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Review

Rational design of lipid nanoparticles for enabling gene therapies

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Lipid nanoparticle (LNP) technology is increasingly enabling RNA-based gene therapies that can potentially be used to treat most diseases. Further, these LNP RNA therapeutics can be designed and manufactured in a matter of weeks, allowing personalized medicines that can be produced in a time frame relevant to individuals suffering from terminal diseases. Here, we focus on the rational design principles that have successfully enabled LNP small interfering RNA (siRNA) formulations to silence pathogenic genes in the liver and LNP mRNA formulations to express therapeutic proteins for vaccines and gene therapies. These principles have evolved from over 50 years of research into the physical properties and functional roles of lipids in membranes as well as experience gained developing LNP systems for delivery of small molecule drugs. It is expected that these rational design principles will be successful in enabling most forms of gene therapies.

INTRODUCTION

This review concerns the development of gene therapies using RNA-based therapeutics enabled by lipid nanoparticles (LNPs). We are using the American Society of Gene & Cell Therapy definition[1] of gene therapy as "the use of genetic material to treat or prevent disease" rather than the US Food and Drug Administration (FDA) definition[2] of gene therapy as "a technique that modifies a person's genes to treat or cure disease". This is because it is becoming increasingly apparent that RNA-based therapeutics, which may or may not modify a person's genes, can potentially perform most types of gene therapies.

The practice of gene therapy has historically been limited by the lack of effective delivery systems to introduce genetic material into cells in the body. Delivery systems are necessary because "naked" macromolecular DNA- or RNA-based drugs are usually unstable in biological fluids, do not accumulate in target tissue, and cannot penetrate target cell membranes even if they get there.[3,4] Many different systems have been developed to deliver RNA or DNA into cells, broadly categorized as viral and non-viral vectors. Viral vectors suffer from limited genetic capacity, immunogenicity concerns and problems associated with random integration into the genome of target cells. Non-viral delivery systems have long been associated with toxicity and lack of potency. It is in this context that non-viral lipid nanoparticles are providing remarkable breakthrough technology.

LNP-based delivery of mRNA and small interfering RNA (siRNA) is rapidly enabling the potential of gene therapies with clinically approved therapeutics that have already had enormous impact and many more in pre-clinical and clinical trials.[5–9] These new technologies are transforming medicine as highlighted by the success of the COVID-19 vaccines. Applications range from vaccines[4,9] to treatments for rare genetic diseases,[5,10,11] personalized immunotherapies for cancer and other diseases,[12–14] as well as therapeutics to enhance longevity,[15,16] to name only a few examples. However, despite the explosion in the applications of LNP technology, further development is required. Considerable improvements in potency, therapeutic index, tissue and organ distribution, and cell specificity are both necessary and possible.

Much of the field is exploiting a high throughput approach to identify improved LNP RNA formulations.[17–21] However, we believe that a rational design approach based on an understanding of the physical properties and functional roles of lipids in biological membranes as well as an understanding of biology as it relates to intracellular delivery has been and will be more successful. Here, we first summarize features of lipid biophysics that influence the design of LNP systems for systemic administration and then review experience gained from the design of LNP delivery systems for small molecule drugs. We show how this combined background and experience has led to rational design of LNP systems that enable intra-cellular delivery of nucleic acid-based drugs for hepatic and vaccine applications that have had major impact. Similar approaches provide insight into methods to develop next generation LNP delivery systems that will expand the scope of future gene therapies to include extra-hepatic tissues.

Terminology

In this work we will use the term LNP as a term that distinguishes systems with an interior non-polar core from liposomes. Liposomes

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have long been defined to consist of lipid bilayer(s) separating interior aqueous environment(s) from an aqueous exterior.[22] The term LNP was originally introduced[23] to describe lipid-based systems with a hydrophobic "solid core," such as lipid nanoparticles containing ionizable cationic lipids that adopt a hydrophobic oil droplet at neutral pH, which is surrounded by a lipid monolayer. As discussed later in this review, LNPs also can display hybrid characteristics with both hydrophobic and aqueous internal compartments. To distinguish these alternatives, we will use the terms liposomal LNPs to describe systems that are surrounded primarily by a lipid bilayer but have a solid core either protruding into the aqueous interior or forming a micelle-like structure in the aqueous interior and solid core LNPs that are primarily surrounded by a lipid monolayer.

BACKGROUND

Membrane lipids and model membrane systems

Lipids arose to provide the basic architecture of biological membranes because of their remarkable material properties. Properties that have proven vital to design of LNP systems for delivery of small molecular drugs and nucleic acid-based drugs are lipid self-assembly properties in aqueous media, the permeability properties of lipid bilayers, lipid polymorphism, and lipid diversity/biocompatibility. The self-assembly properties allow particle size, stability, and encapsulation of macromolecular molecules such as nucleic acid-based drugs to be achieved in a reproducible and scalable manner. The permeability properties allow efficient encapsulation and retention of small molecule drugs. The polymorphic properties of lipids enable a rational design approach to membrane fusion and intracellular delivery. The biocompatibility of lipids provides a huge library of non-immunogenic molecules to utilize for the design of systems for delivery to hepatic and extra-hepatic tissues.

The original description of liposomes by Bangham[24,25] and colleagues in the 1960s, which describes the spontaneous formation of multilamellar vesicles by hydration of egg phosphatidylcholine, was immediately followed by a host of biophysical studies aimed at understanding the physical properties and functional roles of lipids in biological membranes. Studies that have proven of particular utility concern: (1) studies to develop model membranes, particularly unilamellar liposomal systems, that could be used to study properties such as the area per lipid molecule and thickness of bilayer configurations of lipids; (2) studies on gel-to-liquid crystalline phase transitions, particularly as they pertain to phospholipid-cholesterol interactions; (3) studies on the properties of LNP systems containing polar and non-polar lipids and (4) studies regarding the polymorphic properties of lipids.

With regard to model membranes, early efforts to develop unilamellar liposomal systems included detergent dialysis,[26] sonication,[27] extrusion of multilamellar liposomes through polycarbonate filters,[28] and the Batzri and Korn procedure[29] involving dilution of solutions of lipid in organic solvents into aqueous media to produce "limit size" small unilamellar vesicles. The extrusion technique was the first scalable method to generate unilamellar liposomes in the 100 nm size range[28] and is used routinely for generating liposomes used for approved cancer therapies. As noted later, refined versions of the Batzri and Korn process have proven essential for formulation of LNP systems containing nucleic acid-based drugs as they allow encapsulation of macromolecular hydrophilic cargo into an LNP.[30]

Studies on the gel-liquid crystalline phase behavior of membrane lipids also have direct relevance to the rational design of LNP formulations of nucleic acid-based drugs. As reviewed elsewhere,[31] lipids such as phospholipids can exhibit a large range of gel-to-liquid crystalline phase behaviors. The presence of cholesterol is well known to eliminate the phase transition at 30 mol% or higher concentrations to induce the relatively impermeable "liquid ordered" bilayer state[32,33] that mixtures of phosphatidylcholine (PC) and cholesterol such as 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol can adopt. The maximum level of incorporation of cholesterol into PC bilayers is approximately equimolar.[34] Equimolar DSPC/cholesterol and sphingomyelin (SPM)/cholesterol bilayers constitute particularly robust permeability barriers and excellent drug retention as well as extended circulation lifetimes when used for small molecule drug delivery systems.[35] Such behavior carries through to liposomal LNP mRNA systems containing high levels of bilayer lipid for which significantly longer circulation lifetimes are observed.[36]

The self-assembly behavior of systems achieved by mixing an ethanol solution of bilayer lipids such as PC with non-polar lipids such as triolein in ethanol with an aqueous medium offers substantial insight into the structure of LNP RNA systems containing ionizable lipids. Triolein is relatively insoluble in lipid bilayers.[37] At low proportions of PC to triolein, micelle-like structures are formed where the PC is arranged in a monolayer around a triolein core.[38] Alternatively, at high proportions of bilayer lipid, hybrid structures are observed with an aqueous and hydrophobic compartment largely surrounded by a lipid bilayer,[30] mirroring the behavior of LNP mRNA systems with high helper lipid contents.[36] The morphology of these systems can be modeled by assuming that the triolein is contained in the hydrophobic core surrounded by a monolayer of bilayer forming lipids.[30] These modeling efforts are enabled by studies on lipid bilayer systems to evaluate parameters such as the area per molecule of lipids in a bilayer configuration[39] as well as the thickness of lipid bilayers.[40] Similarly, the structure of LNP siRNA and mRNA systems can be modeled assuming the neutral form of the ionizable lipid is relatively insoluble in a lipid monolayer or bilayer and forms an internal hydrophobic oil droplet, imaged as a "solid core" by cryogenic transmission electron microscopy. The bilayer-forming "helper" lipids are localized to an external lipid monolayer surrounding the oil droplet and a lipid monolayer surrounding internalized nucleic acid cargo. At high proportions of bilayer lipid, bilayer membranes form "blebs" that partially or completely surround the hydrophobic solid core[39,41,42] and an aqueous compartment containing the nucleic acid cargo.

The polymorphic phase properties of aqueous lipid dispersions are of particular relevance to the rational design of LNP formulations of

nucleic acid-based drugs as it relates to fusion with/destabilization of the endosomal membrane to allow nucleic acid cargo to access the cytoplasm. As noted in numerous reviews,[43–46] the presence of membrane lipids that, in isolation, adopt "inverted" non-bilayer structures such as the hexagonal H_{II} phase on hydration correlates with the ability of lipid bilayers to fuse.[43] It is now generally accepted that membrane fusion proceeds via non-bilayer intermediates; indeed, it is impossible to envision fusion without a local departure from bilayer structure. The ability of cationic lipids to combine with negatively charged lipids found in biological membranes to form the hexagonal H_{II} phase[47] provides a rational framework for the design of the ionizable cationic lipids with maximum "cone" shape.[23] This ensures maximum destabilization of/fusion with the endosomal membrane during acidification following endocytosis to engender maximum intracellular delivery of nucleic acid cargo.

Liposomal systems for delivery of small molecule drugs

LNP delivery technology for siRNA and mRNA technology builds on more than 50 years of work to use liposomal systems as drug delivery systems for chemotherapeutic drugs. This work has been successful in that more than 10 such drugs have received regulatory approval (Table 1).[46,48]

This reflects decades of work by legions of investigators as reviewed elsewhere[45] and has resulted in a remarkable body of knowledge that can be used to guide the rational development of LNP systems for delivery of nucleic acid-based drugs. Lessons learned concern the LNP components to use, the design of LNP systems for systemic applications and possible techniques for co-loading hybrid LNP systems with small molecule drugs among others. The potential of liposomes as drug delivery vehicles was immediately recognized following their initial description by Bangham and colleagues.[25] Gregoriadis and colleagues soon demonstrated that small molecules and proteins could be loaded into the internal aqueous compartments exhibited by liposomes[49,50] during formation of these structures. However, these early systems were micron-sized multilamellar systems; the "passive" encapsulation efficiencies for loading drug cargo were low[51] and none of the manufacturing processes used were scalable. Solutions to these problems came in the form of extrusion and pH-dependent drug loading. Extrusion, which involves forcing multilamellar liposomes through polycarbonate filters with pore sizes of 100 nm diameter to generate unilamellar systems,[22,28] provided a rapid, reproducible, and scalable method to generate unilamellar liposomal LNP with a size range of 100 nm diameter or less.[28] Loading of small molecule drugs was achieved by establishing a pH gradient (inside acidic) across the liposomal membrane which leads to the loading of small molecule drugs that are weak bases.[52,53] Over half of the drugs in the Merck index are weak bases, including many anti-cancer drugs.[53] Drugs that are not weak bases can be converted to weak base pro-drugs that can be loaded using pH-dependent techniques.[54] The most popular version of the pH loading technique involves entrapment of ammonium sulfate to generate a pH gradient.[55]

Much effort has been spent on developing long circulating liposomal systems for delivery of anti-cancer drugs as long-circulating systems exhibit enhanced accumulation at tumor sites[56] via an effect[57] known as the "enhanced penetration and retention (EPR) effect." Early attempts using larger liposomal systems noted rapid clearance from the circulation by the reticuloendothelial system (RES), resulting in short (<10 min) circulation half-lives in rodent models.[49,50] These short circulation times resulted in poor drug delivery to tumor target sites[58–60] as circulation halftimes of 10 h or longer are required in order to achieve significantly enhanced accumulation at tumor sites via the EPR effect.[52,61] The circulation lifetimes of liposomal systems were improved in stages. The inclusion of cholesterol in liposome formulations increased circulation lifetimes significantly.[62–64] Subsequently, Allen and Chonn showed that inclusion of gangliosides such as GM1 resulted in additional gains.[65] Inclusion of polyethylene glycol (PEG) lipids such as distearoylphosphatidylethanolamine (DSPE)-PEG_{2000} enabled circulation lifetimes as long as 24 h in mice.[66] These developments led to the concept of "stealth" liposomes, a term implying long circulation lifetimes achieved by evading immune cell detection. Stealth liposome technology satisfied the requirement of sufficient circulation time for liposomal formulations of small molecule drugs to reach and accumulate at disease sites such as tumors.[67] However, stealth qualities can also be achieved without the use of PEG-lipids. Liposomes containing egg SPM or dihydrosphingomyelin[35] can lead to stealth-like circulation lifetimes, as can the use of high lipid doses in excess of 100 mg lipid/kg body weight.[35,68] This is important for the design of long-circulating formulations of LNP formulations of nucleic acid-based drugs as the presence of a PEG-coating inhibits uptake into cells and thus prevents transfection.[69] Lipids such as PC and SPM do not suffer such limitations.

The achievement of straightforward ways of manufacturing and loading long-circulating liposomes led to the first generation of LNP anticancer drugs[70] such as Myocet (liposomal doxorubicin), Doxil (liposomal doxorubicin),[70] and Marqibo (liposomal vincristine)[71] all of which are in clinical use today (see Table 1). Two other lipid-based formulations of amphotericin B (Ambisome and Abelcet)[72,73] have also been approved for clinical use to buffer the toxicity of amphotericin B in the treatment of fungal infections.

The experience gained from liposomal delivery of small molecule drugs emphasizes two major points. First, in order to affect any tissue in the body, the delivery system has to actually get there. The macrophages and monocytes of the immune system constitute the first line of defense and will induce rapid clearance of any delivery system that is recognized as foreign.[74] Lipid-based delivery systems enjoy an advantage as lipids such as PC and cholesterol are non-immunogenic, naturally occurring components of biological membranes. Further, the most straightforward way of avoiding rapid clearance is to present an exterior that is similar to long-circulating blood components such as erythrocytes, which last for over 100 days in the circulation. The exterior monolayer of the erythrocyte membrane consists primarily of PC and SPM together with equimolar cholesterol.[75]

Table 1. Clinically approved liposomal systems containing small molecule drugs and LNP RNA drugs that have received regulatory approval are in phase 3 clinical development or are of particular note in stage 1 trials

Disease indication	Type of therapeutic	Developer(s)	Stage	Trial identifier	Trade name
Small molecule drugs					
Breast cancer, ovarian cancer, and Kaposi's sarcoma	doxorubicin	Janssen Products	approved (FDA)	N/A	Doxil/Caelyx
Kaposi's sarcoma (AIDs-related)	daunorubicin	Glidead Sciences	approved (FDA)	N/A	DanuXome
Lymphomatous meningitis	cytarabine/ara-C	Pacira Pharmaceuticals	approved (FDA)	N/A	Depocyt
Metastatic breast cancer; w/ cyclophosphamide	doxorubicin	Sopherion Therapeutics	approved (FDA)	N/A	Myocet
Metastatic osteosarcoma, resectable high-grade	muramyl tripeptide-phosphatidyl ethanolamine	Takeda Pharmaceuticals	approved (FDA)	N/A	Mepact
Acute lymphblastic leukemia	vincristine	Talon Therapaeutics	approved (FDA)	N/A	Marquibo
Metastatic adenocarcinoma of pancreas w/ fluorouracil + leucovorin	irinotecan	Ipsen Biopharmaceuticals	approved (FDA)	N/A	Onivyde
Acute myeloid leukemia	daunorubicin and cytarabine	Jazz Pharmaceuticals	approved (FDA)	N/A	Vyxeos
Choroidal neovascularization (wet macular degeneration)	verteporphin	Alcami Carolinas Corporation	approved (FDA)	N/A	Visudyne
Invasive severe fungal infections	amphotericin B	Lediant Biosciences	approved (FDA)	N/A	Abcelet
Presumed fungal infections	amphotericin B	Gliead Sciences	approved (FDA)	N/A	Ambisome
Severe fungal infections	amphotericin B	Alkopharma	approved (FDA)	N/A	Amphotec
Lung disease (antibiotic)	amikacin sulfate	Insmed Incorporated	approved (FDA)	N/A	Arikayce
Shingles vaccine/post-herpetic neuralgia (adjuvant)	monophosphoryl lipid A (MPL) and QS-21 (AS01b; adjuvant)	GlaxoSmithKline	approved (FDA)	N/A	Shingrix
Malaria vaccine (adjuvant)	3-O-desacyl-4′-monophosphoryl lipid A (MPL) and QS-21 (AS01; adjuvant)	GlaxoSmithKline	approved (FDA)	N/A	Mosquirix
Pain management (anesthetic)	morphine	Pacira Pharmaceuticals	approved (FDA)	N/A	DepoDur
Pain management (anesthetic)	bupivacaine	Pacira Pharmaceuticals	approved (FDA)	N/A	Exparel
RNA drugs					
SARS-CoV-2	viral vaccine (mRNA)	Pfizer, BioNTech and Acuitas Therapeutics	approved (FDA)	N/A	Comirnaty
SARS-CoV-2	viral vaccine (mRNA)	Moderna Therapeutics	approved (FDA)	N/A	SpikeVax
Hereditary transthyretin amyloidosis	gene silencing (RNAi)	Alnylam Therapeutics and Acuitas Therapeutics	approved (FDA)	N/A	Onpattro
Respiratory syncytial virus	viral vaccine (mRNA)	Moderna Therapeutics	approved (FDA)	N/A	mRESVIA
SARS-CoV-2	viral vaccine (saRNA)	Gennova Biopharmaceticals	approved (India)	N/A	Gemcovac
SARS-CoV-2	viral vaccine (saRNA)	Arcturus Therapeutics	approved (EU)	N/A	Zapomeran
Melanoma	personalized cancer vaccine	Merck Sharp & Dohme Ltd. and Moderna Therapeutics	3	NCT05933577	V940 (previously mRNA-4157)
Non-small cell lung cancer	personalized cancer vaccine	Merck Sharp & Dohme Ltd and Moderna Therapeutics	3	NCT06077760	MK-3475
Herpes zoster	viral vaccine	GlaxoSmithKline	3	NCT05047770	217670
Melanoma	personalized cancer vaccine	Merck Sharp & Dohme Ltd. and Moderna Therapeutics	3	NCT05933577	V940 (previously mRNA-4157)
Cytomegalovirus	viral vaccine	Moderna Therapeutics	3	NCT05085366	mRNA-1647
Influenza	viral vaccine	Moderna Therapeutics	3	NCT05415462	mRNA-1010
Influenza	viral vaccine	Pfizer	3	NCT05540522	qlRV
Familial hypercholesteremia	CRISPR editor	Verve Therapeutics	1	NCT06164730	Verve-102
B cell disorders	*In vivo* CAR T	Capstan Therapeutics	1	NCT06917742	CPTX2309

For a comprehensive list of LNP mRNA drugs in clinical trials, we refer the reader to a recent review.[9]

Liposomal systems composed of PC or SPM and equimolar cholesterol can exhibit long circulation lifetimes of 12 h or more halftimes in mice[35] which corresponds to more than 24 h in people.[76] As noted later, this also applies to LNP mRNA formulations containing high levels of DSPC or SPM which are surrounded by a lipid bilayer.[36]

The other basic point is the extreme difficulty of using targeting information attached to the exterior of an LNP to achieve tissue and cell specific delivery, particularly extrahepatic targets. Despite 50 years of trying, no liposomal system has ever entered clinical trials to target distal tumors. Attempts are being made for LNP systems for targeting to hepatocytes and T cells (see Table 1, Verve and Capstan). The reasons for the difficulties are manifold. First, any attempt to attach a targeting molecule such as a monoclonal antibody, an Fab fragment, a small molecule on the end of a PEG lipid, a nanobody, an aptamer, or other targeting agent to the exterior of an LNP inevitably results in a novel antigen and a potential immune response.[77,78] Second, all LNP systems adsorb a "protein corona" when in the circulation; this corona can obscure targeting information on the LNP surface.[79] Third, scalable techniques for attaching targeting ligands to achieve a reproducible and defined number of targeting agents per LNP are lacking. Fourth, one can never get more material to a target site than would get there as a result of the size and circulation lifetime of the native carrier. Attaching targeting ligands to an otherwise long-circulating system can often decrease circulation lifetimes and tissue penetration for a variety of reasons (aggregation, larger size, and different surface properties), reducing arrival at target tissue.[77,80] All this is not to say that targeting LNPs will never work; targeting to cells in the blood compartment or the liver remains possible.[12,80] But the record of clinical success is dismal.

To date, the most clinically successful methods of targeting LNP systems employ what is usually termed "passive" or "endogenous" targeting. As noted for liposomal systems containing anti-cancer drugs, the EPR effect for long-circulating systems is a prime example.[81] Certain tumors exhibit neovasculature that is "leaky," enabling small long-circulating LNP containing anti-cancer drugs to preferentially accumulate at tumor sites.[82,83] This feature, combined with the fact that long-circulating liposomal systems are often not taken up into chemotherapy-sensitive organs such as the heart, thus reducing toxicity,[84] accounts for the relative success of approved liposomal systems containing cancer drugs. In the case of LNPs for delivery of siRNA to silence genes in hepatocytes the LNP siRNA systems associate with apolipoprotein E (ApoE) following (intravenous) i.v. administration, leading to uptake by hepatocytes through the low-density lipoprotein (LDL) and scavenging receptors.[85] In our view, it is this approach where we follow the biology and take advantage of natural targeting processes that will continue to be the most successful route for targeting LNP systems.

Rational design and LNP delivery systems for delivery of nucleic acids

Following their discovery in the 1960s, liposomes were seen as potential delivery systems for DNA- and RNA-based molecules. Shown in Figure 1 is a complete timeline of the evolution of LNP systems up to their current form. Initial efforts showed that nucleic acid polymers could be encapsulated in liposomal systems[86] and elicited transfection *in vitro*[87] and *in vivo*.[88] However transfection levels were low and methods used to generate these systems were not scalable as they relied on processes where encapsulation is achieved during vesicle formation. This is very inefficient as the trapped volume in liposomal systems is usually only a small fraction of the total aqueous medium.[89] In addition, the liposomal systems were large and transfection efficacy was poor.[87,90–95]

Lipoplexes

Lipoplexes represent the first application of rational design principles to the delivery of nucleic acids. Obviously, if the lipid nanocarrier was not able to efficiently encapsulate the nucleic acid cargo then subsequent delivery was moot. Equally obviously, cationic lipids should facilitate encapsulation of RNA and DNA into lipid-based systems. Felgner and colleagues[96] showed that small unilamellar liposomes composed of the cationic lipid 1,2-di-O-ocatdecenyl-3-trimethylammonium propane (DOTMA) and a helper lipid such as 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) spontaneously complexed with plasmid DNA through electrostatic interactions between the positively charged lipid and the negatively charged DNA phosphate backbone, resulting in entrapment efficiencies approaching 100%. Surprisingly, when added to cells *in vitro*, these "lipoplexes"[97] enabled intracellular delivery of pDNA.[96,98] Importantly, the nucleic acid in these complexes is protected from nuclease degradation in complex media and upon administration to cells.[96,98]

Lipoplexes found rapid applications as nucleic acid delivery vectors for *in vitro* applications. An early example is lipofectin, a liposomal composition of DOTMA and DOPE. Compared to other methods such as calcium phosphate or dextran-induced transfection, Lipofectin was 5- to 100-fold more transfection potent.[99] This finding stimulated the synthesis of many other cationic lipids such as dioctadecylamidoglycylspermine, dipalmitoyl phosphatidylethanolamidospermine,[100] dioleoyl-3-trimethylammonium propane (DOTAP), N-(2-(2,5-bis ((3-aminopropyl)amino)pentanamido)ethyl)-N,N-dimethyl-2,3-bis ((Z)-octadec-9-en-1-yloxy)propan-1-aminium chloride tetrahydrochloride (DOSPA), and various lipids containing spermine in the head group. Lipofection reagents such as Lipofectamine (DOSPA: DOPE 3:1 w/w) and CellFectin (tetramethyltetrapalmitylspermine: DOPE, 1:1.5 w/w) are commonly used to deliver DNA and RNA into eukaryotic cells in preclinical research today[98,101,102] with highly efficient intracellular delivery.[98,103]

The use of lipoplexes is almost entirely limited to *in vitro* applications due to major limitations for *in vivo* applications. The fundamental limitation is toxicity. There are no permanently positively charged bilayer forming lipids in nature. Positively charged lipid molecules can combine with the negatively charged lipids found in biological membranes to form non-bilayer structures that

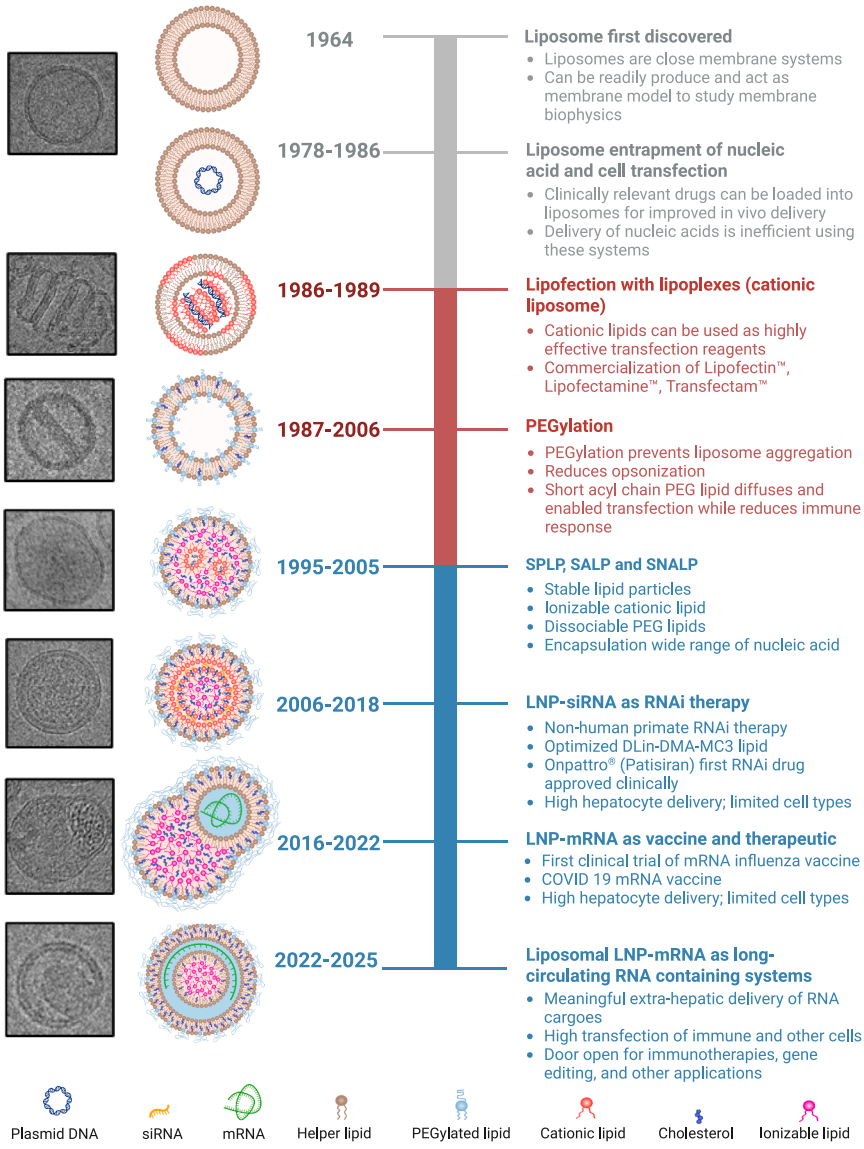

Figure 1. Schematic timeline of the evolution of current lipid nanoparticle RNA systems starting from the discovery of liposomes

Colors of the central bar indicate different epochs of technology development. Gray indicates an early era dominated by liposomal small molecule delivery; red indicates an intermediate era marked by discovery of cationic lipids to enable transfection but limited to *in vitro* uses; blue indicates the current era of *in vivo* transfection enabled by discovery of ionizable cationic lipids. Key technological development events are indicated on the right, and the structures of the particles illustrated both schematically and with sample cryo-TEM images are indicated on the left. Cryo-TEM images generously provided by NanoVation Therapeutics. Note the cryo-TEM image for "PEGyation" is an image of Doxil.

disrupt membranes[47] resulting in cytotoxicity[104] and immune activation[105] (activation of complement and coagulation pathways as well as cytokine stimulation[104,106]). This results in rapid clearance by the RES system, inflammation, and other side effects such as thrombosis,[107] lymphopenia, hepatic necrosis, and an overactive innate immune response.[108–110] Additional problems associated with lipoplexes concern their size and stability. Lipoplex preparations can be heterogeneous with particle diameters range from nanometers to micrometers due to DNA condensation and lipoplex restructuring.[111,112] The resultant complexes are variable in size and morphology and have poor colloidal stability.[113–115] Finally, the rapid clearance of lipoplexes following i.v. administration[116] results in a short circulation time and low systemic bioavailability of the encapsulated nucleic acid payload.[105]

Lipid nanoparticles containing siRNA

Rational design and loading of siRNA into LNP

The second stage of rational design of lipid-based delivery systems for nucleic acid delivery required the development of LNP systems that achieved efficient loading but were compatible with *in vivo* administration. Efforts were first made to develop lipid-based systems that contained very little cationic lipid to reduce toxicity concerns. These efforts resulted in "stabilized plasmid-lipid particles" (SPLP) using a detergent dialysis procedure to encapsulate plasmid DNA complexed with small amounts (~6 mol%) of cationic lipids such as diethyldimethylammonium chloride (DODAC).[117] When these components were mixed with detergents and an excess of a "helper" lipid such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or DOPE, encapsulation efficiencies of up to 70% could be achieved. In order to control the size of these systems and prevent aggregation, SPLP included a PEG lipid (PEG-CerC_{20}) as a third component. These SPLP particles contained one plasmid molecule per LNP that was surrounded by a bilayer as evidenced by calculation[117] and supported by cryogenic transmission electron microscopy (cryo-TEM).[118] After injection, SPLP showed greatly improved properties compared to lipoplexes, with circulation half-lives of at least 6 h and importantly, induction of reporter gene expression in distal tumor tissues.[118] However, while this process produced very well-defined nanoparticles and good *in vivo* activity, detergent dialysis is a tedious method and encapsulation is very dependent on the ratios of various components, particularly the cationic lipid. Thus, the SPLP approach was not pursued.

The next rational design attempt was aimed at reducing the toxicity of cationic lipids by using *ionizable* cationic amino lipids that are

protonated and positively charged at acidic pH values but are neutral and potentially less toxic at physiological pH. The first ionizable cationic lipid employed for encapsulation of nucleic acid-based drugs was 1,2-dioleoyl-3-dimethylammonium propane (DODAP) to produce stabilized antisense lipid particles (SALP).[119] DODAP was originally developed to study the effects of transbilayer lipid asymmetry on the fusogenic properties of liposomes.[120] Structurally, DODAP is an ionizable version of DOTAP,[103] the cationic lipid used in Lipofectin. The key difference between them is that the tertiary amine in DODAP has a pKa of ~6.6, whereas DOTAP has a quaternary amine and is permanently positively charged. DODAP is positively charged at pH values below ~6.6 but is near neutral at physiological pH (~7.4).[120] This endows DODAP with unique features; at low pH (below ~6.6), it has a positive charge and can complex with negatively charged DNA or RNA molecules, while at physiological pH it is relatively uncharged, thus reducing toxicity. Further, when positively charged, DODAP adopts bilayer structure in an aqueous environment whereas when in the neutral form at physiological pH values it forms an oil.[121]

Ionizable cationic lipids provided convenient methods of efficiently encapsulating nucleic acids. The "preformed vesicle" approach[121] for formulating antisense oligonucleotides into LNP was the first scalable approach for achieving LNP antisense systems called "stabilized antisense lipid particles" (SALP) with up to 90% encapsulation efficiencies.[121] SALP were formed from DODAP/DSPC/cholesterol/PEG-lipid mixtures dissolved in ethanol added to citrate buffer (pH 4) to form small vesicles following the Batzri and Korn procedure,[29] and then extruded through a 100-nm pore size filter to ensure small vesicle formation. The extrusion step could be omitted under conditions of rapid mixing of the ethanol-citrate solutions.[121] These vesicles were then added to a solution of antisense oligonucleotides in ethanol (pH 4, 40% ethanol by volume). The ethanol was then removed and the pH adjusted to 7.4 by dialysis against PBS. It was found that an oligonucleotide trapping efficiency of up to 90% could be achieved for the lipid compositions DODAP/DSPC/cholesterol/PEG-lipid (25/20/45/10, mol%).[119,121] SALP displayed long circulation lifetimes and an improved toxicity profile compared to formulations containing permanently charged cationic lipids when injected intravenously.[119]

In 2005, Jeffs et al.[122] reported an improved method of SALP formulation (using the ionizable cationic lipid DLinDMA (1,2-dilinoleyloxy-3-dimethylaminopropane)) that combined the particle formation and nucleic acid encapsulation processes into a single step to produce LNP that they called stabilized nucleic acid lipid nanoparticles (SNALP). Based again on the Batzri and Korn[29] technique of producing small unilamellar vesicles by rapidly mixing an ethanol solution of lipids with an aqueous medium, they showed that mixing an ethanolic solution of lipids with an acidic (pH 4) aqueous solution of nucleic acid using a T-tube,[123] followed by a dialysis step to remove ethanol and raise the pH, generated particles with encapsulated nucleic acid. This was followed in 2012 by a related process using microfluidic mixing rather than T-tube mixing to generate potent LNP siRNA systems.[124]

Rational design and choice of PEG-lipids

An important feature of the SPLP results reported by Wheeler et al.[117] was the identification of a PEG-lipid that would separate from the SPLP following formulation. The PEG-lipid (PEG-ceramide; PEG-Cer) component in SPLP was required during formulation to prevent aggregation during the detergent dialysis step. However, the presence of a permanent PEG coating such as PEG-CerC_{20} inhibits uptake into target cells and, thus, techniques to remove it were required in order to maximize transfection potency. This was achieved by using PEG-CerC_{14}; it was shown that SPLP prepared using PEG-CerC_{14} were much more transfection potent *in vitro* than SPLP containing PEG-CerC_{20} due to the ability of PEG-lipids with short C_{14} acyl chains to dissociate from the nanoparticle.[69] A number of PEG-C_{14} lipids have now been developed such as 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG-DMG)[125,126] and play an important role in the clinically approved LNP siRNA and mRNA formulations. A common feature of these lipids is the use of a lipid anchor that is not negatively charged so that association with cationic lipids is minimized.

Rational design and development of ionizable cationic lipids

As noted, ionizable cationic lipids such as DODAP provided a method of efficiently loading nucleic acid-based drugs into LNP using scalable techniques such as the preformed vesicle approach, the T-tube mixing approach or microfluidic mixing. The relatively non-toxic nature of ionizable lipids meant that these systems could be tested for *in vivo* activity following i.v. administration. LNP systems incorporating DODAP or dilinoleoylDAP (DLinDAP) and containing siRNA to silence a gene in the liver gave signs of gene silencing capabilities following an i.v. administration; however, a high dose of >40 mg siRNA/kg was required (see supplementary information in Semple et al.[23]). In 2005, using a rational design approach to develop "cone shaped"[43] ionizable lipids that maximized membrane disruption when in the positively charged form, the lipid DLinDMA, a more stable and more unsaturated version of DODAP, was identified. LNP containing DLinDMA exhibited potent *in vitro* siRNA-based gene-silencing activity in Neuro2A cells.[127] *In vivo* studies showed that LNP containing DLinDMA could achieve over 90% ApoB gene silencing at a 2.5 mg/kg dose in the liver of non-human primates.[128] Unfortunately, the tolerability of this formulation in the clinic was not sufficient to warrant clinical development.[5]

Further work aimed at reducing the toxicity of the DLinDMA ionizable lipid established that the gene silencing potency of LNP siRNA systems was highly sensitive to the pKa of the ionizable lipid component. This work went through two stages. First, attempts to reduce toxicity by introducing ketal linkages in the head group resulted in the ionizable lipid DLinKC2DMA (KC2). Remarkably, LNP containing KC2 were not only less toxic than LNP containing DLinDMA but were also more potent as assayed by the ability to silence factor VII (FVII) in animal models.[23] LNP siRNA systems containing KC2 demonstrated *in vivo* gene silencing activity at LNP-siRNA doses as low as 0.01 mg/kg in rodents and 0.1 mg/kg in nonhuman primates, a greater than 10-fold improvement over LNP containing

DLinDMA.[23] This finding stimulated a race to develop further optimized ionizable cationic lipids. In 2012, Jayaraman et al.[129] identified DLin-MC3-DMA (MC3) (pKa 6.4) as a "gold standard" ionizable cationic lipid and also identified an optimized lipid composition of MC3/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol%) to achieve maximum gene silencing in the liver following an i.v. injection. LNP siRNA systems containing MC3 exhibited gene silencing at doses as low as 0.005 mg siRNA/kg and therapeutic indices of over 1,000, meaning that the dose at which toxic effects were noted in mice was 1,000 times higher than the dose required to see a biological gene silencing effect.[129] Perhaps the most remarkable finding was the marked dependence of LNP siRNA gene silencing potency on the pKa of the ionizable lipid. The ionizable lipids giving rise to the most potent gene silencing had an apparent pKa of approximately 6.4, a pKa difference of as little as 0.5 pH units resulted in up to 100-fold reduction in gene silencing potency.[129] The parallel with the pH-dependent mechanisms employed by viruses for intracellular delivery of nucleic acid is clear.

Efforts have been made to reduce the toxicity of LNP RNA systems by enhancing the biodegradability of the ionizable cationic lipid component. Clearly, the lipid should not be broken down too rapidly or it cannot fulfill the requirement for endosomal escape. It is likely that ester-containing lipids such as DODAP and DLinDAP are readily hydrolyzed *in vivo* due the esterases encountered following uptake into endosomes,[130] resulting in poor intracellular delivery when compared to more stable lipids such as DLinDMA, which contain ether linkages.[23] Conversely, it could be suggested that MC3 is too stable as a large hydrophobic domain of the molecule persists for days.[131] However, Onpattro has been dosed clinically once every 3 weeks at 0.3 mg siRNA per kg body weight (~3 mg/kg lipid) for over 10 years without toxicity issues arising from the extended presence of MC3 and MC3 metabolites. In any event, many potent ionizable lipids have been synthesized that exhibit improved biodegradability compared to MC3.[132] Ionizable lipids containing esters within the hydrophobic domain exhibit enhanced biodegradability.[133] Structures with the esters closer to the lipid head group resulted in increased pKa and decreased gene silencing when employed in the FVII screening model. The most potent and biodegradable ionizable lipid synthesized was virtually eliminated (~1,000-fold reduction in detected levels) within 24 h of administration.[134]

Toxicity can also potentially be reduced by reducing the amount of ionizable lipid in the LNP. However, LNP siRNA systems containing just enough ionizable lipid to enable encapsulation of siRNA cargo (amino lipid to oligonucleotide phosphate ratio N/P of 1) are not transfection competent, N/P values of at least 3 are required for siRNA.[23,135] For LNP mRNA systems higher proportions (N/P = 6) of ionizable cationic lipids are required, although this remains a relatively un-optimized variable. The overall finding that an excess of ionizable lipid over that required to complex with nucleic acid cargo is consistent with the rational design hypothesis that has driven optimization of the ionizable lipid. Excess ionizable lipid is required that can progressively adopt the protonated cationic form as the pH in the endosome is lowered by inwardly directed proton pumps as the endosome matures.[136] These positively charged lipids can then associate with endogenous negatively charged lipids to induce fusion with the limiting membrane of the endosomal membrane or the multivesicular bodies (MVB) formed in the late endosome.[136–138] Either route can lead to delivery of the nucleic acid cargo to the cytoplasm.

Rational design and the role of helper lipids

The role of helper lipids such as DSPC and cholesterol were elucidated long after the LNP siRNA systems were developed so it is hard to say that rational design principles were employed in the original formulations. However, this understanding is highly useful for the design of advanced LNP RNA delivery systems. DSPC and cholesterol were originally incorporated in early LNP systems containing DODAP and antisense oligonucleotides from a starting DSPC/cholesterol/PEG-lipid (molar ratio 1:1:0.2) lipid composition. DSPC/cholesterol (1:1) lipid vesicles are commonly used to encapsulate small molecule drugs.[61] DODAP was added at the expense of DSPC, and it was found that high antisense encapsulation levels could be achieved for 30 mol% DODAP (20 mol% DSPC) when incubated in the presence of ethanol at pH 4.0 followed by dialysis against PBS.[119] Interestingly, later studies showed that the presence of at least 40 mol% helper lipid (20 mol% DSPC and 20 mol% cholesterol) is required to achieve stable encapsulation of siRNA in LNP systems[42] and that some of these bilayer lipids are internalized into the LNP. It was shown that this is consistent with the requirement that enough bilayer lipid is present to provide at least a monolayer around a hydrophobic core of ionizable cationic lipids in the neutral oil droplet form as well as monolayers around entrapped nucleic acid ("inverted micelles") to provide a polar environment for the hydrophilic cargo. Thus, it may be concluded that the major reason for the need for bilayer "helper" lipids is to maintain encapsulation of nucleic acid cargo when the ionizable lipid is in the neutral form.

DSPC, which forms robust bilayer structures in combination with equimolar cholesterol, is the predominant helper phospholipid used in LNP formulations of RNA. It could be argued that "fusogenic" helper lipids such as DOPE that promote formation of nonbilayer structures and thus membrane fusion,[43] should enhance the intracellular delivery properties of LNP formulations of nucleic acid-based drugs. Results to support this contention are ambiguous. Kauffman et al.[139] showed improved mRNA expression with the incorporation of DOPE instead of DSPC at high ionizable lipid: mRNA charge ratios in an LNP using lipidoid ionizable lipids; however, the same LNP formulation did not improve siRNA delivery. Improvements resulting from substituting DOPE for DSPC have not been observed for LNP containing MC3 or KC2 ionizable lipids. For example, Kulkarni et al.[41,42] showed that LNP plasmid DNA systems containing KC2 or MC3 and PC exhibited superior transfection properties compared to LNPs containing PE *in vitro*. The lamellarity of mRNA-LNPs can be modulated by inclusion of cholesterol derivatives such as stigmastanol to replace cholesterol[140] and many other variants of helper lipid have been characterized. Overall, while transfection potency can occasionally be improved by varying helper lipid

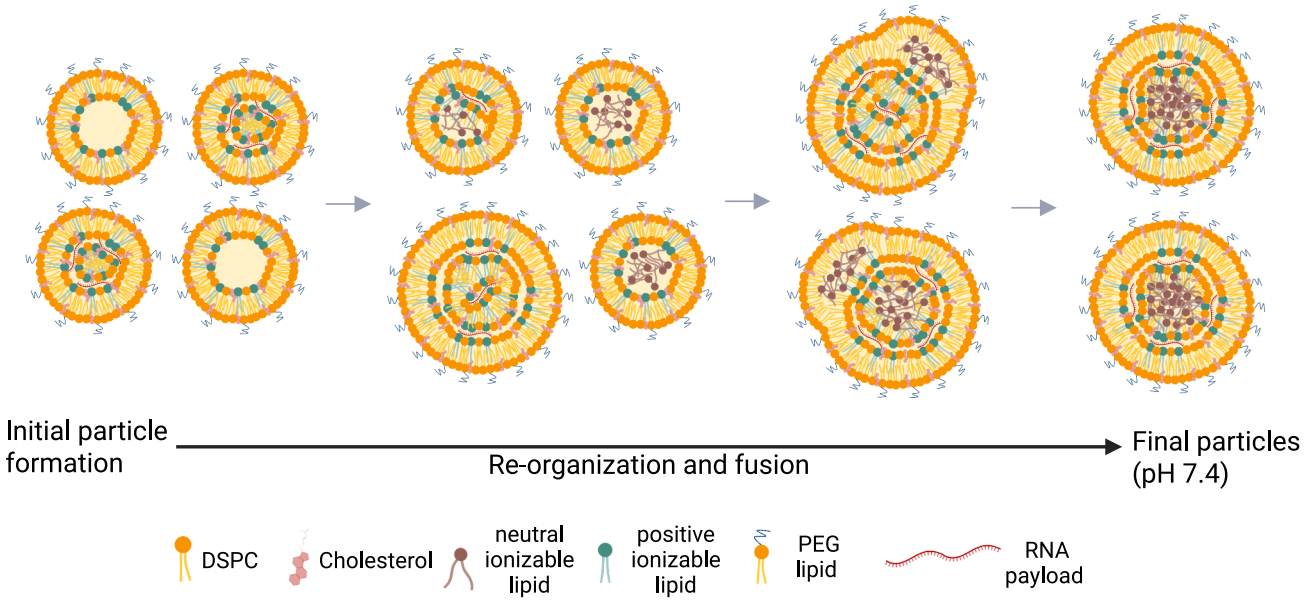

Figure 2. Schematic of the assembly of LNP RNA systems in stages ranging from mixing at pH 4.0 to final particle formation at physiological pH 7.4

composition, efforts to replace cholesterol or DSPC are not generally successful except for other bilayer lipids such as SPM.[36]

The proportions of DSPC have been adjusted as LNP systems have evolved. The original LNP antisense formulations added DODAP at the expense of DSPC in DODAP/DSPC/cholesterol/PEG-lipid formulations to arrive at SALP formulations that contained 25 mol% DSPC,[119] which was further reduced to 20 mol% in SNALP siRNA formulations.[127] The related preformed vesicle formulation process[121] that was used to develop the optimized lipids DLinKC2DMA[23] and DLinMC3DMA[129] consisted of ionizable lipid/DSPC/cholesterol/PEG-lipid (40/10/40/10 mol%) which was then further optimized to the Onpattro formulation with molar ratios 50/10/38.5/1.5.[129] The amount of cholesterol in LNP RNA formulations using the Onpattro molar ratios for liver transfection has certainly not been optimized. It was originally included in liposomal systems due to its ability to induce the relatively impermeable "liquid ordered" bilayer state in equimolar combinations with DSPC.[141] As the proportion of DSPC was replaced by DODAP in LNP antisense formulations to achieve maximum encapsulation, the cholesterol content was not reduced in concert, a feature that has not been changed in subsequent formulations.[119] Thus, given that the maximum solubility of cholesterol in PC bilayers is approximately 1:1,[34] it is likely that there is excess cholesterol in formulations such as the Onpattro formulation. This would be consistent with two studies indicating that Onpattro LNP formulations exhibit the presence of cholesterol crystals in the hydrophobic core.[41,142]

Rational design and LNP siRNA structure and composition

As noted, early cryo-TEM studies on LNP siRNA systems for hepatic applications showed a "solid core" electron dense structure and molecular modeling approaches suggested that this solid core corresponded to a hydrophobic core consisting of neutral ionizable lipid together with siRNA in polar environments inside inverted micelles.[124] ^{31}P-NMR studies showed that encapsulated siRNA is immobilized in similar LNP systems; molecular modeling utilized in the same study indicated the presence of inverted micellar structures containing the nucleic acids.[135] Subsequently, it was shown that the LNP formed following rapid mixing of ethanol solutions of lipid with a pH 4 buffer containing nucleic acid are only intermediates in the formation of the final LNP siRNA particles. At pH 4, very small bilayer vesicles are formed. As the pH is raised during the dialysis against PBS, the small bilayer vesicles fuse together, encapsulating associated siRNA in the process. The fusion is attributed to conversion of the bilayer-forming charged form of the ionizable lipid to the neutral, oil-forming state.[41,42,143,144] The extent of fusion is regulated by the PEG-lipid content; when the concentration of PEG-lipid on the LNP exterior reaches a critical value, further fusion is inhibited. This finding accounts for the fact that the three formulation methods for LNP siRNA systems using a pre-formed vesicle approach, T-tube-mixing, or microfluidic mixing techniques all lead to very similar LNP structures with similar potencies.

The structure of LNP RNA systems resulting from these studies is shown in Figure 2. A feature of the structure is that the RNA resides in a polar environment surrounding an inner "solid core" of ionizable lipid in the neutral oil droplet form in the finished LNP at pH 7.4. This structure is particularly visible at high siRNA contents, where a "small multilamellar vesicle" morphology is observed at N/P values of one.[42] This morphology was first seen for high encapsulation levels of antisense oligonucleotides.[119] On a molecular level, An et al. showed differences in LNP morphology with the

inclusion of phosphorothioate modifications in nucleic acid constructs compared to unmodified nucleic acid constructs, demonstrating that even minor modifications to the payload can result in structural differences.[145]

Rational design and LNP targeting

The optimized LNP siRNA systems clearly display a pronounced tropism for hepatocytes even though there is no targeting information on the LNP surface. The reason for this was elucidated by Akinc et al.[85] who showed that the targeting was due to association of ApoE with the LNP *in vivo*. LNP siRNA systems to silence FVII in the liver were not effective in ApoE$^{-/-}$ mice; however, activity could be restored by pre-incubation of the LNP with ApoE. It would appear that the LNP systems with the ionizable lipid/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol%) lipid composition are treated *in vivo* much like chylomicron remnants that associate with ApoE to trigger uptake into hepatocytes via the LDL and scavenging receptor pathways.[146]

The role of the PEG-lipid in ApoE dependent targeting to hepatocytes also reflects a rational design approach. The PEG-lipid is required during formulation in order to regulate the size of the LNP,[124] however as noted previously the continued presence of the PEG-lipid *in vivo* inhibits LNP uptake and transfection. Further, longer circulation times correlate with reductions of surface-bound protein,[147] indicating that PEG coatings lead to reduced protein corona levels, Thus the use of PEG-lipids that dissociate from the LNP *in vivo* encourages association of serum proteins such as ApoE. Mechanistically, the PEG-DMG lipid dissociates from the LNP, exposing a surface composed primarily of cholesterol, DPSC, and possibly a small fraction of neutral ionizable lipid.[148] Following LNP exposure to serum, proteins can bind to the surface to form a protein corona which mediates receptor mediated endocytosis into cells.[85,149] Within this corona, ApoE plays a major role due to the strong interaction with the LDL receptor, resulting in nearly complete liver accumulation and hepatocyte targeting[10,11,150,151] within 30 min.[85] The role of other serum proteins within the protein corona has not been studied as extensively; however, it is likely that the protein corona plays a central role in passive tissue/cell type targeting of all LNPs.[79,149,152]

Rational design and intracellular delivery

It is likely that LNP nucleic acid systems employ similar intracellular delivery mechanisms as enveloped viruses infecting eukaryotes.[153,154] Other mechanisms such as the "proton sponge" hypothesis[155] have been proposed, but a rational design approach suggests emulation of the way that viruses deliver genetic material into cells may be more productive. Enveloped viruses are of similar size to LNPs (50–200 nm) and consist of a host-derived bilayer membrane surrounding an aqueous compartment containing the viral genome and associated proteins.[156] This membrane is decorated with multiple proteins including host receptor binding proteins which stimulate cellular uptake and endosomal escape.[153,157] Viral uptake occurs via receptor-mediated endocytosis induced by receptor binding by a host receptor, such as hemagglutinin A (HA) binding sialic acid residues for influenza A, the G protein binding LDL for vesicular stomatitis virus (VSV), or the spike protein binding ACE2 for SARS-CoV-2.[158,159] To achieve endosome escape, enveloped viruses have evolved proteins with built-in pH-dependent fusogenic properties to enable fusion with the endosomal membrane.[157] These proteins either undergo a conformational change in response to lowered pH, or are sensitive to pH-dependent ligand binding/proteolytic cleavage by endosomal enzymes to induce structural changes[160,161] that expose a hydrophobic region which inserts into the endosomal membrane leading to membrane fusion and release of the viral genome into the cytosol.[153,156,160,162] The influenza HA protein hemagglutinin subunit contains a hydrophobic domain in the N terminal region that is hidden at neutral pH. Exposure to acidic conditions (pH ~5.0)[163] within the maturing endosome triggers a conformational change from a coil to an extended hydrophobic helix which inserts into the endosomal membrane to induce fusion and viral genome release.[160,164] The efficiency and maturity of the endosomal compartment from which escape occurs vary between viruses but the mechanism by which they function is conserved.[157] Some enveloped viruses such as VSV first fuse to MVB within maturing endosomal compartments, and subsequently undergo a back-fusion event to release the viral genome into the cytoplasm.[161] This represents a potential viral strategy for surviving the harsh degradative endosomal environment prior to escape.[165] See Figure 3A for a comparison of LNP and a representative viral-mediated endosomal escape process.

We note that exosomes are other nanoscale vectors being developed for delivery of nucleic acids or other cargoes. These are complex biological carriers composed of a cell-derived lipid bilayer and endogenous proteins capable of delivering RNA cargoes to tissues distal in the body potentially in a more tissue-specific manner than LNP RNAs.[166] No exosome-based therapeutics have been clinically approved, however, and this may be due to the requirement for live cells to produce these particles, difficulty loading, and challenges with defining the precise composition of each batch—all significant barriers for generating consistent therapeutics. Long-term, we anticipate that there will be rational design principles to be derived from exosome-based delivery systems for both cell targeting and cargo delivery that may be applicable to LNP RNA; however, the field is still in an emergent stage. For detailed reviews comparing exosomes to LNP systems, we refer the reader to studies by Bader et al.[166] and Hagedorn et al.[167]

Design of LNP for intracellular delivery of nucleic acids mimics the design of enveloped viruses and provides rational design parameters for the ionizable cationic lipid.[23] This entails a maximum "cone" shape to induce non-bilayer structure when in the cationic form as well as a need to adopt the protonated positively charged form at a vulnerable stage of endocytosis.[132] The pronounced pKa dependence of ionizable lipids for effective intracellular delivery[129] and subsequent gene silencing or expression mirrors the pH-dependent features of infection by viral vectors and presumably reflects fusion at some vulnerable stage of endocytosis. Three potential results of

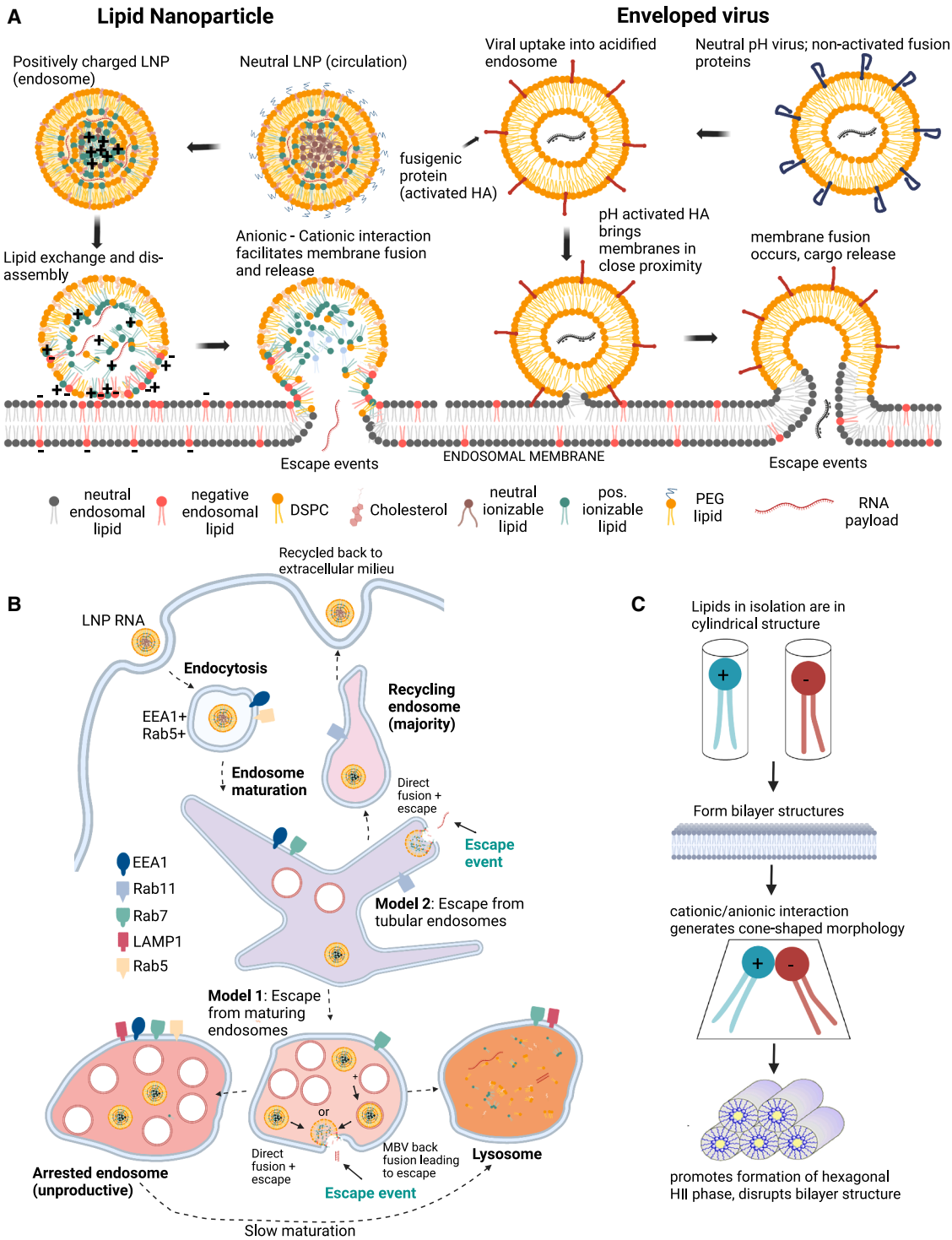

Figure 3. Depiction of various aspects of LNP mediated RNA delivery across the endosomal membrane

(A) Schematic of LNP RNA-based membrane fusion to enable endosomal escape of RNA cargo (left) compared to an enveloped virus endosomal escape process using pH sensitive surface proteins. (B) Cellular uptake and potential points for endosomal escape. (C) Depiction of formation of non-bilayer structures for inducing membrane fusion to enable endosomal escape.

endocytosis of LNP RNA systems are recycling to the extracellular medium, degradation in the lysosome or delivery into the cytoplasm. Obviously, approaches that maximize cytoplasmic delivery are desired. It has been suggested that up to 70% of endocytosed LNP-RNA is recycled into the extracellular medium through the endosomal recycling pathway.[168–170] Current estimates are that only 1%–5% of the nucleic acid is actually released into the cytoplasm,[171–173] the rest being recycled to the extracellular medium[170,173,174] or eventually degraded in lysosomes (see Figure 3B for a summary).

The stage of endocytosis at which LNP nucleic acid cargo is released into the cytoplasm remains ambiguous. Evidence supporting release in early endocytic compartments (pH ∼6.4),[175] is consistent with the presence of LNP-mRNA in tubular structures likely to be part of the recycling network.[168] Related studies show a strong correlation of escape events with the presence of Rab11, a marker of recycling endosomes, and EEA1 and AAPL1, both markers of early endosomes.[172] Inhibition of Niemann-Pick protein (NPC1) reduces endosome recycling and enhanced retention of endocytosed LNP and improved siRNA-mediated gene knockdown were observed in an NPC1−/− cell line.[170] Inhibition of NPC1 by incorporation of a small molecule inhibitor (NP3.47) into LNP-siRNA particles similarly improved LNP-siRNA retention and siRNA-mediated gene silencing.[174] However, other studies suggest that escape occurs in late endosomes (pH 5.5). Rab7, a protein localized to late endosomes, has been linked to endosomal escape events.[171] A haploid Rab7−/− cell line had reduced LNP-mRNA transfection whereas knockouts of Rab4/5 had no effect.[176] Other studies have shown a strong co-localization of Gal8 (which is associated with sites of endosomal damage) and Rab7 when evaluating LNP-mRNA escape dynamics[173] (see Figure 3B).

Regardless of the precise endosomal compartment from which nucleic acid escape occurs, the mechanism by which release occurs is likely conserved. In order for the LNP to destabilize/fuse with the endosomal membrane, cationic lipids must come into close contact with the negatively charged endosomal membrane. During endosomal maturation and associated pH changes, positively charged ionizable lipids are generated on the LNP and increasingly exposed to anionic lipids in the inner leaflet of endosomal membrane(s), such as phosphatidylserine, or endosome-specific lipids such as lysobisphosphatidic acid in multi-vesicular bodies,[137] bringing them in close contact through electrostatic interaction. Formation of membrane disruptive hexagonal phase structures has been demonstrated by mixing of cationic lipids with anionic endosomal lipids and mixing of anionic liposomes and cationic nucleic acid containing liposomes results in complete release of the nucleic acid cargo.[47,97,127,177] This correlates with disruption/fusion of the LNP with the endosomal membrane in the presence of ionizable lipids in the positively charged form and subsequent delivery of nucleic acid cargo to the cell cytoplasm (Figure 3C).

Therapeutic applications of LNP siRNA systems

The clinical potential of LNP siRNA systems with the MC3/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol%) lipid composition was first investigated using siRNA to silence transthyretin (TTR), a retinol binding protein secreted by hepatocytes.[5] Mutations in TTR can cause the hereditary disease known as TTR-induced amyloidosis (hATTR), which is usually fatal within five years of diagnosis. Remarkably positive clinical trial results were reported on 2017, leading to FDA approval of Onpattro in 2018.[5,144] Subsequent uses of LNP for delivery of siRNA have been limited due to the success of "naked" siRNA coupled to a GalNAc targeting ligand, which can be administered subcutaneously.[178] However, this approach is limited to liver applications; it is probable that extrahepatic targets will benefit from LNP delivery approaches.

Lipid nanoparticles containing mRNA

Following the development of LNPs for siRNA delivery which resulted in the clinical development of Patisiran (Onpattro), attention turned to the use of LNP technology for mRNA delivery to the liver to express proteins. Remarkably, it was found that LNP mRNA systems formulated using the same ethanol dilution/rapid mixing process used for siRNA payloads and with the Onpattro lipid composition could lead to gene expression in the liver following an i.v. administration.[151,179] However, there are important differences. First, the mRNA payload has a considerably larger molecular weight and is much more susceptible to breakdown. For example, a standard LNP siRNA formulation at an N/P value of three contain approximately 700 copies of siRNA in a 50 nm diameter particle. An LNP system containing 2 kb mRNA at an N/P ratio of six on the other hand may contain only 3–4 copies of mRNA in a 50 nm diameter LNP. Further, whereas the siRNA is usually chemically modified and thus not susceptible to breakdown by nucleases, the mRNA is less stable, a single nick can thus reduce the potency of the payload by 30% or more. A second difference is that whereas siRNA have a constant molecular weight and structure, mRNAs can have significantly different molecular weights and secondary structures that are likely to influence LNP loading and transfection properties.

Rational design of LNP mRNA systems for liver transfection

As noted, initial studies showed that encapsulation of mRNA coding for luciferase using the same formulation process and lipid composition as Onpattro could transfect hepatocytes following i.v. administration.[151,179] Subsequent studies have largely been aimed at improving the transfection potency of LNP mRNA systems by further optimization of the ionizable lipid component using luciferase expression in the liver as a measure of potency. There has been an enormous synthetic chemistry effort to develop more active ionizable lipids to improve mRNA expression. The large range of ionizable lipids that have been investigated has been reviewed elsewhere[132]; here, we summarize overall trends. With regard to head group modifications, the inclusion of an ethanolamine moiety has proven advantageous, a feature that has been attributed to improved hydrogen bonding with mRNA.[131] Active squaramide ionizable amino lipids have been identified that exhibit hydrogen bonding and pi stacking with mRNA.[180] Improvements in the acyl chain composition of ionizable lipids have led to an alkyne version of MC3,[152] and dilinoleoyl acyl tail derivatives.[181] Branched acyl chains

contribute to a more pronounced cone shape when in the positively charged protonated form[182] to promote membrane fusion events. Similarly, trialkyl ionizable lipids also give enhanced activity.[183] Two notable examples that exemplify general trends are SM-102 and ALC-0315, the lipids that are in the Moderna and Pfizer/BioNTech vaccines, respectively. These lipids maintain a pKa in the region of 6.5, contain an ethanolamine head group, and exhibit branched acyl chain structures. However, it can be suggested that the potential for achieving new ionizable cationic lipids that have dramatically improved potency is becoming increasingly less likely. Further, a major difficulty is that while some lipids may show greater transfection capability, the toxicity profiles *in vivo* (which are not usually screened) may be less favorable.

It is usually assumed that the more potent an ionizable lipid is in an LNP formulation, the more effective it is at facilitating cytoplasmic delivery. However, there is evidence to suggest that the influence of the ionizable lipid on LNP structure and mRNA stability also plays an important role. Recent work has shown that formulation of LNP mRNA using high concentrations of pH 4 buffers such as Na-citrate can result in induction of "bleb" structures under some conditions[39,184] containing segregated mRNA where the mRNA appears to be more stable than in LNP formulated in low concentrations of pH 4 buffer such as Na-acetate.[39] This could arise in part due to reduced association with ionizable lipids and thus reduced formation of ionizable amino lipid-mRNA adducts that can compromise mRNA activity.[185] One study showed that induction of bleb structure by high ionic strength pH 4 buffers improved LNP mRNA systems containing "first generation" lipids such as MC3 and KC2 to roughly equivalent activity for liver transfection as second-generation lipids such as those use in the COVID-19 vaccines.[39] Bleb structures are also sensitive to the ionizable cationic lipid employed in addition to the ionic strength of the pH4 buffer. LNP containing ALC-0315, for example, exhibit bleb structure even when formulated in low concentrations of Na-acetate, whereas others have noted high ionic strength did not improve transfection potency when formulating with SM-102.[186] It is therefore possible that optimization of ionizable lipids to achieve maximum transfection potency may achieve this by favoring bleb structures and thus improving mRNA stability rather than enhancing intracellular delivery properties. Bleb structures also likely arise from an imbalance in the amount of "surface lipid," such as PC and cholesterol, relative to the amount of "core lipid" such as the deprotonated ionizable lipid. If the amount of bilayer lipid exceeds the amount needed to form a monolayer around the hydrophobic core, blebs will form as has been observed for empty LNP (eLNP).[42,187]

The size and nature of the RNA cargo also influences morphology, with a notable influence on the frequency and size of bleb structures. They have not been observed for LNP siRNA systems. They are, however, readily observed for mRNA cargoes formulated in high ionic strength buffers during the mixing stage at pH 4 or in the presence of highly cone-shaped lipids containing branched acyl chains.[184] Both situations appear to drive the ionizable lipid that is not associated with nucleic acid cargo into separate domains at pH 4, leading to more complete separation of mRNA cargo from ionizable cationic lipid and improved mRNA stability. The putative association of mRNA within the bleb regions correlates with studies showing that the number of mRNA per LNP can be calculated from the proportion of bleb structure observed by cryo-TEM per LNP when formulated in high citrate solutions.[39] This technique agrees well with theory assuming a diameter of 2 nm for mRNA and a length per nucleotide of 0.3 nm and that the mRNA is condensed into a sphere occupying the bleb region. The presence of very large RNA molecules, such as self-amplifying RNA (saRNA) further promotes bleb structure. A study of LNP saRNA systems shows pronounced bleb structure in LNPs that exhibit an oval shape with one end containing the neutral oil droplet ionizable lipid and the other end a bleb containing the saRNA.[188]

The rational design of LNP mRNA systems would clearly design against the presence of empty LNPs. In this regard, there is wide disparity in the reports of the number of mRNAs encapsulated per LNP and the proportion of eLNP. Several studies have indicated a weighted average of 4–6 mRNA constructs within a single particle,[189,190] while Arteta et al. reported around 10 erythropoietin (EPO) mRNA copies per particle[142]; however, a recent study utilizing multi-laser cylindrical illumination confocal spectroscopy reported 2 mRNAs per particle with a presence of 40%–80% empty LNPs depending on the assembly conditions.[191] This is clearly an area ripe for exploration.

Therapeutic applications of LNP mRNA systems

LNP mRNA vaccines

The first studies that established the potential of LNP RNA-based vaccines used self-amplifying RNA[192] in combination with LNP containing DLinDMA developed for delivery of siRNA to the liver. Other investigators established the utility of similar LNP systems containing mRNA to produce protein *in vivo*; however, this was largely confined to the patent literature.[193] All these studies built off the success of Onpattro and had very similar lipid compositions. Weissman and colleagues showed that strong expression of marker genes could be observed following administration of LNP-mRNA via various routes, including intramuscular (i.m.) in 2015.[179] This was followed by a seminal publication in 2017 demonstrating the potential of LNP mRNA systems as vaccines for the Zika virus.[4] The clinical utility of an LNP mRNA vaccine was subsequently demonstrated by Moderna for a flu vaccine[194] and then by CureVac for a rabies vaccine.[195] These efforts culminated in the efforts of Acuitas, BioNTech, and Pfizer to develop Comirnaty and those of Moderna to develop SpikeVax as vaccines for COVID-19.[6,7,196]

The success of LNP mRNA systems as COVID-19 vaccines has led to extensive efforts to develop mRNA vaccines against a variety of infectious diseases and cancer as summarized elsewhere.[8,9] These include influenza,[197,198] HPV-induced tumors,[199] melanoma,[13] Congo hemorrhagic virus,[200] herpes,[201] HIV,[202,203] malaria,[204] Zika virus,[205] metapneumovirus,[206] respiratory syncytial virus,[207] Chikungunya virus,[208] yellow fever,[209] rabies,[210] and encephalamyelitis.[211] LNP

mRNA vaccines have become a major technology for future vaccine development.

It is highly likely that improvements can be made in LNP mRNA systems for vaccine applications. Current versions have similar lipid compositions to those used for liver expression. However, i.m. administration allows the use of LNP systems that can differ substantially from LNP systems administered intravenously. Parameters such as LNP size, circulation lifetime, systemic toxicity, and manufacturing are less constraining. For example, LNP mRNA systems to achieve therapeutic levels of protein production in the liver are usually operating at dose levels in the range of 0.3 mg mRNA/kg body weight.[151] For an 80 kg man, this corresponds to a dose of 24 mg of mRNA. Vaccine applications on the other hand require as little as 30 μg per individual.[4,205] The major issues for vaccines are how to maximize and extend protection against infection for longer periods, how to improve storage stability, and how to eliminate components such as the PEG-lipid that may cause unwanted immunogenic reactions.

Improvements in potency/duration can be expected from variations in the ionizable lipid. Work by Alameh et al.[14] has shown that the ionizable lipid within the LNP drives a strong adjuvant effect resulting in T follicular helper (Tfh) cell activation driving germinal center B cell differentiation as a key pathway driving this response. eLNP with no mRNA encapsulated functioned as a potent adjuvant when co-administered with an equivalent recombinant protein antigen and induced long-term immunity similar to LNP mRNA encoding the same protein, demonstrating independence of the LNP adjuvant effect from encapsulated mRNA. Notably, replacing the ionizable lipid with the permanently cationic lipid DOTAP resulted in complete loss of adjuvant activity of eLNP. A study by Moderna for optimal ionizable lipids for i.m. vaccine applications showed little correlation between protein expression and immunogenicity and that the optimal pKa of ionizable lipids for immunogenicity of vaccines was between 6.6 and 6.8. However, the immunogenicity observed was highly variable,[212] possibly due to differential sensing of lipid structures by the innate immune system.[213] In this regard, the adjuvant activity of SARS-CoV-2 LNP mRNA vaccines could be improved by inclusion of an adjuvant lipidoid that endowed Toll-like receptor 7/8 agonistic activity, thereby improving innate immune activation resulting in a longer lasting humoral immune response.[214]

Lastly, a key goal is to develop LNP formulations that have similar stability and potency to current formulations but do not contain PEG at all. Multiple approaches are being explored including replacing PEG with different polymers or outright eliminating the moiety through other formulation methods. Unpublished results suggest PEG-lipids can be eliminated.

Therapeutic applications of LNP mRNA systems for protein replacement, gene editing, or cell reprogramming

The remarkable finding that i.v. administration of LNP mRNA systems could result in expression by the liver of the protein the mRNA codes for has led to a legion of potential LNP mRNA therapeutics. Early studies showed that biologically significant levels of proteins such as erythropoietin could be secreted into the circulation at LNP mRNA dose levels as low as 0.03 mg mRNA/kg body weight in non-human primates.[151] A wide range of applications have followed,[8,9] including secreting monoclonal antibodies to target cancer cells or viruses.[215] For example, the systemic administration of LNP encapsulating mRNA encoding the light and heavy chains of the anti-HIV-1 antibody VRC01 fully protected humanized mice from intravenous HIV-1 challenge.[216] A huge range of potential LNP mRNA therapeutics relying primarily on liver transfection are currently being pursued. These include treatments for rare diseases (up to 6% of the population has a disease in this category[217]) such as propionic acidemia,[11] acyl CoA dehydrogenase deficiency,[218] glycogen storage disease,[10] phenylketonuria[219] to more common disorders such as obesity,[16] liver cancer,[220] liver fibrosis,[221] and liver failure.[222] Transfection of other tissues following i.v. or intra-target tissue administration are finding utility to treat cystic fibrosis.[223,224] spinal cord injury,[225] heart failure,[12,226,227] and lymphedema[228] among others.

LNP mRNA enabled gene editing *in vivo* using CRISPR-based approaches is also becoming a reality. LNP mRNA delivery of CRISPR-Cas9-base editor fusions were able to introduce a missense mutation into the PCSK9 gene in the hepatocytes of non-human primates (NHP), resulting in long-term lowering of LDL cholesterol.[227,229] Alternatively, the use of CRISPR-Cas9 gene editing to disable production of TTR in the liver to treat hATTR has resulted in an 80%–96% response rate for this potentially deadly genetic disease.[230]

Finally, the potential for cell reprogramming in areas such as chimeric antigen receptor (CAR) T cell therapy is immense. CARs are artificial receptors expressed on T cells which allow them to recognize and destroy essentially any target cell.[231–235] Broad implementation of CAR T therapies has been limited by logistical problems such as leukapheresis and use of viral vectors for engineering.[236,237] *In vivo* delivery of CAR mRNA to T cells via LNP would transform this powerful treatment modality from an expensive and privileged procedure of last resort[236,238,239] to a generally available therapeutic. *In vivo* CAR T cell generation using LNP-mRNA has been demonstrated in a study in which mRNA encoding a CAR targeting fibroblast activation protein (FAP) was successfully delivered to up to 25% of T cells using a CD5 targeting ligand.[12] These *in vivo* generated CAR T cells were subsequently shown to have a functional response in reducing cardiac fibrosis after damage by destroying cardiac fibroblasts expressing the FAP protein. Of note, an LNP mRNA encoding a CD19 targeting CAR has recently entered phase 1 clinical trials for B cell disorders (see Table 1). The potential downstream applications of this technology are immense, including in the emerging fields of CAR macrophages[240] and CAR natural killer cells,[231,241] among others.

The applications of LNP mRNA systems have been extensively reviewed elsewhere[8,9,211,242–245] and there is little point in reiterating

all applications. However, we note three areas where rapid progress is expected. These concern treatment of rare diseases, cancer therapies, and gene editing applications. If a child is born with a rare metabolic disease due to a mutated protein, there is now often a simple solution. Construct an mRNA coding for that protein, package it in an LNP, and get the liver to make it. Or get it made by direct injection into privileged organs such as the eye or brain for treatment of ocular[246] and neurological disorders.[247] For more prevalent diseases, transfection of bone marrow[248,249] is increasingly enabling treatment of myeloid and erythroid pathologies. The fact that these personalized medicines can be developed in a matter of weeks means that the therapy can be constructed on a timescale relevant to the patient. Similar considerations apply to the rapidly growing field of cancer vaccines. LNP mRNA systems coding for tumor neoantigens are now proving effective vaccines for pancreatic cancer,[250] lung cancer,[251] and melanoma.[252] Further advances for cancer therapeutics are likely to include *in vivo* CAR-T therapeutics as well as personalized immunotherapies. Finally, gene editing for atherosclerosis[253] and amyloid disease[230] among many other possibilities are having increasing impact.

Next generation LNP delivery systems

Rational design for extrahepatic delivery

Most LNP mRNA systems currently used for extra-hepatic applications employ the "Onpattro" formulation (ionizable lipid/DSPC/cholesterol/PEG-lipid in molar ratios 50/10/38.5/1.5), which compromises activity in extra-hepatic tissues as such formulations are rapidly sequestered to the liver following i.v. administration. In order to access extrahepatic tissues such as bone marrow, lung, skin, or other organs with meaningful quantities of LNP, much longer circulation lifetimes are required. Classic solutions to this problem such as incorporating PEG-lipids that do not dissociate are not viable as the presence of a PEG coating inhibits uptake into target cells and thus prevents transfection.

There have been extensive efforts made to identify LNP mRNA systems with tropism for various organs following i.v. administration using high through-put approaches. One of these has been to co-encapsulate a unique DNA barcode with a reporter mRNA within a specific formulation to generate an LNP library. Screens are done by pooling 30+ formulations followed by i.v. delivery. Transfected cells in each organ are then separated, and high-throughput sequencing performed to determine the relative abundance of each unique barcode sequence within a cell population, implying transfection by the specific LNP composition formulated with the barcode.[17,254] While this allows screening of multiple formulations, it is unclear whether a much larger dose of any specific composition to achieve therapeutic benefit will have the same biodistribution, transfection or toxicity profiles. Another strategy for endowing LNP with altered *in vivo* transfection profiles is to include a 5th lipid component to LNPs, such as the selective organ targeting (SORT) approach. Inclusion of a permanently cationic lipid DOTAP (up to 50% mol/mol) to canonical LNP formulations skews transfection to the lungs, whereas inclusions of the anionic 18PA enables spleen-specific transfection,[20] demonstrating organ-specific transfection can be achieved in this manner. The mechanism by which SORT functions is proposed to be via an altered biomolecular corona composition due to altered charge-based interactions with passive targeting serum proteins.[149] However, as already noted, the inclusion of toxic molecules such as permanently charged cationic lipids raises tolerability concerns.

From a rational design point of view, the obvious solution is to surround the LNP mRNA system with a lipid bilayer consistent with longer circulation lifetimes. Due to the remarkable self-assembly properties of lipids this is increasingly possible. As the proportion of bilayer helper lipids is increased in LNP systems containing ionizable lipids, the morphology changes from the solid core with a monolayer of lipid to systems with an apparent solid core suspended in the aqueous interior surrounded by a lipid bilayer (see Figure 1, bottom). These hybrid LNP are highly transfection competent. Further, consistent with the hypothesis that an exterior bilayer consisting of bilayer lipids such as DSPC/Chol or SPM/Chol, these LNP exhibit significantly longer circulation lifetimes following i.v. administration[36] and are increasingly enabling extrahepatic transfection, particularly for tissues such as the spleen and bone marrow.

CONCLUSIONS

LNP RNA delivery systems are enabling a host of gene therapies. There are over 400 LNP RNA therapeutics in preclinical or clinical development. The huge advantages of LNP technology for delivery of nucleic acid-based drugs include unlimited payload capacity, lack of immunogenicity enabling repeat dosing, extremely rapid development times for highly personalized therapeutics, ever improving tolerability profiles and straightforward manufacturing processes. We contend that LNP technology will become the dominant method for enabling gene therapies of all types.

This review illustrates the power of the rational design approach as it applies to development of LNP RNA therapeutics. A summary timeline of development from liposomes to liposomal LNP is shown in Figure 1 highlighting this process. Essentially all of the advances that have led to approved LNP RNA products such as Onpattro, Comirnaty, and SpikeVax have stemmed from a rational design approach based on an understanding of membrane lipid biophysics and an understanding of biology as it relates to LNP interactions with serum proteins and intracellular delivery following endocytosis. We expect that this situation will continue. This applies particularly to the development of LNP systems that efficiently transfect extrahepatic tissue. We make the point that while LNPs are highly promiscuous transfection systems for many different tissues *in vivo*,[255] in order for them to transfect such tissues effectively they must actually get there in reasonable quantities, which demands long-circulating systems such as the liposomal LNPs achieved by employing high levels of bilayer lipids. These systems combine excellent transfection properties with an external bilayer that can be optimized to achieve circulation lifetimes of many hours. Once transfection of tissues of interest is achieved, methods to achieve more specific transfection can be pursued as necessary.

In summary, the potential of gene therapies enabled by LNP technology is revolutionary. There is every reason to believe that LNP RNA systems to efficiently transfect most if not all tissues in the body to enable treatment of most diseases are forthcoming. It is a most exciting time in medicine.

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AUTHOR CONTRIBUTIONS

C.A.B., writing, review and editing, writing – original draft and final draft, and conceptualization. J.A.K., writing, review and editing, writing – original draft and final draft, and conceptualization. M.H.Y.C., writing – original draft, review and editing, and conceptualization. K.A., writing – original draft. D.W., review and editing, and writing – final draft. P.R.C., writing, review and editing, conceptualization, and supervision.

DECLARATION OF INTERESTS

C.A.B. and P.R.C. are co-founders of Resolve Nanotherapeutics. J.A.K., D.W., and P.R.C. are co-founders at NanoVation Therapeutics.

NanoVation therapeutics has the filed patent application US20240285544A1 for LNP mRNA delivery systems.

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