

Lipid nanoparticle delivery systems for siRNA-based therapeutics

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Abstract Therapeutics based on small interfering RNA (siRNA) have a huge potential for the treatment of disease but requires sophisticated delivery systems for in vivo applications. Lipid nanoparticles (LNP) are proven delivery systems for conventional small molecule drugs with over eight approved LNP drugs. Experience gained in the clinical development of LNP for the delivery of small molecules, combined with an understanding of the physical properties of lipids, can be applied to design LNP systems for in vivo delivery of siRNA. In particular, cationic lipids are required to achieve efficient encapsulation of oligonucleotides; however, the presence of a charge on LNP systems can result in toxic side effects and rapid clearance from the circulation. To address these problems, we have developed ionizable cationic lipids with pK_a values below 7 that allow oligonucleotide encapsulation at low pH (e.g., pH 4) and a relatively neutral surface at physiological pH. Further optimization of cationic lipids to achieve maximized endosomal destabilization following uptake has resulted in LNP siRNA systems that can silence genes in hepatocytes at doses as low as 0.005 mg siRNA/kg body weight in mouse models. These systems have been shown to be highly effective clinically, with promising results for the treatment of hypercholesterolemia and transthyretin-induced amyloidosis among others. More LNP siRNA therapeutics, targeting different tissues and diseases, are expected to become available in the near future.

Keywords Lipid nanoparticles · Therapeutics · Small interfering RNA · Drug delivery systems · LNP siRNA systems

Introduction

RNA interference (RNAi) is a natural cellular process that can be exploited to silence specific genes by intracellular delivery of small interfering RNAs (siRNAs) and microRNAs [1]. Initial work on RNAi was conducted in flies and worms; however, it was soon demonstrated that synthetic siRNAs could induce gene silencing in mammalian cell lines [2]. Numerous examples of siRNA knockdown have now been published with the potential for the treatment of diseases ranging from viral infections [3–7], cancer [8–10], dominant genetic disorders [11–13], and autoimmune disease [14].

Theoretically, siRNAs can be designed to silence any gene of interest. However, a number of challenges exist for the delivery of free siRNA into target cells in vivo. First, the large, anionic, hydrophilic structure of siRNA molecules inhibits intracellular delivery. Second, siRNA molecules are not stable in serum and are prone to nuclease degradation [15]; the half-life for unmodified/unprotected siRNAs in serum ranges from minutes to an hour [6, 16, 17]. Finally, side effects such as immunogenicity and off-target effects can also occur [18]. Attempts have been made to improve the therapeutic profile of siRNAs by chemical modifications such as by the introduction of phosphorothioate linkages [15, 19]. These modifications protect the siRNA molecules from nuclease degradation in serum and can diminish off-target effects; however, dose levels required to achieve gene silencing in vivo are very high and effects are limited to the liver [20].

Another approach to improving siRNA therapy is to incorporate siRNAs into nonviral delivery systems. Here, we focus on lipid (or liposomal) nanoparticles (LNP). Here, we briefly review LNP delivery of small molecule drugs and subsequently

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summarize recent advances in the design of LNPs for the delivery of siRNA and the promising clinical results that have been achieved using this new class of therapeutics.

Generation of lipid nanoparticle delivery systems

Bangham and colleagues first showed that phospholipids dispersed in water spontaneously formed closed bilayer structures [21, 22] and introduced the term “liposomes” [23] to describe these systems, which were initially used as models of biological membranes. The potential of liposomes to be used as drug delivery systems was soon recognized [24–27] due to their bilayer structure that allowed the encapsulation of biologically active materials.

Considerable efforts have been made to produce small (diameter <100 nm) unilamellar liposomes [28, 29]. The extrusion technique, which uses high pressure to force preformed large multilamellar liposomes through polycarbonate filters of defined pore size, is now the preferred procedure for making LNP in the 100-nm size range [30–33]. LNPs can also be formed by diluting organic solutions of lipids with aqueous media under rapid mixing procedures, LNPs form as the polarity is raised [34]. Microfluidic mixing techniques can also be used to generate liposomal LNPs using the staggered herringbone mixer (SHM) [35]. “Limit size” LNPs can be formed with diameters of 20–100 nm depending on the lipid composition [36, 37]. This technique is readily scalable; previous techniques for producing limit size systems, such as sonication [38, 39], are difficult to scale up.

LNPs for the delivery of conventional drugs

Drugs that are weak bases and weak acids can be loaded into LNP in response to transmembrane pH gradients [40–45]. This technique can be applied to drugs that are not weak bases by synthesizing weak base prodrugs [46]. LNPs that have circulation half-lives of several hours or more preferentially accumulate at disease sites such as tumors and sites of infection and inflammation due to the enhanced vascular permeability in these regions (Fig. 1). This is known as the enhanced permeability and retention effect [47–49]. Long-circulating LNP loaded with therapeutics are, therefore, able to extravasate into target tissue [50], and subsequent drug release leads to a therapeutic benefit [51]. Healthy tissues such as muscle that have non-fenestrated vascular endothelia have limited permeability [52, 53], which reduces the toxic side effects of the encapsulated drugs in these tissues.

For LNPs to accumulate in diseased tissues such as tumours, they must have long circulation half-lives. Opsonization by serum proteins can result in rapid clearance of the LNPs by the mononuclear phagocyte system (MPS) consisting of liver

Kupffer cells and spleen macrophages [54–59]. The presence of polyethylene glycol (PEG) polymers on the surface of LNPs can reduce opsonization, leading to longer circulation lifetimes and increased tumor accumulation [60–66]. A PEGylated liposomal doxorubicin formulation has been approved for the treatment of AIDS-related Kaposi’s sarcoma [67], ovarian cancer [68], and multiple myeloma (in combination with bortezomib) [69]. Alternatively, the use of saturated lipids such as sphingomyelin results in reduced opsonization and long circulation lifetimes, leading to an approved LNP formulation of vincristine [70–74] for the treatment of acute lymphoblastic leukemia at second or greater relapse. At least eight LNP formulations of conventional drugs have been approved by the FDA, primarily for the treatment of cancer and its complications. At least ten LNP formulations of other small molecule drugs are in clinical trials [75].

LNPs for the delivery of siRNA

The potential of cationic lipids with a permanent positive charge to mediate the intracellular delivery of nucleic acid was first demonstrated by Felgner [76, 77]. Liposomes containing high proportions of cationic lipids were mixed with plasmid DNA, resulting in the formation of DNA–lipid complexes that facilitated efficient transfection of cells in vitro. It has been shown that cationic lipids interact with naturally occurring anionic lipids to induce non-bilayer H_{II} phase structure which is consistent with the ability of cationic lipids to destabilize biological membranes and facilitate intracellular delivery of macromolecules such as DNA and RNA polymers [78]. Complexes have proven of limited use in vivo due to their large size (microns) and high surface charge, resulting in rapid clearance and toxic side effects. Despite the synthesis of hundreds of different cationic lipids [79, 80], gene expression using plasmid–cationic lipid complexes could only be observed following local administration, and the toxic side effects of cationic lipids became increasingly problematic [81, 82].

A key advance was the development of ionizable cationic lipids with apparent pK_a’s in the region of 7 or lower [83, 84], coupled with the development of techniques to encapsulate nucleic acid polymers into stable LNP with diameters of 100 nm or less. At acidic pH values, below the pK_a of the ionizable lipid (e.g., pH 4), the cationic charge on the lipid allows efficient encapsulation of oligonucleotides into LNPs. At physiological pH, above the pK_a of the ionizable lipids, the LNPs acquire a near neutral surface charge that reduces their toxicity. Ionizable cationic lipids also play an important role in stimulating intracellular release from endosomes [36]. At the acid pH of the endosomal compartments, the ionizable lipids acquire a positive charge and can interact with the anionic lipids (such as lysobisphosphatidic acid) in endosomes to

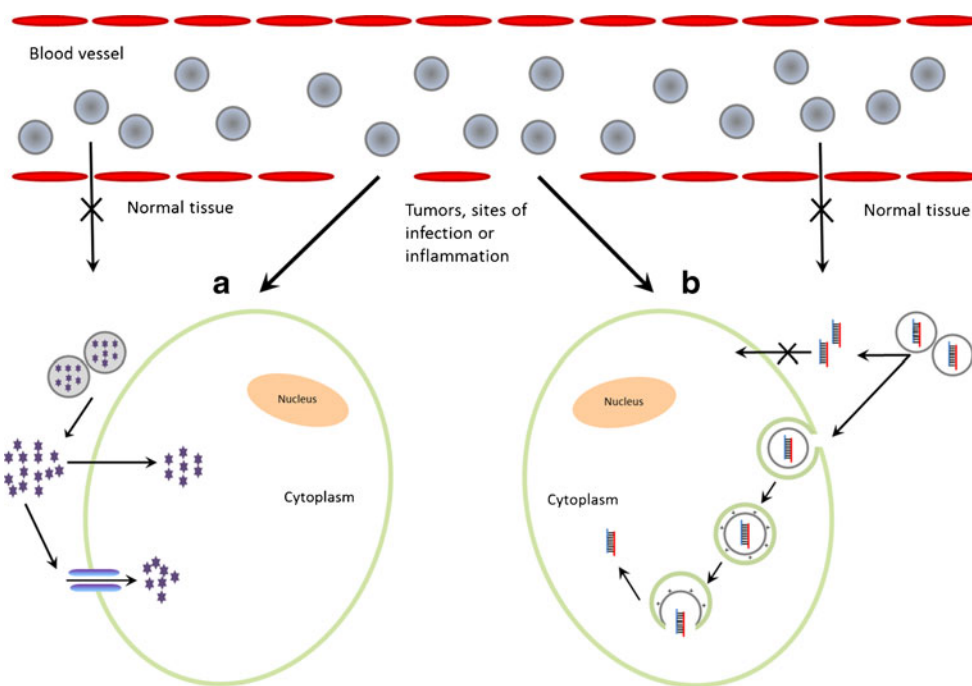


Fig. 1 Mechanism of the intracellular delivery of small molecule drugs (**a**) and siRNA (**b**) to target cell compartments by LNP. LNPs with sufficiently long circulation lifetimes can extravasate through the leaky vasculature in diseased tissues, leading to LNP accumulation in the interstitial fluid surrounding the target cells. **a** LNPs containing small molecule therapeutics. Following extravasation into the interstitial fluid, LNPs release their encapsulated drug over a period of time and the released (free) drug is taken up either by passive diffusion across the target cell membrane or by active transport. LNPs targeted via ligands against target cell surface receptors can be taken up via receptor-mediated endocytosis,

leading to intracellular drug release (not shown). **b** LNPs containing nucleic acid polymers. Following extravasation into the interstitial fluid, released (free) nucleic acids are unable to cross the target cell membrane due to their large size. Attachment of ligands against cell surface receptors to the LNP surface or spontaneous association of serum proteins such as apolipoprotein E to the LNPs can result in endocytotic uptake into target cells. Once in endosomes, the acidic environment causes the ionizable phospholipids to become positively charged and combine with endogenous anionic lipids, which destabilizes the endosomal membrane, releasing the nucleic acids (e.g., siRNA) into the cytoplasm

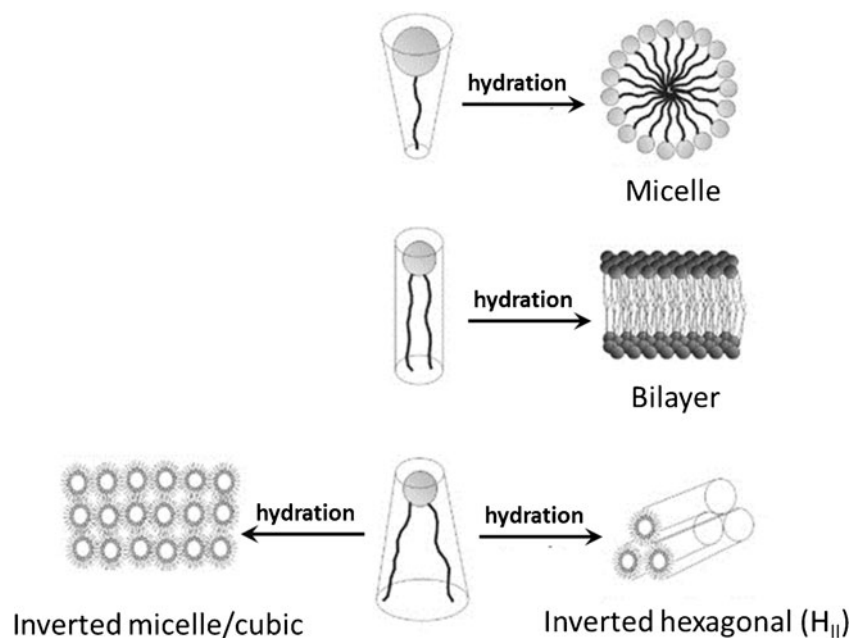
destabilize the endosomal membrane and promote the intracellular release of nucleic acids [78, 85, 86] (see Fig. 2).

The first ionizable cationic lipid used to encapsulate (antisense) oligonucleotides in LNP was 1,2-dioleoyl-3-dimethylaminopropane (DODAP) [83, 84]. The encapsulation procedure involved the formation of 100-nm-diameter LNP consisting of a mixture of DODAP, PEG-lipid, cholesterol, and distearoylphosphatidylcholine (DSPC) at pH 4 in the presence of 40 % ethanol, followed by the addition of oligonucleotides to the preformed vesicles under rapid mixing and then removal of ethanol by dialysis (see Fig. 3a). Encapsulation efficiencies of approximately 60 % could be achieved with these systems [83]. Oligonucleotides could also be added at the time of hydration, leading to encapsulation efficiencies as high as 80 % [84]. Encapsulation of siRNA oligonucleotides was then demonstrated for a similar lipid composition, consisting of the ionizable cationic lipid dilinoleoyl-DMA (DLinDMA), DSPC, cholesterol, and PEG-lipid, using an in-line T-tube mixing procedure [87].

Further improvements in the effectiveness of LNPs for siRNA delivery came from studies to optimize the gene silencing potency of the ionizable cationic lipids by optimizing their bilayer destabilizing capabilities and apparent pK_a

values. These studies were stimulated by two observations. First, more unsaturated acyl chain compositions may be expected to lead to improved membrane destabilization on complexing of the cationic lipid with endogenous anionic lipids due to an enhanced “cone” shape [84]. Second, potency may also be expected to depend on the pK_a of the ionizable cationic lipid. In particular, the pK_a should be as low as possible to avoid rapid clearance by the MPS (due to the positive charge on the LNP) but not so low that the cationic lipid cannot become protonated at endosomal pH values. A large number of ionizable lipids have been screened, using an intravenous (i.v.) murine factor VII (FVII) model, for their ability to inhibit FVII production by hepatocytes [88, 89] following i.v. administration of LNP containing FVII siRNA. Two particularly potent ionizable cationic lipids were identified, 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) [89] and heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLinMC3-DMA) [88] (for lipid structures, see Fig. 4). Both lipids have pK_a values of approximately 6.4. LNP siRNA formulated with DLin-KC2-DMA were 100-fold more potent in the murine FVII model compared to DLinDMA. DLinMC3-DMA led to a further tenfold improvement, resulting in a 50 % gene

Fig. 2 Molecular geometry of lipids and the predicted morphological self-assembly structures. The formation of non-bilayer structures by LNP lipids at the acid pH of the endosomes explains the release of nucleic acids from the endosomes into the cellular cytoplasm. Reprinted from [85] with permission



silencing of FVII at a dose level of 0.005 mg siRNA/kg body weight [88] (Fig. 5). LNP siRNA systems containing DLinMC3-DMA have led to notably successful LNP siRNA formulations in the clinic (see the “Clinical development of LNP siRNA systems” section).

PEG-lipids play a critical role in the formation of LNP particles; in the absence of PEG-lipid, large aggregates are formed [83]. However, when the PEG-lipids remain associated with the LNPs, they inhibit LNP association with cells and prevent the intracellular delivery of the therapeutic cargo. Hence, PEG-lipids with short acyl chains have been developed that quickly dissociate from the LNPs following injection, allowing them to interact with target cells [90, 91]. The acyl chain length determines the rate at which the PEG-lipids dissociate from the nanoparticles. For example, PEG-lipids with C₁₄ acyl chains dissociate from LNPs with half times of around 1 h, versus PEG-lipids with C₂₀ acyl chains with half times of 24 h or longer [92]. The circulation half-lives for LNP containing PEG-lipid with C₁₄ and C₂₀ acyl chains can range from an hour to 10 h, respectively [84]. When PEG-lipid dissociates from the surface of LNPs, serum proteins such as apolipoprotein E (ApoE) can absorb to the LNP surface, facilitating uptake into hepatocytes through the scavenger receptor and low-density lipoprotein (LDL) receptors [93].

Studies on the structure of LNP siRNA systems show that LNP siRNA systems can exhibit a nanostructured core [94] (Fig. 6). A combination of cryo-transmission electron microscopy, ³¹P NMR, membrane fusion assays, density measurements, and molecular modeling studies were employed to characterize the internal organization of LNP siRNA systems containing DLin-KC2-DMA, DSPC, cholesterol, and PEG-lipids. The LNP siRNA systems were generated

using the SHM microfluidic mixing apparatus to mix a lipid in ethanol solution with an aqueous solution of siRNA (see Fig. 3b). The experimental results, which agree with molecular modeling simulations, indicate that the LNPs have a novel nanostructured core containing siRNA duplexes complexed to cationic lipids in inverted micelles inside the LNP. The structural studies offer insights into the mechanism of formation of LNP siRNA and help explain how encapsulation efficiencies that approach 100 % can be achieved. In particular, it can be envisaged that the siRNA molecule first interacts with the cationic lipid to form hydrophobic complexes that serve as nucleating particles for the subsequent deposition of surface lipids such as PEG-lipids and DSPC.

LNP siRNA systems clearly have potential for silencing genes and treating diseases in a wide variety of tissues in the body; however, the most effective systems currently available are primarily useful for silencing genes in the liver (hepatocytes). This is due to the ability of these particles to associate with ApoE, which triggers uptake into hepatocytes via the LDL and other receptors. Effective LNP-dependent delivery of siRNA to extrahepatic tissues has been challenging. Recent studies have shown that LNP siRNA systems optimized for liver gene silencing can also silence genes in other tissues, but at much higher doses. For example, gene silencing in antigen-presenting cells, such as primary bone cell macrophages and dendritic cells [95], has been observed at dose levels of 5 mg siRNA/kg body weight. Alternatively, LNP siRNA systems can be used to silence the androgen receptor (an oncogene driving the progression of prostate cancer) in xenograft distal tumors in vivo, resulting in decreased serum PSA levels, following i.v. injection [96]. However, the dose levels required to achieve this are very high (10 mg siRNA/kg body weight, six doses). Other

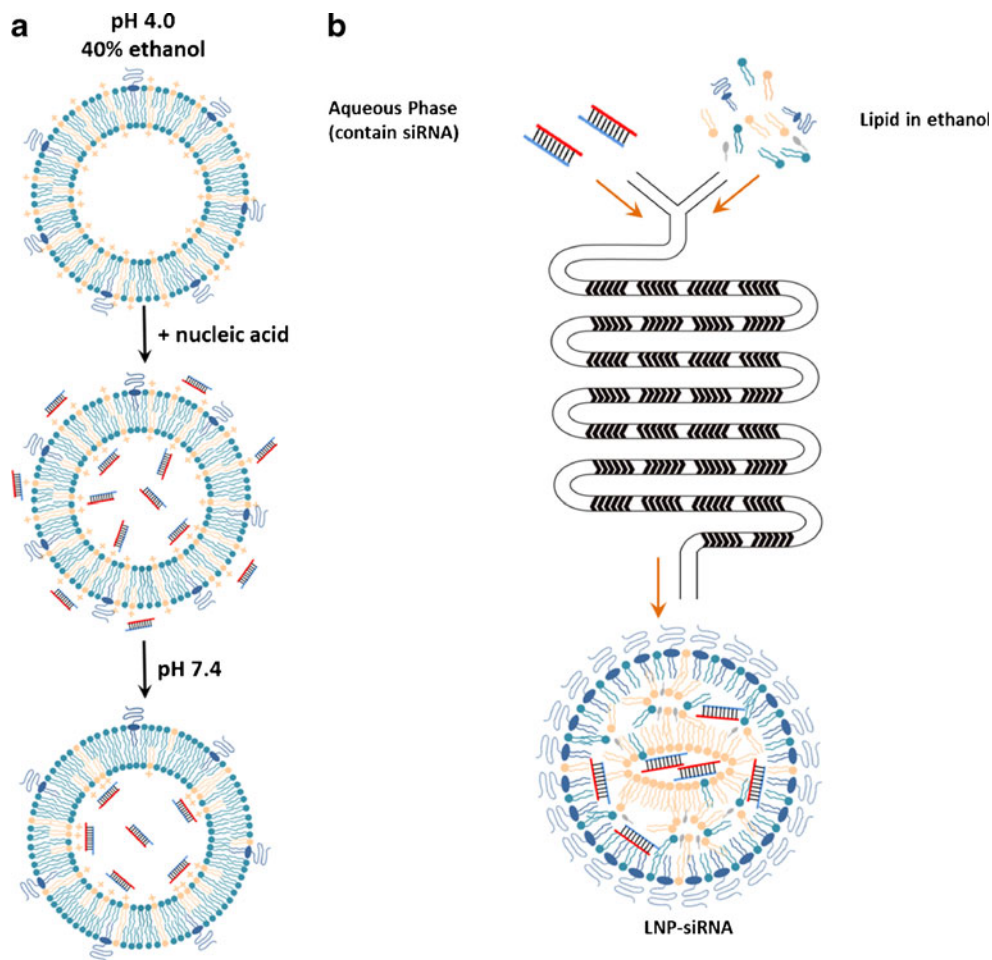


Fig. 3 Schematic demonstration of LNP formulation by the preformed vesicle method (**a**) or by in-line mixing using microfluidics (SHM) (**b**). **a** Preformed vesicles containing PEG and ionizable cationic phospholipids are formed in 40 % ethanol at pH 4.0 by extrusion. The addition of nucleic acid results in structural reorganization of the lipids and internalization of the nucleic acid. Removal of ethanol and increasing the external pH to 7.5 result in LNP with a neutral surface and

encapsulated nucleic acids. **b** SHM in-line mixing method. One inlet of SHM contains lipids in ethanol and the other inlet contains nucleic acids in an aqueous solution. When these two streams meet in the SHM, rapid (millisecond) mixing occurs that results in the rapid precipitation of lipid–siRNA complexes from solution and spontaneous formation of LNP siRNA nanoparticles. The SHM channel is $200 \times 80 \mu\text{m}$, and the herringbone structures are $30 \mu\text{m}$ high and $50 \mu\text{m}$ thick

investigators have demonstrated a ligand-targeted LNP formulation of ALK kinase siRNA that, at a dose level of 1.25 mg siRNA/kg body weight twice a week for a total of five doses, was able to induce apoptosis and inhibit angiogenesis in a murine neuroblastoma model, with a substantial increase in life span [97].

Clinical development of LNP siRNA systems

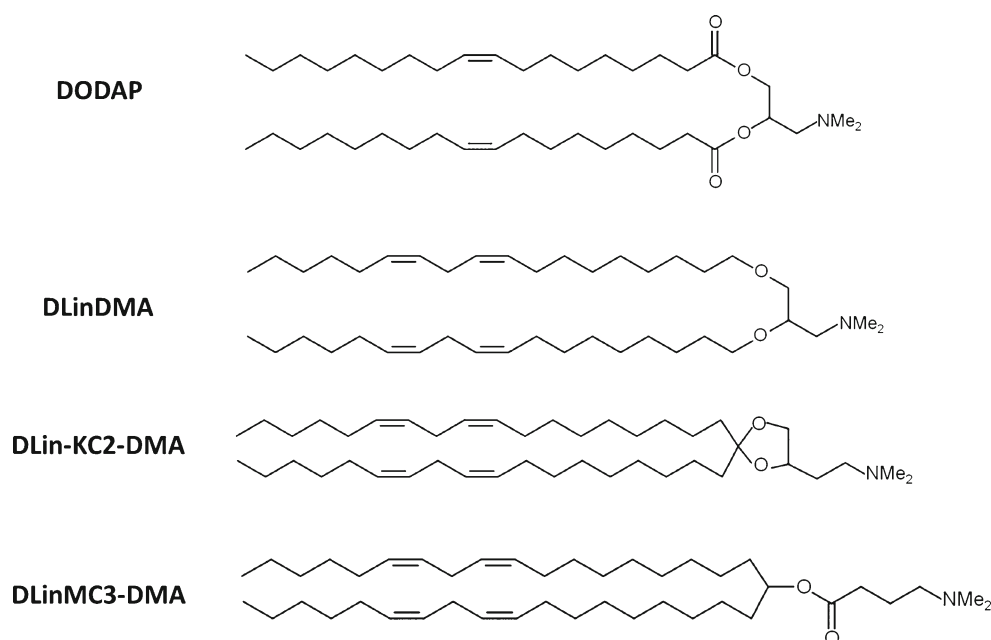
As discussed in previous sections, LNPs containing ionizable cationic lipids have a number of features necessary for the systemic delivery of polynucleic acids, including small sizes, serum stability, low surface zeta potentials at physiological pH, and cationic charge at acidic pH values (e.g., in endosomes). Further, by taking advantage of “endogenous” targeting processes due to association with ApoE following

administration, highly efficient uptake into hepatocytes can be achieved following i.v. administration, leading to excellent gene silencing capabilities in preclinical animal models. These characteristics have led to considerable success in the clinical development of LNP siRNA systems. This section focuses on four LNP siRNA clinical candidates using the ionizable cationic lipids summarized in previous sections.

LNP VEGF, KSP siRNA

LNP vascular endothelial growth factor (VEGF), kinesin spindle protein (KSP) siRNA is an LNP siRNA candidate to treat primary hepatocellular carcinoma (HCC), one of the most common cancers worldwide with more than 600,000 people diagnosed each year [98]. The LNP VEGF, KSP siRNA formulation in the clinic (ALN-VSP) contains the first generation cationic lipid DLinDMA and two species of

Fig. 4 Chemical structures of the ionizable cationic lipids DODAP, DLinDMA, DLin-KC2-DMA, and DLinMC3-DMA. The potency of LNP containing these lipids to silence FVII in mice following i.v. injection increases from *top* to *bottom*



siRNA that target two key genes involved in the HCC disease pathway: KSP and VEGF. KSP is important to cell division [99–101], and VEGF is vital for the growth of new blood vessels that feed the tumor cells [102, 103].

A phase I clinical trial for ALN-VSP was initiated in 2009, and 41 patients have been treated at dose levels ranging from 0.1 to 1.5 mg/ml (see <http://www.alnylam.com/Programs-and-Pipeline/Partner-Programs/Liver-Cancer.php>). The results to date show that ALN-VSP is generally well tolerated, and the recommended dose for phase II studies is 1.0 mg/ml. The therapeutic response data is encouraging; multiple

patients achieved stable disease or better, including a patient with liver metastases from primary endometrial carcinoma who has achieved a 70 % tumor regression by the end of the phase I study and a complete response at the phase I extension study. Tumor biopsy samples obtained in the phase I trial included both hepatic and extrahepatic tumours. The siRNAs targeting VEGF and KSP, respectively, were detected in most biopsy samples [104]. The analysis using 5' rapid amplification of cDNA ends in biopsy samples showed clear evidence of RNAi-mediated target messenger RNA (mRNA) cleavage.

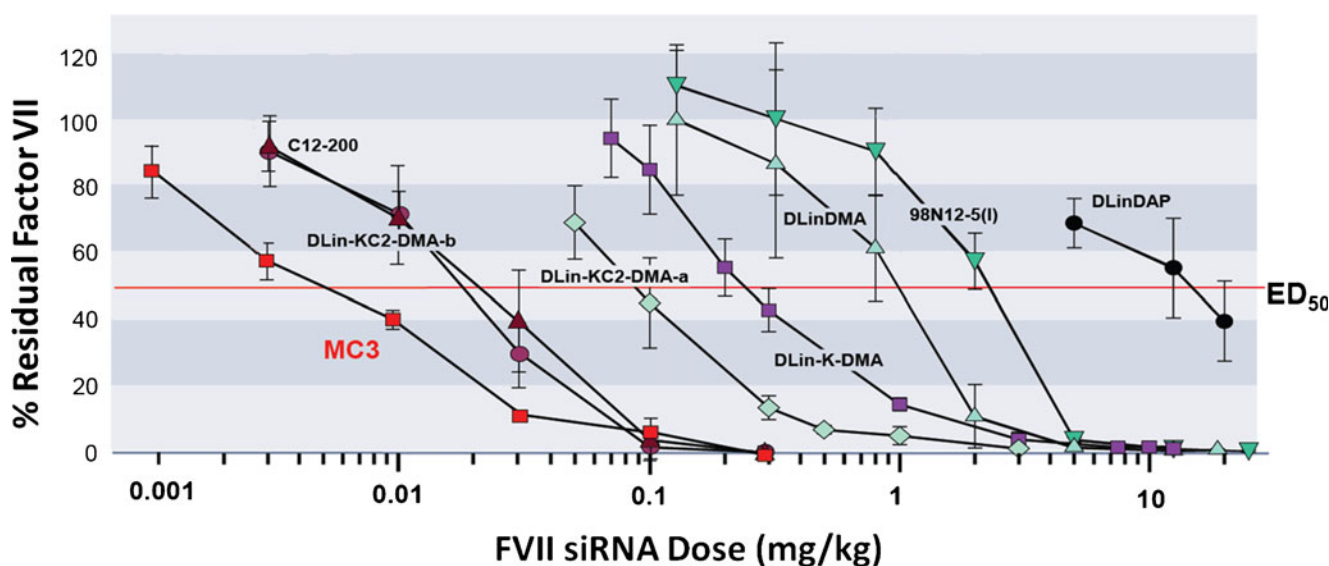


Fig. 5 Dramatic improvement of the efficacy of LNPs containing different cationic lipids in silencing of the FVII gene of hepatocytes in mice

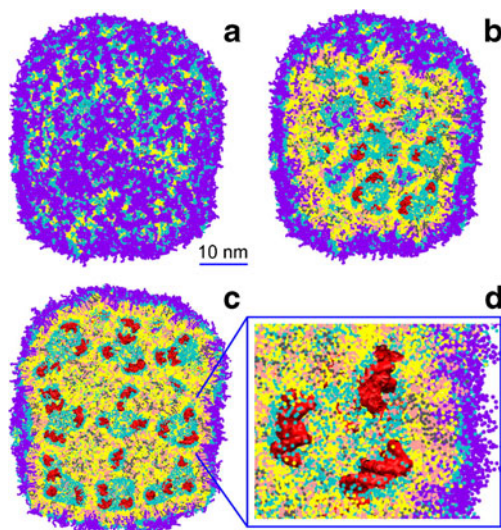


Fig. 6 Molecular modeling simulation of LNP siRNA structure. Prediction of an LNP contains irregular water-filled cavities separated by bilayer membranes, with nucleic acids bound to membrane surfaces. Shown are side (a), cross-section (b, c), and zoom-in (d) views. The cationic lipid DLin-KC2-DMA is shown in *yellow*, cholesterol in *pink*, DSPC in *gray*, lipid polar moiety in *cyan*, PEG-lipid in *violet*, and duplex DNA in *red*; water is not shown. The lipid composition was DLin-KC2-DMA/DSPC/cholesterol/PEG-lipid (4:1:4:1; mol/mol) and DNA to lipid ratio of 0.05 (*w/w*). Reprinted with permission from [94]. Copyright (2012) American Chemical Society

LNP TTR siRNA

The LNP transthyretin (TTR) siRNA formulation targets the TTR gene and is being developed to treat TTR-mediated amyloidosis (ATTR) [105]. ATTR is fatal disease characterized by neuropathy and/or cardiomyopathy. It is caused by mutations in the TTR gene that lead to amyloid protein accumulation in various tissues, including peripheral nerves and heart. Familial amyloidotic polyneuropathy (FAP) [106] and familial amyloidotic cardiomyopathy (FAC) [107] affects approximately 10,000 and 40,000 people worldwide, respectively. FAP patients have a life expectancy of 5 to 15 years from symptom onset, and the disease is currently treatable only by liver transplantation; the mean survival for FAC patients is approximately 2.5 years, and there are no approved therapies.

The LNP TTR siRNA formulation in the clinic (ALN-TTR02) is an intravenously delivered LNP siRNA formulation that contains the optimized ionizable cationic lipid DLinMC3-DMA (see <http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/TTR-Amyloidosis.php>). A phase I clinical trial of ALN-TTR02 in healthy volunteers has been conducted with doses ranging from 0.01 to 0.5 mg siRNA/kg body weight; preliminary results were announced in July 2012. The data showed that a single dose could mediate a rapid, dose-dependent, specific knockdown of up to 94 % in serum TTR protein levels, and a near 80 % level of suppression could be

sustained for up to 1 month. In addition, no significant toxicity issues were found. ALN-TTR02 is currently in a phase II trial in Europe, and the results from this trial are expected in mid-2013.

LNP PCSK9 siRNA

The LNP proprotein convertase subtilisin/kexin type 9 (PCSK9) siRNA formulation targets the PCSK9 gene, which regulates the expression of the LDL receptor in the liver [108–110]. Silencing PCSK9 leads to higher LDL receptor levels in the liver and, as a consequence, lower circulating LDL levels. Although hypercholesterolemia can be treated through dietary restriction and medicines such as the statins, many patients are statin-resistant or statin-intolerant and could benefit from this RNAi therapy.

The clinical formulation of LNP PCSK9 (ALN-PCS) contains the optimized ionizable cationic lipid DLinMC3-DMA (see <http://www.alnylam.com/Programs-and-Pipeline/Alnylam-5x15/Hypercholesterolemia.php>). Preclinical results in nonhuman primates have shown specific silencing of PCSK9 mRNA and reductions in PCSK9 serum protein levels of up to 90 %; the ED₅₀ is approximately 0.06 mg siRNA/kg body weight. A phase I clinical trial of ALN-PCS in healthy volunteers has been reported and shows significant and durable reductions in plasma PCSK9 levels of up to 84 % and reductions in LDL cholesterol plasma levels of up to 50 % following a single i.v. administration at a dose level of 0.4 mg siRNA/kg body weight. No significant toxicities were observed.

LNP PLK1 siRNA

LNP polo-like kinase 1 (PLK1) siRNA contains siRNA targeting PLK1. The formulation in clinical development (TKM-PLK1) contains the first generation ionizable cationic lipid DLinDMA. PLK1 is a serine/threonine kinase that is involved in many types of tumor cell proliferation. The downregulation of PLK1 prevents cell cycle progression into mitosis and induces apoptosis in tumor cells [111]. In pre-clinical studies, TKM-PLK1 has been shown to effectively reduce PLK1 mRNA level in vitro and in tumor xenograft models in mice.

Phase I clinical trials on TKM-PLK1 were initiated in 2010 in patients with advanced solid tumors that are resistant to current therapies. The trial is evaluating the safety, tolerability, and pharmacokinetics of TKM-PLK1, as well as determining the maximum tolerated dose (MTD). To date, 23 patients have been treated with TKM-PLK1 at doses ranging from 0.15 to 0.9 mg/kg body weight/week [112]. Results show that the most common drug-related adverse events included mild to moderate levels of fever, chills, nausea, vomiting, and fatigue, leading to an MTD of 0.75 mg/kg body weight/week. Two

patients have received TKM-PLK1 for more than 6 months with no cumulative toxicity. One of these patients has stable disease (colon), and the other has a durable partial response (carcinoid tumor).

Summary

LNP delivery systems for small molecule drugs have proven clinical utility, and the accumulated knowledge has informed the development of LNP formulations to deliver nucleic acid polymers, in particular siRNAs. The key advances made for LNP siRNA systems for liver targets were the use of dissociable PEG-lipids that stabilize the LNP formulations during formation but dissociate following i.v. injection to allow the association of ApoE, which triggers hepatocyte uptake, and the development of optimized ionizable cationic lipids for endosomal escape. LNP siRNA systems currently available have demonstrated utility for silencing any gene in hepatocytes and may be expected to result in siRNA-based therapeutics for a wide range of liver diseases. Long-circulating LNP siRNA systems on the horizon will allow siRNA-dependent gene silencing in a variety of extrahepatic tissues, such as the immune system, tumors, endothelial cells, and bone marrow, leading to further therapeutic opportunities.

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Conflicts of interest C.W. and T.M.A. have no conflict of interest. P.R.C. has a financial interest in Tekmira and has received research support from Alnylam and Tekmira Pharmaceuticals.

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