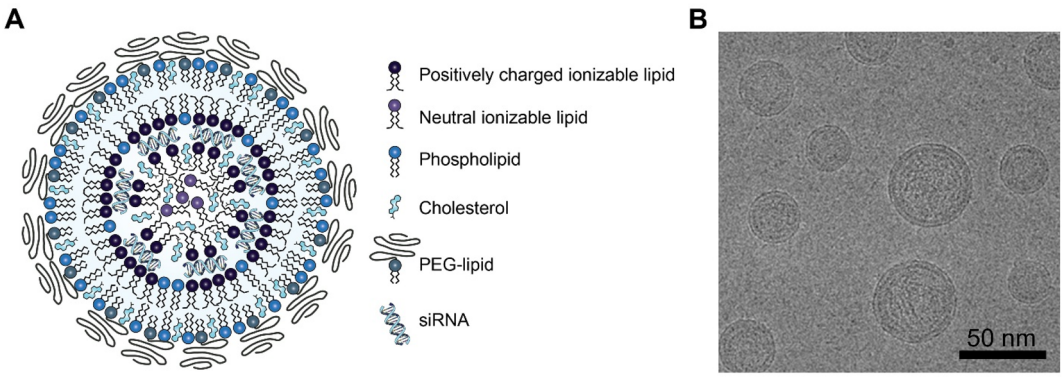

Figure 1. Lipid nanoparticle delivery platform. (A) Schematic representation and (B) cryogenic electron micrograph of a lipid nanoparticle containing siRNA including key lipid components. Adapted with permission from ref 35. Copyright 2018 American Chemical Society.

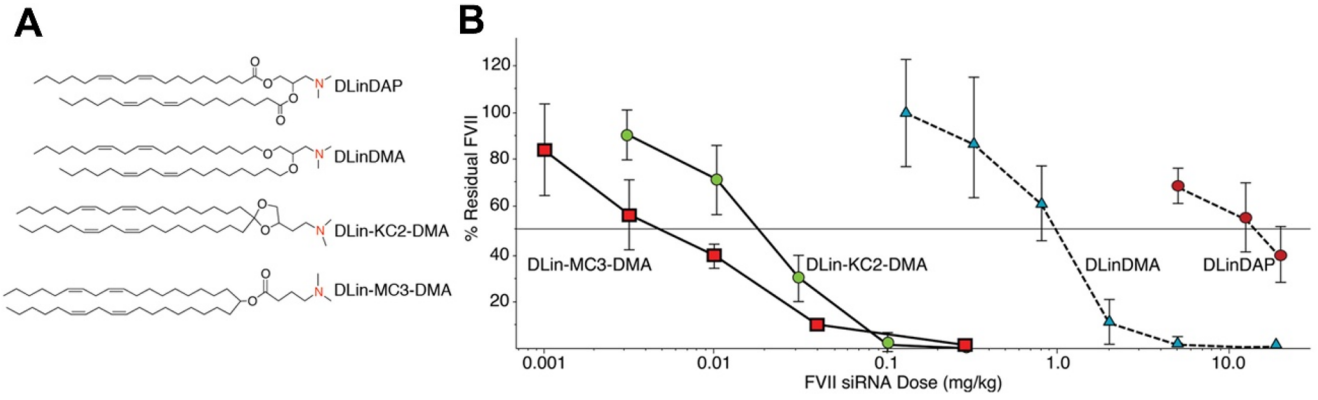

Figure 2. Evolution of ionizable cationic lipids enabling gene silencing *in vivo*. (A) DLinDMA, an ether-linked variant of DLinDAP, was the first lipid that enabled appreciable LNP-siRNA gene silencing activities *in vivo* following intravenous administration. Subsequent studies identified KC2 and MC3 lipids as potent successors with the optimal structure and pK_a necessary for siRNA delivery. (B) *In vivo* optimization of LNP-siRNA systems using the FVII mouse model. Over 300 species of ionizable cationic lipids were synthesized, formulated in LNP-siRNA, and screened in the FVII model. This led to the identification of the current "gold standard" lipid MC3, which resulted in improved LNP-siRNA potency by 4 orders of magnitude compared to DLinDAP, with no increase in toxicity. MC3 was subsequently employed in the patisiran formulation.[4,5,17]

Table 1. Influence of PEG-Lipid on Pharmacokinetics and Liver Accumulation[a]

PEG-lipid type	mol %	composition	payload	blood $t_{1/2}$ (h)	liver accumulation (24 h, %)	ref
PEG-DMG	1.5	DMAP-BLP/DSPC/Chol/PEG-DMG (50:10:38.5:1.5 mol %)	siRNA	<0.25	91.4	20
	5	DMAP-BLP/DSPC/Chol/PEG-DMG (50:10:35:5 mol %)	siRNA	<0.25	83.6	20
	10	DODAC/DOPE/PEG-s-DMG (7.5:82.5:10 mol %)	plasmid	1	35[b]	18
PEG-DSG	1.5	DMAP-BLP/DSPC/Chol/PEG-DSG (50:10:38.5:1.5 mol %)	siRNA	<0.5	68.6	20
	5	DMAP-BLP/DSPC/Chol/PEG-DSG (50:10:35:5 mol %)	siRNA	10.9	34	20
	10	DODAC/DOPE/PEG-s-DSG(7.5:82.5:10 mol %)	plasmid	15	20[b]	18

[a]Chol, cholesterol; DMAP-BLP, 3-(dimethylamino)propyl(12*Z*,15*Z*)-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]henicosa-12,15-dienoate; DODAC, *N*,*N*-dioleoyl-*N*,*N*-dimethylammonium chloride; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; PEG-DMG, 1,2-dimyristoyl-*rac*-glycero-3-methoxy-poly(ethylene glycol); PEG-DSG, distearoyl-*rac*-glycerol-poly(ethylene glycol). [b]Assuming 1 g of liver.

LNP formulations that improved FVII gene silencing potency by nearly 4 orders of magnitude compared to DLinDAP (Figure 2B). A key advance was the identification of heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (MC3, Figure 2A). MC3 enabled extremely potent hepatic gene silencing with an ED_{50} as low as 0.005 mg of siRNA per kilogram of body weight for mice and less than 0.03 mg/kg for non-human primates.[5]

In addition to the ionizable cationic lipid structure, the LNP's overall composition also evolved. Previous generations of LNPs were composed of ionizable cationic lipids, phospholipid, cholesterol, and PEG-lipids in a ratio of 20/25/45/10 mol %, respectively.[16] While these formulations displayed high entrapment efficiencies, they performed poorly in terms of gene silencing (especially for hepatocyte targets). Modifications to the formulations led to particles with the molar composition of 40/10/40/10 mol %, which improved liver accumulation and gene silencing activity.[4] Further adjustments of the composition to 50/10/38.5/1.5 mol % resulted in a 6-fold improvement in hepatocyte gene silencing.[5]

2.2. Diffusible PEG-lipids: Endogenous Hepatocyte Targeting and Immune Reactions

Another key component that determines LNP−siRNA systems' transfection competency is the PEG-lipid. Optimization of the

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PEG-lipid has benefited from earlier work on LNP systems for plasmid and antisense oligonucleotide delivery. The role of these lipids in LNP formulations was found to be 2-fold. First, PEG-lipid content dictated particle size, where an increased PEG-content decreased particle size.[6] Second, a PEG-lipid was required to prevent aggregation during particle formation and in complex biological medium.[18] However, for the purposes of transfection, PEG-lipids were counter-productive, inhibiting uptake into target cells and preventing endosome destabilization.[19] To find an optimal balance between stability and transfection competency, diffusible PEG-lipids that contain C_{14} acyl chains were developed.[18] These lipids rapidly dissociated from the LNP in the presence of a lipid reservoir (such as serum lipoproteins), generating transfection competent LNPs.[20] Thus, the use of diffusible PEG-lipids resulted in LNP systems that were stable in storage and retained their transfection competency upon administration.

In preclinical studies, it was determined that LNPs containing diffusible PEG-lipids rapidly accumulate in the liver, with circulation half-lives of less than 15 min (Table 1).[20] LNP hepatocyte accumulation and transfection potency stemmed from apolipoprotein E (ApoE) adsorption to the particles' surface, resulting in uptake by ApoE-dependent low density lipoprotein receptors (LDLR) on the sinusoidal surface of hepatocytes. This is supported by the observation that LNP−siRNA activity was compromised in an ApoE knockout model ($ApoE^{-/-}$) but could be rescued with ApoE supplementation prior to administration.[21]

A further benefit of the diffusible PEG-lipid component is the abrogation of PEG-induced antibodies that can affect clearance behavior. Accelerated blood clearance of PEGylated nanoparticles has been observed following repeated administrations. This results from an antibody response against the PEGylated material after the first dose.[22] It should be noted that patisiran was well-tolerated with reproducible pharmacokinetics following repeated administrations in a clinical setting.[23] Thus, diffusible PEG-lipids played a major role in determining the preclinical and clinical success of LNP−siRNA.

2.3. Persistent PEG-lipids and Extrahepatic Targets

While LNP technology has been optimized for siRNA-mediated hepatic gene silencing, the ability to knockdown target genes in extrahepatic tissues has been pursued by modulating the formulation's PEG-lipid component. For example, a preliminary study demonstrated *SOST* silencing in compact bone.[24] At a dose of 15 mg/kg (3000-fold higher than for hepatocytes; nearing the maximum tolerated dose) with LNP containing PEG-C_{14}, only modest knockdown was achieved. Following intravenous administration, clinical LNP−siRNA formulations are heavily entrenched in the ApoE−LDLR pathway. As such, modifying the LNP biodistribution to extrahepatic targets required changes to their size and composition. Thus, the aim was to increase circulation time and accumulation at target tissues without loss of efficacy. A key development was incorporation of persistent PEG-lipids (composed of C_{18} acyl chains rather than C_{14}) at higher molar ratios than previously used, though this was deemed to be a "double-edged sword". Merely replacing 1.5 mol % PEG-C_{14} with PEG-C_{18} did not sufficiently alter hepatic gene silencing or pharmacokinetic properties of LNPs.[25] However, increasing the PEG-C_{18} content to 2.5 or 5 mol % resulted in drastically improved circulation times, up to ∼10−12 h (Table 1).[20] While this approach improved LNP circulation, the particle functionality was compromised with a 10-fold drop in potency (ED_{50} of 0.8 mg/kg).[20,25] Partially rescuing the performance of such particles required an increase in the amount of ionizable lipid from 50 to 60 mol % and an increase in the amine-to-phosphate ratio from 3 to 6. This allowed a 3-fold improvement of the ED_{50} to 0.3 mg/kg.[20]

While the transfection potency of such systems has not reached levels that justify clinical translation, preliminary studies have established their potential in distal tumor models. Systems containing 2.5−5 mol % PEG-lipid have been used to knockdown the androgen receptor in a prostate cancer xenograft model with effective reduction in serum prostate specific antigen levels.[26] Another study combined LNP−siRNA against clusterin with antisense oligonucleotides against the androgen receptor to treat an enzalutamide-resistant model of prostate cancer.[27]

3. PRODUCTION, CHARACTERIZATION, AND FUNCTIONALIZATION OF LIPID NANOPARTICLES

3.1. Production: Emergence of Rapid-Mixing Techniques

Clinical application of LNP−siRNA systems required formulation processes that afforded rigorous control over manufacturing, high entrapment efficiencies, high-throughput synthesis, and reproducibility.[10] As such, the methods of generating LNP−nucleic acid formulations have undergone significant improvements. Starting with the "dump and mix" methods in a test tube[28] or the detergent-dialysis technique,[29] various iterations suggested rapid-mixing technologies as likely to fulfill all criteria. The rapid-mixing procedures currently used have evolved (alongside the lipid composition) to enhance entrapment of nucleic acids, limit tedious manufacturing steps, and improve LNP physicochemical properties all-the-while maintaining and improving LNP potency.

The first iteration of LNP formulations for encapsulating nucleic acid relied on detergent dialysis. This resulted in ∼70% plasmid DNA entrapment but the use of detergents limited translation. Further developments showed that similar particles could be generated using an ethanol-loading technique. This involved mixing preformed liposomes with oligonucleotides (at pH 4) in the presence of high (∼40% by volume) ethanol concentrations.[30] Drawing from this knowledge, and an older technique introduced by Batzri and Korn[31] that involved forming vesicles by introducing lipids dissolved in ethanol into an aqueous medium, high-throughput processes were developed. These procedures, generally termed "rapid-mixing", bring together two fluid streams where one contains lipids in an organic phase, and the second stream contains the nucleic acid in an aqueous phase. At first, a T-junction apparatus was employed.[32] Later, studies suggested that microfluidic approaches (also based on rapid-mixing) can also be used to generate LNP composed of triglycerides[7] and then siRNA.[6,33] In all cases, LNP formation relies on dilution of the organic phase into the aqueous phase.

The key determinant for the success of such mixing techniques was controlling the local mixing environment. While macroscopic mixing methods resulted in heterogeneous particles with broad size distributions, rapid-mixing methods provided a high-throughput and continuous approach for synthesizing nanoparticles from the bench-scale to clinical volumes (through parallelization of mixers). These technological advances together culminated with the first report of successful RNAi in non-human primates in 2006.[12]

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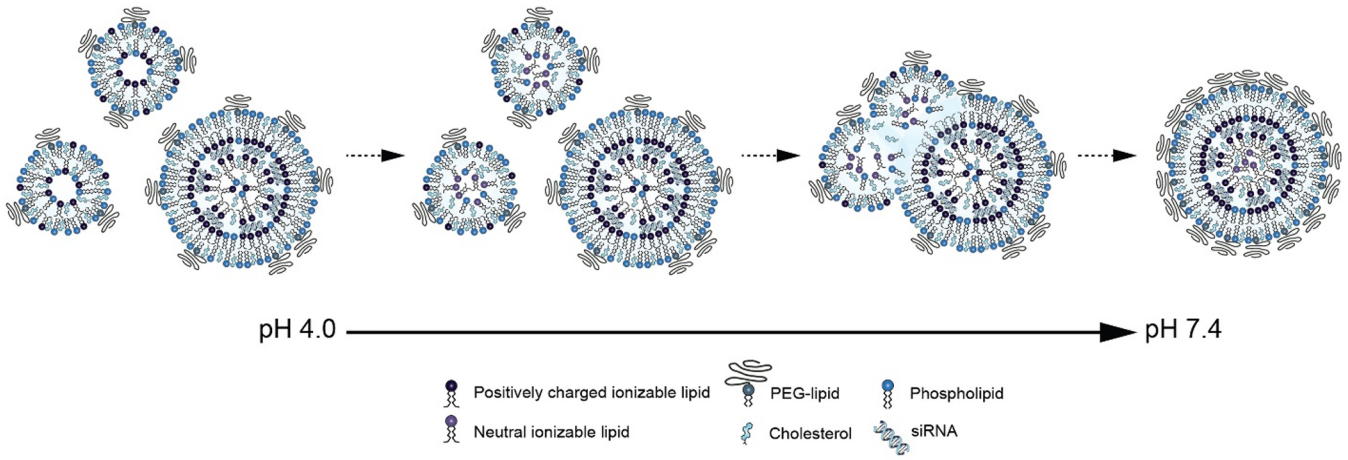

Figure 3. Lipid nanoparticle formation and structure. (A) Proposed LNP-siRNA structure consisting of an oil core. Rapid-mixing techniques generate small liposomal structures and particles containing siRNA in a lamellar arrangement in acidic buffer (pH 4.0; left). Through neutralization of the pH to generate the final LNP systems, the insolubility of the neutralized ionizable cationic lipid within the membrane generates an oil-phase (left-center). These metastable particles continue to fuse (right-center) until the surface of the LNP is coated with PEG-lipid. The final LNP-siRNA structure consists of siRNA sandwiched between layers of lipid and a lipid core consisting of neutralized ionizable cationic lipid and cholesterol. Reproduced from ref 35. Copyright 2018 American Chemical Society.

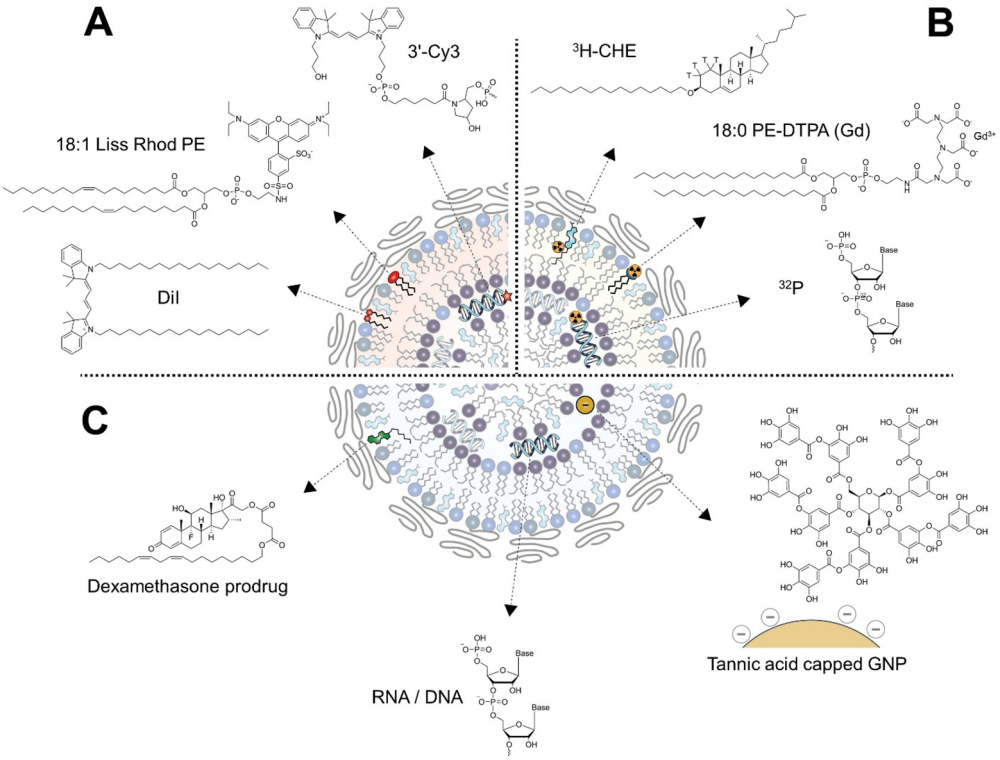

Figure 4. Lipid nanoparticle functionalization. (A) Fluorescence tagging of LNP systems is enabled using dialkylcarbocyanine derivatives (e.g., DiI) or fluorophore-conjugated phospholipids (e.g., 18:1 Liss Rhod PE) or by encapsulating fluorescently labeled siRNA (e.g., Cy3-labeled). (B) LNP systems are amenable to radiolabeling using tritiated lipid components (e.g., ^{3}H-CHE), incorporation of phospholipids chelating radiotracers (e.g., 18:0 PE-DTPA Gd), or radiolabeled siRNA (e.g., ^{32}P). (C) Different payloads can be entrapped in LNP systems including lipid-modified prodrugs (e.g., dexamethasone prodrugs), different nucleic acids (e.g., siRNA, mRNA, mcDNA, pDNA), or negatively charged nanoparticles (e.g., tannic acid capped gold nanoparticles, GNPs).

3.2. Characterization: Studies on LNP Morphology

Initial studies on the structure of LNP formulations revealed that particles are observed as electron-dense structures under cryogenic transmission electron microscopy (cryo-TEM).[33] The ability of rapid-mixing methods to generate electron-dense structures was attributed to a bottom-up assembly of the particles (as compared to traditional top-down manufacturing).[6] An initial proposal suggested that the mixing efficiency afforded by these methods results in an increase in the polarity of the medium achieving a state of supersaturation of lipid monomers.[6] Such events lead to nucleation and homogeneous particle formation on time scales that are much faster than those required for aggregation.[6,33] The resulting particle was hypothesized to contain a nanostructured core of inverted

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micellar structures encasing the siRNA, and the proposal was later extended to mRNA, plasmid DNA, and gold nanoparticles (GNPs).[34]

Recent studies have suggested that while entrapment and particle formation initially occur during the mixing of an organic−lipidic phase with the nucleic acid in aqueous buffer, the final structure of the LNP is only formed following neutralization of the pH.[35,36] More importantly, a fusion process is involved in the formation of such nanoparticles, and one component (i.e., the PEG-lipid) largely dictates the final particle size. This work also suggests that the LNP structure includes a hydrophobic oil-core consisting primarily of neutral ionizable lipid surrounded by siRNA complexed to lipids in a bilayer arrangement.[35] In addition to these observations, two separate studies determined that the amount of cholesterol in a typical LNP formulation is in excess of the amount that is soluble within the membrane, resulting in cholesterol crystals.[35,37] The proposed mechanism of LNP formation and the resulting structure is shown in Figure 3. This mechanism of formation has since been extended to the formation of particles containing mRNA, minicircle DNA, or plasmid DNA and to GNPs.[36] It should be noted that this new proposal is consistent with all previously generated empirical data except molecular modeling approaches (Figure 3).[38]

3.3. Functionalization: Modifications to Provide Additional Utility

Given their straightforward manufacturing and design process, LNP systems are particularly amenable to modifications for various applications. Here, we provide an overview of established LNP modifications for adding functionalities and imaging or tracking applications. We focus on the use of fluorophores, radiolabels, and various payloads.

Fluorescently labeled LNPs can generated by using fluorophore-conjugated lipids, (hydrophobic) fluorophore incorporation, or entrapment of a fluorescent (siRNA) payload (Figure 4A). Fluorescent tags offer several advantages such as enabling routine imaging methods[39] and providing structural and mechanistic insights.[33] The most commonly used hydrophobic tracers are dialkylcarbocyanine derivatives (DiO, DiI, DiD, DiR). These tracers cover a broad range of excitation (484−750 nm) and emission (501−780 nm) wavelengths. A key benefit of these dyes is that very little dissociation from the LNP is observed (even in complex media) making them powerful tracking tools.[26,39] Fluorophore-conjugated phospholipids (cyanine, rhodamine, nitrobenzoxadiazole-based) also readily associate with LNPs although their stability within the particles has been less well characterized. An alternative is labeling the siRNA with cyanine, fluorescein, or Alexa-Fluor-based dyes to assess successful intracellular delivery or transfection efficiency.[40] The use of fluorescently labeled siRNA has been essential in understanding cellular processing of LNP−siRNA.[15] Previous studies suggest that up to 70−80% of internalized siRNA is exocytosed.[41,42] Using Förster resonance energy transfer, labeled siRNAs were used to monitor the disassembly of LNP−siRNA within the cell as well.

LNPs are also amenable to incorporation of radiolabels, which offer significant advantages that make such labels irreplaceable (Figure 4B). Radiolabeling offers unparalleled sensitivity, enables robust quantification (regardless of environment), and allows for tracking each LNP's component without drastically modifying the chemical structure or composition. Given the low aqueous volume of LNPs, only hydrophobic or amphipathic tracers are used. As lipid remodeling or dissociation occurs in the complex biological environment, it is important to verify that the tracer remains associated with the LNP over the time frame of interest. Tritiated-cholesteryl hexadecyl ether (^{3}H-CHE) has been widely used as it satisfies two essential requirements: it is nonexchangeable and non-biodegradable.[43] In LNP systems, ^{3}H-CHE displays no dissociation while other radiolabeled components such as MC3, DSPC, and PEG-lipid dissociate at variable rates.[20] An additional benefit is that only trace amounts of ^{3}H-CHE (<0.2 mol %) are needed to study the LNPs' pharmacokinetic parameters and biodistribution following parenteral administration. Other radioisotope labeling strategies involving positron or γ-emitters, such as ^{111}In-, ^{99m}Tc-, or ^{68}Ga-bound diethylenetriamine pentaacetate (DTPA) conjugated phospholipids,[44] are useful for noninvasive radionuclide imaging. Furthermore, the siRNA payload can also be radiolabeled. The most common method is to label the RNA 5′ end with ^{32}P through a phosphate transfer with ^{32}P-ATP. Structurally, the siRNA remains unchanged. Other methods of radiolabeling siRNA require the conjugation of chelators such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or DTPA.

Finally, LNPs can entrap a variety of payloads ranging from hydrophobic compounds to anionic macromolecules (Figure 4C). Hydrophobic small molecules can be conveniently formulated into LNPs, similar to the aforementioned fluorescent tags. For compounds that are not sufficiently hydrophobic, a lipid-modified prodrug strategy could be used to improve LNP association.[45] Furthermore, LNP composition and manufacturing allow for the simple replacement of the siRNA with larger nucleic acids (mRNA,[34] minicircle DNA,[36] and plasmid DNA[34,46]) or negatively charged GNP. Entrapment of alternative nucleic acids has demonstrated preclinical utility in protein replacement, vaccine, and gene editing applications. Metallic nanoparticles with unique electronic and optical properties allowed LNP to be used for imaging and therapeutic applications. Entrapment of GNP allowed for structural and mechanistic characterization of the LNP[34,36] but also improved the intracellular delivery of GNP for enhancing radiotherapy.[47]

4. CLINICAL APPLICATION OF LNP−siRNA: ONPATTRO (PATISIRAN)

Last year, the FDA approved Onpattro (patisiran) for treatment of hereditary (ATTRv) amyloidosis. Patisiran is the first approved siRNA drug and provides a treatment for an otherwise fatal hereditary disease that affects an estimated 50 000 patients worldwide. TTR is a serum protein produced in the liver that is responsible for the transport of retinol in the circulation. The disease is characterized by deposition of mutated TTR as amyloid fibrils in multiple organs, particularly nerve tissue, resulting in progressive neurodegeneration. Patisiran, which halts and reverses this neurodegeneration, provides the first definitive hope for patients suffering from hereditary TTR amyloidosis with polyneuropathy.

Following the optimization and preclinical evaluation of LNP−siRNA systems, two formulations were evaluated in a placebo-controlled phase I trial for treatment of ATTRv amyloidosis to determine their safety and efficacy.[17] A single dose of DLinDMA-based ALN-TTR01 (0.01 to 1 mg/kg) was infused intravenously to 32 (24:8) ATTRv amyloidosis patients, while MC3-based ALN-TTR02 (0.01 to 0.5 mg/kg) was administered to 17 (13:4) healthy volunteers. Results showed

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that ALN-TTR01 at a dose of 1 mg/kg was able to significantly suppress TTR levels by a mean reduction of 38% compared to placebo after 7 days. The ALN-TTR02 formulation was more effective and at doses of 0.15 and 0.3 mg/kg suppressed TTR levels >80% compared to placebo, with reductions >50% after 28 days.[17]

In a phase II study, a total of 29 ATTRv amyloidosis patients received 2 systemic infusions of patisiran (ALN-TTR02) at a dose of 0.01−0.3 mg/kg every 4 weeks or 0.3 mg/kg every 3 weeks (Q3W). Multiple patisiran administrations were generally well tolerated with most adverse events being infusion-related. A mean level of TTR knockdown >85% was achieved after the second dose for the Q3W protocol.[23]

In the randomized, double-blind, placebo-controlled phase III APOLLO study, 148 patients received patisiran at a dose of 0.3 mg/kg Q3W and 77 patients received placebo infusions. The primary end point was the change from baseline in the modified Neuropathy Impairment Score+7 (mNIS+7), used to quantify polyneuropathy, after 18 months. Results indicated that in patients who received patisiran, the median reduction in serum TTR levels during the 18 months was >80%. The sustained reduction in TTR levels resulted in a change from baseline in the mNIS+7 that was significantly lower for the patisiran treatment group compared to the placebo treatment group, indicating a beneficial effect regarding polyneuropathy and halting disease progression. These effects were observed after 9 months of treatment. Importantly, significant favorable differences of patisiran treatment compared to placebo were also observed for all secondary end points, such as quality of life (Norfolk Quality of Life Diabetic Neuropathy questionnaire score), motor strength, disability, gait speed, nutritional status, and patient-reported autonomic symptoms. Both treatment groups reported adverse events that were mostly mild or moderate in severity. The frequency of severe and serious adverse events in both treatment groups was comparable.[3,49]

A critical consideration during patisiran's clinical translation was the role of infusion-related reactions (IRSs), which are well-known for macromolecular drugs such as micellar complexes, monoclonal antibodies, and LNPs.[48] IRSs are most common following the first dose, with patients becoming more tolerant of subsequent doses. Notably, there are no preclinical models to precisely predict IRSs in humans. In the clinic, specific pretreatment regimens involving corticosteroids, antihistamines, and acetaminophen are used to mitigate these reactions. Furthermore, IRSs are significantly reduced by simply slowing the infusion rate. In the case of patisiran, patients are premedicated before infusion with dexamethasone, oral acetaminophen/paracetamol, an H_2 blocker, and an H_1 blocker.[49] The main symptoms include flushing, backpain, abdominal pain, and nausea, all of which were described as mild-to-moderate in the patisiran trials, and the frequency of the reactions decreased over time as expected. While this is typically associated with administration of nanoparticles, strategies to overcome these side effects are already under development. Chen et al. have recently demonstrated that a hydrophobic prodrug version of dexamethasone can readily be incorporated in LNPs containing nucleic acids and provide effective immunosuppression.[45]

5. CONCLUSIONS AND OUTLOOK

Developing LNP delivery technology has proven to be instrumental for translating the first siRNA therapeutic to the clinic,[3] 20 years following the discovery of RNAi.[1] Patisiran's approval clearly demonstrates that LNP technology can be applied to achieve robust RNAi-mediated therapeutic effects for disorders caused by production of pathological proteins in the liver. With LNP design parameters, scalable production methods, and structure−function relationship now well established, it is expected that additional LNP−siRNA therapeutics will be developed for silencing disease-causing genes in hepatocytes, for example, knockdown of proprotein convertase subtilisin/kexin type 9 for treatment of hypercholesterolemia.[50] A considerable challenge that remains is achieving clinically relevant gene silencing levels in non-hepatic tissues, which would significantly increase the range of indications that can be treated with siRNA therapeutics.

At the same time, LNP technology is now being exploited for developing treatments that express therapeutic proteins or edit genes by delivering mRNA or components of the CRISPR/Cas9 system, respectively. Harnessing the efficient ApoE-mediated hepatocyte transfection, LNP−mRNA can be employed to convert the liver into a "bioreactor" for producing therapeutic proteins. For example, a single intravenous administration of LNP−mRNA encoding for erythropoietin (EPO) resulted in high serum EPO levels, increased reticulocyte levels, and elevation of the hematocrit in pigs and non-human primates.[51] LNP-mediated mRNA delivery is gaining particular traction for vaccine development, given its advantages compared to viral- or DNA-based vaccines: it is noninfectious, nonintegrating, and only requires cytoplasmic delivery. As an example, Pardi et al. recently showed that a single intravenous administration of LNP−mRNA encoding a broadly neutralizing anti-HIV-1 antibody resulted in sufficient antibody production levels to protect humanized mice from HIV challenge.[52] Alternatively, LNP technology is utilized for immunization approaches via delivery of mRNA encoding antigens to immune cells following subcutaneous, intramuscular, or intradermal administration. This approach has shown considerable potential for developing a broadly protective influenza virus vaccine[53] and induced complete protection from Zika virus challenge in mice and non-human primates.[54] Notably, comparable strategies are also utilized to develop vaccines for cancer immunotherapy. For example, Oberli et al. showed potent antitumor effects after subcutaneously administering LNP−mRNA encoding tumor antigens in a melanoma mouse model.[55] Demonstrating the potential for developing personalized cancer vaccines, Kreiter et al. showed in three murine tumor models that nonsynonymous cancer mutations are immunogenic and that corresponding mRNA-based vaccines significantly inhibited tumor growth or even induced complete rejection of established tumors following intravenous administration.[56]

Owing to LNPs' capability to accommodate larger payloads than, for example, viral systems, the technology is most suitable for developing gene editing therapeutics. Recently, Finn and colleagues reported the design of an LNP formulation for coencapsulating Cas9 mRNA and single guide RNA. A single intravenous injection of LNPs targeting the TTR gene in mice resulted in ∼70% editing of hepatocyte DNA and >90% reduction in serum TTR levels that persisted for a year.[57]

While LNP technology is rapidly enabling the possibility to silence, express, or edit genes in human patients, it should be noted that important issues need to be addressed in the future including cost-effectiveness, off-target effects, and toxicity. The experience gained from the development and use of the first LNP−siRNA drug product in the clinic provides valuable insights to improve the technology for future applications.

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Related to this, other approaches such as GalNAc–siRNA conjugates or antisense oligonucleotides may prove attractive alternative options for therapeutic gene silencing.[8,9] It is clear, however, that LNP technology's advantageous features, such as the possibility to encapsulate various (large) nucleic acid payloads, will likely enable a range of genetic drugs to become embedded in mainstream treatment regimens.

AUTHOR INFORMATION

Corresponding Author

*Pieter R. Cullis. E-mail: pieterc@mail.ubc.ca.

ORCID

Jayesh A. Kulkarni: 0000-0002-3622-6998
Dominik Witzigmann: 0000-0002-8197-8558
Sam Chen: 0000-0002-3738-6600
Pieter R. Cullis: 0000-0001-9586-2508

Author Contributions

#J.A.K. and D.W. made equal contributions.

Notes

The authors declare no competing financial interest.

Biographies

Jayesh Kulkarni is a postdoctoral fellow at UBC.

Dominik Witzigmann is an honorary postdoctoral fellow at UBC.

Sam Chen is a senior scientist at Integrated Nanotherapeutics.

Pieter Cullis is a Professor at UBC (Biochemistry and Molecular Biology). He is the Director of the Nanomedicines Research Group, which focuses on the development of lipid nanoparticle technology for delivery of nucleic acids.

Roy van der Meel is an Assistant Professor at Eindhoven University of Technology. His research is focused on developing nanomedicines for immunotherapy.

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