



Lipid Nanoparticles for Short Interfering RNA Delivery

Alex K.K. Leung, Yuen Yi C. Tam, Pieter R. Cullis¹

Department of Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, BC, Canada

¹Corresponding author: E-mail: pieterc@mail.ubc.ca

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Abstract

The discovery of RNA interference (RNAi) in mammalian cells has created a new class of therapeutics based on the reversible silencing of specific disease-causing genes. This therapeutic potential depends on the ability to deliver inducers of RNAi, such as short-interfering RNA (siRNA) and micro-RNA (miRNA), to cells of target tissues. This chapter reviews various challenges and delivery strategies for siRNA, with a particular focus on the development of lipid nanoparticle (LNP) delivery technologies. Currently, LNP delivery systems are the most advanced technology for systemic delivery of siRNA, with numerous formulations under various stages of clinical trials. We also discuss methods to improve gene silencing potency of LNP-siRNA, as well as application of LNP technologies beyond siRNA to the encapsulation of other nucleic acids such as mRNA and clustered regularly interspaced short palindromic repeats (CRISPR).



1. INTRODUCTION

Since the demonstration of RNA interference (RNAi) in mammalian model systems, much interest has arisen in the utilization of various promoters of RNAi, such as short-interfering RNA (siRNA) and microRNA (miRNA), for the treatment of diseases. RNAi is an endogenous process in eukaryotic cells that uses RNA molecules to catalyze degradation of specific, complementary messenger RNA (mRNA) sequences, possibly as a mechanism to protect against foreign pathogens such as viruses. When long double-stranded RNA is introduced into cells, a large, 200-kDa nuclease of the RNaseIII family known as Dicer (Filipowicz, Jaskiewicz, Kolb, & Pillai, 2005) cleaves the RNA into shorter fragments of approximately 21–23 nucleotides long. These fragments, or short-interfering RNA (siRNA) (Bernstein, Caudy, Hammond, & Hannon, 2001), are then loaded into the RNA-induced gene silencing complex (RISC) (Rand, Ginalski, Grishin, & Wang, 2004), a complex comprising many different proteins. One of these proteins is Argonaute 2 (Ago2), which is responsible for unwinding the siRNA and degradation of the sense or passenger strand (Matranga, Tomari, Shin, Bartel, & Zamore, 2005; Rand, Petersen, Du, & Wang, 2005). The single-stranded antisense strand then allows the RISC complex to actively seek out mRNA complementary to the antisense strand of the siRNA (Ameres, Martinez, & Schroeder, 2007). Once the mRNA is bound to the antisense strand of the siRNA, Ago2 mediates the cleavage of the mRNA between nucleotides 10 and 11 from the 5' end of the antisense strand (Tomari & Zamore, 2005). The RISC complex can then seek out and degrade additional mRNA, leading to remarkable silencing of the target gene (Hutvagner & Zamore, 2002).

In theory, siRNA is able to specifically block the synthesis of any protein responsible for any disease; however, the actual translation of siRNA into clinical use has been hampered by several major difficulties. These include the need to protect siRNA from degradation by nucleases in biological fluids, the delivery of siRNA to target tissues, and the intracellular delivery of siRNA to the target cell's cytoplasm where RNAi takes place. Lipid nanoparticles (LNPs) are the leading delivery systems for enabling the therapeutic potential of siRNA for systemic applications (Semple et al., 2010; Zimmermann et al., 2006). LNP-siRNA systems containing optimized ionizable cationic lipids exhibit remarkable in vivo potencies in silencing liver (hepatocyte) target genes at doses as low as

0.005-mg siRNA/kg body weight following intravenous (i.v.) injection in rodents (Jayaraman et al., 2012; Semple et al., 2010). With therapeutic indices in mice approaching 1000, these systems are relatively nontoxic and demonstrate promising clinical utility. Here we review the challenges in siRNA delivery and different strategies to deliver siRNA, with a focus on LNP systems and their formulation methods. We also discuss the current LNP-siRNA formulations in clinical testing. It is important to note that technologies for the production of LNP systems are not restricted for siRNA but are also applicable to plasmid DNA, mRNA and potentially, clustered regularly interspaced short palindromic repeat (CRISPR) DNA. This versatility would not only pave the way for the translation of nucleic acid-based therapeutics into the clinic but would also expand our current arsenal of nucleic acid therapeutics.



2. CHALLENGES AND STRATEGIES FOR DELIVERY OF siRNA

Delivery of siRNA for therapeutic purposes is hampered by several major hurdles. First, “naked” siRNA is unstable and rapidly degraded by nucleases in biological fluids (Choung, Kim, Kim, Park, & Choi, 2006; Layzer, Mccaffrey, Tanner, Huang, & Kay, 2004). It has been shown that chemical modifications of nucleic acids, such as 2' O-methyl (Sproat, Lamond, Beijer, Neuner, & Ryder, 1989), 2' fluoro-pyrimidine (Pieken, Olsen, Benseler, Aurup, & Eckstein, 2014), and phosphothioate linkages (Chowrira, & Burke, 1992) can protect siRNA from nuclease degradation. Second, siRNA cannot penetrate the cell membrane to enter the cytoplasm due to its large size, which ranges from 13 to 15 kDa, and negative charges (de Fougères, Vornlocher, Maraganore, & Lieberman, 2007; Whitehead, Langer, & Anderson, 2009). Moreover, despite evidence that naked siRNA can be taken up by endocytosis in neuronal cells, it faces an additional challenge of escaping the endocytosis-based degradation to mediate effective RNAi (Lingor, Michel, Schöll, Bähr, & Kügler, 2004). Finally, systemically administered siRNA accumulates in the liver and kidney and fails to accumulate at the site of disease (Braasch et al., 2004). As the glomerular filtration pore size is roughly 8 nm, naked siRNA are removed from circulation by the kidneys and excreted (Huang et al., 2011). Delivery vehicles are therefore necessary to overcome the above hurdles and realize the potential of siRNA as a therapeutic.

2.1 Localized Delivery of siRNA

Despite these challenges, accumulating evidence suggests that siRNA can be delivered locally to some tissues without the use of a sophisticated delivery system. One of the simplest strategies by which to administer unprotected, free siRNA for therapeutic purposes is directly to the localized tissue. It has been shown that intranasal administration of free siRNA against the P protein of the respiratory syncytial virus (RSV) and parainfluenza virus localizes siRNA to the lungs for up to 2 days and inhibits subsequent infections by these two viruses (Bitko, Musiyenko, Shulyayeva, & Barik, 2005). Furthermore, the siRNA shows effective antiviral properties when administered after RSV infection, with the best therapeutic response observed when the siRNA is administered at the time of viral infection (Bitko et al., 2005). Notably, the antiviral effects of this siRNA when complexed to the transfection reagent TransIT-TKO (Mirus Bio, Madison, WI) were significantly better than that of the naked form. A chemically modified siRNA targeting the nucleocapsid protein of RSV for intranasal delivery has since been developed by Alnylam Pharmaceuticals (Cambridge, MA). The siRNA formulation, named ALN-RSV01, was effective in inhibiting RSV when administered either prophylactically or therapeutically (Alvarez et al., 2009). In a phase II clinical trial, ALN-RSV01 was demonstrated to be safe and provided modest protection against RSV infection compared to placebo treatment (DeVincenzo et al., 2008, 2010). Another example of clinical applicability is the intranasally administered siRNA against the SARS coronavirus, which was shown to alleviate SARS-like symptoms in rhesus macaque (Li et al., 2005).

The eye is another popular tissue for local administration of siRNA. Age-related macular degeneration is a disease characterized by the development of choroidal neovascularization (CNV) in the eye. Intravitreal injection of naked siRNA against the vascular endothelial growth factor (VEGF) receptor has been shown to decrease the extent of CNV in mice (Shen et al., 2006). The siRNA was localized in the ganglion layer of the retina by 6 h after injection and the mRNA levels of VEGF receptor were significantly reduced by 7 days after injection. An intravitreal dose of as low as 0.5 μ g siRNA was enough to suppress the development of laser-induced CNV in mice (Shen et al., 2006). In a similar study, intravitreal injection of siRNA targeting erythropoietin was effective at reducing neovascularization induced by hypoxia (Chen et al., 2009). In a nonhuman primate model of laser-induced CNV, an intravitreal dose of 70- μ g siRNA targeting

VEGF suppressed the development of CNV for 36 days after laser induction (Tolentino et al., 2004).

The presence of the blood-brain barrier makes it extremely difficult for drugs, especially large molecules such as siRNA, to reach the central nervous system from the blood compartment. Direct administration is therefore the preferred route of drug entry. It has been shown that siRNA can be directly administered through either the intraventricular or intrathecal route to mediate gene silencing in the brain. Intraventricularly injected siRNA has been shown to mediate the gene silencing of enhanced green fluorescent protein (EGFP), dopamine transporter and serotonin transporter in the brain (Thakker et al., 2004, 2005). Also, intrathecally administered siRNA targeting the pain-related cation channel P2X₃ has been shown to reduce both P2X₃ expression and pain perception in treated mice (Dorn et al., 2004).

Despite the successes in the above examples, many tissues in the body remain inaccessible by local administration. Systemic delivery of drugs to these tissues would require the use of an appropriate delivery system.

2.2 Strategies for Systemic Delivery of siRNA

One of the simplest and earliest examples of systemic delivery of siRNA is the conjugation of cholesterol onto the 3' end of the sense or passenger strand of the siRNA (Soutschek et al., 2004). Intravenous administration of cholesterol-conjugated siRNA against apolipoprotein B (apoB) effectively decreased levels of apoB mRNA in the liver as well as plasma apoB protein and serum cholesterol (Soutschek et al., 2004). Modifications such as 2'-O-methyl at the ribose and phosphothioate at the siRNA backbone offer general protection for the siRNA against hydrolysis by nucleases. Other modifications for siRNA are available and reviewed elsewhere (de Fougères et al., 2007; Kanasty, Dorkin, Vegas, & Anderson, 2013; Whitehead et al., 2009). The effective uptake of cholesterol-conjugated siRNA by hepatocytes is mediated via its association with serum lipoproteins (Wolfrum et al., 2007). Moreover, the reconstitution of cholesterol-conjugated siRNA into purified mouse high density lipoprotein (HDL) showed higher gene silencing efficacy than free cholesterol-conjugated siRNA (Wolfrum et al., 2007). Others also demonstrated that mimetic lipoprotein particles prepared from recombinant apolipoprotein A1 and apolipoprotein E3 can effectively deliver cholesterol-conjugated siRNA to the liver and mediate effective gene silencing (Nakayama et al., 2012). These examples highlight the importance of endogenous lipoproteins in

mediating the uptake of cholesterol-conjugated siRNA. Indeed, it was later revealed that LNP-siRNA also depends on the lipoprotein pathway for effective uptake in the liver (Akinc et al., 2010).

While cholesterol conjugation is a delivery strategy specifically designed for siRNA, most cationic polymers were initially used to deliver plasmid DNA or antisense oligonucleotides and later applied to siRNA. Polyethylenimine (PEI) is a polycationic polymer widely used for condensing nucleic acids into a transfection-competent polyplex (Baker et al., 1997; Utsuno & Uludağ, 2010; Zheng et al., 2012). It has been shown that polyplexes of PEI and siRNA targeting the *N*-methyl-D-aspartate (NMDA) receptor reduced the expression of the receptor and decreased pain perception in rats following intrathecal administration (Tan, Yang, Shih, Lan, & Cheng, 2005). After administration of 5 µg of siRNA, significant reduction in NMDA receptor mRNA was observed in 3 days and protein levels of the NMDA receptor was suppressed for 14 days (Tan et al., 2005). In addition to condensing nucleic acids, PEI has been shown to protect siRNA from degradation by serum nucleases (Kim et al., 2005; Mahato, Kumar, & Sharma, 2013; Scheule et al., 1997; Urban-Klein, Werth, Abuharbeid, Czubayko, & Aigner, 2005). PEI has also demonstrated efficacy through other means of administration. Intravenous administration of polyplexes of PEI and siRNA against VEGF has shown intratumoural localization of the polyplexes as well as silencing of VEGF in mice (Schiffelers et al., 2004). In a xenograft mouse tumor model, intraperitoneal injection of PEI-siRNA targeting the HER2 receptor led to the downregulation of HER2 expression and inhibition of tumor growth (Urban-Klein et al., 2005). Despite these successes, the toxicity of high molecular weight PEI, such as PEI87 (87 kDa) and PEI217 (217 kDa), remains a major hurdle for its clinical application (Thomas, Ge, Lu, Chen, & Klibanov, 2005; Thomas et al., 2005; Tseng, Mozumdar, & Huang, 2009; Zintchenko, Philipp, Dehshahri, & Wagner, 2008).

Cyclodextrin polymer (CDP) is another popular cationic polymer used for the delivery of siRNA in vivo. Each cyclodextrin monomer comprises five to eight glucopyranose molecules linked together into a ring by α 1 \rightarrow 4 linkages (Kanasty et al., 2013). These cyclodextrin monomers are then joined together by a linker containing cationic amidine groups, which bind nucleic acids including siRNA (Gonzalez, Hwang, & Davis, 1999). When mixed with plasmid DNA, CDP has been shown to self-assemble into a spherical complex of approximately 120 nm in diameter (Gonzalez et al., 1999; Hwang, Bellocq, & Davis, 2001). Interaction of CDP particles with plasma components can be prevented by coating the

particle surface with polyethylene glycol (PEG) conjugated to adamantane, which is a hydrophobic moiety that form an inclusion complex with cyclodextrin by inserting into the cyclodextrin core (Davis et al., 2004). Targeting ligands, such as transferrin, can be coupled to the adamantane-PEG to target the CDP nanoparticle to specific cells (Bellocq, Pun, Jensen, & Davis, 2003; Davis et al., 2004). In a mouse model of Ewing's sarcoma, transferrin-adamantane-PEG CDP nanoparticles were able to reduce metastatic tumor size after three consecutive daily doses of 2.5 mg/kg siRNA (Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche, 2005). Although no measurable innate immune response or toxicity in the liver and kidney was observed, tumor volume rebounded soon after cessation of treatment (Hu-Lieskovan et al., 2005). In another study, CDP nanoparticles also did not show significant signs of toxicity or innate immune response in cynomolgus monkeys at doses up to 9 mg/kg siRNA; however, kidney and liver damage as well as elevated levels of pro-inflammatory cytokines were observed at a siRNA dose of 27 mg/kg (Heidel et al., 2007). Finally, in a phase I clinical trial involving melanoma patients, CDP nanoparticles containing siRNA against the ribonucleotide reductase M2 subunit (RRM2) were effective at reducing both the mRNA and protein levels of RRM2 in tumors (Davis et al., 2010). In addition to PEI and cyclodextrin, other synthetic polymers such as chitosan and poly(lactic-co-glycolic acid) are also under intensive investigation for their potential in siRNA delivery in vivo (Patil, Swaminathan, Sadhukha, Ma, & Panyam, 2010; Pillé et al., 2006; Yuan, Naguib, & Wu, 2011).

In comparison to cationic polymers, lipid nanoparticles (LNP) are currently the most mature technology enabling the delivery of siRNA in vivo, with at least four formulations of LNP-siRNA in various phases of clinical trials for the treatment of hypercholesterolemia, transthyretin-mediated amyloidosis and liver cancers (Allen & Cullis, 2013). LNP exhibit potent gene silencing activity in hepatocytes at doses as low as 0.005 mg siRNA/kg body weight in mice and 0.03 mg siRNA/kg in nonhuman primates following a single i.v. injection (Jayaraman et al., 2012). This is approximately 1000 times more effective than the examples of PEI and cyclodextrin delivery systems mentioned above. Furthermore, recent clinical results indicate that LNP-siRNA systems provide prolonged suppression of human transthyretin at a single dose of 0.3 mg siRNA/kg body weight, with full recovery occurring by day 70, the current gold standard for potency of a siRNA-based drug (Coelho et al., 2013). The following sections will discuss various formulation methods for these leading edge delivery systems.



3. LIPID-BASED DELIVERY SYSTEMS

Current development of the encapsulation technology for siRNA stems from early work on liposomes for small molecule delivery. In the early 1960s, Alec Bangham and colleagues realized that lecithin (egg yolk phosphatidylcholine) assembles into concentric lamellae when dispersed in water (Bangham & Horne, 1964). Later it was reported that these lipid vesicles are capable of maintaining a concentration gradient of ions, which can be disrupted by the addition of detergents (Bangham, Standish, & Watkins, 1965; Bangham, Standish, & Weissmann, 1965). Studies on the properties of lipid vesicles intensified soon after its discovery and it was quickly realized that the physical properties of these vesicles such as osmotic swelling and ability to trap solutes including ions and glucose were qualitatively similar to that of the cell membrane (Sessa & Weissmann, 1968). These lipid vesicles were initially termed “Bangasomes” due to their discovery by Alec D. Bangham; however, the term “liposome” was adopted subsequently (Sessa & Weissmann, 1968). In one of the first applications of liposomes as model biological membranes, Bangham and colleagues demonstrated that liposomes exposed to anesthetics were more permeable to ions (Johnson, Miller, & Bangham, 1973). Subsequently, liposomes have been widely used as drug delivery vehicles.

3.1 Lipid Systems for Small Molecule Drugs

The potential of the liposome as a drug carrier was recognized soon after its discovery in the early 1960s. Gregory Gregoriadis was one of the pioneers to recognize the drug delivery potential of liposomes and apply them in enzyme replacement therapy (Gregoriadis, Leathwood, & Ryman, 1971; Gregoriadis & Ryman, 1971). These systems were made by hydrating the dry lipid film with an aqueous buffer containing the enzyme amyloglucosidase or albumin. The resulting encapsulation efficiency was extremely poor, with less than 10% of the original enzymes and proteins being encapsulated. Encapsulation of antibiotics such as penicillin and actinomycin was also very poor (Gregoriadis, 1973).

The major breakthrough for improving drug encapsulation efficiencies in liposomes came with the development of the transmembrane pH gradient loading technique to entrap weakly basic drugs, which can be accomplished by three different methods (Figure 4.1). First, a pH gradient is generated by hydrating the lipid film with a low pH buffer followed by a buffer exchange

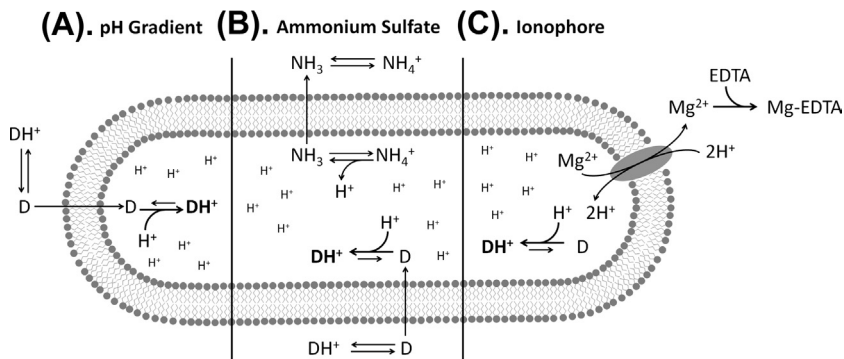


Figure 4.1 *Encapsulation of small molecule drugs in response to transmembrane pH gradient.* (A) In the standard pH gradient method, the lipid film is first hydrated with buffer of low pH (typically citrate buffer, pH 4), followed by extrusion generating large unilamellar vesicles. A pH gradient is generated by exchanging the external buffer with a buffer of higher pH (typically HEPPES, pH 7.5). Weakly basic drugs enter the vesicle in their neutral form and then become protonated and trapped in the acidic interior of the vesicle. (B) In the ammonium sulfate method, a transmembrane ammonium sulfate gradient is first generated by hydrating and extruding the lipid in a buffer containing ammonium sulfate, followed by an exchange of the external buffer. The interior of the vesicle is acidified as the neutral NH_3 exits the vesicle, leaving behind a proton in the process. This pH gradient is then used for the encapsulation of weakly basic drugs. (C) In the ionophore method, a transmembrane gradient of Mg^{2+} or Mn^{2+} is first generated. The ionophore A23187 couples the export of Mg^{2+} with the import of two protons, therefore acidifying the interior of the vesicle. A chelator of Mg^{2+} , such as EDTA, is usually required to remove the Mg^{2+} as it is transported out of the vesicle.

using a solution of neutral pH (Figure 4.1(A)). It was demonstrated that weakly basic compounds such as catecholamines can be concentrated inside liposomes with an acidic interior (Nichols & Deamer, 1976). Later, it was shown that drugs that are weak bases, such as doxorubicin, could also accumulate in high amounts inside liposomes with an acidic interior (Mayer, Bally, & Cullis, 1986; Mayer, Bally, Hope, & Cullis, 1986). The encapsulation of drugs in response to a transmembrane pH gradient can be readily explained. At neutral pH, weakly basic drugs are a mixture of the protonated, membrane-impermeable form (DH^+) and the deprotonated, membrane-permeable form (D). The deprotonated form of the drug (D) can readily diffuse across the membrane and then becomes protonated (DH^+) and trapped inside the liposome where the pH is lower than the pK_a of the drug (Figure 4.1(A)). At equilibrium, the drug concentration gradient mirrors the proton gradient; thus a pH gradient of 3 units results in a 1000-fold higher drug concentration inside the liposome than that of the exterior medium.

In the second method, the loading of weakly basic drugs can be accomplished using ammonium sulfate (Bolotin et al., 1994). In this scenario, a pH gradient is generated by encapsulating ammonium sulfate inside the liposome followed by exchanging the external buffer (Figure 4.1(B)). An equilibrium of positively charged ammonium ion (NH_4^+) and neutral ammonia (NH_3) exists inside the liposome. Since the lipid membrane is highly permeable to the uncharged NH_3 , one proton remains inside the liposome as NH_3 travels down its concentration gradient to the outside of the liposome, resulting in acidification of the liposome interior (Figure 4.1(B)). Loading of weak base drugs in response to pH gradient can result in interior drug concentrations that are so high that nanocrystals of drugs are formed, leading to a characteristic “coffee bean” appearance in electron micrographs (Abraham et al., 2005).

The use of an ionophore offers a third method to generate a pH gradient needed for the encapsulation of weakly basic drugs (Fenske et al., 1998). A transmembrane gradient of divalent cations such as Mn^{2+} or Mg^{2+} is first generated by preparing liposomes in solutions of MnSO_4 or MgSO_4 followed by an exchange of the external buffer with a sucrose solution. After generating the divalent cation gradient, the weakly basic drug, the ionophore A23187 and Ethylenediaminetetraacetic acid (EDTA) are added to the large unilamellar vesicle (LUV). The ionophore couples the transport of one divalent cation out of the LUV with the import of two protons, resulting in acidification of the vesicle interior (Figure 4.1(C)) (Fenske et al., 1998). The exported divalent cations are then chelated by EDTA while the drug enters the vesicles. This method showed very high levels of encapsulation (>80%) for both vincristine and ciprofloxacin with drug retention properties similar to other pH gradient loading methods (Fenske et al., 1998).

The goal of using liposomes as drug delivery vehicles is to enhance the potency of drugs and reduce their side effects by increasing their delivery to the sites of disease while avoiding the healthy tissues. Many studies have noted that the vasculature at sites of disease such as tumors, sites of infection and sites of inflammation is “leakier” than that of healthy tissues (Skinner, Tutton, & Brien, 1990; Steinberg, Konerding, & Streffer, 1990). For example, the neovasculature formed in tumors lacks the smooth muscle wall of normal vasculature and is relatively permeable to particles of 200-nm diameter or larger (Yuan et al., 1994, 1995). As a result, long-circulating liposomes with a diameter of 100 nm preferentially escape in the region of tumors, resulting in large increases in the amount of drug that is delivered to

the tumor. In addition, lymphatic drainage in the tumor is often impaired, causing liposomes to be retained in the interstitial space (Maeda, 2001). This combined phenomenon is called the enhanced permeation and retention (EPR) effect (Maeda, 2001; Maeda, Wu, Sawa, Matsumura, & Hori, 2000). It is important to note that the encapsulated drug is not available to target cells and must be released from the liposome to exert its effect. For certain drugs, the rate of drug release strongly influences the anticancer potency of the formulation. This is particularly true of cell cycle-specific drugs such as vincristine (Mayer et al., 1993; Webb, Harasym, Masin, Bally, & Mayer, 1995). To achieve optimal therapeutic activity, the rate of drug release can be controlled by changing the lipid composition of the liposome (Charrois & Allen, 2004) or by varying the drug-to-lipid ratio (Johnston et al., 2006).

Serum lipoproteins have been shown to play a critical role in both the circulation lifetime and cellular uptake of lipid nanoparticles. It has been shown that the apolipoproteins apoA-I, apoA-IV, and apoE bind to the surface of neutral phospholipid liposomes (Bisgaier, Siebenkas, & Williams, 1989). In particular, only apoE, but not apoA-I and apoA-IV, was shown to mediate cellular uptake of these liposomes by hepatocytes in vitro (Bisgaier et al., 1989). Similarly, apoE was shown to mediate hepatic uptake and blood clearance of neutral liposomes in vivo (Yan et al., 2005). ApoE is an apolipoprotein found on the surface of cholesterol-enriched lipoproteins, such as chylomicrons, very-low density lipoproteins (VDLs) and high density lipoproteins (HDLs) (Mahley, 1988; Mahley & Ji, 1999). The uptake of these lipoproteins by hepatocytes is mediated by receptor-mediated endocytosis of low density lipoprotein (LDL) receptor, LDL receptor (LDLR)-related protein and scavenger receptor BI (Beisiegel, Weber, Ihrke, Herz, & Stanley, 1989; Krieger, & Herz, 1994; Mahley & Ji, 1999). The role of apoE in mediating the hepatocellular uptake of LNP-siRNA system comprising ionizable cationic lipid was demonstrated in vivo with the use of ApoE-deficient mice (Akinc et al., 2010). The authors demonstrated that both ionizable LNP uptake by hepatocytes and silencing of the hepatic gene Factor VII were greatly reduced in ApoE-deficient mice compared to wild-type animals. The results were similar in *LDLR*^{-/-} mice, which lack the LDLR to mediate hepatocellular uptake of the LNP. Interestingly, efficacy of LNP in ApoE-deficient mice can be rescued by conjugating the targeting ligand N-acetylgalactosamine (GalNAc) onto the PEG lipid of the LNP. GalNAc binds to the asialoglycoprotein receptor expressed on the surface of the hepatocyte with high affinity, providing an alternative pathway for LNP-siRNA to enter the hepatocytes of ApoE-deficient mice (Akinc et al., 2010). In contrast, LNP composed of a permanent positively charged cationic

lipid does not seem to depend on apoE for liver uptake, and its gene silencing efficacy *in vivo* was lower than that of ionizable LNP (Akinc et al., 2010).

3.2 Lipid Systems for siRNA

Unlike the entrapment of soluble drugs in neutral liposomes, cationic lipids are essential for efficient encapsulation of nucleic acids; lipid formulations without cationic lipids result in poor encapsulation of antisense oligonucleotides (Maclachlan, 2007). The use of cationic lipid was first described by Felgner and coworkers (Felgner et al., 1987), who used the permanently positively charged cationic lipid, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) to mediate cellular transfection of DNA. The positive charges of cationic lipids facilitate the encapsulation of anionic nucleic acids via electrostatic interactions. Cationic lipids can also provide a positive surface charge to the LNP, promoting close association of the LNP with the negatively charged cell surface. It has been shown that cell surface proteoglycan and sialic acids interact with cationic liposomes and facilitate their cellular uptake via the endocytic pathway (Mislick & Baldeschwieler, 1996; Mounkes, Zhong, Cipres-Palacin, Heath, & Debs, 1998; Stamatatos, Leventis, Zuckermann, & Silvius, 1988; Wrobel & Collins, 1995). Following endocytosis, cationic lipids of the LNP are hypothesized to form membrane disruptive ion pairs with anionic lipids of the endosomes, thereby facilitating intracellular release of nucleic acids (Hafez, & Cullis, 2001; Hafez, Maurer, & Cullis, 2001). However, positively charged liposomes are rapidly eliminated by the mononuclear phagocyte system (MPS) (Litzinger, 1997). Because most permanently charged cationic lipids are highly toxic, this has severely limited their *in vivo* application (Audouy, de Leij, Hoekstra, & Molema, 2002; Liebert et al., 2000; Scheule et al., 1997; Zhang, Liu, & Huang, 2005).

To avoid toxicity issues, ionizable cationic lipids with primary, secondary, or tertiary amines in the headgroup and apparent pK values of less than seven have been developed for the purposes of encapsulating nucleic acids when the lipid is positively charged at pH values below the pKa (e.g. pH 4), and for almost neutral LNP at physiological pH values. The first ionizable cationic lipid used for the encapsulation of nucleic acids was 1,2-dioleoyl-3-dimethylammonium propane (DODAP) (Maurer et al., 2001; Semple et al., 2001) with a pKa of 6.6 (Bailey & Cullis, 1994). Antisense oligonucleotides LNP containing DODAP exhibited a relatively long circulation lifetime following *i.v.* administration, as expected for a lipid particle with little surface charge (Semple et al., 2001).

In the past several years, significant improvement in the potency of LNP-siRNA was achieved by varying the chemical structure of the ionizable cationic lipid. This is noted in Figure 4.2 where the progression from DODAP to DODMA, DLinKDMA, DLinKC2DMA, and DLinMC3DMA is shown. These lipids were identified using an *in vivo* screening model employing LNP systems containing siRNA to silence Factor VII (FVII), one of the proteins in the blood-clotting cascade. Factor VII is made in hepatocytes and secreted into the circulation, thus the potency of the LNP-siRNA systems can be monitored by

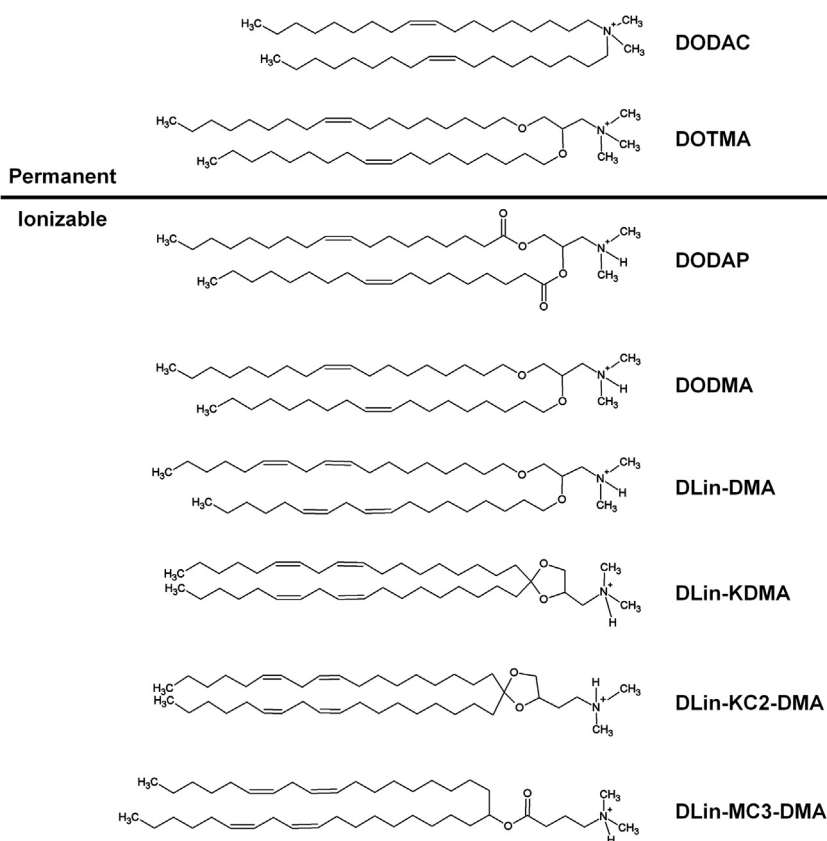


Figure 4.2 Evolution of cationic lipids. Early cationic lipids, such as DOTMA and DODAC, contain a permanently positive quaternary amine headgroup. Ionizable cationic lipids with tertiary amines in the headgroup have an apparent pKa less than seven, which allowed encapsulation of nucleic acids to be performed at acidic pH (pH 4.0) while the resulting LNP will exhibit a net neutral charge at physiological pH.

measuring FVII levels in the circulation 24 h after i.v. administration of the LNP. Remarkably, the potency of LNP-siRNA systems improved from ~10 mg siRNA/kg body weight to result in 50% gene silencing for LNP containing DODAP to 0.005 mg siRNA/kg body weight for the LNP containing DLinMC3DMA (Jayaraman et al., 2012; Semple et al., 2010). This improvement in potency is attributed to the use of ionizable cationic lipids with optimized pKa values and maximized bilayer destabilizing capabilities given the presence of anionic lipids that exist within the endosome. DLinMC3DMA, with a pKa of 6.4, is currently the most potent ionizable cationic lipid for hepatic gene silencing in vivo (Jayaraman et al., 2012).

The type of PEG lipid can also influence the potency of LNP-siRNA systems. PEG lipids are incorporated into LNP-siRNA formulations to prevent particle aggregation during the formulation process as the PEG moiety provides a hydrophilic steric barrier on the particle surface (Maurer, Fenske, & Cullis, 2001; Maurer et al., 2001). Without the PEG-lipid coating, it is impossible to produce small LNP-siRNA systems with diameters of less than 100 nm, and large micron sized aggregates are instead formed. Although the PEG coating is important for forming small and stable particles, it can also inhibit LNP association with the plasma membrane of target cells, indicating a need to remove the coating before cellular uptake can proceed. One potential solution is to use acid-labile PEG lipids where the PEG moiety dissociates from the lipid anchor at low pH (Choi, MacKay, & Szoka, 2003; Guo, & Szoka, 2001; Shin, Shum, & Thompson, 2003). However, the pH required is too low (pH < 5) to exist in the extracellular medium and thus the PEG lipids remain on the LNP surface, preventing close association with the cell membrane. Another approach is to use a PEG lipid that dissociates from the LNP in circulation following i.v. administration. The length of lipid chains anchored on the PEG lipid influences how long it stays on liposomes (Parr, Ansell, Choi, & Cullis, 1994), with longer acyl chains showing longer retention times than their shorter counterparts. Recently it has been shown that PEG lipids with short (C₁₄) acyl chains dissociate from LNP-siRNA in vivo with a half-time of approximately 1 h (Mui et al., 2013), whereas PEG lipids with longer (C₁₈) acyl chains exhibit dissociation rates of days or longer. This offers the possibility of employing LNP stabilized by PEG lipids with short acyl chains that rapidly dissociate following i.v. administration, allowing the LNP-siRNA systems to become potent transfection agents.

In addition to cationic lipids and PEG lipids, LNP formulations of siRNA typically contain other structural lipids. The most commonly used lipids are cholesterol and a saturated phosphatidylcholine (PC) such as distearoyl-PC (DSPC) or dipalmitoyl-PC (DPPC). The reasons as to why these lipids are required are unclear. Cholesterol is included because of historical data for bilayer liposomal systems indicating that liposomes consisting of PC alone accumulate cholesterol from serum components (Rodriguez, Pritchard, & Hope, 1993); consequently, systems containing approximately equimolar cholesterol are in better equilibrium with their surroundings. The need for DSPC is counter-intuitive as the presence of DSPC would be expected to mitigate against fusion with the endosomal membrane following uptake into target cells due to its strong bilayer-stabilizing properties (Cullis, Hope, & Tilcock, 1986). However, recent data from our group suggested that elimination of DSPC compromises the gene silencing potency of LNP-siRNA systems for reasons yet to be determined (unpublished data).

There has been extensive work as well to incorporate targeting ligands into LNP systems. Targeting moieties such as antibody fragments and small molecule ligands have been coupled onto the surface of LNP in hopes of increasing cellular uptake of the LNP in target tissues through receptor-mediated endocytosis (Allen & Cullis, 2013; Sapra & Allen, 2003). It has been reported that liposomes with monoclonal antibody fragment against HER2 showed better cellular uptake in HER2-overexpressing breast cancer xenografts as compared to nontargeted liposomes (Kirpotin et al., 2006). Also, antibody-bearing liposomes containing antisense oligonucleotides against viral mRNA have showed better efficacy against viral infection compared to the same liposomes without the antibody (Leonetti, Machy, Degols, Lebleu, & Leserman, 1990). In addition to using antibodies as the targeting moiety, small molecules have also been coupled to the PEG lipid for enhancing LNP uptake in cells and subsequent gene silencing activity. For example, anisamide, a small compound that interacts with the sigma receptors, increases gene silencing activity of siRNA nanoparticles in lung tumors and metastases (Chen et al., 2009; Li & Huang, 2006; Li, Chono, & Huang, 2008). In addition, we have shown that strophanthidin, a cardiac glycoside that binds to the ubiquitously expressed cell surface receptor Na^+/K^+ ATPase, enhances delivery of LNP-siRNA to cells originating from a number of tissues such as the prostate, ovary, breast, lung, and pancreas (Tam et al., 2013). In many of these cases, the targeting moiety is conjugated to the distal end of the PEG lipid and the resulting targeting lipid is added along with the rest of the lipid components during the formulation process.

Alternatively, the targeting lipid is added to the stable, preformed LNP in a process called postinsertion (Iden & Allen, 2001). The use of postinsertion technique prevents the targeting lipid from interfering with the formulation process and provides a relatively simple method for preparing targeted LNP formulations.

3.3 Formulation Methods for LNP-siRNA

Similar to the development of cationic lipids, formulation technologies for siRNA were often designed for the delivery of plasmid DNA and later adopted for siRNA. The formulation used by Felgner and coworkers (Felgner et al., 1987) for cellular transfection was a simple mixture of DOTMA and dioleoyl-phosphatidylethanolamine (DOPE), which together form a complex (lipoplex) with plasmid DNA. Lipoplexes are often microns in diameter and are unstable and difficult to reproduce. They are often formulated with excess cationic charge not only to promote interaction with the plasmid DNA but also to facilitate association with the negatively charged cell surface and subsequent uptake by endocytosis (Stamatatos et al., 1988). Incorporation of helper lipids such as DOPE is thought to facilitate the release of plasmid DNA into the cytoplasm due to its propensity to adopt the nonbilayer, membrane lytic hexagonal H_{II} phase (Cullis & de Kruijff, 1978). Despite intensive efforts, however, lipoplexes have not proven to be useful for in vivo applications. The chief reason for this shortcoming is that they are highly toxic due to their positive charge and are quickly removed from the circulation by the fixed and free macrophages of the MPS due to their size and charge. As a result, they do not distribute efficiently to target cells such as tumor cells.

These difficulties have led to attempts to encapsulate nucleic acids within an enclosed bilayer membrane that exhibits low surface charge. In the “stabilized antisense lipid particle” method, a solution of the lipids in ethanol is added to an aqueous solution of nucleic acid at pH 4.0 (Semple et al., 2001). The relatively low pH of the buffer ensures that the ionizable cationic lipid, 1,2-dioleoyl-3-dimethylammonium propane (DODAP, pK_a 6.6), is positively charged for interaction with the nucleic acid. The vesicles are subsequently extruded through polycarbonate membranes to ensure uniform size distribution. Dialysis in buffer at pH 7.0 removes residual ethanol in the mixture and deprotonates the ionizable cationic lipid, creating a relatively neutral system with prolonged circulation lifetimes (Figure 4.3). Encapsulation efficiencies of over 70% and particle diameter of about 100 nm were achieved with this method (Semple et al., 2001).

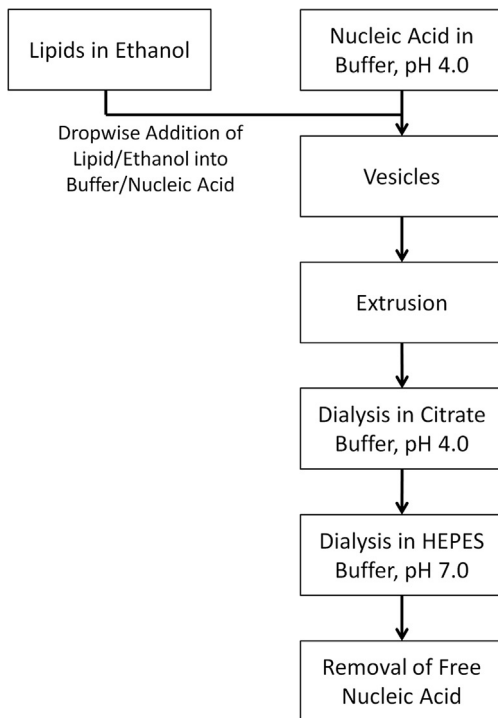


Figure 4.3 Encapsulation of nucleic acid by the “stabilized antisense lipid particle” method. This method involves the dropwise addition of an ethanolic solution of lipids into an aqueous buffer containing nucleic acid. The drop in ethanol concentration facilitates the formation of vesicle as the ethanol concentration is now below the solubility limit of the lipids. The vesicles are subsequently extruded, followed by dialysis to remove residual ethanol and to raise the pH of the buffer. Encapsulation efficiencies are typically in the range of 70%. The resulting particles are approximately 80–140 nm in diameter and show a range of lamellarity, depending on the initial nucleic acid-to-lipid ratio.

Interestingly, the resulting particles are mainly unilamellar at a low nucleic acid-to-lipid ratio, but adopt a multilamellar configuration at higher nucleic acid-to-lipid ratios. These observations led to the suggestion that nucleic acid bridges between individual lipid vesicles promoting the formation of multilamellar structures.

A variation of the ethanol-drop method is the preformed vesicle (PFV) method (Maurer et al., 2001). PFVs containing DODAP, cholesterol, DSPC and a PEG lipid at low pH, typically pH 4, where the DODAP is protonated, are prepared by extrusion in the presence of up to 40% ethanol (Figure 4.4). Extrusion is usually very rapid in the presence of ethanol.

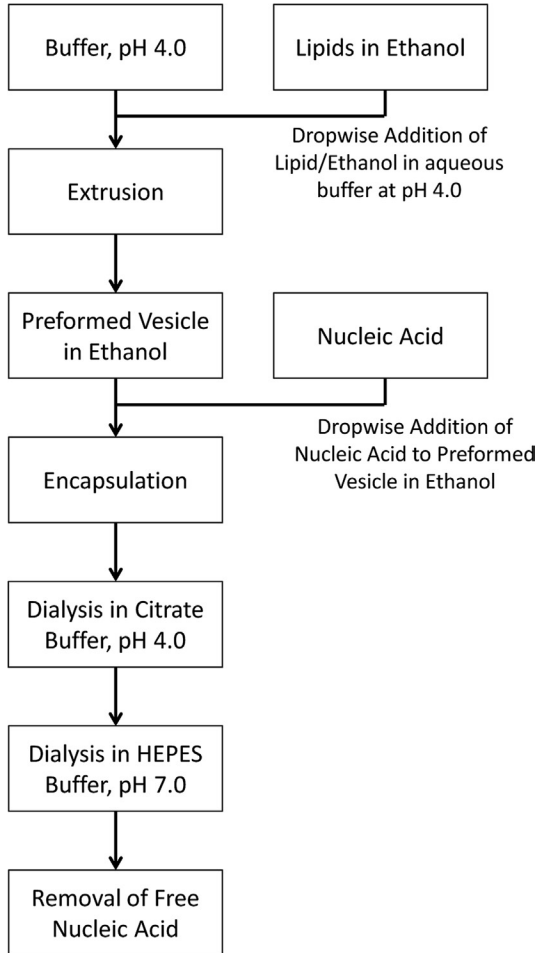


Figure 4.4 Encapsulation of nucleic acids by the preformed vesicle method. Lipid vesicles are first produced by dropwise addition of an ethanolic solution of lipids into an aqueous buffer, followed by extrusion. An aqueous solution of nucleic acid is added slowly to the resulting vesicle solution, which usually contains approximately 40% ethanol. The mixture is incubated for 1 h at 37°C to allow encapsulation to take place. Residual ethanol is removed by dialysis and the pH of the solution is raised to 7.0. This method usually results in multilamellar particle of approximately 100 nm in diameter with an encapsulation efficiency of approximately 80%.

A solution of nucleic acid at pH 4.0 is then slowly added to the ethanol-destabilized liposomes to avoid particle aggregation and the mixture is then incubated for 1 h to allow encapsulation of the nucleic acid. Ethanol is removed by dialysis after the encapsulation step and the pH of the solution

is adjusted to 7.0 to create a charge neutral system. This method generated multilamellar particles of approximately 100 nm in diameter with nucleic acid encapsulation efficiency of over 80% (Maurer et al., 2001). It was suggested that the attachment of nucleic acid onto the surface of the extruded liposome creates an adhesion point between liposomes, facilitating the formation of the multilamellar vesicles. This also explains the high nucleic acid encapsulation efficiency achieved with this method as the nucleic acid appears to be entrapped between lamellae of the multilamellar particle.

An alternative method to extrusion is the spontaneous vesicle formation (SVF) by ethanol dilution method (Jeffs et al., 2005). Similar to many methods of encapsulation, this method was first developed for plasmid DNA and later adopted for the encapsulation of siRNA. In this method, nucleic acid is prepared in an acidic buffer, while cationic lipid, DSPC, cholesterol and PEG lipid are dissolved in ethanol. The nucleic acid and lipid mixtures are then combined using a T-tube mixer, with the flow of the two streams of fluid controlled via a peristaltic pump (Figure 4.5) (Jeffs et al., 2005). Vesicles form spontaneously as the lipids precipitate from solution as the polarity of the medium is raised. Residual ethanol in the final mixture is then removed by dialysis. For siRNA encapsulation, this method uses low amounts of PEG lipid, typically in the range of 1–5% (mol% lipid), and results in particles ranging from 70 to 80 nm in diameter (Judge, Bola, Lee, & MacLachlan, 2006; Zimmermann et al., 2006). The encapsulation of siRNA is highly efficient with efficiencies of over 90% routinely achieved. The resulting LNP-siRNA are termed “stable nucleic acid lipid particles”. Limitations of T-tube mixer formulation include the difficulty in applying the process to laboratory scale formulations due to the high flow rates needed to achieve rapid mixing, and the limited mixing rate which results in an inability to formulate LNP with lipid compositions that require very rapid mixing to achieve stable systems.

The Cullis laboratory has developed an in-line mixing method employing a microfluidic herring-bone micromixer (Belliveau et al., 2012). The herring-bone micromixer has two inlets, one for an ethanolic mixture of lipids and the other for a buffered solution of siRNA at pH 4.0. A dual syringe pump is used to drive the two streams of fluid into the herring-bone micromixer (Figure 4.6). As the two streams of fluid meet at the herring-bone micromixer, they fold and wrap around each other, exponentially decreasing the diffusion length between the two streams. This allows for rapid mixing of the two streams of fluid on a millisecond timescale. The rapid decrease in solvent polarity results in the precipitation of lipids in accordance to their solubility.

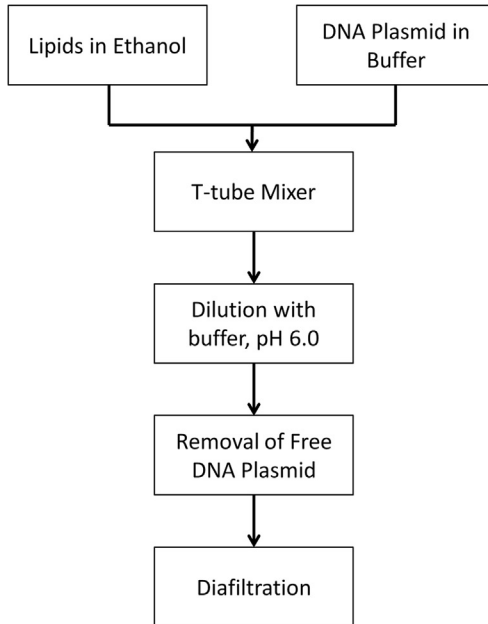


Figure 4.5 Encapsulation of nucleic acid with spontaneous vesicle formation by ethanol dilution. This method is also commonly referred as the “T-tube” method. It involves mixing of lipid components dissolved in ethanol with nucleic acid in aqueous buffer using a T-tube connector. The flow of the two streams of fluid is controlled with a peristaltic pump. Particles are formed as the ethanol is diluted below the solubility limit of the lipid. The ethanol content is further diluted upon exiting the T-tube connector to stabilize the resulting particles. Unencapsulated nucleic acid can be removed by using an ion exchange column. Residual ethanol is removed by diafiltration against phosphate buffered saline (PBS). This method has been used for encapsulation of plasmid DNA (stabilized plasmid-lipid particles), antisense oligonucleotides and siRNA (stable nucleic acid lipid particle).

This technique has resulted in greater than 90% siRNA encapsulation efficiency over a wide range of siRNA-to-lipid ratios (Leung et al., 2012). Cryo-transmission electron microscopy has indicated that LNP-siRNA produced by microfluidic mixing exhibit an electron dense core similar to the ones produced by T-tube in-line mixing (Figure 4.7). Additional biochemical characterizations strongly suggest that the solid, electron dense core is comprised of distorted inverted micelles of cationic lipids complexed to the encapsulated siRNA. Furthermore, molecular dynamics simulation of LNP-siRNA systems suggests the particles contain a nanostructured, hydrophobic core with the siRNA surrounded by an inverted micelle of ionizable cationic lipids (Figure 4.8). These results are consistent with the initial condensation of

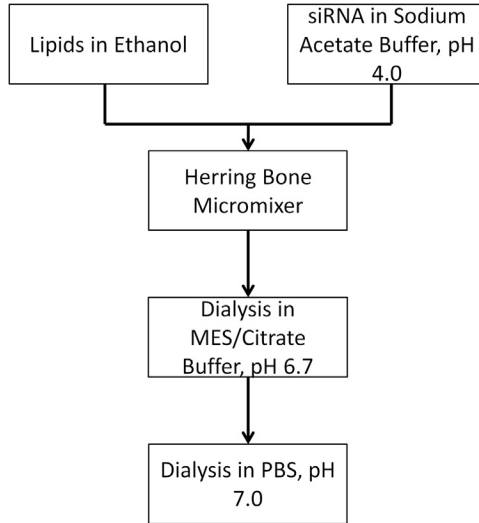


Figure 4.6 Encapsulation of siRNA by microfluidic mixing. This method employs a herringbone micromixer to facilitate the mixing of lipid components dissolved in ethanol and siRNA in aqueous buffer. The flow of the two streams of fluid is controlled using a dual syringe pump. The herringbone micromixer exponentially increases the surface area between the two streams of fluid, resulting in rapid mixing on a millisecond timescale. Residual ethanol is removed by dialysis and the pH of the solution is raised to 7.0. This method results in over 90% siRNA encapsulation efficiencies. Particle size is adjustable from 20 to 50 nm by varying the PEG-lipid content from 5% to 1%.

inverted micelles of ionizable cationic lipids around the siRNA, which serve as nucleating structures for the rest of the lipids to assemble into a solid core LNP (Figure 4.9). The most hydrophilic lipid, the PEG lipid, would be the last component to be deposited on the nascent LNP thus providing an outer shell for the stabilized particle (Figure 4.9). The results are also consistent with the PEG lipid, as the last component to be deposited on the nascent LNP, providing the outer shell of the stabilized particle (Figure 4.8). Based on this model, one would expect the size of the resulting LNP to be dictated by the ratio of “core” lipid to “surface” lipid in the lipid mix. Indeed, the size of the particle is freely adjustable between 20 and 100 nm in diameter simply by altering the PEG-lipid content of the formulation (Belliveau et al., 2012). In comparison with the PFV technique, microfluidic mixing results in higher encapsulation efficiency (>90%), generates smaller particles, and permits small scale production with little loss due to dead volume. This method allows for well-defined and reproducible mixing between the solutions of lipids and siRNA, resulting in lower batch-to-batch variation (Belliveau et al., 2012).

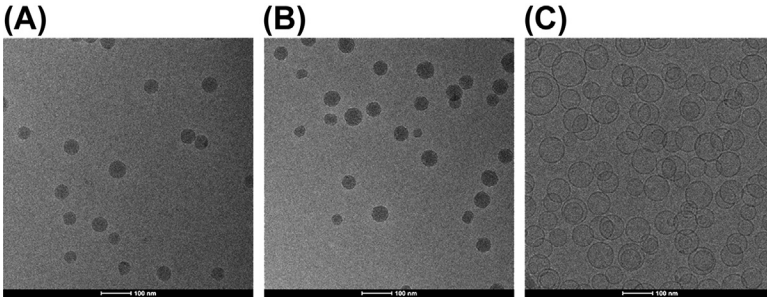


Figure 4.7 Cationic LNP produced by microfluidic mixing exhibit electron dense cores both in the presences and absence of siRNA as indicated by cryo-TEM. (A), Cryo-TEM micrograph obtained from siRNA-LNP with the lipid composition cationic lipid/DSPC/Cholesterol/PEG lipid (40/11.5/47.5/1; mol/mol) at a siRNA/lipid ratio of 0.06, wt/wt. (B), LNP with the same lipid composition as (A) but prepared in the absence of siRNA. (C), POPC/cholesterol (50/50; mol/mol) bilayer vesicles prepared by extrusion through polycarbonate filters with 80 nm pore size. Reproduced with permission from Leung et al. (2012).

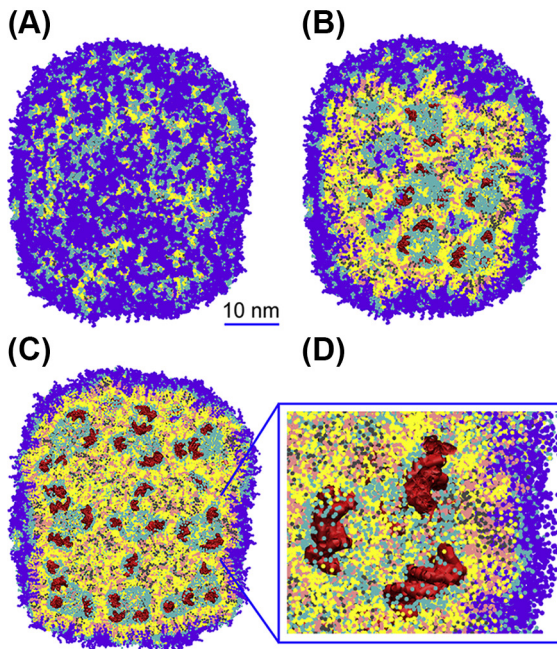


Figure 4.8 A lipid nanoparticle (LNP) contains irregular water-filled cavities separated by bilayer membranes, with nucleic acids bound to the membrane surface. (A) side view, (B,C) cross-section, and (D) zoom-in views. Cationic lipid DLin-KC2-DMA is shown in yellow, cholesterol in pink, DSPC in grey, lipid polar moiety in cyan, PEG lipid in violet, nucleic acids (duplex DNA) in red, water not shown for clarity. The lipid composition was DLin-KC2-DMA/DSPC/cholesterol/PEG lipid (4:1:4:1; mol/mol) and DNA to lipid ratio ~ 0.05 wt/wt. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.) Reproduced with permission from Leung et al. (2012).

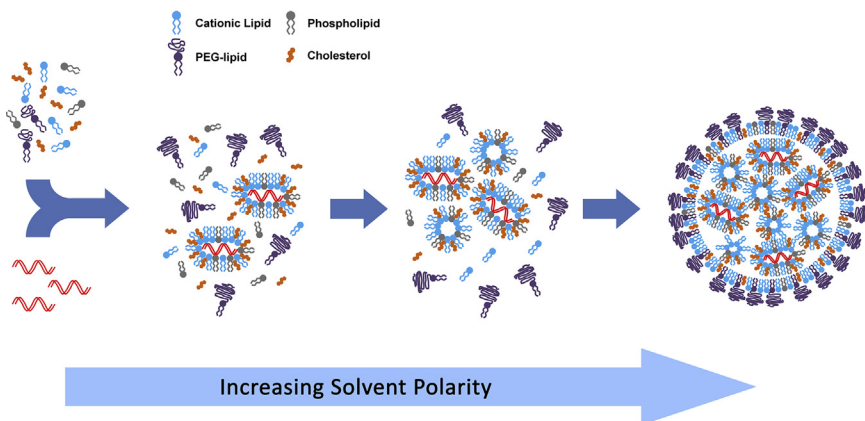


Figure 4.9 Assembly of LNP-siRNA by microfluidic mixing. Lipid components reach their individual solubility limit and precipitate out of solution as the ethanol in the lipid stream is being diluted by the aqueous stream. The acidic sodium acetate buffer protonates the ionizable cationic lipids (blue), which then form an inverted micelle around the siRNA (red) via electrostatic interaction. As the polarity of the solvent increases, inverted micelles begin to aggregate, which is followed by coating by PEG lipids (purple) to form the LNP-siRNA. (See the color plate.)

The above-mentioned examples of formulation rely on ionizable cationic lipids to condense the siRNA for encapsulation into LNP. Alternatively, a lipid-coated calcium phosphate (LCP) nanoparticle that utilizes calcium phosphate to condense siRNA before encapsulation by a lipid layer was developed (Li, Chen, Tseng, Mozumdar, & Huang, 2010). The fabrication process involves combining calcium chloride, sodium phosphate, and siRNA in a cyclohexane/Triton-X-100/hexanol solution to form a reversed-phase, water-in-oil micro-emulsion (Figure 4.10). Aqueous sodium citrate is then added to the emulsion to form calcium phosphate precipitates with the entrapped siRNA. The calcium phosphate precipitate is then purified using silica gel and combined with liposomes made of the permanently charged cationic lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and cholesterol to form the LCP nanoparticle (Figure 4.10). This LCP formulation was later modified to include the anionic lipid dioleoyl-phosphatidic acid (DOPA), which serves as an inner leaflet lipid to coat the calcium phosphate precipitate (Li, Yang, & Huang, 2012). Electron micrographs of DOPA coated calcium phosphate showed a hollow core of approximately 20 nm in diameter. This is consistent with the hypothesis that the negatively charged DOPA headgroup interacts with the positively charged calcium phosphate core in a way that the acyl chains of DOPA face the exterior of the particle, forming an inverted micelle-like structure.

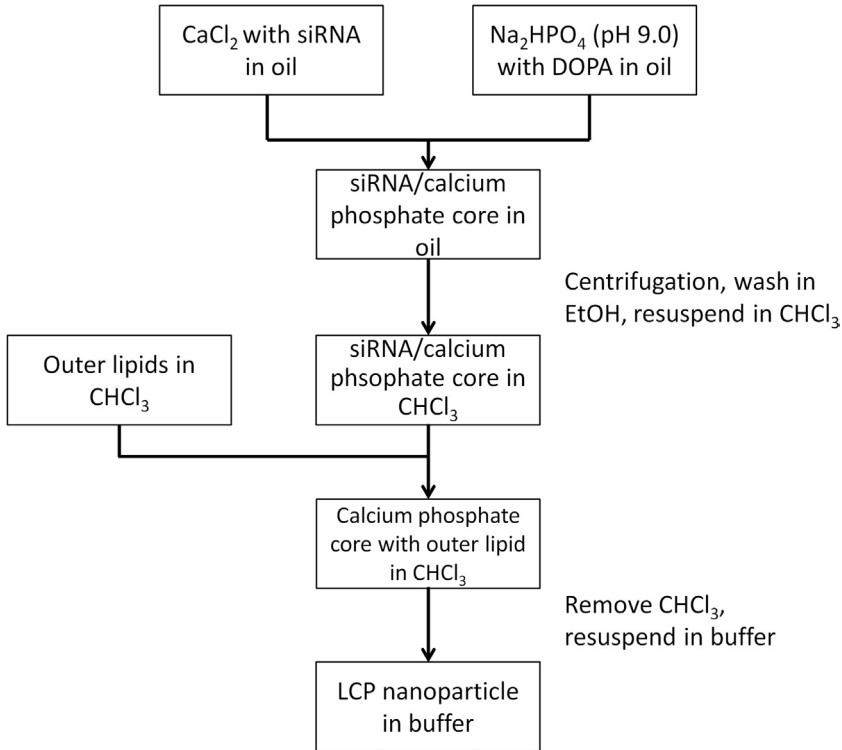


Figure 4.10 Preparation of lipid-coated calcium phosphate (LCP) nanoparticle. This method consists of first preparing a solution containing calcium chloride and siRNA dispersed in a cyclohexane/Igepal oil, as well as a solution of dioleoyl-phosphatidic acid (DOPA) and sodium phosphate dibasic (pH 9) similarly dispersed in a cyclohexane/Igepal oil. These two solutions are then mixed for 20 min and the calcium phosphate cores containing the siRNA are isolated by centrifugation followed by a wash with ethanol. A mixture of outer lipids containing DOTAP, cholesterol and PEG lipid are added to the cores resuspended in chloroform. The chloroform is later removed by evaporation and the resulting LCP nanoparticle solution is resuspended in aqueous buffer.

The surface of the particle is then coated with a variety of surface lipids to adjust the surface charge of the final LCP from positive to negative. In addition, PEG lipids can be included to prolong the circulation lifetime of the resulting LCP nanoparticle (Li et al., 2012). siRNA encapsulation efficiency of the LCP nanoparticle is typically over 90% with a particle size of approximately 40 nm in diameter including the PEG coating (Li et al., 2012). An LCP nanoparticle constructed with the cationic lipid DOTAP and an anisamide targeting PEG lipid has demonstrated efficacy in inhibiting lung metastasis in a melanoma mouse model (Yang, Li, Liu, & Huang,

2012). This nanoparticle contains three different siRNA sequences targeting MDM2, c-myc, and VEGF in order to achieve a greater therapeutic effect by inhibiting multiple pathways simultaneously. Mice treated with this targeted LCP particle have shown significantly improved survival rates and have markedly reduced numbers of lung metastatic nodules compared to either control mice or mice treated with LCP without the anisamide targeting ligand (Yang et al., 2012). In addition, the LCP technology has been used to co-encapsulate siRNA with gemcitabine, a nucleoside analog used as a first-line chemotherapeutic for advanced non-small cell lung carcinomas (Zhang, Schwerbrock, Rogers, Kim, & Huang, 2013). The mechanism of action of LCP nanoparticle is still under investigation; however, it is hypothesized that the acidic environment inside the endosome dissolves the calcium phosphate core of these particles, which causes an increase in the endosomal osmotic pressure that eventually breaks the endosome and release the siRNA into the cytoplasm (Li et al., 2010). It is important to note that divalent cations such as calcium and magnesium are known to induce membrane-disrupting hexagonal H_{II} phases in membranes containing anionic lipids, such as the ones found in the endosomal membrane (Hope, Walker, & Cullis, 1983; Tilcock, & Cullis, 1981). It is possible that the calcium released from the LCP particle induces hexagonal H_{II} phases with anionic lipids of the endosomal membrane, thereby disrupting the integrity of the endosomal membrane and facilitating the intracellular release of the encapsulated siRNA.



4. LNP-SIRNA FORMULATIONS IN CLINICAL TRIALS

Recent advances in lipid-based formulation technologies have led to numerous LNP-siRNA formulations in various stages of clinical trials. One of the most advanced formulations is ALN-TTR02 from Alnylam Pharmaceuticals (Cambridge, MA), which is currently in a phase III clinical trial for the treatment of transthyretin (TTR) amyloidosis. In nonhuman primates, ALN-TTR02 was shown to cause a 75% decrease in serum TTR levels 7 days after a single i.v. dose of 0.1 mg siRNA/kg body weight (Coelho et al., 2013). For animals receiving a single 0.3 mg/kg dose of siRNA, over 70% suppression of serum TTR levels was observed for 28 days after LNP-siRNA injection. Similarly in humans, the authors observed 50% knock-down of TTR levels by day 3 after a single 0.3 mg/kg dose of siRNA and over 50% reduction in protein levels for 28 days (Coelho et al., 2013). The suppression of serum TTR levels by ALN-TTR02 was specific as siRNA

targeting an unrelated protein had no effects on serum TTR levels. Furthermore, the formulation was well tolerated, with only minor infusion-related reactions and no liver and kidney toxicity.

Another promising formulation from Alnylam Pharmaceuticals is ALN-PCS, which is designed to specifically target proprotein convertase subtilisin/kexin type 9 (PCSK9) produced by liver for the treatment of hypercholesterolemia. Mice lacking PCSK9 was shown to have increased expression of the LDLR in the liver and decreased levels of cholesterol in the plasma (Rashid et al., 2005). Knockdown of PCSK9 in nonhuman primates by LNP-siRNA was shown to lower serum LDL cholesterol levels and increase the expression of LDLR in the liver (Frank-Kamenetsky et al., 2008). In a recent phase I clinical trial, a single dose of ALN-PCS at 0.4 mg siRNA/kg body weight caused a 70% decrease in serum PCSK9 levels 3 days after injection (Fitzgerald et al., 2014). This has consequently resulted in a 40% reduction in serum LDL cholesterol level that required 30 days to recover to the pre-treatment level. Similar to the ALN-TTR02 formulation, ALN-PCS was well tolerated.

In addition to targeting intrinsic proteins of the body, the same LNP-siRNA technology is also actively pursued as a therapeutic against viral infections. Tekmira Pharmaceuticals (Burnaby, BC) has recently launched a Phase I clinical study for TKM-Ebola, an LNP-siRNA formulation for the treatment of infection from the Zaire strain of Ebola virus (ZEBOV). This formulation was produced using the T-tube in-line mixing technique mentioned above and contained three different siRNA sequences targeting the L polymerase, viral protein 24 and viral protein 35 of ZEBOV (Geisbert et al., 2010). In a preclinical study utilizing nonhuman primates, all animals challenged with ZEBOV by intramuscular inoculation survived the infection following seven daily doses of TKM-Ebola at 2 mg siRNA/kg body weight per dose. The investigators were unable to detect ZEBOV in any of the treated animals after 14 days. Control animals treated with LNP-siRNA formulated with an unrelated siRNA against luciferase succumbed to the viral infection 10 days after viral inoculation. TKM-Ebola was granted Fast Track designation from the U.S. Food and Drug Administration (FDA) in March 2014.

Although LNP-siRNA systems are currently the most advanced siRNA delivery technology in clinical trials, lipoplexes of lipid and siRNA are also under active clinical investigations as means to deliver therapeutic siRNA in vivo. One such formulation is Atu027 from Silence Therapeutics GmbH (Berlin, Germany), a lipoplex formulation targeting protein kinase N3

(PKN3) for the treatment of advanced solid tumors (Aleku et al., 2008). This formulation contains a polycationic lipid, AtuFECT01, and is prepared by a method very similar to the PFV method described above (Santel et al., 2006). One very important difference from the PFV method is the lack of ethanol during the formulation process. Ethanol is an important factor in facilitating the encapsulation of siRNA as it allows remodelling of PFVs into multilamellar vesicles after membranes are bridged together by electrostatic interactions with the siRNA (Maurer et al., 2001). Without the ethanol, the formulation is likely a complex of cationic liposomes linked together by siRNA on the surface. Regardless of the formulation procedure, Atu027 was effective at silencing PKN3 mRNA levels in the lung and liver of mice following tail vein injections of four daily doses of Atu027 at 0.7 mg siRNA/kg per dose (Aleku et al., 2008). The formulation was successful at reducing the volume of xenografted tumor in the prostate and lowering the amount of metastatic tumors in the lymph node of mice. The company is currently conducting Phase Ib/IIa clinical trials of Atu027 in combination with gemcitabine for the treatment of advanced pancreatic cancer. Interestingly, localization of Atu027 to the lungs following i.v. administration has prompted the company to develop a lipoplex formulation, DACC, to treat lung metastases (Fehring et al., 2014). Effective reduction of CD31 expression in lung tumors and an increased survival rate were observed in a mouse model of lung metastases injected via tail vein with DACC complexed with siRNA against CD31 (Fehring et al., 2014). It remains to be seen whether this formulation would be advanced to clinical testing for the treatment of lung metastases.



5. FUTURE PROSPECTS

5.1 Avenues for Improving LNP Delivery

As mentioned above, tremendous efforts have been invested in the design of ionizable cationic lipids for maximizing the gene silencing capability of LNP-siRNA (Jayaraman et al., 2012; Semple et al., 2010). Although hepatic gene silencing resulted from LNP-siRNA containing the new generation of ionizable cationic lipids was greatly improved as compared to systems made with earlier generations of lipids, a recent study suggested that the intracellular release of siRNA from the current LNP-siRNA systems is a highly inefficient process. Following uptake of LNP-siRNA into the cell via endocytosis, only 1–2% of the total siRNA is released into the cytosol within a small window of time when LNP-siRNA accumulate in a specific

endocytic compartment with both early and late endosomal characteristics (Gilleron et al., 2013). In addition, the authors did not detect any major disruption of the endosomal membrane. Another recent report on the intracellular trafficking of LNP-siRNA systems revealed that approximately 70% of internalized particles eventually exits the cell through a recycling mechanism dependent on the protein Niemann-Pick Type C-1 (NPC1) (Sahay et al., 2013). Cells lacking the NPC1 gene were shown to have reduced endosomal recycling activities between late endosomes or lysosomes to the extracellular medium. As a result, these cells retain more LNP-siRNA following endocytosis and show more potent gene silencing than wild-type cells. It is therefore possible to improve the gene silencing potency of LNP-siRNA systems by manipulating the endocytic pathways to increase the intracellular accumulation of LNP-siRNA and/or the release of siRNA into the cytosol. Coincidentally, cellular entry of Ebola virus also requires functional NPC1 and treatment of cells with small molecular inhibitors of NPC1 greatly attenuates infections by the virus (Carette et al., 2011; Côté et al., 2011). Such small molecule inhibitors of NPC1 can be used in conjunction with LNP-siRNA to inhibit LNP-siRNA recycling and thus enhance the bioavailability of the internalized siRNA and gene silencing potency.

Incorporation of metallic nanoparticles in LNP-siRNA systems may offer an attractive method to disrupt the endosomal membrane. By exciting the metallic nanoparticle embedded in a lipid membrane through light radiation or a magnetic field, it is possible to increase the temperature of the lipid membrane to its gel-to-liquid crystal phase transition temperature (T_M) at which the permeability of the membrane is the greatest. Liposomes with gold nanoparticles embedded in their membrane have been shown to release their aqueous content following irradiation with ultra-violet light (Paasonen et al., 2007). In addition to gold, other metallic nanoparticles such as iron oxide were utilized in generating thermo-sensitive liposomes (Amstad et al., 2011). Formulations of gold nanoparticles with liposome have been prepared by several different methods: (1) reverse-phase evaporation with gold nanoparticles suspended in the organic phase (Hong, Friend, Glabe, & Papahadjopoulos, 1983; Paasonen et al., 2007); (2) physical adsorption of gold nanoparticles onto the surface of liposomes (Kojima, Hirano, Yuba, Harada, & Kono, 2008); (3) postinsertion of gold nanoparticle-conjugated phospholipids onto preformed liposomes (Chithrani, Dunne, Stewart, Allen, & Jaffray, 2010) and (4) embedding gold nanoparticle in a dry lipid film followed by hydration (Park, Oh, Mun, & Han, 2006). Recently, our laboratory

has demonstrated encapsulation of gold nanoparticles in LNP by mixing the commonly used lipids in LNP-siRNA formulation with commercially available negatively charged gold nanoparticles in a microfluidic mixer (unpublished). This method is straightforward as the ionizable cationic lipids bind to the negatively charged gold nanoparticles in a manner similar to the encapsulation of siRNA. Using the same method, LNP encapsulation of iron oxide nanoparticles is potentially possible when they are modified with functional groups such as carboxylic acids or silica to render their surface negatively charged (Laurent et al., 2008). A thermo-sensitive LNP-siRNA system can be generated by co-encapsulating metallic nanoparticles with siRNA using ionizable cationic lipids. Enhanced release of siRNA following cellular uptake may be made possible by excitation of cells or tissues with ultra-violet light or alternating magnetic field to generate enough heat to disrupt or permeabilize the endosomal membrane.

In addition to the potential benefit of enhancing endosomal release of siRNA, metallic nanoparticles co-encapsulated with siRNA in the same LNP system can also serve as a contrast agent for bioimaging. Gold nanoparticles, a good absorber of X-ray radiation, have been shown to provide therapeutic effects in tumor-bearing mice after radiotherapy and enhance contrast of tumors during X-ray imaging (Hainfeld, Slatkin, & Smilowitz, 2004; Hainfeld, Slatkin, Focella, & Smilowitz, 2006; Hainfeld, Smilowitz, O'Connor, Dilmanian, & Slatkin, 2013). However, the use of gold nanoparticles in radiation medicine or bioimaging suffers from poor accumulation of gold nanoparticles in tumors and rapid excretion through the kidneys following i.v. injection (Hainfeld et al., 2004). Thus, a LNP carrier system has the potential to improve the accumulation of the metallic nanoparticle to tumor sites by the aforementioned EPR effect. Co-encapsulation of siRNA with metallic nanoparticles can therefore generate an LNP system with dual functionality that allows both bioimaging of the tumor and silencing of the target gene for therapeutic purposes. It is anticipated that such “theranostic” system would have tremendous impact in the biomedical applications of LNP technologies.

5.2 Delivery of Other Nucleic Acids

The ionizable cationic lipid-based encapsulation technology for siRNA described above is fully applicable to other nucleic acid polymers such as plasmids and mRNA. A major challenge for LNP-plasmid systems is sufficient target gene expression since the nuclear envelope remains the barrier to the translocation of plasmid DNA into the nucleus. Efficient transgene

expression is usually only observed in rapidly dividing cell where the nuclear envelope disintegrates during mitosis allowing the plasmid DNA to enter the nucleus (Bally et al., 1999; Mortimer et al., 1999). In contrast, mRNA-LNP systems eliminate the need for nuclear translocation for gene expression (Hecker, 2013; Zou, Scarfo, Nantz, & Hecker, 2010). It has been shown that mRNA delivered by electroporation can result in the expression of target gene (Van Tendeloo et al., 2001). The therapeutic potential of mRNA was demonstrated by the fact that chemically modified mRNA can mediate the expression of the target protein, SP-B, in the lungs following intratracheal administration in SP-B deficient mice (Kormann et al., 2011). However, many tissues are inaccessible by local administration and the LNP-mRNA systems should allow systemic delivery with particular potency for tissues such as the liver (hepatocytes). Recently, it has been demonstrated that chemically modified mRNA condensed by protamine can be encapsulated inside of preformed cationic vesicles (Wang et al., 2013). The microfluidic mixing method presented previously would eliminate the need for nucleic acid-condensing agents such as protamine and the need for the time-consuming extrusion process. As LNP-mRNA systems produced by microfluidic mixing would contain the same lipid components as used for the delivery of siRNA, it is expected that these LNP-mRNA would display biodistribution and pharmacokinetic properties similar to that of LNP-siRNA systems. The ability to systemically deliver mRNA has considerable potential for the treatment of cancer and a variety of genetic disorders.

Another genetic material that can benefit from lipid-based delivery for therapeutic purposes is the recently discovered clustered, regularly interspaced, short palindromic repeats (CRISPR). CRISPR were originally discovered in *Escherichia coli* and was subsequently recognized as an important adaptive immunity pathway found in bacteria and archaea (Ishino, Shinagawa, Makino, Amemura, & Nakata, 1987; Marraffini, & Sontheimer, 2010). The mechanism of bacterial adaptive immunity by CRISPR has been reviewed extensively elsewhere (Marraffini, & Sontheimer, 2010; Rusk, 2012; Sorek, Kunin, & Hugenholz, 2008; Waters & Storz, 2009; Wiedenheft, Sternberg, & Doudna, 2012). Recent interests in CRISPR technology stemmed from the possibility of using CRISPR to modify genes in order to tackle human diseases. CRISPR, along with the endonuclease CRISPR-associated protein 9 (Cas9), facilitate genome editing by introducing double-strand breaks at a specific genetic locus that has sequence complementary to the guide RNA (gRNA) (Jinek et al., 2012, 2013). A gene can therefore be deleted

or replaced using this novel technology. The CRISPR system has been used to produce genetically modified animals from numerous species, including fruit fly, zebrafish, rabbit, rat and mouse (reviewed in [Sander & Joung, 2014](#)). Genetically modified mice have been produced by injecting mRNA encoding the endonuclease Cas9, along with guide RNA (gRNA) targeting the gene to be modified, into mouse embryos ([Wang et al., 2013](#)). The CRISPR system can also be used to correct disease-causing mutations. Cataract-free mice have been generated by injecting Cas9 mRNA and gRNA targeting the mutated *Crygc* gene locus into mouse zygotes followed by implantation in surrogate female mice ([Wu et al., 2013](#)). Also, mutated cystic fibrosis transmembrane conductor receptor (CFTR) from the intestinal stem cells of cystic fibrosis patients has been corrected by the CRISPR/Cas system using gRNA targeting the mutated *CFTR* locus along with a plasmid DNA encoding the correct *CFTR* sequence as a template for homology-directed repair ([Schwank et al., 2013](#)). These preliminary examples support CRISPR-mediated genetic modifications as novel strategies to treat genetic diseases.

Proper genetic modification by CRISPR requires at least two components to be introduced to the target cells: first, the gRNA specific to the genetic locus to be modified; and second, the Cas9 endonuclease, which is usually introduced as a plasmid vector or mRNA. If the cell to be modified contains a homozygous mutation of the gene, an additional plasmid encoding the correct version of the gene is necessary in order to facilitate homology-directed repair of the mutation. It is obvious that all these nucleic acids require an efficient method to enter the target cells. The examples of CRISPR-mediated genetic modifications mentioned above used either commercially available transfection reagent, viral vectors or direct injection of CRISPR components into the target cells, which all have inferior in vivo performances as compared to lipid-based delivery systems. It is clear that our understanding of CRISPR/Cas system is still at the early stage; however, numerous companies have begun aggressive programs to develop research tools for the CRISPR field (reviewed in [Baker, 2014](#)). The race for the first CRISPR-based therapeutic for genetic diseases are currently underway and much like the development of siRNA-based drugs 10 years ago, the development of an effective delivery system for CRISPR components will be the key to this race. Based on its proven track record in nucleic acid delivery, it is possible that lipid-based systems will be at the forefront of this CRISPR technology. It remains to be seen whether CRISPR technologies will enjoy as much success as siRNA has in the clinic and it is

certain that we will see many innovations in both formulation method and delivery material for CRISPR in the near future.

REFERENCES

- Abraham, Waterhouse, Mayer, Cullis, Madden, & Bally. (2005). The liposomal formulation of doxorubicin. *Methods in Enzymology*, *391*, 71–97.
- Akinc, Querbes, De, Qin, Frank-Kamenetsky, Jayaprakash., et al. (2010). Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Molecular Therapy*, *18*, 1357–1364.
- Aleku, Schulz, Keil, Santel, Schaeper, Dieckhoff., et al. (2008). Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. *Cancer Research*, *68*, 9788–9798.
- Allen & Cullis. (2013). Liposomal drug delivery systems: from concept to clinical applications. *Advanced Drug Delivery Reviews*, *65*, 36–48.
- Alvarez, Elbashir, Borland, Toudjarska, Hadwiger, John., et al. (2009). RNA interference-mediated silencing of the respiratory syncytial virus nucleocapsid defines a potent antiviral strategy. *Antimicrobial Agents and Chemotherapy*, *53*, 3952–3962.
- Ameres, Martinez, & Schroeder. (2007). Molecular basis for target RNA recognition and cleavage by human RISC. *Cell*, *130*, 101–112.
- Amstad, Kohlbrecher, Müller, Schweizer, Textor, & Reimhult. (2011). Triggered release from liposomes through magnetic actuation of iron oxide nanoparticle containing membranes. *Nano Letters*, *11*, 1664–1670.
- Audouy, de Leij, Hoekstra, & Molema. (2002). In vivo characteristics of cationic liposomes as delivery vectors for gene therapy. *Pharmaceutical Research*, *19*, 1599–1605.
- Bailey & Cullis. (1994). Modulation of membrane fusion by asymmetric transbilayer distributions of amino lipids. *Biochemistry*, *33*, 12573–12580.
- Baker, Saltik, Lehrmann, Killisch, Mautner, Lamm, G., et al. (1997). Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. *Gene Therapy*, *4*, 773–782.
- Baker. (2014). Gene editing at CRISPR speed. *Nature Biotechnology*, *32*, 309–312.
- Bally, Harvie, Wong, Kong, Wasan, & Reimer. (1999). Biological barriers to cellular delivery of lipid-based DNA carriers. *Advanced Drug Delivery Reviews*, *38*, 291–315.
- Bangham & Horne. (1964). Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *Journal of Molecular Biology*, *8*, 660–668.
- Bangham, Standish, & Watkins. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, *13*, 238–252.
- Bangham, Standish, & Weissmann. (1965). The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *Journal of Molecular Biology*, *13*, 253–259.
- Beisiegel, Weber, Ihrke, Herz, & Stanley. (1989). The LDL-receptor-related protein, LRP, is an apolipoprotein E-binding protein. *Nature*, *341*, 162–164.
- Belliveau, Huft, Lin, Chen, Leung, Leaver., et al. (2012). Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA. *Molecular therapy. Nucleic Acids*, *1*, e37.
- Belloq, Pun, Jensen, & Davis. (2003). Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. *Bioconjugate Chemistry*, *14*, 1122–1132.
- Bernstein, Caudy, Hammond, & Hannon. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, *409*, 363–366.
- Bisgaier, Siebenkas, & Williams. (1989). Effects of apolipoproteins A-IV and A-I on the uptake of phospholipid liposomes by hepatocytes. *The Journal of Biological Chemistry*, *264*, 862–866.

- Bitko, Musiyenko, Shulyayeva, & Barik. (2005). Inhibition of respiratory viruses by nasally administered siRNA. *Nature Medicine*, *11*, 50–55.
- Bolotin, Cohen, Bar, Emanuel, Ninio, Danilo., et al. (1994). Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes. *Journal of Liposome Research*, *4*, 455–479.
- Braasch, Paroo, Constantinescu, Ren, Oz, Mason., et al. (2004). Biodistribution of phosphodiester and phosphorothioate siRNA. *Bioorganic & Medicinal Chemistry Letters*, *14*, 1139–1143.
- Carette, Raaben, Wong, Herbert, Obernosterer, Mulherkar., et al. (2011). Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature*, *477*, 340–343.
- Charrois & Allen. (2004). Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer. *Biochimica et Biophysica Acta*, *1663*, 167–177.
- Chen, Connor, Aderman, Willett, Aspegren, & Smith. (2009). Suppression of retinal neovascularization by erythropoietin siRNA in a mouse model of proliferative retinopathy. *Investigative Ophthalmology & Visual Science*, *50*, 1329–1335.
- Chen, Sen, Bathula, Yang, Fittipaldi, Huang., et al. (2009). Novel cationic lipid that delivers siRNA and enhances therapeutic effect in lung Cancer cells. *Molecular Pharmaceutics*, *6*, 696–705.
- Chithrani, Dunne, Stewart, Allen, & Jaffray. (2010). Cellular uptake and transport of gold nanoparticles incorporated in a liposomal carrier. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *6*, 161–169.
- Choi, MacKay, & Szoka. (2003). Low-pH-sensitive PEG-stabilized plasmid-lipid nanoparticles: preparation and characterization. *Bioconjugate Chemistry*, *14*, 420–429.
- Choung, Kim, Kim, Park, & Choi. (2006). Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochemical and Biophysical Research Communications*, *342*, 919–927.
- Chowrira & Burke. (1992). Extensive phosphorothioate substitution yields highly active and nuclease-resistant hairpin ribozymes. *Nucleic Acids Research*, *20*, 2835–2840.
- Coelho, Adams, Silva, Lozeron, Hawkins, Mant., et al. (2013). Safety and efficacy of RNAi therapy for transthyretin amyloidosis. *The New England Journal of Medicine*, *369*, 819–829.
- Côté, Misasi, Ren, Bruchez, Lee, Filone., et al. (2011). Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. *Nature*, *477*, 344–348.
- Cullis & de Kruijff. (1978). The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. A 31P NMR study. *Biochimica et Biophysica Acta*, *513*, 31–42.
- Cullis, Hope, & Tilcock. (1986). Lipid polymorphism and the roles of lipids in membranes. *Chemistry and Physics of Lipids*, *40*, 127–144.
- Davis, Pun, Bellocq, Reineke, Popielarski, Mishra., et al. (2004). Self-assembling nucleic acid delivery vehicles via linear, water-soluble, cyclodextrin-containing polymers. *Current Medicinal Chemistry*, *11*, 179–197.
- Davis, Zuckerman, Choi, Seligson, Tolcher, Alabi., et al. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*, *464*, 1067–1070.
- De Fougerolles, Vornlocher, Maraganore, & Lieberman. (2007). Interfering with disease: a progress. *Nature Reviews Drug Discovery*, *6*, 443–453.
- DeVincenzo, Cehelsky, Alvarez, Elbashir, Harborth, Toudjarska., et al. (2008). Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). *Antiviral Research*, *77*, 225–231.
- DeVincenzo, Lambkin-Williams, Wilkinson, Cehelsky, Nochur, & Walsh. (2010). A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. *Proceedings of the National Academy of Sciences of the United States of America*, *107*, 8800–8805.

- Dorn, Patel, Wotherspoon, Hemmings–Mieszczyk, Barclay, Natt., et al. (2004). siRNA relieves chronic neuropathic pain. *Nucleic Acids Research*, *32*, e49.
- Fehring, Schaeper, Ahrens, Santel, Keil, Eisermann., et al. (2014). Delivery of therapeutic siRNA to the lung endothelium via novel lipoplex formulation DACC. *Molecular Therapy: the Journal of the American Society of Gene Therapy*, *22*, 811–820.
- Felgner, Gadek, Holm, Roman, Chan, Wenz., et al. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America*, *84*, 7413–7417.
- Fenske, Wong, Maurer, Maurer, Leenhouts, Boman., et al. (1998). Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients. *Biochimica et Biophysica Acta*, *1414*, 188–204.
- Filipowicz, Jaskiewicz, Kolb, & Pillai. (2005). Post-transcriptional gene silencing by siRNAs and miRNAs. *Current Opinion in Structural Biology*, *15*, 331–341.
- Fitzgerald, Frank–Kamenetsky, Shulga–Morskaya, Liebow, Bettencourt, Sutherland., et al. (2014). Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial. *Lancet*, *383*, 60–68.
- Frank–Kamenetsky, Grefhorst, Anderson, Racie, Bramlage, Akinc., et al. (2008). Therapeutic RNAi targeting PCSK9 acutely lowers plasma cholesterol in rodents and LDL cholesterol in nonhuman primates. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 11915–11920.
- Geisbert, Lee, Robbins, Geisbert, Honko, Sood., et al. (2010). Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet*, *375*, 1896–1905.
- Gilleron, Querbes, Zeigerer, Borodovsky, Marsico, Schubert., et al. (2013). Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nature Biotechnology*, *31*, 638–646.
- Gonzalez, Hwang, & Davis. (1999). New class of polymers for the delivery of macromolecular therapeutics. *Bioconjugate Chemistry*, *10*, 1068–1074.
- Gregoriadis. (1973). Drug entrapment in liposomes. *FEBS Letters*, *36*, 292–296.
- Gregoriadis, Leathwood, & Ryman. (1971). Enzyme entrapment in liposomes. *FEBS Letters*, *14*, 95–99.
- Gregoriadis & Ryman. (1971). Liposomes as carriers of enzymes or drugs: a new approach to the treatment of storage diseases. *Biochemical Journal*, *124*, 58P.
- Guo, & Szoka. (2001). Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG–diortho ester–lipid conjugate. *Bioconjugate Chemistry*, *12*, 291–300.
- Hafez & Cullis. (2001). Roles of lipid polymorphism in intracellular delivery. *Advanced Drug Delivery Reviews*, *47*, 139–148.
- Hafez, Maurer, & Cullis. (2001). On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Therapy*, *8*, 1188–1196.
- Hainfeld, Slatkin, Focella, & Smilowitz. (2006). Gold nanoparticles: a new X-ray contrast agent. *The British Journal of Radiology*, *79*, 248–253.
- Hainfeld, Slatkin, & Smilowitz. (2004). The use of gold nanoparticles to enhance radiotherapy in mice. *Physics in Medicine and Biology*, *49*, N309–N315.
- Hainfeld, Smilowitz, O'Connor, Dilmanian, & Slatkin. (2013). Gold nanoparticle imaging and radiotherapy of brain tumors in mice. *Nanomedicine*, *8*, 1601–1609.
- Hecker. (2013). Synthetic messenger RNA and cell metabolism modulation. In P. M. Rabinovich (Ed.), *Synthetic messenger RNA and cell metabolism modulation: Methods and protocols* (Vol. 969) (pp. 73–88). Totowa, NJ: Humana Press.
- Heidel, Yu, Liu, Rele, Liang, Zeidan., et al. (2007). Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 5715–5721.

- Hong, Friend, Glabe, & Papahadjopoulos. (1983). Liposomes containing colloidal gold are useful probe of liposome-cell interactions. *Biochimica et Biophysica Acta*, 732, 320–323.
- Hope, Walker, & Cullis. (1983). Ca²⁺ and pH induced fusion of small unilamellar vesicles consisting of phosphatidylethanolamine and negatively charged phospholipids: a freeze fracture study. *Biochemical and Biophysical Research Communications*, 110, 15–22.
- Hu-Lieskovan, Heidel, Bartlett, Davis, & Triche. (2005). Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Research*, 65, 8984–8992.
- Huang, Hong, Zheng, Ding, Guo, Zhang., et al. (2011). Elimination pathways of systemically delivered siRNA. *Molecular Therapy: the Journal of the American Society of Gene Therapy*, 19, 381–385.
- Hutvagner & Zamore. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, 297, 2056–2060.
- Hwang, Bellocq, & Davis. (2001). Effects of structure of beta-cyclodextrin-containing polymers on gene delivery. *Bioconjugate Chemistry*, 12, 280–290.
- Iden & Allen. (2001). In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochimica et Biophysica Acta*, 1513, 207–216.
- Ishino, Shinagawa, Makino, Amemura, & Nakata. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology*, 169, 5429–5433.
- Jayaraman, Ansell, Mui, Tam, Chen, Du., et al. (2012). Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angewandte Chemie (International Ed. in English)*, 51, 8529–8533.
- Jeffs, Palmer, Ambegia, Giesbrecht, Ewanick, & MacLachlan. (2005). A Scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. *Pharmaceutical Research*, 22, 362–372.
- Jinek, Chylinski, Fonfara, Hauer, Doudna, & Charpentier. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 337, 816–821.
- Jinek, East, Cheng, Lin, Ma, & Doudna. (2013). RNA-programmed genome editing in human cells. *eLife*, 2, e00471.
- Johnson, Miller, & Bangham. (1973). The opposing effects of pressure and general anaesthetics on the cation permeability of liposomes of varying lipid composition. *Biochimica et Biophysica Acta*, 307, 42–57.
- Johnston, Semple, Klimuk, Edwards, Eisenhardt, Leng., et al. (2006). Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations. *Biochimica et Biophysica Acta*, 1758, 55–64.
- Judge, Bola, Lee, & MacLachlan. (2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Molecular Therapy*, 13, 494–505.
- Kanasty, Dorkin, Vegas, & Anderson. (2013). Delivery materials for siRNA therapeutics. *Nature Materials*, 12, 967–977.
- Kim, Park, Lee, Kim, Park, & Kim. (2005). Polyethylenimine with acid-labile linkages as a biodegradable gene carrier. *Journal of Controlled Release*, 103, 209–219.
- Kirpotin, Drummond, Shao, Shalaby, Hong, Nielsen., et al. (2006). Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Research*, 66, 6732–6740.
- Kojima, Hirano, Yuba, Harada, & Kono. (2008). Preparation and characterization of complexes of liposomes with gold nanoparticles. *Colloids and Surfaces. B, Biointerfaces*, 66, 246–252.
- Kormann, Hasenpusch, Aneja, Nica, Flemmer, Herber-Jonat., et al. (2011). Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nature Biotechnology*, 29, 154–157.

- Krieger & Herz. (1994). STRUCTURES and FUNCTIONS of receptors: macrophage scavenger receptors and LDL. *Annual Review of Biochemistry*, *63*, 601–637.
- Laurent, Forge, Port, Roch, Robic, Vander Elst., et al. (2008). Magnetic iron oxide nanoparticles: synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications. *Chemical Reviews*, *108*, 2064–2110.
- Layzer, Mccaffrey, Tanner, Huang, & Kay. (2004). In vivo activity of nuclease-resistant siRNAs in vivo activity of nuclease-resistant siRNAs. *RNA*, *10*, 766–771.
- Leonetti, Machy, Degols, Lebleu, & Leserman. (1990). Antibody-targeted liposomes containing oligodeoxyribonucleotides complementary to viral RNA selectively inhibit viral replication. *Proceedings of the National Academy of Sciences of the United States of America*, *87*, 2448–2451.
- Leung, Hafez, Baoukina, Belliveau, Zhigaltsev, Afshinmanesh., et al. (2012). Lipid nanoparticles containing siRNA synthesized by microfluidic mixing exhibit an electron-dense nanostructured core. *The Journal of Physical Chemistry C*, *116*, 18440–18450.
- Li, Chen, Tseng, Mozumdar, & Huang. (2010). Biodegradable calcium phosphate nanoparticle with lipid coating for systemic siRNA delivery. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, *142*, 416–421.
- Li, Chono, & Huang. (2008). Efficient oncogene silencing and metastasis inhibition via systemic delivery of siRNA. *Molecular Therapy*, *16*, 942–946.
- Liebert, Tousignant, Gates, Ingram, Johnson, Nietupski., et al. (2000). Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid: plasmid DNA complexes in mice. *Human Gene Therapy*, *11*, 2493–2513.
- Li & Huang. (2006). Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Molecular Pharmaceutics*, *3*, 579–588.
- Lingor, Michel, Schöll, Bähr, & Kügler. (2004). Transfection of “naked” siRNA results in endosomal uptake and metabolic impairment in cultured neurons. *Biochemical and Biophysical Research Communications*, *315*, 1126–1133.
- Li, Tang, Cheng, Qin, Xie, Wei., et al. (2005). Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nature Medicine*, *11*, 944–951.
- Litzinger. (1997). Limitations of cationic liposomes for antisense oligonucleotide delivery in vivo. *Journal of Liposome Research*, *7*, 51–61.
- Li, Yang, & Huang. (2012). Calcium phosphate nanoparticles with an asymmetric lipid bilayer coating for siRNA delivery to the tumor. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, *158*, 108–114.
- Maclachlan. (2007). Liposomal formulations for nucleic acid delivery. In *Antisense drug technologies*, 2nd ed. (pp. 237–270). Taylor & Francis Group, LLC.
- Maeda. (2001). The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Advances in Enzyme Regulation*, *41*, 189–207.
- Maeda, Wu, Sawa, Matsumura, & Hori. (2000). Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, *65*, 271–284.
- Mahato, Kumar, & Sharma. (2013). Amphiphilic polyethylenimine polymers mediate efficient delivery of DNA and siRNA in mammalian cells. *Molecular BioSystems*, *9*, 780–791.
- Mahley. (1988). Apolipoprotein E: cholesterol protein with expanding role in cell. *Science*, *240*, 622–630.
- Mahley & Ji. (1999). Remnant lipoprotein metabolism: key pathways involving cell-surface heparan sulfate proteoglycans and apolipoprotein E. *Journal of Lipid Research*, *40*, 1–16.
- Marraffini, & Sontheimer. (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nature Reviews Genetics*, *11*, 181–190.
- Matranga, Tomari, Shin, Bartel, & Zamore. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell*, *123*, 607–620.

- Maurer, Fenske, & Cullis. (2001). Developments in liposomal drug delivery systems. *Expert Opinion on Biological Therapy*, 1, 923–947.
- Maurer, Wong, Stark, Louie, McIntosh, Wong., et al. (2001). Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes. *Biophysical Journal*, 80, 2310–2326.
- Mayer, Bally, & Cullis. (1986). Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. *Biochimica et Biophysica Acta*, 857, 123–126.
- Mayer, Bally, Hope, & Cullis. (1986). Techniques for encapsulating bioactive agents into liposomes. *Chemistry and Physics of Lipids*, 40, 333–345.
- Mayer, Nayar, Thies, Boman, Cullis, & Bally. (1993). Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. *Cancer Chemotherapy and Pharmacology*, 33, 17–24.
- Mislick & Baldeschwieler. (1996). Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 12349–12354.
- Mortimer, Tam, MacLachlan, Graham, Saravolac, & Joshi. (1999). Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Therapy*, 6, 403–411.
- Mounkes, Zhong, Cipres-Palacin, Heath, & Debs. (1998). Proteoglycans mediate cationic liposome-DNA complex-based gene delivery in vitro and in vivo. *Journal of Biological Chemistry*, 273, 26164–26170.
- Mui, Tam, Jayaraman, Ansell, Du, Tam, & Hope. (2013). Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticles. *Molecular Therapy Nucleic Acids*, 2, e139.
- Nakayama, Butler, Sehgal, Severgnini, Racie, Sharman., et al. (2012). Harnessing a physiologic mechanism for siRNA delivery with mimetic lipoprotein particles. *Molecular Therapy: the Journal of the American Society of Gene Therapy*, 20, 1582–1589.
- Nichols, & Deamer. (1976). Catecholamine uptake and concentration by liposomes maintaining pH gradients. *Biochimica et Biophysica Acta*, 455, 269–271.
- Paasonen, Laaksonen, Johans, Yliperttula, Kontturi, & Urtti. (2007). Gold nanoparticles enable selective light-induced contents release from liposomes. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 122, 86–93.
- Park, Oh, Mun, & Han. (2006). Loading of gold nanoparticles inside the DPPC bilayers of liposome and their effects on membrane fluidities. *Colloids and Surfaces B Biointerfaces*, 48, 112–118.
- Parr, Ansell, Choi, & Cullis. (1994). Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles. *Biochimica et Biophysica Acta*, 1195, 21–30.
- Patil, Swaminathan, Sadhukha, Ma, & Panyam. (2010). The use of nanoparticle-mediated targeted gene silencing and drug delivery to overcome tumor drug resistance. *Biomaterials*, 31, 358–365.
- Pieken, Olsen, Benseler, Aurup, & Eckstein. (2014). Kinetic characterization of ribonuclease-resistant 2' -Modified hammerhead ribozymes kinetic characterization of ribonuclease-resistant 2' -modified hammerhead ribozymes. *Science*, 253, 314–317.
- Pillé, Li, Blot, Bertrand, Pritchard, Opolon., et al. (2006). Intravenous delivery of anti-RhoA small interfering RNA loaded in nanoparticles of chitosan in mice: safety and efficacy in xenografted aggressive breast cancer. *Human Gene Therapy*, 17, 1019–1026.
- Rand, Ginalski, Grishin, & Wang. (2004). Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 14385–14389.
- Rand, Petersen, Du, & Wang. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell*, 123, 621–629.

- Rashid, Curtis, Garuti, Anderson, Bashmakov, Ho., et al. (2005). Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 5374–5379.
- Rodrigueza, Pritchard, & Hope. (1993). The influence of size and composition on the cholesterol mobilizing properties of liposomes in vivo. *Biochimica et Biophysica Acta*, 1153, 9–19.
- Rusk. (2012). Microbiology: prokaryotic RNAi. *Nature Methods*, 9, 220–221.
- Sahay, Querbes, Alabi, Eltoukhy, Sarkar, Zurenko., et al. (2013). Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. *Nature Biotechnology*, 31, 653–658.
- Sander & Joung. (2014). CRISPR–Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*, 32, 347–355.
- Santel, Aleku, Keil, Endruschat, Esche, Fisch., et al. (2006). A novel siRNA–lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene Therapy*, 13, 1222–1234.
- Sapra & Allen. (2003). Ligand-targeted liposomal anticancer drugs. *Progress in Lipid Research*, 42, 439–462.
- Scheule, St George, Bagley, Marshall, Kaplan, Akita., et al. (1997). Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Human Gene Therapy*, 8, 689–707.
- Schiffelers, Ansari, Xu, Zhou, Tang, Storm., et al. (2004). Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Research*, 32, e149.
- Schwank, Koo, Sasselli, Dekkers, Heo, Demircan., et al. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*, 13, 653–658.
- Semple, Akinc, Chen, Sandhu, Mui, Cho., et al. (2010). Rational design of cationic lipids for siRNA delivery. *Nature Biotechnology*, 28, 172–176.
- Semple, Klimuk, Harasym, Dos Santos, Ansell, Wong., et al. (2001). Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochimica et Biophysica Acta*, 1510, 152–166.
- Sessa & Weissmann. (1968). Phospholipid spherules (liposomes) as a model for biological membranes. *Journal of Lipid Research*, 9, 310–318.
- Shen, Samul, Silva, Akiyama, Liu, Saishin., et al. (2006). Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Therapy*, 13, 225–234.
- Shin, Shum, & Thompson. (2003). Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG–lipids. *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 91, 187–200.
- Skinner, Tutton, & Brien. (1990). Microvascular architecture of experimental colon tumors in the rat. *Cancer Research*, 50, 2411–2417.
- Sorek, Kunin, & Hugenholtz. (2008). CRISPR—a widespread system that provides acquired resistance against phages in bacteria and archaea. *Nature Reviews Microbiology*, 6, 181–186.
- Soutschek, Akinc, Bramlage, Charisse, Constien, Donoghue., et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*, 432, 173–178.
- Sproat, Lamond, Beijer, Neuner, & Ryder. (1989). Highly efficient chemical synthesis of 2'-O-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases. *Nucleic Acids Research*, 17, 3373–3386.
- Stamatatos, Leventis, Zuckermann, & Silvius. (1988). Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry*, 27, 3917–3925.

- Steinberg, Konerding, & Streffer. (1990). The vascular architecture of human xenotransplanted tumors: histological, morphometrical, and ultrastructural studies. *Journal of Cancer Research and Clinical Oncology*, *116*, 517–524.
- Tam, Chen, Zaufman, Tam, Lin, Ansell., et al. (2013). Small molecule ligands for enhanced intracellular delivery of lipid nanoparticle formulations of siRNA. *Nanomedicine: Nanotechnology, Biology, and Medicine*, *9*, 665–674.
- Tan, Yang, Shih, Lan, & Cheng. (2005). Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. *Gene Therapy*, *12*, 59–66.
- Thakker, Natt, Hüsken, Maier, Müller, van der Putten., et al. (2004). Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 17270–17275.
- Thakker, Natt, Hüsken, van der Putten, Maier, Hoyer., et al. (2005). siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. *Molecular Psychiatry*, *10*, 782–789 714.
- Thomas, Ge, Lu, Chen, & Klibanov. (2005). Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. *Pharmaceutical Research*, *22*, 373–380.
- Thomas, Lu, Ge, Zhang, Chen, & Klibanov. (2005). Full deacylation of polyethylenimine dramatically boosts its gene delivery efficiency and specificity to mouse lung. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 5679–5684.
- Tilcock & Cullis. (1981). The polymorphic phase behaviour of mixed phosphatidylserine-phosphatidylethanolamine model systems as detected by ³¹P-NMR. Effects of divalent cations and pH. *Biochimica et Biophysica Acta*, *641*, 189–201.
- Tolentino, Brucker, Fosnot, Ying, Wu, Malik., et al. (2004). Intravitreal injection of vascular endothelial growth factor small interfering RNA inhibits growth and leakage in a non-human primate, laser-induced model of choroidal neovascularization. *Retina*, *24*, 660.
- Tomari & Zamore. (2005). Perspective: machines for RNAi. *Genes & Development*, *19*, 517–529.
- Tseng, Mozumdar, & Huang. (2009). Lipid-based systemic delivery of siRNA. *Advanced Drug Delivery Reviews*, *61*, 721–731.
- Utsuno & Uludağ. (2010). Thermodynamics of polyethylenimine-DNA binding and DNA condensation. *Biophysical Journal*, *99*, 201–207.
- Urban-Klein, Werth, Abuharbeid, Czubayko, & Aigner. (2005). RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Therapy*, *12*, 461–466.
- Van Tendeloo, Ponsaerts, Lardon, Nijs, Lenjou, van Broeckhoven., et al. (2001). Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. *Blood*, *98*, 49–56.
- Wang, Su, Yang, Hu, Zhang, Blancafort., et al. (2013). Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy. *Molecular Therapy*, *21*, 358–367.
- Wang, Yang, Shivalila, Dawlaty, Cheng, Zhang., et al. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, *153*, 910–918.
- Waters & Storz. (2009). Regulatory RNAs in bacteria. *Cell*, *136*, 615–628.
- Webb, Harasym, Masin, Bally, & Mayer. (1995). Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. *British Journal of Cancer*, *72*, 896–904.

- Whitehead, Langer, & Anderson. (2009). Knocking down barriers: advances in siRNA delivery. *Nature Reviews Drug Discovery*, 8, 129–138.
- Wiedenheft, Sternberg, & Doudna. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature*, 482, 331–338.
- Wolfrum, Shi, Jayaprakash, Jayaraman, Wang, Pandey., et al. (2007). Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nature Biotechnology*, 25, 1149–1157.
- Wrobel & Collins. (1995). Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochimica et Biophysica Acta*, 1235, 296–304.
- Wu, Liang, Wang, Bai, Tang, Bao., et al. (2013). Correction of a genetic disease in mouse via use of CRISPR–Cas9. *Cell Stem Cell*, 13, 659–662.
- Yang, Li, Liu, & Huang. (2012). Systemic delivery of siRNA via LCP nanoparticle efficiently inhibits lung metastasis. *Molecular Therapy: the Journal of the American Society of Gene Therapy*, 20, 609–615.
- Yan, Kuipers, Havekes, Havinga, Dontje, Poelstra., et al. (2005). The role of apolipoprotein E in the elimination of liposomes from blood by hepatocytes in the mouse. *Biochemical and Biophysical Research Communications*, 328, 57–62.
- Yuan, Dellian, Fukumura, Leunig, Berk, Torchilin., et al. (1995). Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Research*, 55, 3752–3756.
- Yuan, Leunig, Huang, Berk, Papahadjopoulos, & Jam. (1994). Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Research*, 54, 3352–3356.
- Yuan, Naguib, & Wu. (2011). Recent advances of siRNA delivery by nanoparticles. *Expert Opinion on Drug Delivery*, 8, 521–536.
- Zhang, Liu, & Huang. (2005). Implications of pharmacokinetic behavior of lipoplex for its inflammatory toxicity. *Advanced Drug Delivery Reviews*, 57, 689–698.
- Zhang, Schwerbrock, Rogers, Kim, & Huang. (2013). Codelivery of VEGF siRNA and gemcitabine monophosphate in a single nanoparticle formulation for effective treatment of NSCLC. *Molecular Therapy: the Journal of the American Society of Gene Therapy*, 21, 1559–1569.
- Zheng, Pavan, Neeb, Schaper, Danani, Klebe., et al. (2012). Targeting the blind spot of poly-cationic nanocarrier-based siRNA delivery. *ACS Nano*, 6, 9447–9454.
- Zimmermann, Lee, Akinc, Bramlage, Bumcrot, Fedoruk., et al. (2006). RNAi-mediated gene silencing in non-human primates. *Nature*, 441, 111–114.
- Zintchenko, Philipp, Dehshahri, & Wagner. (2008). Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. *Bioconjugate Chemistry*, 19, 1448–1455.
- Zou, Scarfo, Nantz, & Hecker. (2010). Lipid-mediated delivery of RNA is more efficient than delivery of DNA in non-dividing cells. *International Journal of Pharmaceutics*, 389, 232–243.