LIPID NANOPARTICLES FOR DELIVERY OF BIOACTIVE MOLECULES

by

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Abstract

Nanoparticle drug delivery vehicles can be used for in vivo delivery of a variety of bioactive molecules including nucleic acid polymers and small molecule drugs for applications ranging from gene therapy to improving the efficacy of anticancer drugs. This thesis explores different variations of lipid nanoparticles (LNP) formulated with microfluidic mixing method, for encapsulation of a wide range of bioactive agents.

The first part of the thesis focuses on the effect of cationic lipid content of the LNP and its effect on encapsulation of negatively charged small cargo. The ability of the rapid mixing/cationic lipid protocol to encapsulate small negatively charged molecules and short oligonucleotides in LNP systems containing ionizable cationic lipids and/or permanently positively charged cationic lipids is explored. It was found that encapsulation of small cargo is dependent on molecule size and charge and that permanently charged LNP are more efficacious at encapsulating small, charged cargo than ionizable LNP. It is also shown that charged prodrug forms of molecules that are neutral in their native form can be encapsulated and delivered using this method. The results of these studies can be used as general guidelines for entrapment and delivery of novel bioactive molecules that are typically difficult to formulate.

While the cationic lipid plays an obvious role in entrapment and delivery of negatively charged molecules, the role of helper lipids is not very clear. The second chapter of the thesis looks at the effect of helper lipid titration and variation in in vitro cellular uptake and knockdown and in vivo hepatocyte gene silencing, using ionizable-LNP containing siRNA. It was found that while in vitro knockdown might not be greatly affected when the conventional 10 mol% DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) is varied, in vivo hepatocyte gene silencing was almost fully disrupted by helper lipid variations. The results of these studies could lead to a more
targeted LNP simply by varying the helper lipid that enables avoiding certain cell types or targets specific tissues.
Lay Summary

Nanoparticles are drug carriers that can improve drugs by making them more efficient and/or less toxic. In brief, drug molecules can be encapsulated in these nano-carriers and stay intact until they reach their target tissues such as tumors. Nanoparticles can have different applications depending on their type and composition. This thesis focuses on lipid-based nanoparticles that can deliver a wide range of bioactive molecules for different purposes. The effect of cationic lipid type and content on encapsulation of small drug molecules was studied and it was found that larger molecules with a higher negative charge are more readily entrapped in cationic lipid nanoparticles. Furthermore, the role of the helper lipid content of lipid nanoparticle on gene silencing efficacy was investigated. The findings presented here can be used to further specialize and target nanoparticles for therapeutic or diagnostic purposes.
Preface

I was responsible for writing the entire thesis including all revisions. Experimental designs and data analyses were performed by myself with valuable input and feedback from Dr. Ismail Hafez, Ms. Joslyn Quick, Dr. Robert Young and Dr. Genc Basha. All of the in vitro studies were performed by myself other than FACS processing performed by Genc Basha. Cryogenic transmission electron microscopy of the nanoparticles was done by Jayesh Kulkarni. Synthesis and radiolabeling of C1 and related compounds were done by Dr. Gang Chen and Dr. Marion Thevanin. The in vivo experiments were designed by me and performed with assistance from Dr. Genc Basha, Dr. Ying Tam and Ms. Yan Liu. All the in vivo experiments were performed at the University of British Columbia with the exception of the C1-LNP pharmacokinetic-biodistribution study (Chapter 2), which was done at Simon Fraser University animal facility and in collaboration with Dr. Robert Young’s group. All the animal work and procedures in this thesis have been approved by the Animal Care Committee at The University of British Columbia and Simon Fraser University and were performed in accordance with guidelines established by the Canadian Council on Animal Care. I was responsible for formulation and analysis of all the LNP discussed in this dissertation, and Nisha Chander assisted with formulation and analysis of siRNA-LNP for the pharmacokinetic study in Chapter 3.

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List of Abbreviations

A4pA       P1,P4-Di(adenosine-5’) tetraphosphate
A5pA       P1,P5-Di(adenosine-5’) pentaphosphate
ADP        Adenosine diphosphate
AMP        Adenosine monophosphate
ApoB       Apolipoprotein B
ApoE       Apolipoprotein E
ATP        Adenosine triphosphate
BP         Bisphosphonate
cDNA       Complementary DNA
CHE        Cholesteryl hexadecylether
Chol       Cholesterol
CRISPR     Clustered regularly interspaced short palindromic repeats
Cryo-TEM   Cryogenic transmission electron microscopy
Dex-P      Dexamethasone-21-phosphate
Dex-Phosphate  Dexamethasone-21-phosphate
DiI        1,1’-dilinoleyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate
DLin-KC2-DMA 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane
(6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl4-
DLin-MC3-DMA (dimethylamino)butanoate (or dilinoleylmethyl-4-
dimethylaminobutyrate)
DLS        Dynamic light scattering
DMEM  Dulbecco's modified eagle medium
DNA   Deoxyribonucleic acid
DODMA N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride
DOPE 1, 2 dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP 1,2-dioleoyl-3-trimethylammonium-propane
DOTMA N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride
DSPC 1,2-distearoyl-sn-glycero-3-phosphocholine
EDTA Ethylenediaminetetraacetic acid
eGFP Enhanced green fluorescent protein
EP4 Prostaglandin E2 receptor 4
EP4-A Prostaglandin E2 receptor 4 agonist
EP4-tri Prostaglandin E2 receptor 4 triglyceride
EPR Enhanced permeability and retention
ESM Egg sphingomyelin
FACS Fluorescence-activated cell sorting
FBS Fetal bovine serum
FDA Food and drug administration
FVII Factor VII
GFP Green fluorescent protein
HEPES 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
HPLC High-performance liquid chromatography
hprt Hypoxanthine-guanine phosphoribosyltransferase
i.v. Intravenous
LNP Lipid nanoparticle
MC3 DLin-MC3-DMA
mRNA Messenger ribonucleic acid
PBS Phosphate buffered saline
Pdi Polydispersity index
PEG Polyethelene glycol
PEG-DMG (R)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate
PEG-DSG (R)-2,3-bis(stearlyloxy)propyl-1-(methoxy poly(ethylene glycol 2000) carbamate
Pen/Strep Penicillin/streptomycin
PGE2 Prostaglandin E receptor 2
pK\textsubscript{a} Acid dissociation constant
Poly-A Poly adenosine oligonucleotide
POPC 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
RBC Red blood cells
RNA Ribonucleic acid
RPMI Roswell Park Memorial Institute
rT-qPCR Real time quantitative polymerase chain reaction
SHM Staggered herringbone mixer
siCtr Control small interfering ribonucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>siGFP</td>
<td>Green fluorescent protein small interfering ribonucleic acid</td>
</tr>
<tr>
<td>siLuc</td>
<td>Luciferase small interfering ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>Tbp</td>
<td>Tata-binding protein</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TT</td>
<td>N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide</td>
</tr>
<tr>
<td>TNT</td>
<td>1,3,5-triazinane-2,4,6-trione</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
</tbody>
</table>
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Chapter 1. Introduction

1.1 Common methods of nanoparticle production

Owing to recent advances in the nanomedicine field, nanoparticles are now well-known as delivery vehicles for a wide range of diverse bioactive agents (Singh, Chakrapani, and O’Hagan 2007; Singh and Nalwa 2011; Tian, Chen, and Chen 2013; Wan, Allen, and Cullis 2014a; Perrie et al. 2016; Allen and Cullis 2012). When encapsulated in nanoparticles, bioactive molecules show enhanced circulation lifetimes, improved efficacy and reduced side effects (Mayer, Bally, and Cullis 1990; Tam, Chen, and Cullis 2013; Allen and Cullis 2012). Traditional methods of nanoparticle production include sonication (Johnson et al. 1971), extrusion (Hope et al. 1985), ethanol injection (Batzri and Korn 1973), rapid solvent exchange (Buboltz and Feigenson 1999), and phase evaporation (Akbarzadeh et al. 2013; Mozafari 2010; Szoka and Papahadjopoulos 1978; Olson et al. 1979). While these methods can be effective for small-scale experiments, they are often associated with problems such as sample contamination, material loss or degradation, and batch-to-batch variability (Garg et al. 2016). With the exception of extrusion, these methods provide little control over particle size and often cannot be easily scaled-up for clinical applications. Moreover, these methods can lead to a heterogeneous nanoparticle population (Quintanar-Guerrero et al. 1998; Bilati, Allémann, and Doelker 2005; Phapal and Sunthar 2013). As the nanomedicine field gains more clinical utility, reliable and scalable methods are required to facilitate the bench-to-clinic transition of newly developed therapeutics.
1.2 Microfluidic methods of manufacturing nanoparticles

Microfluidic formulation technology is an option that can overcome difficulties experienced with other methods of nanoparticle synthesis. This method has been used to generate a wide range of nanoparticles (Van Swaay and Demello 2013; Sun et al. 2013; Xu et al. 2017; Wang et al. 2014; Yang, Bteich, and Li 2017; Karnik et al. 2008; Lim et al. 2014; Min et al. 2014; Jahn et al. 2004, 2007; Pradhan et al. 2008; Laouini et al. 2013; Kulkarni, Cullis, and van der Meel 2018; Tam, Chen, and Cullis 2013). The microfluidic mixing method rapidly and reproducibly mixes two miscible solvents with widely differing polarities (such as water and ethanol) that contain dissolved nanoparticle components, resulting in nanoparticle precipitation as the components in the less polar solvent fall out of solution (Figure 1.1). This nanoprecipitation process builds on an initial nucleation event such as formation of a hydrophobic nanoparticle. Microfluidic mixing leads to reproducible nanoparticle synthesis with robust control over particle size and population homogeneity. Multiple microfluidic devices have been developed for nanoparticle synthesis, amongst which hydrodynamic flow focusing and Y-junction mixing are commonly used methods. Flow focusing utilizes a T-shaped junction consisting of a main channel and two perpendicular channels arranged as a 4-way junction. In flow focusing, the organic phase is injected into the main channel and the aqueous phase is injected into the two perpendicular channels. Alternatively, in the Y-junction mixing, two streams of organic and aqueous solvents are mixed and diffused through a Y or T-shaped junction (Jahn et al. 2004). This dissertation focuses on a Y-junction microfluidic mixer known as staggered herringbone micromixer (SHM) (Figure 1.1). SHM microfluidic mixing has been successfully used for development of a wide range of nanoparticles including lipid-based nanoparticles that entrap small molecule drugs, nucleic acids, proteins and peptides that can be
used for different therapeutic purposes such as treatment of cancer, microbial infections and vaccines (Perrie et al. 2016; Singh and Nalwa 2011; Wan, Allen, and Cullis 2014b; Tian, Chen, and Chen 2013).

Nanoparticles formulated using the SHM microfluidic mixing technique are generated by the rapid mixing of two streams consisting of an organic solvent (containing the hydrophobic nanoparticle forming materials) and an aqueous solvent (containing the hydrophilic nanoparticle forming materials) (Belliveau et al. 2012). The mixing of the two streams leads to formation of hydrophobic nucleating intermediates and nanoparticle formation (Belliveau et al. 2012; Leung et al. 2012). The ratio of aqueous/organic streams and the total rate at which the streams are mixed can be adjusted, allowing for a flexible formulation process and precise control over particle size and polydispersity. This microfluidic technology has been used to develop several different types of nanoparticles including lipid nanoparticles (LNP), and polymeric nanoparticles.

1.3 Design of lipid nanoparticles for drug delivery

Many different biomolecules can be delivered using LNPs. Initially used as delivery vehicles for small molecule drugs (Bangham and Horne 1964; Bangham, Standish, and Watkins 1965), LNPs have been developed that are now capable of delivering a wide range of biomolecules including siRNA, mRNA and plasmid DNA. LNP manufacturing by microfluidic mixing has been demonstrated for different payloads as discussed below. A list of select FDA-approved nanoparticle therapeutics can be found in Table 1.1.
Figure 1.1. Schematic of microfluidic mixing for formation of lipid-based nanoparticles.

Microfluidic mixing allows for generation of nanoparticles by rapid mixing of formulation components through a microfluidic chamber. The example shown in this figure is a staggered herringbone micromixer (SHM) which employs a Y-junction followed by a serpentine microfluidic channel which facilitates laminar flow and repeated folding of solvents as they pass over the herringbone vanes. This method is easily scalable by utilizing a multi-mixer device where the SHM devices are placed in parallel and can provide precise and reproducible mixing parameters to eliminate batch-to-batch variation as well as providing full control over particle size and polydispersity.
Table 1.1. FDA-approved liposomal/LNP drugs

<table>
<thead>
<tr>
<th>Name</th>
<th>Formulation type</th>
<th>Indications</th>
<th>Year Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diprivan</td>
<td>Liposomal (emulsion) Propofol</td>
<td>Anesthesia</td>
<td>1989</td>
</tr>
<tr>
<td>Abelcet</td>
<td>Liposomal Amphotericin B lipid complex</td>
<td>Fungal infections</td>
<td>1995</td>
</tr>
<tr>
<td>Doxil</td>
<td>Liposomal doxorubicin</td>
<td>Karposi’s Sarcoma; Ovarian cancer; multiple myeloma</td>
<td>1995 2005 2008</td>
</tr>
<tr>
<td>DaunoXome</td>
<td>Liposomal Daunorubicin</td>
<td>Karposi’s Sarcoma</td>
<td>1996</td>
</tr>
<tr>
<td>AmBisome</td>
<td>Liposomal Amphotericin B</td>
<td>Fungal/protozoal infections</td>
<td>1997</td>
</tr>
<tr>
<td>Lipovert</td>
<td>Liposomal Verteporfin</td>
<td>Macular degeneration, myopia; ocular histoplasmosis</td>
<td>2012</td>
</tr>
<tr>
<td>Marqibo</td>
<td>Liposomal Vincristine</td>
<td>Acute Lymphoblastic Leukemia</td>
<td>2012</td>
</tr>
<tr>
<td>Onpattro</td>
<td>siRNA-LNP</td>
<td>Hereditary transthyretin-mediated amyloidosis</td>
<td>2018</td>
</tr>
</tbody>
</table>
1.3.1 Lipid nanoparticles for delivery of nucleic acids

Gene therapy has great clinical potential. Genetic drugs such as siRNA, mRNA and plasmids can enable in vivo gene silencing, gene expression or gene editing and can potentially treat a large number of diseases. However, in their native forms, nucleic acid polymers are unstable in biological fluids and cannot penetrate into the cytoplasm of target cells, thus requiring delivery systems. LNP formulations are among the most advanced non-viral delivery systems. Encapsulation of the genetic drug in LNP occurs through electrostatic interactions between the negatively charged nucleic acid and either a permanently charged cationic lipid or an ionizable cationic lipid. Generally, formulations containing permanently cationic lipids result in LNPs with positive surface charge and are toxic in vivo (Lv et al. 2006). A largely positive surface charge on LNPs also leads to accumulation in macrophages (Chonn, Semple, and Cullis 1992) and subsequently rapid clearance from the circulatory system. Ionizable cationic lipids, specifically those with pKₐ < 7, can be used to achieve efficient encapsulation of nucleic acid polymers at low pH but result in largely neutral systems at physiological pH values and thus alleviate problems associated with permanently-charged cationic lipids.

LNP formulations of genetic drugs using ionizable lipids can be generated by dissolving lipids in an organic solvent (usually ethanol), which is then mixed through the micromixer with the nucleic acid dissolved in an acidic buffer (usually pH 4). At this pH the ionizable cationic lipid is positively charged and interacts with the negatively-charged nucleic acid polymers. The nanostructures containing the genetic drugs are then converted to neutral LNPs when dialyzed against a pH 7.4 buffer during the ethanol removal step. LNPs containing ionizable cationic lipids and encapsulated RNA or DNA have a distinct electron-dense nanostructured core (Figure 1.2) as opposed to the traditional bilayer liposomal structures (Leung et al. 2012; Kulkarni et al.)
A more recent study has suggested that the inverted micelle solid core structure is perhaps not the most accurate, proposing a siRNA/lipid sandwich model with the excess cationic lipid forming oil-droplets in conjugation with cholesterol (Kulkarni et al. 2018).

Figure 1.2. Computational simulation of a lipid nanoparticle (LNP) structure.

Nucleic acids are trapped in water cavities that are formed inside lipid membranes. (A) Side view, (B, C) Cross-section, and (D) the zoomed-in view of the cationic lipid nanoparticle.

Adapted with permission from https://pubs.acs.org/doi/abs/10.1021/jp303267y (Leung et al. 2016).
The size and potency of LNP formulations of genetic drugs are sensitive to the lipid components employed. The lipid composition usually consists of an ionizable amino lipid, a helper lipid (usually a phospholipid), cholesterol, and a polyethylene glycol-lipid conjugate (PEG-lipid) at a typical mole ratio of 50/10/38.5/1.5. PEG-lipid can be varied from ~1 to 5 mol% to modify particle properties such as size, stability and circulation time. A list of common ionizable cationic lipid structures and helper lipids is shown in Figure 1.3. The cationic lipid plays a crucial role both in nucleic acid encapsulation through electrostatic interactions and intracellular release by disrupting endosomal membranes (Akinc et al. 2010). Cholesterol and the helper lipid have structural roles that are not well-defined and could relate to binding of apolipoproteins, however the absence of the helper lipid can severely compromise activity (Semple et al. 2010; Cheng and Lee 2016). The PEG-lipid forms the surface lipid; the size of the LNP can be readily varied by varying the proportion of surface (PEG) lipid to the core (ionizable cationic) lipids. LNP siRNA systems for silencing genes in the liver use a PEG-lipid with short (C_{14}) acyl chains, this form of PEG-lipid dissociates rapidly from the LNP after administration in vivo (Mui et al. 2013), facilitating adsorption of apolipoprotein E (ApoE) onto the surface of the LNP (Cullis and Hope 2017).
Figure 1.3. Structures of lipids that are commonly used in formulation of lipid nanoparticles.

DLin-MC3-DMA (heptatriaconta-6,9,28,31-tetraen-19-yl4-(dimethylamino)butanoate), DLin-KC2-DMA (2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane), TNT (1,3,5-triazinane-
2,4,6-trione) and TT (N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide) are used as cationic lipids. DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), POPC (2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine) and DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) are used as helper lipids, and cholesterol and PEG-DMG ((R)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate) play roles in stability, circulation and size of LNP. R represents amino lipid chains from C8 to C14.

The presence of ApoE on the LNP surface leads to uptake by the LDL and scavenging receptors found on numerous cell types (Akinc et al. 2010) and subsequent internalization via endocytosis. In the low pH endosomal environment, the ionizable lipid contained in the LNP becomes protonated, cationic (positively charged) and is hypothesized to interact with endogenous anionic lipids in the endosomal compartment, inducing formation of “non-bilayer” membrane disruptive structures leading to disruption of the endosomal bilayer and intracellular release of the genetic drug cargo (Figure 1.4) (Hafez, Maurer, and Cullis 2001). Formulations employing ionizable cationic lipids are preferred over those with permanently charged cationic lipids as they are generally both more potent and better tolerated.

In order to deliver LNP to sites other than the liver, such as distal tumor sites, PEG-lipids that form a more long-lived coat must be employed. PEG-lipids with C_{18} acyl chains exhibit LNP residence times of days or longer and can result in long LNP circulation times (Mui et al. 2013; Chen et al. 2016) and thus tumor delivery. Long-circulating LNP accumulate in tumors through the enhanced penetration and retention (EPR) effect due to the leaky vasculature found in tumors, leading to enhanced tissue accumulation (Maeda 2012).
Figure 1.4. A simplified overview of nucleic acid-LNP cellular uptake.

Following systemic administration and blood circulation, (A) the polyethylene glycol (PEG)-lipid anchor of the LNP diffuses, leaving the LNP ready for cellular uptake (Ambegia et al. 2005). (B, C) The LNP is then engulfed and taken up by an endosome. (D) The acidic endosome renders the ionizable lipids strongly positively charged which in turn disrupts the LNP structure. This leads to rupture of LNP and release of cargo into the cytoplasm.

1.3.1.1 siRNA Delivery by LNPs

LNP systems for the delivery of siRNA to silence target genes in vivo are one of the most advanced nanoparticle formulations of genetic drugs. Following the demonstration that antisense oligonucleotides could be encapsulated in LNP systems in the presence of the ionizable cationic
lipid DODAP (Semple et al. 2001; Maurer et al. 2001), the potential application for delivery of siRNA was investigated in a gene silencing study (targeting the apolipoprotein B (ApoB) gene in hepatocytes) demonstrating that in non-human primates, LNP siRNA systems containing the ionizable lipid DLinDMA (dilinoleoyl-DMA) (Zimmermann et al. 2006) could enable effective gene silencing. Subsequent studies have shown the ionizable cationic lipid assists with endosomal release (Belliveau et al. 2012; Hafez, Maurer, and Cullis 2001; Hafez and Cullis 2001; Koltover et al. 1998), leading to extensive lipid synthesis efforts to identify the most potent and least toxic ionizable lipids for siRNA delivery. These studies culminated in the identification of DLin-MC3-DMA (heptatriaconta-6,9,28,31-tetraen-19-yl4-(dimethylamino)butanoate) as highly potent for hepatic gene silencing (Jayaraman et al. 2012). A typical LNP siRNA formulation consists of DLin-MC3-DMA/DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine)/cholesterol/PEG-lipid at a mole ratio of 50/10/38.5/1.5 (mole ratio). Particle potency not only depends on the ionizable amino lipid (Jayaraman et al. 2012; Basha et al. 2011), but also on the PEG-lipid coating. High PEGylation leads to smaller and less potent particles; this effect is also seen for longer-chain PEG-lipid molecules (Mui et al. 2013; Chen et al. 2016). It is likely that the presence of high levels of PEG-lipid on the LNP surface reduces LNP interaction with target cells and ApoE association (Cullis and Hope 2017).

LNP siRNA systems with maximum in vivo gene silencing potency in hepatocytes have an excess of cationic lipid as compared to siRNA, N/P charge ratios of (protonated) cationic amino (N) lipid to oligonucleotide phosphate (P) values of three or higher are commonly employed. The siRNA is dissolved in an aqueous buffer at low pH (pH=4-5) and is mixed 3:1 (v/v) with the lipids dissolved in an organic solvent (usually ethanol).
While LNP siRNA systems containing DLin-MC3-DMA are highly effective agents for silencing genes in the liver (hepatocytes), following intravenous (i.v.) administration (ED$_{50}$<10µg siRNA/kg body weight) such systems are considerably less effective for gene silencing in other tissues, even though most cells exhibit receptors for ApoE (Cullis and Hope 2017). LNP siRNA systems have been administered systemically to target human cancer cells such as leukemic cells (Jyotsana et al. 2015, 2017), malignant B-cells (Weinstein et al. 2016), glioblastomas (Cohen et al. 2015) and prostate cancer (Lee et al. 2012, 2016; Yamamoto et al. 2015) as well as murine bone osteocytes (Basha et al. 2016) and macrophages (Basha et al. 2011; Lin et al. 2013), however ED$_{50}$ values are well in excess of 1 mg siRNA/kg body weight. While the LNP systems described here are not capable of crossing the blood-brain barrier, when injected intracortically, particle uptake and gene silencing can be observed in brain neurons (Rungta et al. 2013, 2015). In addition to neurons, LNP siRNA systems have also been used for treatment of intracerebral glioma in mice through immune cell modulation with the formulation also tested on a canine model for assessment of toxicity and immunogenicity (Yaghi et al. 2017). LNP siRNA are usually administered intravenously (Chen et al. 2016; Zhigaltsev et al. 2012), however, with some modifications to the formulation, subcutaneous administration can also lead to efficacious gene silencing in the liver (S. Chen et al. 2014). Moreover, several LNP siRNA formulations using the rapid mixing formulation techniques are currently undergoing clinical development (Ragelle et al. 2017); for instance, Alnylam Pharmaceuticals (Boston, USA) and Sanofi (Gentilly, France) have recently successfully completed Phase 3 clinical trials for Patisiran which is an LNP siRNA formulation to treat amyloidogenic transthyretin (ATTR) amyloidosis by silencing the TTR gene in hepatocytes. Patisiran (trade name ONPATTRO) is the
first US FDA-approved non-viral gene therapy drug administered systemically and validates the clinical potential of the LNP delivery approach for enabling genetic drugs.

### 1.3.1.2 mRNA Delivery by LNPs

The ability to express proteins *in vivo* using mRNA delivery has led to exciting opportunities in the protein replacement therapy and vaccine development fields. The microfluidic formulation method principles described earlier can be used to encapsulate larger nucleic acid polymers such as mRNA and plasmids. LNPs can shield the readily degradable mRNA molecules from nucleases and facilitate intracellular delivery and protein expression, particularly in the liver. Advantages of LNP delivery as compared to other vectors such as viruses include ease of manufacture and, importantly, the lack of immunogenicity which inhibits repeat dosing of viral vectors (Minor 2015; Sahin, Karikó, and Türeci 2014; Weissman 2015).

Additionally, therapeutics based on mRNA have advantages of transitory gene expression and lack of integration into host genome and correspondingly low risk of mutagenesis and potential oncogenesis (Pardi et al. 2017). LNP mRNA systems appear to have significant potential as vaccines where the packaged mRNA codes for antigens such as viral proteins. The non-replicating modified LNP mRNA vaccine against the Zika virus (ZIKV), developed by Richner *et al* is an example of a microfluidic-manufactured LNP mRNA vaccine (Richner, Himansu, et al. 2017; Richner, Jagger, et al. 2017). ZIKV has recently become a global concern due to its rapid spread and severe health consequences for those infected. In brief, ZIKV has been associated with congenital birth defects such as microcephaly and miscarriage (Brasil et al. 2016; van der Eijk et al. 2016; Rasmussen et al. 2016) and is in some cases linked to an autoimmune disorder known as Guillain-Barre’ syndrome in adults (Oehler et al. 2014; Cao-
Lormeau et al. 2016). Using the microfluidic-formulated LNPs with a proprietary cationic lipid and modified mRNA sequences, Richner et al. have developed a versatile vaccine platform that can induce antibody response with minimal antibody cross-reactivity (Richner, Himansu, et al. 2017). Antibody cross-reactivity is a side effect that can lead to lethal shock syndrome and, in the case of ZIKV, this phenomenon occurs when the ZIKV vaccine recipient is exposed to a virus with a similar sequence such as dengue virus (Dejnirattisai et al. 2016; Stettler et al. 2016; Sapparapu et al. 2016). The vaccine platform developed by Richner et al. allows for safer alternatives that do not pose the risk of antibody cross-reactivity. Furthermore, this vaccine induced high levels of viral structural proteins upon intramuscular injection in mice and provided immunity in ZIKV-challenged groups. The ZIKV LNP mRNA formulation was also tested in pregnant mice and shown to prevent fetal infection in the majority of fetuses (Richner, Jagger, et al. 2017).

A related LNP mRNA vaccine formulated against H10N8 and H7N9 influenza viruses, has recently advanced to phase 1 clinical trials and represents the first published nucleic acid vaccine used in humans without electroporation (Bahl et al. 2017). H7N9 and H10N8 are avian influenza virus A subtypes that have been reported to infect humans with a high fatality rate (Gao et al. 2013; Wei et al. 2013; Wei et al. 2014; H. Chen et al. 2014; WHO 2017). The LNP mRNA influenza vaccines against these subtypes were administered intramuscularly or intradermally and generated a robust immune response. H10N8 or H7N9 vaccines were tested in mice, ferrets and cynomolgus monkeys where high titers of neutralizing antibodies were observed (Bahl et al. 2017). More importantly, the LNP mRNA vaccine against H10N8 was administered to humans with minor adverse reactions and induced a strong immune response (Bahl et al. 2017) suggesting that LNP mRNA vaccines can be an effective platform for rapidly
emerging infectious diseases. Other immunomodulatory LNP mRNA applications are also being explored for immunotherapy for treatment of cancers such as leukemia (Oberli et al. 2017).

In addition to vaccine development for infectious diseases, the LNP mRNA platform is employed for conditions where a mutation leads to dysfunctional or reduced levels of protein. Gene replacement therapy for example has been successfully used for Friedreich’s Ataxia, by intrathecal injection of Frataxin mRNA in mice (Nabhan et al. 2016). Hemophilia B as another gene replacement therapy candidate, occurs due to a mutation that renders coagulation factor IX (FIX) dysfunctional, leading to clotting defects. Treatment for this condition is limited to use of recombinant or serum purified FIX both of which are costly and associated with complications such as anaphylactic shock, desensitization to treatment and short lived effects of treatment (Srivastava et al. 2013; Peyvandi, Garagiola, and Young 2016; Mancuso et al. 2009). The LNP mRNA system developed by Ramaswamy et al. was found to express human FIX in mice and could alleviate hemophilia B clotting defects (Ramaswamy et al. 2017). The systemic administration of the LNP mRNA in the FIX knock-out mouse model indicated blood FIX levels that significantly exceeded the acceptable clinical levels with the effects lasting 3 days longer than the conventional treatment (Ramaswamy et al. 2017). Additionally, it was found that the proprietary ionizable lipid, provided by Arcturus Therapeutics, was up to two-fold superior to its counterpart DLin-MC3-DMA (Ramaswamy et al. 2017) which is the gold standard for siRNA delivery (Jayaraman et al. 2012). This finding suggests that depending on the nature and size of the nucleic acid, some changes to the standard LNP siRNA formulation might be required. Studies have shown that LNP siRNA lipid composition as well as formulation parameters need to be altered for optimal mRNA (Li et al. 2015, 2016; Luo et al. 2017) and plasmid DNA (Kulkarni et al. 2016) delivery.
To this effect, a group from Ohio State University has done extensive screening to optimize their LNP mRNA formulations for *in vivo* delivery (Li et al. 2015, 2016; Luo et al. 2017). This group synthesized seven variants of N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide (TT) with different length amino lipid chains (TT2-8) to be used as the cationic lipid for LNP mRNAs. Different helper lipids were also tested and based on particle size, encapsulation efficiency, cytotoxicity and *in vitro* knockdown, DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) was found to be superior to the commonly used DSPC helper lipid (Li et al. 2015, 2016). Orthogonal optimization and varying the LNP lipid ratios led to an optimal formulation of TT3/DOPE/Cholesterol/PEG-DMG ((R)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate) of 20/30/40/0.75 mole ratio which was significantly more effective than their original formulation of TT3/DSPC/Cholesterol/PEG-DMG (50/10/38.5/1.5) (Li et al. 2015). Not surprisingly, it was found that similar to the LNP siRNA (Mui et al. 2013; S. Chen et al. 2016), in LNP mRNA PEGylation leads to smaller size, increased particle stability and reduced potency of nanoparticles (Li et al. 2015, 2016). TT3-based LNP mRNA have also been used as dual-functioning nanoparticles for delivering an MRI contrast agent, gadolinium (Luo et al. 2017). By including a gadolinium-lipid conjugate, LNP mRNA was optimized and used as a theranostic agent to express mRNA and also enable non-invasive imaging. This formulation is likely the first of a new class of nanoparticle theranostics that are safe and efficacious for clinical purposes.

Further in-depth optimization of cationic lipid for LNP mRNA has suggested that local structural transformation of functional groups in cationic lipids can significantly affect *in vivo* delivery (Li et al. 2016). Various derivatives of 1,3,5-triazinane-2,4,6-trione (TNT) tested as cationic lipid for mRNA delivery indicated that functional group chain length and position
dramatically alters LNP mRNA efficacy (Li et al. 2016). In addition to modifying cationic and helper lipids of LNP mRNA, it has been shown that *in vivo* mRNA delivery can be improved by incorporating boosters that assist with endosomal release and mRNA transcription (Patel et al. 2017). When incorporated into LNPs, leukotriene antagonists, specifically MK-571, have been shown to significantly enhance *in vivo* mRNA delivery and expression (Patel et al. 2017).

### 1.3.1.3 CRISPR Technology and LNPs

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology enables genome editing in a wide range of organisms using specific nucleases that are steered by a guide RNA (gRNA) sequence (Hsu, Lander, and Zhang 2014; Sander and Joung 2014; Doudna and Charpentier 2014). It is currently the most advanced gene therapy tool owing to its ability to mediate gene editing for providing curative treatment of genetic disorders. Unlike viral delivery vehicles, mRNA LNPs do not pose the risk of undesirable genome integration and thus do not risk non-specific genome modification. CRISPR technology utilizing mRNA gene delivery therefore, is thought to impose a lower risk of genome integration. The CRISPR technology combined with LNP formulation for effective *in vivo* genome editing has been recently through screening of a library of biodegradable amino-ester lipids for effective Cas9 mRNA delivery to eGFP- and the corresponding (gRNA)-expressing tumor xenograft-mice (Zhang et al. 2017). The results indicated decreased eGFP expression in tumors without any significant hepatotoxicity. Moreover, Cas 9 mRNA and gRNA can be co-encapsulated to achieve a more efficacious *in vivo* gene editing tool. Forming a ribonucleoprotein complex, Cas9 and gRNA have been simultaneously loaded in the same LNP formulation (Miller et al. 2017). Through lipid screening and optimization, the Cas9/gRNA-containing nanoparticle formulation was able to activate a
reporter molecule upon administration in a transgenic mouse model that contained a homozygous LoxP-flanked Stop cassette upstream of a tdTomato reporter. The two cleavages needed to delete the Stop cassette and allow the reporter to be expressed were performed successfully using the Cas9/sRNA-LNP (Miller et al. 2017). These findings demonstrate the potential for microfluidic production of LNP mRNAs to simplify the development CRISPR/Cas9 treatments that have the potential for long-lasting and curative effects for genetic disorders.

1.3.1.4 Plasmid Delivery by LNPs

In addition to mRNA and siRNA, plasmids are another potential means of gene therapy. Like mRNA, plasmids can be utilized to express genes of interest and thus compensate for protein loss-of-function or to introduce new genes into the system. Plasmids are advantageous over viral vectors since they are easier to engineer and modify, usually less immunogenic and pose a lower risk of integration into host genome. However, due to plasmid DNA’s inability to cross the nuclear membrane (MacLachlan, Cullis, and Graham 1999; Hu et al. 2013; Miller and Dean 2009; Vaughan, DeGiulio, and Dean 2006) and its large size, plasmid encapsulation and in vivo delivery can be more challenging than siRNA and mRNA (Kulkarni et al. 2016). The siRNA and mRNA LNP formulations are optimized for cellular uptake and endosomal release but not nuclear delivery (MacLachlan, Cullis, and Graham 1999) and are thus only potent in actively dividing cells (Tseng, Haselton, and Giorgio 1997). Not surprisingly, the LNP lipid composition used for delivery of siRNA and, in some cases, mRNA is not optimal for plasmid DNA delivery. Nucleic acid delivery by LNP typically requires lipids that are highly effective in promoting non-bilayer (HII) lipid structures and thus induce membrane fusion and endosomal escape (Semple et al. 2010; Jayaraman et al. 2012; Hafez, Maurer, and Cullis 2001).
An optimized LNP system for plasmid delivery has been achieved by modifying the well-established LNP siRNA system and replacing the DSPC, a saturated helper lipid, with DOPE (an unsaturated variety), and DLin-MC3-DMA cationic lipid with DLin-KC2-DMA (2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane). Both changes improved plasmid DNA translation efficiency likely due to their superiority in promoting structures that enhance membrane fusion and intracellular delivery. Moreover, the plasmid-LNPs exhibit electron dense core structures (Kulkarni et al. 2016) similar to that observed for LNP siRNA (Leung et al. 2012). Successful plasmid transfection in vivo (in chick embryo) indicates that plasmid-LNP can potentially provide a flexible and efficacious tool for genome-editing in chick embryo, an important step toward inserting exogenous genes in therapeutic applications.

1.3.2 LNP for delivery of small molecule drugs

In contrast to LNP systems employed for genetic drugs such as siRNA and mRNA, LNP systems for delivery of small molecule drugs are usually bilayer systems where the drug to be delivered is sequestered in the interior of the LNP. Such LNP systems, usually referred to as liposomes, have been used for delivery of small molecule drugs for many years (Allen and Cullis 2013). The microfluidic formulation method overcomes certain limitations of previous methods used to generate liposomes (Jahn et al. 2004, 2007; Pradhan et al. 2008; Laouini et al. 2013). A particular advantage is the ability to generate “limit size” nanoparticles in a straightforward and scalable manner. Limit size lipid nanoparticles — defined as the “smallest achievable aggregates compatible with the packing of the molecular constituents in a defined and energetically stable structure” (Zhigaltsev et al. 2012) — are smaller than can be generated using techniques such as extrusion and could be more effective for penetrating tumors, lymphatic tissue and bone marrow.
The SHM microfluidic method has been used to generate limit size LNPs composed of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) only and POPC/triolein LNPs (20 nm diameter), as well as POPC/cholesterol LNPs (40 nm diameter) (Zhigaltsev et al. 2012). As observed by cryo-TEM, the POPC or POPC/cholesterol LNPs exhibited a single bilayer surrounding an aqueous core (liposomal structure).

Limit size POPC-LNP were used to encapsulate doxorubicin (an anticancer drug), using the pH gradient loading method (Mayer, Bally, and Cullis 1986; Cullis et al. 1997a; Li et al. 1998) and even though the very small size of limit size LNP can potentially restrict efficient drug loading, 100% of doxorubicin was encapsulated at drug-to-lipid ratios of 0.15 (w/w) or lower with longer than 8-week retention time (Zhigaltsev et al. 2012). This encapsulation efficiency is comparable with the commercial doxorubicin formulation of 0.125 drug-to-lipid (w/w). The system can be even further optimized by addition of saturated phospholipids to achieve longer circulating particles for \textit{in vivo} applications (Zhigaltsev et al. 2015) indicating that the limit size particles can be therapeutically practical systems.

The POPC/triolein LNP formed using microfluidic mixing resulted in limit size systems with a nonpolar lipid core surrounded by a POPC monolayer. The POPC/triolein lipid core system described by Zhigaltzev \textit{et al.} demonstrates the ability of microfluidic mixing to produce such emulsions at a size range significantly smaller than previously possible (Zhigaltsev et al. 2012). Other microfluidic devices were reported to generate lipid droplets in the micrometer size range (Thorsen et al. 2001; Nisisako, Torii, and Higuchi 2002; Sugiura, Nakajima, and Seki 2002; Anna, Bontoux, and Stone 2003; Tan et al. 2004; Tice, Ismagilov, and Zheng 2004) and emulsions made by sonication and other methods have only achieved particle sizes of 50 nm or larger (Handa, Saito, and Miyajima 1990; Lundberg 1994; Liu and Liu 1995; Sonnevile-
Aubrun, Simonnet, and L’Alloret 2004; Tadros et al. 2004; Constantinides, Chaubal, and Shorr 2008; Shah, Bhalodia, and Shelat 2010). The limit size emulsion LNP can potentially be used for encapsulation of highly hydrophobic drugs by incorporating the lipophilic molecules in the LNP lipid core.

The preferred method for loading small molecule drugs into liposomal systems is to load weak base drugs (or weak base pro-drug derivatives) into the interior aqueous space using transmembrane pH gradients, interior acidic (Mayer, Bally, and Cullis 1986; Cullis et al. 1997b; Li et al. 1998) (Figure 1.5). This procedure offers excellent drug loading capacities and drug retention properties. However, in many cases formulations are required for hydrophobic drugs that are not weak bases and that have limited aqueous solubility. These low-solubility drugs are often challenging to formulate for systemic administration and can have poor bioavailability (Williams III, Watts, and Miller 2011; Savjani, Gajjar, and Savjani 2012). Such drugs can benefit from LNP entrapment which leads to enhanced bioavailability and solubilization in aqueous media (Kastner et al. 2015). Using a rapid one-step microfluidic process, many hydrophobic molecules can be encapsulated in LNP (Kastner et al. 2015).

When encapsulated, hydrophobic drugs can associate with the LNP lipophilic component, the lipid bilayer in liposomes (Kulkarni, Betageri, and Singh 1995) and the lipid core in emulsions. For example, Propofol is a drug molecule with low solubility that has been successfully entrapped using microfluidic mixing at a clinical drug-to-lipid ratio range and with superior encapsulation efficiency compared to sonication (Kastner et al. 2015); the superior encapsulation efficiency can be explained by highly efficient microfluidic mixing and thus, more efficacious incorporation of the drug in the bilayer (Altomare et al. 2003). Other hydrophobic molecules including Atenolol have also been successfully encapsulated in LNP using this

The one-step microfluidic LNP formulation has also been applied for encapsulation of the hydrophilic molecule quinine (Guimarães Sá Correia et al. 2017); and interestingly it can also be used to simultaneously entrap hydrophilic and hydrophobic molecules in the same LNP (Joshi et al. 2016). For instance, metformin (hydrophilic) and glipizide (lipophilic) are two drugs used in combination for treatment of diabetes type 2; and their dual incorporation can facilitate co-treatment which is otherwise challenging. The two drugs can be encapsulated into LNP during a one-step process. Surprisingly, double-loading does not negatively affect metformin and glipizide’s individual encapsulation efficiencies but increases their \textit{in vitro} release rates (Joshi et al. 2016). Metformin and glipizide simultaneous encapsulation is the first report of a hydrophilic and lipophilic drug dual-loading on a scalable system (Joshi et al. 2016) and opens up new opportunities where co-treatment with two or more drugs can be beneficial. Combined with high encapsulation efficiencies, tunable particle size, narrow population dispersity, stability and drug release profiles of small molecule drug-LNPs (Leung et al. 2012; Zhigaltsev et al. 2015; Guimarães Sá Correia et al. 2017; Kastner et al. 2015; Joshi et al. 2016) qualify them for potential clinical applications.
Figure 1.5. A schematic of pH gradient-induced drug loading.

The pH gradient loading method works best for nanoparticles with a bilayer membrane and aqueous core, known traditionally as liposomes. To establish the proton (pH) gradient, liposomes are formulated in a neutral pH buffer and dialyzed against a basic buffer (A). The liposomes are then incubated with desired drug while maintaining the gradient (B) and the mixture is loaded on a gel filtration column (C). The free drug molecules are separated by size exclusion leaving the liposomes with the entrapped cargo in the final solution (D).
1.4 Thesis Objective

Lipid nanoparticle (LNP) systems are showing considerable promise as effective delivery systems to enable gene therapies employing short interfering RNA (siRNA) (Zimmermann et al. 2006; Semple et al. 2010). The LNP siRNA systems employ ionizable cationic lipids and a rapid microfluidic mixing procedure and exhibit a “solid core” structure (Belliveau et al. 2012; Leung et al. 2012) where the siRNA is encapsulated in inverted micelles in the hydrophobic interior. This process, which results in effectively complete encapsulation of the negatively charged polymer, has been extended to larger nucleic acid polymers such as mRNA and plasmids (Kauffman et al. 2015; Pardi et al. 2017; Kulkarni et al. 2016; Ramaswamy et al. 2017). It is of interest to examine whether the same process can be applied to smaller negatively charged molecules as LNP encapsulation and delivery can result in decreased toxicity, enhanced stability and, in some cases enhanced efficacy for small molecule drugs. The results of this examination can be useful for encapsulation of small negatively charged molecule drugs (weak acids) that do not readily precipitate and are more challenging to encapsulate (Zhigaltsev et al. 2010) compared to weak bases which can be efficiently entrapped using the pH gradient remote loading technique (Deamer, Prince, and Crofts 1972; Mayer, Bally, and Cullis 1986; Madden et al. 1990). It is also of interest to determine the limitations of the cationic-lipid dependent loading technique for oligonucleotides smaller than siRNA.

In the work presented in Chapter 2, the cationic-lipid dependent loading of oligomers as small as a single stranded 5-mer was characterized, as well as smaller molecules containing between one to five phosphate groups. It is shown that if permanently positively charged cationic lipids are employed, highly efficient encapsulation and retention can be achieved for molecules as small as adenosine monophosphate. However, permanently charged cationic lipids are known
to be fairly unstable and toxic \textit{in vivo} (Lv et al. 2006) and thus, are not ideal for systemic
delivery. Alternatively, if ionizable cationic lipids are used, which have advantages of enhancing
intracellular delivery and reducing toxicity, efficient encapsulation and retention is only observed
for single stranded oligonucleotides of 7-mer or larger. Appropriate mixtures of permanently
positively charged cationic lipids and ionizable cationic lipids can result in improved
encapsulation for smaller negatively charged molecules. These techniques were applied to a
small molecule EP4 agonist that promotes bone growth and can be potentially used for treatment
of osteoporosis (Arns et al. 2012; Liu et al. 2015; Chen, Arns, and Young 2015). It is shown that
an EP4-bisphosphonate prodrug can be efficiently loaded in LNP systems. The bisphosphonate
prodrug strategy can potentially be used for treatment of other bone conditions and the overall
encapsulation platform can be applied for other hard-to-encapsulate small molecule drugs. In
conclusion, the results included in Chapter 2 provide new insight into encapsulation of small
cargo with negative charge, explore the molecular charge density impact on loading efficiency,
and provides information to improve LNP design.

While Chapter 2 mostly explores the impact of type and quantity of cationic lipid on
encapsulation of negatively charged molecules, in Chapter 3 the impact of helper lipid type and
quantity on LNP was explored. siRNA-LNP system was chosen for this purpose due to its robust
efficacy readout assays and the massive body of literature exploring this well-established
delivery system. Traditionally, the siRNA-LNP systems are made with about 10\% of DSPC
(molar ratio to the total lipid content of the LNP). Previous studies have shown that lack of
helper lipids can obliterate siRNA-LNP activity (Semple et al. 2010; Cheng and Lee 2016). In
Chapter 3 a more in-depth look was taken into the role of DSPC on siRNA-LNP gene silencing
efficacy both \textit{in vitro} and \textit{in vivo}. DSPC was titrated and varied to other potential helper lipids
and the effect was assessed on particle uptake and gene silencing on different cells and also on \textit{in vivo} hepatic gene silencing following systemic administration in mice. Finally, the pharmacokinetics-biodistribution profile of high DSPC content LNP was compared with the benchmark siRNA-LNP formulation. The findings presented in Chapter 3 can lead to better understanding of how the helper lipid content of LNP can impact its efficacy both \textit{in vitro} and \textit{in vivo}.
Chapter 2: Influence of Cargo Molecular Size and Negative Charge on Encapsulation in LNP Systems Containing Cationic Lipids

2.1 Synopsis

LNPs have been used to deliver bioactive molecule drugs for many years (Table 1.1). The majority of small molecule drugs encapsulated using LNPs, however, are loaded either via the pH gradient loading method as weak bases (Fenske et al. 1998; Haran et al. 1993; Madden et al. 1990; Mayer, Bally, and Cullis 1986) or incorporation in the lipid bilayer as lipophilic molecules (Kastner et al. 2015; Joshi et al. 2016). Larger nucleic acids (siRNA, mRNA, plasmids) are encapsulated using an entirely different approach; they are trapped within the LNP by the ionic interactions they form with the positively charged cationic (or ionizable) lipid. Here, it is shown that the same principle—ionic interaction-based encapsulation—can be applied to non-nucleic acid small molecules and smaller nucleic acid molecules. Table 2.1 lists molecules of different charges and sizes encapsulated here using this approach. The results suggest that the type of cationic/ionizable lipid affects encapsulation efficiency. Moreover, cargo molecule size and charge greatly impacts encapsulation. In brief, larger molecules, or those with a higher negative charge can be more readily entrapped in cationic/ionizable LNPs.

2.2 Materials and methods

2.2.1 Materials

1,2-dioleoyloxy-N,N-dimethyl-3-aminopropane (DODMA) was obtained from CordenPharma International (Germany). 1,2-dioleoyloxy-N,N,N-trimethyl-3-aminopropane (DOTMA), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids (Alabaster, AL).
(R)-2,3-bis(stearyloxy)propyl-1-(methoxy poly(ethylene glycol)2000) carbamate (PEG-DMG) and DLin-MC3-DMA were purchased from Biofine International Inc (Vancouver, BC). Cholesterol, HEPES free acid, acetic acid, and sodium hydroxide were from Sigma-Aldrich (St. Louis, MO). Adenosine 5’-monophosphate sodium salt (AMP), Adenosine 5’-diphosphate sodium salt (ADP), Adenosine 5’-triphosphate disodium salt hydrate (ATP), P1,P4-Di(adenosine-5’) tetraphosphate ammonium salt (Ap4A), P1,P5-Di(adenosine-5’) pentaphosphate pentasodium salt (Ap5A), and Dexamethasone 21-phosphate disodium salt were purchased from Sigma-Aldrich (St. Louis, MO). Single stranded DNA (ssDNA) oligonucleotides 5’-AAAAA-3’ (5-mer), 5’-AAAAAAAA-3’ (7-mer), 5’-AAAAAAAAAA-3’ (10-mer), 5’-AAAAAAAAAAAAA-3’ (13-mer) were obtained from Integrated DNA Technologies (Coralville, IA). Cold and radiolabeled C1 conjugate, EP4-A, and EP4-tri were provided by Dr. Young’s group at Simon Fraser University. Radiolabeled Prostaglandin E2 ([5,6,8,11,12,14,15-3H(N)]-Prostaglandin E2) was purchased from Perkin Elmer (Boston, MA).

TLC silica gel 60 plates and Sephadex G-50 were purchased from Sigma-Aldrich. Spectra/Por 2 (12-14KDa cut-off) dialysis bags were from Spectrum Laboratories Inc. 14C-DSPC was purchased from American Radiolabeled Chemicals (St Louis, MO). 3H-cholesteryl hexadecyl ether (3H-CHE) was purchased from Perkin Elmer (Boston, MA). Radiolabeled C1 was synthesized as previously described (Arns et al. 2012; Chen, Arns, and Young 2015). Cholesterol E assay kit was from Wako Chemicals (Richmond, VA). Ion exchange filter plates, AcroPrep™ Advance 96 filter plate Mustang® Q, were courtesy of VWR (Mississauga, ON). Formulations used in vivo were sterilized via 0.2 µm filters (Pall, Ville St. Laurent, QC) and concentrated using Amicon Ultra centrifugal filters (Millipore, Billerica, MA).
2.2.2 Preparation of lipid nanoparticles

All the LNPs were made using the NanoAssemblr benchtop instrument (Precision Nanosystems, Vancouver, BC) at total flow rates of 12-18 mL/min and flow rate ratio of 3:1 aqueous: organic phases (v/v) with initial lipid concentration of 20 µg/mL. Following the mixing, the nascent LNP (in 25% ethanol v/v) were dialyzed against ~40000x volumes of 50 mM HEPES buffer pH 7.4-7.5 for at least 15 hours. Alternatively, for some of the DODMA formulations, the nascent LNP were dialyzed against ~ 40000x volumes of 50 mM acetate buffer pH 5.0 to try to keep the ionizable lipid charged during dialysis.

2.2.3 Encapsulation of Nucleotides (Small Adenosine-Based Cargo)

For the encapsulation of AMP, ADP, ATP, A4pA, A5pA (ANPs) with permanently charged DOTMA- or ionizable cationic lipid DODMA-containing LNP, the anionic cargo series were dissolved in either 50 mM HEPES buffer at pH 7.5 for the DOTMA encapsulation, or 50 mM acetate buffer at pH 5.0 for encapsulation with DODMA. The aqueous stream containing the charged cargo was mixed 3:1 with an ethanol stream containing either DOTMA/Chol/DSPC/PEG-DSG or DODMA/Chol/DSPC/PEG-DSG in 50/37.5/10/2.5 mol %. Cationic lipid amine (N) to total small molecule phosphate (P) ratio was kept at 1 or 3 molar ratio (therefore N/P = 1.0 or 3.0). The small adenosine-based cargoes were quantified before and after dialysis (using HEPES buffer at pH 7.5 or 50 mM acetate buffer at pH 5.0) using absorbance of adenosine at 260nm, while a cholesterol assay was used to assay total lipid concentration. All free AMP, ADP, ATP, Ap4A, and Ap5A rapidly leaked from the 12-14 KDa membranes during dialysis. Comparison of the pre- and post-dialysate A260nm and cholesterol content allowed for calculation of encapsulation efficiency.
2.2.4 Encapsulation of short single stranded DNA oligonucleotides of 5, 7, 10, and 13 bases in length

Single stranded oligonucleotides of 5’-AAAAA-3’ (5-mer), 5’-AAAAAAA-3’ (7-mer), 5’-AAAAAAAAAAAA-3’ (10-mer), and 5’-AAAAAAAAAAAAAAAAA-3’ (13-mer) were diluted in either 50 mM HEPES buffer pH 7.5 for entrapment within DOTMA containing LNP, or 50 mM Acetate buffer pH 5.0 buffer for entrapment using ionizable cationic lipid DODMA containing systems. Lipid composition was DOTMA/Chol/DSPC/PEG-DSG or DODMA/Chol/DSPC/PEG-DSG (50/37.5/10/2.5 mol %) and the cationic lipid amine (N) to total oligonucleotide phosphate (P) molar ratio was kept at 3 (N/P=3.0).

During the course of the short oligonucleotide encapsulation trials, it was empirically determined that even the short 5’-Poly A-3’ oligos (especially the 7-mer, 10-mer and 13-mer) did not efficiently exit the dialysis tubing during extensive dialysis, despite having a molecular weight considerably lower than the stated dialysis cut-off of the Spectra/Por 2 (12-14kDa cut-off) membranes. Therefore, to determine the short oligonucleotide cargo encapsulation efficiency, all oligonucleotide-containing samples were further cleaned and free oligonucleotides were removed from the post-dialysate LNP using a strong anion exchange filter (AcroPrep™ Advance 96 filter plate Mustang® Q) which effectively binds all free non-lipid nanoparticle encapsulated oligonucleotides, while allowing LNP containing encapsulated oligonucleotides to flow through. Oligonucleotides were quantified by measuring absorbance at 260 nm, and the cholesterol assay was used to quantify the lipid concentration. Comparison of the pre- and post-dialysate A260nm and cholesterol content allowed for calculation of encapsulation efficiency.
2.2.5 **Encapsulation of Dexamethasone-21-phosphate**

Dexamethasone-21-phosphate (Dex-phosphate) was diluted in 50 mM HEPES buffer pH 7.5, for the permanently charged DOTMA-LNP and in 50 mM acetate buffer pH 5, for DODMA systems. The ethanol-lipid phase was diluted 1:3 with the Dex-phosphate aqueous phase. The cationic lipid amine (N) to Dexamethasone-21-phosphate ratio (P) was varied from N/P = 0.5 to 3. Systems were also prepared that contained both ionizable and permanently charged lipids DODMA and DOTMA (aqueous phase pH 7.5). Lipid composition was DOTMA/Chol/DSPC/PEG-DSG, DODMA/Chol/DSPC/PEG-DSG or DOTMA+DODMA/Chol/DSPC/PEG-DSG at 50/37.5/10/2.5 mol%. As with the other nanoparticle systems a cholesterol assay was used to measure total lipid and Dexamethasone-21-phosphate was quantified by absorbance at A250nm. Comparison of the pre- and post-dialysate A250nm and cholesterol content allowed for calculation of encapsulation efficiency.

2.2.6 **Encapsulation of PGE2 and its analogs into liposomes, lipid core particles and cationic LNP**

PGE2 or EP4-A were loaded into liposomes consisting of DSPC or POPC/Chol/PEG-DSG LNP (58/40/2 mol%) and 60°C (in the case of DSPC-containing LNP) and in the presence of a proton gradient. For the proton gradient, LNPs were formulated using the extruder or microfluid mixing with a heating block, at 65°C with a basic buffer (20,150 or 450 mM borate, pH=10) and then dialyzed against a neutral buffer (150mM NaCl, 20mM HEPES, pH=7). Once the pH gradient was established, the small molecule drug was incubated with the LNP at high temperature (60°C) to allow encapsulation to occur. LNP and the small molecule drug (PGE2 or EP4-A) were labeled with $^3$H and $^{14}$C, respectively. Malvern Zetasizer NanoZS was used to size
the particles and the encapsulation efficiency was measured as the ratio of small molecule drug to LNP radiolabels. The remote loaded liposomes were then incubated in 10% FBS solution at 37°C. At time points, aliquots were loaded on Sephadex G-50 spin columns and centrifuged to separate the released drug from LNPs. Radiolabeled C1 and LNP were detected by a Beckman LS 6500 liquid scintillation counter through addition of 5 mL of Pico-Fluor Plus scintillation fluid (Perkin Elmer) to LNP samples. Drug retention was measured as ratio of tritiated small molecule drug to 14C-DSPC labeled-LNP.

Alternatively, EP4 was encapsulated as the hydrophobic prodrug form (EP4-tri) into POPC-EP4-tri emulsion nanoparticles using microfluidic mixing and a neutral buffer (PBS) at DSPC/Chol/EP4-tri/PEG-DSG at 50-55/0-35/10-40/5 (ranges were tested for optimization). EP4-tri was included in the organic phase along with other lipids and formulated in one step during the rapid mixing. Following ethanol removal by dialysis, lipids were extracted using the Bligh and Dyer method (Bligh and Dyer 1959) and analyzed on TLC silica gel 60 plates. For a more quantitative analysis, EP4-tri was radiolabeled by Dr. Young’s group and encapsulation efficiency was measured as the ratio of 3H-EP4-tri to 14C-DSPC labeled-LNP.

PGE2 and EP4-A were also encapsulated using the permanently charged cationic lipid, DOTMA/DSPC/PEG-DSG/Cho/PGE-2 at 50/10/2/38-x/x, where x stood for 5, 10, 20% PGE2 in various formulations. PGE2 or EP4-A was dissolved in the organic phase along with lipids and mixed with PBS (pH = 7.4) or borate buffer (pH = 9) as the aqueous phase. Encapsulation efficiency for PGE2 and EP4-A was determined as the ratio of tritiated small molecule drug to 14C-DSPC labeled-LNP.
2.2.7 Encapsulation of C1 conjugate

C1 conjugate was dissolved in different concentrations in 50 mM HEPES buffer at pH 7.4 and mixed 3:1 with lipids dissolved in ethanol. The organic phase included the lipid mix consisting of cationic lipid(s) (DLin-MC3-DMA and DOTMA or DOTAP individually or in combination), DSPC, Cholesterol, PEG-DSG at 50/10/37.5/2.5 molar ratio. The aqueous phase was radiolabeled C1 dissolved at different concentrations in 50 mM HEPES buffer pH 7.4. The formulation was dialyzed against 50 mM HEPES pH 7.4 overnight for in vitro experiments or 4 hours in HEPES buffer and then overnight in 0.9% saline solution for in vivo studies. For detection purposes tritiated or 14C-C1 was used while LNPs were probed by 3H-CHE or 14C-DSPC. Malvern Zetasizer NanoZS (Worcestershire, UK) was used to determine LNP size and population polydispersity (PdI) through dynamic light scattering (intensity and number weighing). Some formulations were also imaged for morphology using cryogenic transmission electron microscopy (cryo-TEM) as described previously (Belliveau et al. 2012). Total lipid concentration was determined by cholesterol content using the Cholesterol E assay. Radiolabeled C1 and LNP were detected by a Beckman LS 6500 liquid scintillation counter through addition of 5 mL of Pico-Fluor Plus scintillation fluid (Perkin Elmer) to LNP samples. Encapsulation efficiency was determined as the ratio of C1/LNP before and after dialysis as the un-encapsulated C1 diffuses out of the dialysis bag during LNP dialysis step.

2.2.8 Hemolysis assay

Human erythrocytes (RBC) (Innovative Research, Novi, MI) were rinsed with 0.9% saline before use. C1-LNP were incubated at a final 2mg/mL lipid concentration with 4% (v/v) RBCs suspended in PBS for 30 minutes at 37⁰C. 0.4% Triton X-100 and saline were used as
positive and negative controls respectively. The samples were then centrifuged for 5 minutes at 1000x g at 4°C and absorbance was measured at 450 nm. Sample hemolysis was normalized to the Triton X-100 control which marked 100% RBC lysis.

2.2.9 C1-LNP pharmacokinetics-biodistribution studies

\(^{14}\)C-C1 encapsulated with \(^{3}\)H-CHE-LNP was injected at 3.5 mg/kg C1 to female CD-1 mice intravenously (i.v., tail vein injection). For some groups, blood samples were taken before the terminal time points. At terminal time points blood was collected via cardiac puncture and tissues and organs were harvested for analysis. Samples were then weighed out and analyzed in a Harvey OX-300 biological oxidizer (R. J. Harvey Instrument Corporation, NJ) using the 4 min dual label program. The resulting scintillation mixture was counted on a Beckman Coulter LS-6500 scintillation counter. For the naked C1 study, dual labeled C1 (\(^{14}\)C and \(^{3}\)H) was injected intravenously through the tail vein to C57BL/6 females at 5 mg/ kg. All procedures were approved by the Animal Care Committee of Simon Fraser University and Animal Care Committee at the University of British Columbia and were performed in accordance with the Canadian Council on Animal Care guidelines.

2.3 Results

2.3.1 LNP containing permanently positively charged lipids and high cationic lipid contents exhibit superior encapsulation efficiencies for small, negatively charged molecules

The cationic lipid-dependent loading process can be employed using permanently positively charged lipids or ionizable cationic lipids to bind to the negatively charged molecules
to be encapsulated. The representative permanently positively charged lipids and ionizable cationic lipids chosen were DOTMA and DODMA, respectively (Figure 2.1A). In the case of the ionizable lipids, loading must be performed at low pH (pH 4) where the lipid is protonated and bears a positive charge. The lipid composition chosen was the same as used for encapsulation of siRNA for maximum gene silencing of siRNA in hepatocytes (Jayaraman et al. 2012) consisting of cationic lipid/cholesterol/DSPC/PEG-lipid in the molar ratios 50/37.5/10/2.5. In the first set of experiments it was sought to identify the smallest anionic molecule that could be loaded into LNP containing DOTMA or DODMA using an amino-lipid to phosphate (N/P) charge ratio of 3. As shown in Figure 2.1, LNP containing DOTMA were able to load and retain ADP and larger adenosine polyphosphates efficiently, whereas the maximum encapsulation achieved employing DODMA was approximately 60%. The reduced encapsulation for DODMA vs DOTMA may arise in part because of the dialysis step to raise the pH of the medium from pH 4 to pH 7.4. In order to determine whether this is the case, retention in media at pH 5 was tested, which lead to significantly improved trapping efficiency (Figure 2.1).
Table 2.1. List of small molecules and nucleic acids encapsulated in Chapter 2.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular weight (g/mol)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine 5’-monophosphate</td>
<td>324.22</td>
<td><img src="image" alt="Adenosine 5’-monophosphate" /></td>
</tr>
<tr>
<td>Adenosine 5’-diphosphate</td>
<td>404.20</td>
<td><img src="image" alt="Adenosine 5’-diphosphate" /></td>
</tr>
<tr>
<td>Adenosine 5’-triphosphate</td>
<td>505.14</td>
<td><img src="image" alt="Adenosine 5’-triphosphate" /></td>
</tr>
<tr>
<td>P1,P4-Di(adenosine-5’) tetraphosphate</td>
<td>818.39</td>
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</tr>
<tr>
<td>P1,P5-Di(adenosine-5’) pentaphosphate</td>
<td>911.28</td>
<td><img src="image" alt="P1,P5-Di(adenosine-5’) pentaphosphate" /></td>
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<tr>
<td>Molecule</td>
<td>Molecular weight (g/mol)</td>
<td>Structure</td>
</tr>
<tr>
<td>-------------------------</td>
<td>--------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>5-mer poly-A</td>
<td>1503.50</td>
<td>5'- AAAA -3'</td>
</tr>
<tr>
<td>7-mer poly-A</td>
<td>2130.50</td>
<td>5'- AAAAAA -3'</td>
</tr>
<tr>
<td>10-mer poly-A</td>
<td>3069.90</td>
<td>5'- AAAAAAAAAAA -3'</td>
</tr>
<tr>
<td>13-mer poly-A</td>
<td>4009.40</td>
<td>5'- AAAAAAAAAAAAAAA -3'</td>
</tr>
<tr>
<td>Dexamethasone 21-phosphate</td>
<td>470.40</td>
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<tr>
<td>EP4-Agonist</td>
<td>395.44</td>
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</tr>
<tr>
<td>C1 conjugate</td>
<td>832.72</td>
<td><img src="image" alt="C1 conjugate" /></td>
</tr>
</tbody>
</table>
Figure 2.1. Influence of small molecule charge and cationic lipid species (ionizable vs. permanently charged) on encapsulation of adenosine polyphosphates in LNP systems.

LNP systems were prepared using microfluidic mixing as indicated in Methods. Lipids in ethanol were mixed with an aqueous solution containing the small molecule to be entrapped and then dialyzed to remove residual ethanol. In the case of formulations containing the ionizable lipid DODMA, the pH of the aqueous mixture was 5, otherwise pH = 7.4. The lipid composition employed was DOTMA/Chol/DSPC/PEG-DSG or DODMA/Chol/DSPC/PEG-DSG (50/37.5/10/2.5; mol %).

(A) Structures of permanently charged lipid DOTMA and ionizable lipid DODMA. (B) Encapsulation of adenosine phosphates in LNP containing either DOTMA or DODMA employing an amino lipid to phosphate ratio (N/P) of 3. (C) Encapsulation of adenosine phosphates in LNP containing either DOTMA or DODMA at N/P=1. Error bars represent standard deviation, n = 3. The values represent the mean.
2.3.2 LNP systems containing permanently positively charged lipids exhibit efficient encapsulation properties for oligonucleotides as short as 5-mers

Previous work has shown that microfluidic mixing using lipid mixtures similar to those employed here results in efficient encapsulation of double-stranded 21-mer siRNAs for ionizable cationic lipids (Belliveau et al. 2012; Jayaraman et al. 2012). It was of interest to determine the shortest oligonucleotide that can be loaded and retained within an LNP. As shown in Figure 2.2, LNP containing the permanently charged lipid DOTMA were able to encapsulate short oligonucleotides with encapsulation efficiencies increasing from 50% for the 5-mer poly-A to about 90% for the 13-mer poly-A. Interestingly the shortest oligonucleotide tested here, the 5-mer poly-A which has 4 acidic phosphate groups, loaded to a similar extent as adenosine tetraphosphate with the DODMA system (Figure 2.1). For the LNP systems formulated with the ionizable cationic lipid DODMA, the oligonucleotide encapsulation efficiencies were consistently lower than the permanently charged DOTMA systems (30-60% for DODMA compared to 50-90% for DOTMA). This is consistent with results obtained for the adenosine polyphosphates showing that LNP containing DODMA are less efficient in encapsulating small charged cargo.

2.3.3 LNP systems containing cationic lipids can be used to encapsulate small molecule pro-drugs containing phosphate groups

It is of obvious interest to see whether the LNP encapsulation technique demonstrated for short oligonucleotides and adenosine polyphosphates can be extended to small molecule drugs. It was first examined whether LNP formulations of dexamethasone phosphate (Dex-phosphate), a commonly employed pro-drug version of dexamethasone could be achieved. As shown in figure
2.2B, it was found that Dex-phosphate could be loaded nearly completely into DOTMA-containing lipid nanoparticles. As the N/P molar ratios were increased from 0.5 to 3.0, the encapsulation efficiency with DOTMA systems improved from 27% to 90%. In the case of DODMA-containing LNP, only formulation and incubation in a low pH (pH = 5.0) environment resulted in loading and retention.

A potential strategy to achieve high encapsulation of molecules such as Dex-phosphate in LNP systems containing ionizable cationic lipids is to include a proportion of permanently positively charged lipid with the ionizable lipid. As shown in Fig 2.2C, LNP prepared with equimolar amounts of DOTMA and DODMA systems exhibit reasonable (~65%) Dex-phosphate encapsulation efficiencies at neutral pH. Such systems can potentially benefit from the intracellular delivery enhancement provided by the presence of ionizable cationic lipids (Hafez et al., 2001).
Figure 2.2. Influence of small molecule size and cationic lipid species (ionizable vs. permanently charged) on encapsulation of poly-adenosine oligonucleotides and dexamethasone-21-phosphate.

LNP systems were prepared using microfluidic mixing as indicated in Methods. Lipids in ethanol were mixed with an aqueous solution containing the small molecule to be entrapped and then dialyzed to remove residual ethanol. In the case of formulations containing the ionizable lipid DODMA the pH of the aqueous mixture was 4, otherwise pH = 7.4. The lipid composition employed was DOTMA/Chol/DSPC/PEG-DSG or DODMA/Chol /DSPC/PEG-DSG (50/37.5/10/2.5; mol %). (A) Encapsulation of poly-A oligonucleotides in LNP containing either DOTMA or DODMA at amino lipid to phosphate ratio (N/P) of 3. (B) Influence of N/P ratio on encapsulation of dex-phosphate (Dex-P) in LNP systems containing DOTMA. (C) Dex-Phosphate was encapsulated in LNP by replacing a portion of DODMA with permanently charged DOTMA. Lipid composition was DOTMA/DODMA/Cholesterol/DSPC/PEG-DSG (50-
X/X/37.5/10/2.5 mol%) with N/P of 3. Error bars represent standard deviation, n = 3. The values represent mean.

2.3.4 **PGE2 analogs are not efficiently entrapped and/or retained without the phosphate group addition.**

In an attempt to encapsulate PGE2 and EP4-A, liposomes were generated with the lipid composition of 58% POPC or DSPC, 40% Cholesterol, 2% DSG-PEG. The pH gradient was established by using an internal pH 10 buffer and an external pH 7 buffer. The small molecules were then incubated with the pre-made liposomes at 60°C. The encapsulation efficiency was also tested for particles without any pH gradient (pH 7 for both internal and external buffers) and at room temperature incubation for control conditions. Also, to improve the encapsulation efficiency, higher buffering capacity was tested for the interior of liposomes. Following optimization of the encapsulation process, the loaded liposomes were incubated with serum to test drug retention capacity.

No PGE2 encapsulation was observed in POPC-based LNP with the proton gradient of pH 10 interior buffer and pH 7 exterior buffer. POPC is an unsaturated phosphocholine that typically constructs leakier membranes. It is likely that POPC-based membranes (58% POPC, 40% Cholesterol, 2% DSG-PEG) could not sufficiently encapsulate and retain PGE2 and its analog Ep4-A. In order to improve the drug retention, proton gradient-containing LNP (containing DSPC as helper lipid instead of POPC) were constructed. DSPC, a saturated phosphocholine, has been shown to reduce the permeability of LNP, leading to higher retention of small molecule drugs. DSPC-LNPs, however, are generally larger in diameter (about 80-100nm) and need to be formulated at 50°C or higher. By replacing POPC with DSPC, the LNPs
were able to achieve a PGE2 or EP4-A encapsulation efficiency of about 50% when incubated using a drug to lipid molar ratio of 1:9 (Figure 2.3). Encapsulation efficiency was increased when higher buffering capacity of the internal buffer was used (Figure 2.3D). A similar trend was observed for the PGE2 and EP4-A. Unfortunately, the release rate of both PGE2 and EP4-A in plasma at 37°C were extremely rapid (Figure 2.3E) and thus the liposomal version of PGE2 and EP4-A would not have the benefits of other stably retained LNP drugs.
Figure 2.3. Encapsulation and release of PGE2 and its analog EP4-A.

(A) Structures of PGE2 (top) and EP4-A (bottom). (B) Schematic demonstration of the pH gradient loading method for small molecule drugs. (C) PGE2 was loaded into LNPs composed of DSPC/Cholesterol/PEG-DSG at 58/40/2 mol% at room temperature or 60°C and in the presence of a proton gradient (drug: lipid of 1:9 molar; internal pH=7 and external pH=10 for pH gradient establishment). (D) PGE2 loading was improved when a higher internal buffering capacity was
used to establish the pH gradient. LNP composition was DSPC/Cholesterol/PEG-DSG at 58/40/2 mol%, LNPs formulated at 60°C and the external buffer was 20 mM Hepes at pH=7 with the internal pH being 10 (drug: lipid of 1:9 molar, 15-minute incubation). (E) More than half of the encapsulated PGE2 or EP4-A was released during the first hour of incubation in 10% FBS solution at 37°C (LNP formulated with DSPC/Cholesterol/PEG-DSG at 58/40/2 mol% and at 60°C with drug:lipid of 1:9 molar). Error bars represent standard deviation, n = 3.

Another approach for encapsulation of small molecule drugs is to modify the molecule structure to obtain certain properties that facilitate the encapsulation process, similar to Dex-phosphate discussed in 2.3.3. The drug molecule can be chemically modified into a highly hydrophobic molecule. Such hydrophobic molecules can form a solid core which is embedded inside the more hydrophilic structural lipids such as POPC (Zhigaltsev et al. 2012). In order to test this approach, a highly hydrophobic derivative of EP4-A was synthesized. EP4-tri (Figure 2.4A) is the hydrophobic prodrug form of EP4-A which mimics a triglyceride structure and is anticipated to reside in the core of emulsion particles due to the presence of highly hydrophobic groups. EP4-tri was then microfluidic-formulated using cholesterol, POPC, and PEG-DSG lipid. In this LNP format, the hydrophobic prodrug molecule will form droplets inside a shell of more hydrophilic lipids (i.e. POPC, PEG, etc.; Figure 2.4B). The encapsulation efficiency of EP4-tri was determined by thin layer chromatography (TLC; Figure 2.4C). The results of these experiments indicated encapsulation of EP4-tri (Figure 2.4D). As a more quantitative method, the experiment was performed using tritiated EP4-tri and 14C labeled-LNP. While the TLC results indicated EP4-tri encapsulation in the LNPs, the quantification of tritiated EP4-tri indicated that only about 10% of the 3H label was retained in all formulations, regardless of the
lipid composition and drug-to-lipid ratios. Further investigation by HPLC analysis indicated that the EP4-tri was unstable and rapidly degraded in ethanol which is an essential solvent of the microfluidic formulation technique.

Figure 2.4. Formulation of the hydrophobic EP4-tri.

(A) Structure of EP4-tri. (B) Hypothetical structure of the hydrophobic prodrug-LNPs based on previous studies (Zhigaltsev et al. 2012). (C) EP4-tri formulations were synthesized using the
microfluidic mixing method and dialyzed overnight. The formulations were then subjected to lipid extraction and thin layer chromatography for detection purposes. (D) As assessed by TLC, EP4-tri was retained in the 3 different tested lipid composition LNPs, this qualitative method however, did not indicate percentage EP4-tri encapsulation via LNP. Formulation 1: 50/35/10/5, Formulation 2: 43/32/20/5, Formulation 3: 32,23,40,5 mol% of POPC, cholesterol, EP4-tri and PEG-DSG.

Since the traditional remote loading and the hydrophobic drug encapsulation methods did not work very well for PGE2 and its analogs, the ionic interaction-based encapsulation method was adapted and tested for encapsulation of PGE2 and EP4-A. This approach is similar to the encapsulation method of nucleic acids using cationic lipids where the negatively charged nucleic acid is entrapped via positively charged lipids and the resulting complex forms a lipid nanoparticle with a solid core structure (Belliveau et al. 2012; Leung et al. 2012). Since PGE2 and EP-4 bear partial negative charges they can hypothetically be encapsulated using permanently charged or ionizable cationic lipids. For this purpose, 5, 10 and 20 mol% drug was mixed with DOTMA 50%, DSPC 10%, PEG 2%, and cholesterol (38-X)% where X stands for drug mol%. PGE2 or EP4-A were included in the organic phase and the formulation was tested at pH = 7.4 or pH = 9. Regardless of the lipid composition and formulation pH, encapsulation efficiency for both drug molecules was about 35%. While encouraging, the results observed here were still not optimal and further improvement of formulation method was needed for PGE2 analogs.
2.3.5 EP4 agonist- bisphosphonate conjugate (C1) can be efficiently encapsulated using cationic lipids

In order to encapsulate the EP4-A molecule, a bisphosphonate derivative of EP4 (named the C1 conjugate) was synthesized (for structure see Figure 2.5A) that has three potential advantages. First, the presence of the negatively charged bisphosphonate should allow encapsulation into LNP containing cationic lipids. Second, the bisphosphonates have been previously used for bone targeting due to their affinity toward the bone (Uludag 2002; Heymann et al. 2004). Finally, the EP4-A-bisphosphonate linker has been designed to degrade over time (Chen, Arns, and Young 2015), potentially resulting in sustained release of the active EP4-A compound after arrival in bone.

In order to achieve encapsulation and adequate retention of a molecule such as C1 (two negative charges) the results to this point indicate that a proportion of the cationic lipid must be permanently charged. However, permanently positively charged lipids such as DOTMA can be toxic (Lv et al. 2006). Previous work has shown that DOTAP, a version of DOTMA that contains ester linkages as opposed to the ether linkages in DOTMA is less toxic, presumably due to rapid breakdown of biodegradable ester bonds by endogenous esterases (Freedland et al. 1996; Leventis and Silvius 1990; Farhood et al. 1992). Therefore, the encapsulation properties of LNP containing either DOTMA and DOTAP was evaluated with a view towards using the DOTAP-containing formulation if carrier toxicity proved limiting.

LNP containing either DOTAP or DOTMA were formulated using the microfluidic formulation technique as indicated in the legend to Figure 2.5. As shown in Figure 2.5D, C1 encapsulation efficiency decreases at higher drug-to-lipid ratios while LNP size increases at higher drug-to-lipid ratios. LNP containing DOTAP exhibited larger particle sizes and
polydispersity indices (Pdl) in comparison to LNP containing DOTMA. Further, as shown in Figure 2.5 D, LNP containing DOTMA exhibited significantly higher encapsulation efficiencies of 80% or greater for drug-to-total lipid ratios ranging from 1:32-1:2 mol/mol, as compared to encapsulation efficiencies that decreased from ~70% to ~40% for LNP containing DOTAP as the drug to lipid ratio was increased. The LNPs with higher C1 content were larger when sized by DLS.
Figure 2.5. EP4-A-bis-phosphonate (C1) can be encapsulated in LNP containing the permanently positively charged lipids DOTMA or DOTAP.

C1 was encapsulated into LNP with lipid composition DOTMA or DOTAP/DSPC/cholesterol/PEG-lipid (50/10/37.5/2.5; mol%) using the microfluidic mixing technique outlined under Methods. The C1 drug-to-total lipid ratio was varied from 0.03-0.5 mol/mol. (A) Structures of EP4-A and C1; Influence of drug-to-lipid ratio on LNP size and polydispersity of (B) C1-DOTMA LNPs and (C) C1-DOTAP LNPs where A, B, C, D, E, F represent 1:32, 1:16, 1:8, 1:4, 1:3, 1:2 of C1: total lipid mol/mol ratio respectively. (D) Encapsulation efficiency of C1 in LNP containing DOTMA or DOTAP as a function of the drug-to-total lipid ratio. Error bars represent standard deviation, n = 3.
It is of interest to determine whether a proportion of the permanently charged DOTMA or DOTAP can be replaced with an ionizable cationic lipid that is potentially less toxic and can also facilitate intracellular delivery (Hafez et al., 2001; Hafez & Cullis, 2001; Koltover et al., 1998). The current “gold standard” ionizable cationic lipid is DLin-MC3-DMA (MC3), which is a component of an LNP siRNA formulation in FDA-approved ONPATTRO. Encapsulation of C1 in LNP containing a combination of MC3 with DOTMA or DOTAP was therefore evaluated. As shown in Figure 2.6C, LNP containing MC3 combined with DOTMA or DOTAP (MC3 of 50, 25, 12.5, 0 mol% and DOTAP or DOTMA = 50 - MC3%) exhibit reasonable C1 encapsulation efficiencies (at a drug-to-total lipid ratio of 1:8). While the DOTMA particles show a significant size increase at higher C1:lipid ratios, the DOTAP particles sustained a relatively consistent size across drug:lipid titrations (Figure 2.6A, 2.6B). Similar to DOTMA-only vs DOTAP-only LNP, C1-LNP formulations with combination of DOTMA/ MC3 had higher encapsulation efficiencies and smaller particle sizes compared to those with DOTAP/MC3.

Overall, the encapsulation efficiency of MC3-only LNP increased from 36% to up to 80% and 60% in the presence of DOTMA or DOTAP respectively, indicating that when insufficient, ionizable lipids’ encapsulation efficiency can be rescued by partial replacement with permanently charged cationic lipids (Figure 2.5B, 2.5C).
Figure 2.6. The ionizable cationic lipid DLin-MC3-DMA (MC3) can be partially substituted for DOTMA or DOTAP to achieve efficient encapsulation of C1 in LNP systems.

C1 was encapsulated into LNP with lipid composition DOTMA or DOTAP/ DSPC/ cholesterol/ PEG-lipid (50/ 10/ 37.5/ 2.5; mol%) using the microfluidic mixing technique outlined under Methods. (A) Influence of drug-to-cationic lipid ratio and lipid composition on LNP size and polydispersity index (PdI) for LNP containing 0, 12.5, 25, 50 mol% DOTMA with DLin-MC3-DMA of 50, 25, 12.5 and 0 mol% and permanently cationic lipid: C1 of 0, 1, 2, 4 mol/mol (A, B, C, D respectively) at C1:total lipid of 1:8 mol/mol. (B) Influence of drug-to-cationic lipid ratio and lipid composition on LNP size and PdI for LNP containing 0, 12.5, 25, 50 mol% DOTAP with DLin-Mc3-DMA of 50, 25, 12.5, and 0 mol% and permanently cationic lipid: C1 of 0, 1, 2, 4 mol/mol (A, B, C, D respectively) at C1:total lipid of 1:8 mol/mol. (C) Influence permanently
charged and ionizable lipid ratio on the encapsulation efficiency of C1 in LNP systems containing 0, 12.5, 25, 50 mol% DOTMA or DOTAP with DLin-Mc3-DMA of 50, 25, 12.5 and 0 mol% and permanently charged cationic lipid: C1 of 0,1, 2, 4 mol/mol (A, B, C, D respectively) at C1: total lipid of 1:8 mol/mol. (D) Structures of cationic lipids DOTMA, DOTAP, and MC3. Error bars represent standard deviation, n = 3.

2.3.6 DLin-MC3-DMA/DOTAP C1-LNP causes minimal hemolysis in vitro and exhibits an electron dense core structure

Three formulations were picked based on encapsulation efficiencies and imaged using Cryo-electron microscopy (Cryo-TEM). All three formulations—C1-LNP made with DLin-MC3-DMA/ DOTAP (25/25 mol%), DLin-MC3-DMA/ DOTMA (37.5/12.5 mol%), and DOTAP (50 mol%) LNPs—exhibited electron dense core structures similar to the previously described siRNA-LNP (Leung et al. 2012) (Figure 2.7B-D). DLin-MC3-DMA/ DOTAP C1-LNP had a well-defined shape, size and structure of typical LNPs compared to the other 2 formulations and the control empty particles.

Particles with permanently charged cationic lipids might not be easily tolerated depending on the lipid composition and payload. Since positively charged lipids can disrupt biological membranes—including the red blood cells in the circulation—the can lead to intolerance and toxicity. The three formulations picked for imaging were also tested for their hemolysis effect on human erythrocytes. As shown in Figure 2.7 E, DLin-MC3-DMA/ DOTAP C1-LNP caused minimal lysis at levels comparable to the saline control. C1-LNP formulated with DOTMA and MC3 as cationic lipid had the highest lysis effect on the red blood cells.
Figure 2.7. C1-LNP exhibit an electron dense core and have negligible toxicity \textit{in vitro}.

Cryo electron microscope images of: (A) Empty MC3/DOTAP (25/25 mol/mol); (B) C1 MC3/DOTAP (25/25 mol/mol) at drug: total lipid of 1:8 (mol%); (C) C1 DOTAP (50 mol%)
and drug:total lipid of 1:8 (mol/mol); and (D) C1 MC3/DOTMA (37.5/12.5 mol%) and C1:total lipid of 1:8 mol/mol. The scale bar shows 100 µ. (E) Hemolysis assay performed with C1 LNP formulations described in B, C and D at neutral pH. Error bars represent standard deviation, n = 3.

2.3.7 DLin-MC3-DMA/DOTAP C1-LNP has a comparable biodistribution profile to C1

DLin-MC3-DMA/ DOTAP C1-LNP was administered via tail vein injection to female CD 1 mice (Jackson Laboratories) and mice were sacrificed at different times and tissues were harvested for analysis. C1 was probed by $^{14}$C and the LNP lipids were probed by $^{3}$H measurement. Analysis of multiple tissues indicated no significant C1-LNP accumulation in muscle, lungs, and kidneys (data not shown). On the other hand, C1-LNP was detected at significant levels in bone, liver and spleen (Figure 2.8A). 18% of injected dose of C1 was initially observed in bone. This measurement dropped gradually over the course of 13 days from 18 to 10%. The biodistribution profile of naked C1 injected i.v. (Figure 2.8 B) was very similar to LNP-C1, indicating that this platform can be helpful for other bone drug conjugates that are not stable in naked form or are severely toxic.
Figure 2.8. Pharmacokinetics and biodistribution studies of C1-LNP and naked C1.

(A) C1 DOTAP-MC3 LNP (25/25 DOTAP/MC3) were intravenously injected to healthy mice and compared to naked C1. C1 was labeled by $^{14}$C and LNP lipids were probed by $^{3}$H. (B)
Naked C1 was dual labeled by both $^{14}$C and $^3$H. The $^{14}$C label resided on the bisphosphonate end and the $^3$H resided on the EP4-A end of C1. (C) The structure of C1 with the location of radiolabels tissues were harvested and counted for activity. Error bars represent standard deviation, n = 3.

2.4 Conclusion

In the studies discussed in this chapter, it was demonstrated that ionic interaction-based entrapment of very small molecules depends on the molecule’s size and charge density. Moreover, the encapsulation efficacy is also highly dependent on the LNP lipid composition, specifically the cationic lipid. While the LNPs have been extensively optimized for siRNA delivery, the encapsulation of small molecules by these systems is still largely unknown. Small molecules can be toxic or insoluble in their native form, and the prodrugs can be highly unstable or not well tolerated. This study highlights a new platform for the delivery of such drugs, where the prodrug can be encapsulated to improve the toxicity, circulation time and/or biodistribution.

An important point taken from this work is the minimum limit number of charges required for a molecule to be encapsulated by conventional cationic lipids. As indicated in the results section, a single phosphate group addition to a small molecule (AMP) such as adenine might not be sufficient to construct the electrostatic interactions required for efficient entrapment and retention. Even with the permanently charged lipid DOTMA, little to no loading could occur pointing to the lower limit of this encapsulation method. The entrapment however, significantly improved upon addition of a second phosphate group (ADP) and an upward trend was observed for encapsulation. Additionally, there was a significant difference in encapsulation efficiencies of the permanently charged and conditionally ionizable cationic lipids with the former having a
greater advantage. This trend was generally observed across all studies performed in this work, indicating that permanently charged lipids are potentially more efficient in encapsulating charged molecules than the conditionally ionizable ones. This could in part be compensated for by dialyzing in an acidic buffer instead of the desired neutral pH, rescuing encapsulation efficiency almost similar to that of a permanently charged lipid. However, for use in biological systems the LNPs need to be in neutral pH, therefore this is not a practical solution for ionizable cationic lipid formulations with low encapsulation efficiencies. It is also worth mentioning that although neutral pH dialysis of ionizable nanoparticles relieves the electrostatic interactions and leads to further shedding of ADP and ATP from the lipid nanoparticle, some stable trapping is still present following complete buffer equilibration. This can be rationalized as the ability of these very small di- and tri-phosphate compounds to become trapped in the hydrophilic cavities of the nanostructured lipid nanoparticle. If this is the case, then larger polyphosphates should be trapped to a greater extent within the LNP, despite the loss of electrostatic interactions that follows a switch to neutral pH. Moreover, a large jump in encapsulation efficiency occurred when the oligonucleotide length was increased from 5 to 7 DNA bases and only marginal increases in efficiency occurred with oligos greater than 7-mer. These data suggest that the shortest oligonucleotide tested was too small to be fully retained within the nanocavities of the lipid nanoparticle, yet some retention did occur. It is suggested that the partial retention could be due to a size-range of nanocavities within the nanoparticle, that allow some of the 5-mer to remain entrapped while a majority is shed during processing of the ionizable LNP. Just a slight increase in oligonucleotide size from 5 to 7 DNA bases (from ~1500Da to ~2100 Da, respectively) almost doubled the retention within the LNP. These data suggest that cargo molecular weight is a critical parameter in the retention of very short single stranded
oligonucleotides. These data mirror findings obtained for the adenosine series, and again suggest that larger molecular weight cargo is retained more completely than smaller cargo and that this is also the case for oligonucleotides. This work defines the lower limit for entrapment of single stranded DNA oligonucleotides in ionizable cationic lipid nanoparticles.

Overall, it was observed that loading and retention within ionizable systems has a strong relationship with anionic cargo charge and size. For permanently charged systems, it was found that maximum loading occurred for compounds with 2 or more strongly acidic phosphate groups. For ionizable systems, it was found that cargo loading increased with increasing negative charge or increasing cargo size. The encapsulation of Dex-21-phosphate was observed to follow said rules and therefore, it could not be encapsulated with ionizable lipids which are usually more desirable for biological applications. To remedy this, a mixture of permanently charged and ionizable lipids can be used, rescuing the encapsulation efficiency to a great extent. This strategy was also applied to encapsulate the C1 conjugate. C1 was first entrapped using two different permanently charged cationic lipids DOTMA and DOTAP. DOTMA was more efficient in encapsulating C1 than DOTAP, possibly due to different packing of the two lipids in LNPs caused by their different structures. To transition to a potent ionizable lipid system, DOTMA or DOTAP were partially replaced by DLin-MC3-DMA and reasonable encapsulation efficiencies were achieved. Further assessment in vitro hemolysis assay indicated that DOTMA containing formulations were overall less biologically compatible than those with pure DOTAP or a mixture of DOTAP and DLin-MC3-DMA. While all of C1 formulations had the LNP characteristic electron dense nanostructure core as observed by Cryo-TEM imaging, the DOTAP/DLin-MC3-DMA LNPs had the most desirable morphology and size (consistent with the results obtained
through dynamic size scattering measurements). These particles showed minimal toxicity in vivo and were able to successfully deliver the C1 conjugate to the bone.

### 2.5 Discussion and future directions

It is speculated that increased anionic charge will lead to increased hydration and molecular space-filling of the larger polyphosphates within the nanocavities of the LNP. Since electrostatic interactions are low between the anionic cargo and the ionizable cationic lipid at neutral pH (pKₐ of DODMA ~ 6.5 (Jeffs et al. 2005)), it is likely that molecular size or hydrophilicity of anionic cargo is a more dominant factor in retention than net cargo molecular charge, although the cooperative interactions between the polycharged anionic components and weakly charged ionizable cationic lipids can also contribute to drug retention. It is possible that anionic molecular cargo elements remain entrapped within nanocavities of the LNP after it is formed and their ability to expel or shed from the LNP is dependent on cargo molecular weight, and cargo charge. These results open up the ability to load a myriad of small anionic molecules into ionizable cationic lipid nanoparticles.

Overall, this study indicates that LNP are capable of encapsulation and delivery of small charged molecular weight cargo and the formulation can be easily engineered to accommodate loading of different small molecules and prodrugs. It would be of interest to further improve these formulations for increased efficiency and decrease toxicity by using other cationic lipids. The overall trend indicated that ionizable cationic lipids are inferior in trapping small molecule drugs, this however needs to be further confirmed by screening other available ionizable lipids. For therapeutic purposes, synthesis of novel cationic lipids might be required that are capable of trapping these very small charged cargoes without causing too much in vivo toxicity. To this end
methods such as that described by Bin et al. (B. Li et al. 2015) can prove beneficial and allow higher throughput lipid library design and screenings.
Chapter 3: The Effect of Helper Lipid Variation on Gene Silencing Efficiency of siRNA-LNP In Vitro and In Vivo

3.1 Synopsis

In Chapter 2 the effects the cationic lipids can have on encapsulation of small molecules are explored. For the standard siRNA-LNP systems, cationic lipid/ionizable lipids have been tested and optimized previously (Semple et al. 2010). The role and impact of helper lipid variation in these systems however, remains mostly unexplored. Helper lipids are mostly neutral lipids that have been associated with increased drug delivery efficiency in nucleic acid LNPs (Felgner et al. 1987; Mok, Lam, and Cullis 1999). DSPC is a widely used helper lipid, usually referred to as a structural lipid (Semple et al. 2001). It has been noticed that replacing DSPC by other lipids negatively impacts gene silencing in vitro (Leung 2014). In this chapter, the significance of DSPC and similar lipids that can potentially be used as helper lipids in siRNA-LNP systems is revisited. DSPC has been previously investigated in vitro in LNP systems with DLin-KC2-DMA (Semple et al. 2010) as ionizable lipid. Here, DSCP was titrated within LNP systems with DLin-MC3-DMA (Jayaraman et al. 2012) both in vitro and in vivo. While both DLin-KC2-DMA and DLin-MC3-DMA have a pKₐ of about 6.4, DLin-MC3-DMA has shown 10-fold higher efficacy in Factor VII silencing, in vivo. Surprisingly, it was observed that the more potent DLin-MC3-DMA is less sensitive to DSPC variations than was previously observed (Leung 2014). The results of this chapter indicated, perhaps more than ever, that each LNP system is unique to its cargo and composition and needs individual optimization.
3.2 Materials and methods

3.2.1 Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), egg sphingomyelin (ESM), and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) were purchased from Avanti Lipids (Alabaster, AL). 1,1’-dilinoleyl-3,3,3’,3’-tetramethyldiindocarbocyanine perchlorate (DiI) was purchased from Invitrogen. (R)-2,3-bis(stearyloxy)propyl-1-(methoxy poly(ethylene glycol)2000) carbamate (PEG-DMG) and DLin-MC3-DMA were purchased from Biofine International Inc (Vancouver, BC). Cholesterol (Chol) was obtained from Sigma-Aldrich (St. Louis, MO). Quant-iT RiboGreen reagent was purchased from Life Technologies (Burlington, ON) and cholesterol assay kit was purchased from Wako Chemicals (Richmond, VA). Triton X-100 was from Sigma-Aldrich (St. Louis, MO). Malvern Zetasizer NanoZS was used to measure particle size. 3H-cholesteryl hexadecyl ether (3H-CHE) was purchased from Perkin Elmer (Boston, MA). Trizol reagent was purchased from Life Technologies (Carlsbad, CA).

3.2.2 siRNA sequences

The sense and antisense strand siRNA sequences against GFP are 5’-aCaMGAAgCAgCAGAcUUT*T-3’ and 5’-AAGUCGMGCUGUCAMGUT*T-3’. The 2’O-methyl-modified nucleotides are represented in lower case, chimeric DNA nucleotides are underlined, and the phosphorothioate bonds are marked by asterisks. The sense and antisense strand siRNA sequences against Factor VII (siFVII) are 5’-GGAucAucucAAGucuuAcT*T-3’ and 5’-GuAAGAcuugAGAugAucT*T-3’, respectively (Akine et al. 2008, 2009; Semple et al. 2001). 2’-Fluoro-modified nucleotides are represented in lower case and phosphorothioate bonds are marked by asterisks.
linkages are represented by asterisks. siRNAs were provided by Alnylam Pharmaceuticals. The sense and antisense strand siRNA sequences against Luciferase (siLuc) are 5’-cuuAcGcugAGaAcuucGAdT*T-3’ and 5’-UCGAAGuACUCAGCGuAGdT*T-3’. The 2’-O-methyl-modified nucleotides are represented in lower case, the chimeric DNA nucleotides are underlined, and the phosphorothioate linkages are represented by asterisks.

3.2.3 Encapsulation of siRNA into LNP

Lipid stocks of DLin-MC3-DMA, Cholesterol, DSPC and PEG-DMG were co-dissolved in ethanol at appropriate molar ratios. Nitrogen-to-phosphate ratio (N/P) was kept at 3:1, and the DLin-MC3-DMA/Cholesterol molar ratio at 1.3:1 while the DSPC content was varied from 0-60 mol%. 1.5 mol% PEG-DMG and 0.2 mol% Dil were used in all formulations tested in vitro for detection purposes. In some cases, DSPC was replaced with other potential helper lipids, DOPE, ESM, or POPC. siRNA was dissolved in 25 mM sodium acetate buffer (pH = 4) and was mixed with the lipids at N/P of 3:1 by the microfluidic mixing method. All the LNPs were made using the NanoAssembler benchtop instrument (Precision Nanosystems, Vancouver, BC) at total flow rate of 12 mL/min and flow rate ratio of 3:1 aqueous: organic phases (v/v) with initial lipid concentration of 20 µg/mL. Following the mixing, the LNPs (in 25% ethanol v/v) were dialyzed against ~40000x volumes of PBS (pH = 7.4) for at least 15 h.

3.2.4 Characterization of siRNA-LNP

Following dialysis, the LNPs were sized and particle lipid concentration was measured using the cholesterol assay kit. siRNA encapsulation efficiency was measured using Quant-iT Ribogreen RNA assay by addition of final 1% Triton X-100 to LNPs and incubating at 37°C for
10 minutes. This was followed by the addition of the RiboGreen reagent and measurement of fluorescent intensity (Ex/Em: 480/520 nm). The fluorescent intensity of Triton X-100-treated samples was compared to those not treated with Triton X-100 to calculate siRNA encapsulation efficiency. Triton X-100-treated samples represent total siRNA present in the LNP solution while untreated samples represent un-encapsulated siRNA. The siRNA concentration of final LNP solution was determined by measuring absorbance at 260 nm following a lipid extraction via lipids were extracted using the Bligh and Dyer method (Bligh and Dyer 1959).

3.2.5 Cell culture, cell lines and reagents

H1299-dGFP cells were used for GFP knockout studies and were a generous donation from the BC Cancer Agency. The H1299-dGFP cells were seeded at 40,000 cells/well in 24-well plates in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. GFP expressing-DC2.4 cells were used to confirm the knockout observations made in H1299-dGFP cells. DC2.4-GFP cells were seeded at 75,000 cells/well in 24-well plates and cultured in RPMI supplemented with 10% FBS and 1% Pen/Strep. Both H1299-dGFP and DC 2.4-GFP cells were sorted to purify the GFP+ cell population and monitored through multiple passages to ensure GFP expression stability.

The ex vivo experiment was done using bone marrow cells isolated from a GFP mouse (C57BLKa-Thy1.1 with the GFP driven by a CMV-IE vector under a chicken β-actin promoter (Wright et al. 2001)) generously donated by the Rossi Lab, University of British Columbia. The bone marrow cells were isolated by placing the long bones (maximum of 2 femurs and 2 tibiae) into Eppendorf tubes, knee-end down, and centrifugation at ≥10,000 x g for 15 s. The bone marrow cells were then counted and seeded at 200,000 cells/well in 24-well plates using DMEM.
basal media supplemented with 10% FBS, and Puromycin 20 µg/mL and 1% Pen/Strep. All cells were trypsinated at time points using 0.25% trypsin/EDTA at 37°C for 4 min, resuspended in FACS buffer, centrifuged at 1000 x g for 5 min, and the pellet was resuspended in FACS buffer for downstream analysis. The FACS buffer consisted of PBS supplemented with 2% FBS, 0.1% sodium azide, 1 mM EDTA and 25 mM Hepes.

3.2.6 Flow cytometry

Cells were treated with Dil-labeled LNP encapsulating either GFP- or Luc-siRNA. Cellular uptake and GFP knockdown were then assessed using a LSRII flow cytometer and FACS Diva Software (BD Bioscience) by measuring the fluorescence intensity of DiI and GFP, respectively and analyzed using FlowJo software following acquisition of 10,000 events.

3.2.7 Factor VII gene silencing using modified siRNA-LNP

*In vivo* factor VII knockdown was tested in female CD-1 mice (from Jackson Laboratories). The mice were injected a single dose of intravenous factor VII siRNA-LNPs at different siRNA doses. Twenty-four hours following injection the mice were sacrificed and blood was collected via cardiac puncture. Serum was separated from whole blood using Microtainer Tubes with Serum Separator (Becton Dickinson, Franklin Lakes, NJ) according to manufacturer’s protocol. Serum FVII level was measured using the Biophen VII chromogenic assay (Aniara, Mason, OH) according to manufacturer’s protocol and normalized to that of control mice injected with PBS. All procedures were approved by the Animal Care Committee at the University of British Columbia and were performed in accordance with guidelines established by the Canadian Council on Animal Care.
3.2.8 Bone marrow and hepatic gene silencing using modified siRNA-LNP

C57BL/6-Tg(UBC-GFP)30SchaJ transgenic mice (from The Jackson Laboratory) were used throughout. They express enhanced Green Fluorescent Protein (GFP) under the direction of the human ubiquitin C promoter in all tissues examined (Schaefer et al. 2001). Mice were intravenously injected with siRNA-LNPs at 10 mg/kg siRNA targeting GFP (siGFP) or luciferase siRNA (siCtrl). Four days after systemic administration of siRNA-LNP mice were euthanized with a lethal dose of isoflurane.

Femurs were dissected and centrifuged at 10,000 x g in a microcentrifuge for 15 s to collect bone marrow. The bone marrow was resuspended in red blood cell lysis buffer for 1 min to deplete the red blood cells, washed with ice-cold PBS and aliquoted. One aliquot was resuspended in FACS buffer for flow cytometry analysis and the other was used for RNA isolation. Bone marrow GFP expression was determined using LSRII flow cytometer. Data were acquired using the FACSDiva software and analyzed by FlowJo software following acquisition of 10,000 events. Fluorescence intensity was normalized against the PBS treated control mice and was expressed as percent decrease of mean fluorescence intensity. For RNA isolation, bone marrow cells were treated using PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA) according to manufacturer’s protocol. Extracted RNA was used to determine GFP, and house-keeping genes Hprt and Tbp mRNA levels. For qPCR analysis, sample RNA was reverse transcribed to cDNA from total RNA from each sample, using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). RT-PCR was performed on a StepONE Plus Real Time PCR system (Applied Biosystems, Austin, TX) using TaqMan Fast Advanced Master Mix (Applied Biosystems, Austin, TX) as well as the forward (GAACCGCATCGAGCTGAA) and reverse primers (ATCGACTTCAAGGAGGACGGCAAC)
designed by Integrated DNA Technologies’ (IDT, San Diego, CA) Scientific Applications Support Team. For determination of Hprt and Tbp expression levels, primers predesigned by IDT were used. The fluorescein amidite (FAM)-labeled probes for the target genes were purchased from IDT. Relative gene expression levels were quantified using the comparative ΔΔCT algorithm and the results were normalized to the housekeeping genes Hprt or Tbp. The mRNA of siGFP treated samples was compared to siCtrl treated mice.

In addition to bone marrow, livers were also harvested and a part of the caudal lobe was dissected and flash-frozen by dipping in liquid nitrogen, and stored at -80°C. RNA was extracted from liver by addition of 1ml of ice-cold Trizol and homogenization using the FastPrep Homogenizer (MP Biomedicals, Solon, OH) at 5.5 speed for two 15 s intervals. The lysate was transferred into an RNase–free tube followed by addition of chloroform. 100% isopropanol was added to the aqueous phase and the mixture was centrifuged to precipitate the RNA which was eluted using the PureLink RNA Mini Kit protocol and cDNA synthesis was performed by qPCR as discussed before. All procedures were approved by the Animal Care Committee at the University of British Columbia and were performed in accordance with guidelines established by the Canadian Council on Animal Care.

3.2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism. One Way ANOVA test was performed followed by Tukey’s multiple comparison test to assess the significance of differences between different groups. Probability (p) values less than 0.05 were considered significant.
3.2.10 Pharmacokinetics of modified siRNA-LNP

LNPs were labeled with $^{3}$H-CHE and intravenously injected to female CD-1 mice (Jackson Laboratories). Mice were euthanized 30 minutes to 8 hours post-injection and blood, liver, and spleen were harvested. Liver (about 600 g portion/mouse) and spleen were homogenized using the FastPrep homogenizer from MP Biomedicals (Santa Ana, CA) and incubated with 0.5 mL of Solvable (Perkin Elmer) at 50°C overnight. 200 µL of 30% (v/v) hydrogen peroxide. For blood LNP concentration measurement, 100 µL of whole blood was added directly to solvable and hydrogen peroxide, and incubated at 50°C overnight. 5 mL of Pico-Fluor Plus scintillation fluid (Perkin Elmer) was added to all tissue samples and the radioactivity was measured on a Beckman LS 6500 liquid scintillation counter. All procedures were approved by the Animal Care Committee at the University of British Columbia and were performed in accordance with guidelines established by the Canadian Council on Animal Care.

3.3 Results

3.3.1 High DSPC content in siRNA-LNP has minimal effect on gene silencing in lung cancer and dendritic cell lines, but decreases knockdown in primary bone marrow cells

Conventional siRNA-LNP with 10 mol% DSPC are highly efficacious for in vitro transfection and gene silencing. in vitro testing thus provides a relatively high throughput method to assess formulation uptake and gene silencing potency. For this purpose, H1299-dGFP cells were chosen as a cell line with destabilized GFP which allows for rapid turnover rate and thus a quicker silencing readout. The cells, were initially sorted to purify the GFP$^+$ population and were monitored over multiple passages for GFP expression homogeneity. The GFP$^+$ cells were then
treated with a variety of LNP each with a different helper lipid content. The DSPC content of the LNP was titrated from 0-60% while keeping the N/P constant at 3:1. N/P is indicative of the cationic lipid/nucleic acid ratio and is typically kept at 3 for siRNA formulations. DSPC was added at the expense of cholesterol and cationic lipid while keeping their molar ratio constant at 1.3:1, and the particles were labeled with DiI, a fluorescent dye for detection purposes and the PEG-DMG content was kept at 1.5 mol%. The resulting siRNA-LNP were efficacious at in vitro GFP silencing regardless of their DSPC content with a slightly lower knockdown for 0% DSPC formulation (Figure 3.1A). However, cellular uptake—normalized to siRNA dose—was significantly lower for higher DSPC-content LNP (Figure 3.1B). These results indicated that siRNA-mediated gene silencing was not significantly affected in siRNA-LNP formulations, while cellular uptake for higher DSPC LNPs was poor. This is possibly because the cationic composition of these LNPs (DLin-MC3-DMA) is potent enough to compensate for the effects of helper lipid variations in vitro.

In order to further investigate how particles are impacted by their type and amount of helper lipid, DSPC was replaced by three other lipids and the effect on in vitro cellular uptake and gene knockdown was studied. DSPC was replaced by DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), ESM (egg sphingomyelin), or POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and the helper lipid content for all formulations was tested at 10 and 40 mol% (Figure 3.2). At 10 mol% helper lipid, DSPC-, DOPE-, and ESM-containing formulations performed similarly and could all efficiently knockdown GFP in the H1299-dGFP cells following 24 hours of incubation (Figure 3.2A). GFP silencing was however partially hampered for the POPC-based siRNA-LNP at the lower siRNA treatment dose of 0.05 µg/mL. This effect was more pronounced in the 40 mol% POPC particles where—even at 1 µg/mL siRNA treatment
dose—GFP knockdown was very minimal. No silencing effect was noticed for this formulation at 0.05 and 0.2 µg/mL and only 50% silencing was observed at 1 µg/mL siRNA (Figure 3.2A). At higher helped lipid concentrations, DOPE-based formulation was also less efficient in in vitro gene knockdown, especially at lower siRNA doses. Interestingly, LNP uptake was also reduced for the 10 mol% POPC formulation as well as the 40% DSPC and DOPE (Figure 3.2B). The 40% ESM LNP uptake however, was not affected (Figure 3.2 B). This suggested that ESM could be a good candidate to replace DSPC in siRNA-LNP.

The experiments presented in Figures 3.1 and 3.2 were both performed in H1299-dGFP cells, a cancer cell line with a high cell proliferation rate. The high proliferation rate of cells assists with particle uptake and therefore facilitates siRNA-mediated gene silencing. Thus, an in vitro gene silencing experiment was performed in non-cancer cells to study the outcome of helper lipid variation. The 10 and 40 mol% DSPC siRNA-LNP formulations were tested in a dendritic cell line (DC2.4-GFP) and also in primary bone marrow cells isolated from a GFP mouse (Figure 3.3). The cells were treated with siRNA LNPs at higher doses (0.1 - 10 µg/mL) for up to 72 hours. The higher treatment dose and longer treatment period were necessary to ensure gene silencing in slow-to-non proliferating (DC2.4-GFP) and primary bone marrow cells. The results indicated no significant difference in GFP knockdown between the 10 and 40 mol% DSPC formulations in the dendritic cell-line (Figure 3.3A), or bone marrow primary cells ex vivo (Figure 3.3B).
Figure 3.1. Effect of DSPC titration on GFP knockdown and LNP uptake in H1299-dGFP cells.

LNP DSPC content was varied from 0-60 mol% while cationic lipid/cholesterol molar ratio was kept at 1.3:1, PEG-DMG at 1.5 mol% and DiI at 0.2 mol%. H1299k-dGFP cells were treated with DiI fluorescently-labeled LNPs at 0.05, 0.2 and 1 µg/mL siRNA for 24 h. The cells were
then trypsinized and subjected to flow cytometry to measure GFP and Dil signals. The fluorescent signals were compared to the siLuc control-LNP (with the standard 10 mol% DSPC formulation)-treated cells. (A) GFP knockdown was not greatly effected by varying the DSPC content. (B) LNP uptake values are presented based on lipid dose and were normalized against 10% DSPC LNP formulation. Uptake was significantly lowered for LNPs with DSPC contents higher than 30 mol%, this however did not seem to affect gene silencing at the siRNA doses tested. Error bars represent standard deviation, n = 3.
Figure 3.2. Effect of DSPC replacement with other helper lipids on GFP knockdown and LNP uptake in H1299-dGFP cells.

siRNA-LNP was formulated by replacing DSCP with DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), ESM (egg sphingomyelin) or POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-
phosphocholine) at 10 or 40 mol% helper lipid while cationic lipid/cholesterol molar ratio was kept at 1.3:1, PEG-DMG at 1.5 mol% and DiI at 0.2 mol%. H1299k-dGFP cells were treated with DiI fluorescently labeled LNPs at 0.05, 0.2 and 1 µg/mL siRNA for 24 h. The cells were then trypsinized and subjected to flow cytometry to measure GFP and DiI signals. The fluorescent signals were compared to the siLuc control-LNP (with the standard 10 mol% DSPC formulation)-treated cells. (A) Structures of helper lipids used in this study. (B) GFP knockdown efficiency was significantly lower across all siRNA doses when DSPC was replaced with POPC, and when DSPC was replaced with DOPE at the lowest siRNA dose treatment. (C) POPC-LNP uptake was significantly lower than the other helper lipids tested. Uptake is normalized to the 10 mol% DSPC baseline. Error bars represent standard deviation, n = 3.
Figure 3.3. The effect of DSPC content increase on GFP knockdown in non-cancer cells.

(A) 0, 10 and 40 mol% DSPC-LNP containing GFP-siRNA were tested in DC2.4-GFP dendritic cells at different siRNA doses. The GFP signal was measured via flow cytometry following 48 h
of incubation with LNP. GFP knockdown was normalized to siLuc-LNP treated cells (B) 10 and 40 mol% DSPC-LNP, fluorescently labeled and containing GFP-siRNA, were tested on primary GFP-mouse bone marrow cells. GFP signal was measured using flow cytometry and was normalized against the siLuc-LNP control group. (C) The LNP uptake of cells in B was also measured using flow cytometry. The 10 mol% DSPC-LNP is assumed to have 100% uptake and the 40 mol% DSPC formulation is compared to this baseline. Error bars represent standard deviation, n = 3.

3.3.2 High DSPC content negatively impacts hepatic Factor VII gene silencing in vivo

After in vitro testing of helper lipid variations in siRNA-LNPs, some variations were tested in a well-established in vivo hepatic gene knockdown model. For this purpose, wild type CD-1 mice were injected with factor VII (FVII) siRNA formulations containing different helper lipid content and their knockdown efficacy was tested by measuring the blood serum FVII levels using a chromogenic assay (Figure 3.4A). The results indicated that formulations containing 10 mol% helper lipid were more efficacious in hepatic knockdown of FVII compared to 40 mol% helper lipid. The 40 mol% DSPC formulation silencing effect was also tested with equimolar cholesterol content (MC3/Chol/DSPC/PEG-DMG, 18.5/40/40/1.5 mol% at N/P = 3) (Figure 3.4A). This was to determine whether increasing the cholesterol content can rescue high DSPC-LNP hepatic gene silencing. However, FVII knockdown efficiency stayed at basal level for high DSPC content, regardless of LNP-cholesterol concentration. Moreover, pharmacokinetics-biodistribution studies performed with radiolabeled CHE (Cholesteryl-1,2-3H(N)) indicated that
10 and 40 DSPC mol% LNP had similar blood circulations (Figure 3.4B) and liver accumulation profiles (Figure 3.4C) and that both are rapidly cleared from the circulation.

Figure 3.4. *In vivo* investigation of helper lipid variations containing Factor VII siRNA.

siRNA-LNPs were formulated using the microfluidic mixing method using variable helper lipid types and concentrations. The resulting LNPs were then injected intravenously into mice for
further assessment. (A) Different variations of helper lipids were tested for hepatic knockdown in vivo efficacy and the results indicated that lower DSPC content LNPs were superior in FVII knockdown. Formulations tested: MC3/Chol/DSPC-or-ESM/PEG-DMG at 50/38.5/10/1.5 or 33/25/40/1.5 mol%, and MC3/Chol/DSPC/PEG-DMG at 18.5/40/40/1.5 mol%, N/P = 3. (B) Pharmacokinetics of 0, 10, and 40 mol% DSPC-LNP was studied by Cholesteryl-1,2-3H(N) labeled LNP and the results indicated a similar pharmacokinetics profile at 0.3 mg/kg siRNA across all formulations. C) 0, 10, and 40 DSPC mol%-LNP had comparable liver and spleen accumulation as detected by Cholesteryl-1,2-3H(N) labeled LNP at 0.3 mg/kg siRNA. Error bars represent standard deviation, n = 3. **** p<0.0001 compared to the standard 10 mol% DSPC-LNP, a One-Way ANOVA test was performed followed by Tukey’s multiple comparison test.

3.3.3 High DSPC siRNA-LNP performs similar to 10 mol% DSPC formulations in bone marrow gene silencing but is significantly less efficient in hepatic GFP silencing in vivo

To further study the in vivo effects of helper lipid variation, siRNA-LNP-mediated gene silencing was assessed in a GFP mouse model using 10 and 40 mol% DSPC formulations. The GFP-expressing mice were injected intravenously with siGFP-LNP and GFP protein and mRNA levels were measured in the bone marrow 4 days following systemic administration. GFP protein level was measured by flow cytometry analysis of bone marrow cells, while GFP mRNA levels were determined for bone marrow and liver cells using qPCR. FACS analysis of bone marrow indicated only marginal GFP protein level reduction regardless of treatment with LNP-DSPC content (Figure 3.5A). Also, administration of siRNA-LNP did not alter the expression of
the housekeeping genes, indicating the absence of siRNA-mediated off-target effects (Figure 3.5B).

![Figure 3.5. LNP-siRNA containing 10 or 40 mol% DSPC induces GFP silencing in bone marrow.](image)

LNP-siRNA targeting GFP composed of 10 and 40 mol% DSPC, or LNP-siCtrl with 10 and 40 mol% DSPC (10 mg/kg total siRNA injected), or PBS were administered intravenously to GFP
expressing mice. 4 days after injection, bone marrow was harvested and GFP protein levels were measured by flow cytometry. GFP, Hprt and Tbp mRNA levels were also determined following RNA extraction from bone marrow cells as described in methods. Bar graphs represent (A) GFP protein level in bone marrow measured by flow cytometry and GFP mRNA level was measured by qPCR. (B) Tbp mRNA levels relative to Hprt. GFP expression levels of were normalized to siCtr injected mice. Error bars represent standard deviation (±) of 3 animals. *: $P > 0.05$ was considered not significant (ns).

In addition to bone marrow, liver GFP silencing was studied upon systemic administration of siRNA-LNP to GFP mice. When GFP mRNA was measured in the liver (4 days post injection), it was found that siRNA could significantly reduce GFP expression when delivered with 10% or 40% DSPC LNPs (Figure 3.6A, $P < 0.001$). LNP-siRNA composed of 10 mol% DSPC was however, significantly more efficient at GFP knockdown than 40 mol% DSPC LNP (Figure 3.6 A). These findings corroborate the observations of the FVII assay (Figure 3.4). Administration of siCtrl or siGFP did not alter the expression of Tbp housekeeping gene, ruling out any siRNA-mediated off-target effects (Figure 3.6B). It appears that increasing the concentration of the helper lipid DSPC reduces the LNP-siRNA-mediated silencing in liver targets while not having a significant effect on bone marrow targeting and gene silencing.
Figure 3.6. LNPs composed of 40 mol% DSPC exhibit reduced activity in liver gene silencing.

LNP-siRNA targeting GFP composed of 10 and 40 mol% DSPC, or LNP-siCtrl 10 and 40 mol% DSPC, or PBS were administered intravenously into GFP expressing mice at 10 mg/kg siRNA. Following 4 days, livers were harvested and GFP mRNA levels were determined by qPCR. Bar graphs represent expression of (A) GFP and (B) Tbp mRNA relative to Hprt. GFP expression
levels of mice injected with siCtr were considered 100%. Bars represent mean of n = 3 mice. Error bars represent standard deviation **: P < 0.01, **** P < 0.0001.

3.3.4 High DSPC siRNA-LNP shows unusual hybrid structures when imaged by Cryo-TEM

Cryo-TEM imaging of the 40% DSPC LNPs indicated a very different morphology to that of a typical 10% DSPC LNP. The abundance of DSPC seems to cause formation of hybrid-multilamellar structures inside the LNPs (Figure 3.7). The presence of these multilamellar structures can affect the uptake of LNP and/or release of cargo and thus reduce potency, leading to hampered gene silencing in liver cells.

Figure 3.7. LNP-siRNA systems containing high levels of DSPC exhibit unusual hybrid lipid structures.

The structure of LNP siRNA systems containing (A) 10 mol% DSPC (DLin-MC3-DMA/DSPC/cholesterol/PEG-lipid; 50/10/38.5/1.5) or (B) 40 mol% DSPC (DLin-MC3-
DMA/DSPC/cholesterol/PEG-lipid; 33/40/25.5/1.5) and loaded with siRNA (N/P = 3). The high DSPC content leads to a multilamellar morphology which differs from the typical solid core structure observed for the standard siRNA-LNP systems.

### 3.4 Conclusion

In this chapter the effect of helper lipid on siRNA-LNP mediated-gene silencing was explored by varying the helper lipid content and type in the nanoparticle formulation and testing particle uptake and silencing efficacy both *in vivo* and *in vitro*. As mentioned earlier, the gold standard for nucleic acid delivery, specifically siRNA, has been a formulation with cationic lipid, cholesterol, a helper lipid (commonly DSPC), and a PEG lipid at 50/38/10/2 mol%. The role of helper lipid in siRNA-LNP efficacy and structure has been relatively un-explored. A better understanding of helper lipid role can provide insights on siRNA-LNP formulation improvement and potentially specializing the LNP for more specific targeting purposes.

To better understand the effect of DSPC variation, DSPC content was first titrated from 0 to 60 mol%. The addition of DSPC was done at the cost of both cholesterol and cationic lipid to keep this ratio constant. Moreover the N/P was kept at 3 for all formulations to ensure a relatively neutral particle charge and high encapsulation efficacy. It was observed that LNP-mediated knockdown was slightly reduced for LNP lacking the helper lipid DSPC. However, even at 0 mol% DSPC, over 50% gene knockdown was observed for the H1299-dGFP cells and it was concluded that 24 hours of incubation with the LNPs was sufficient to generate an efficient gene silencing response from these cells. Interestingly, LNP uptake did not correlate with the knockdown results, indicating a severe drop for LNPs containing more than 30 mol% DSPC. Despite lower cellular uptake, these LNPs are still capable of highly efficient gene silencing and are perhaps just more efficient in lysosomal escape and can compensate for lower
cellular uptake. To further investigate, DSPC was replaced by three other helper lipids, POPC, DOPE and egg sphingomyelin (ESM) at 10 and 40 mol%. Overall, POPC performed poorly in gene silencing compared to DSPC, DOPE and ESM in both 10 and 40 helper lipid-mol% formulations. DOPE was also inefficient at 40 mol%, however the 10 mol% DOPE formulations had comparable silencing efficacy to 10 mol% DSPC and ESM formulations. Cellular uptake at 40 mol% helper lipid was significantly reduced with the exception of 40 mol% ESM (and 40% mol DOPE), hinting that ESM might be a more potent helper lipid for siRNA-mediated gene silencing. Overall, it was observed that while in vitro gene silencing was mostly unaffected by DSPC titration or replacement (with the exception of POPC), cellular uptake was significantly reduced at higher helper lipid content.

H1299-dGFP cells, tested above, are from a cancer cell line with a high proliferation rate which typically facilitates LNP uptake. Therefore it was important to also test the formulations on slower-growing cells to extend the previous observations. For this purpose, the 0, 10, and 40 mol% DSPC LNPs were tested in a dendritic cell line (DC2.4-GFP), this time with a longer time point to allow for the slowly propagating cells to take-up LNPs. The results of treatments indicated no significant difference in gene silencing among the three formulations. The 10 and 40 mol% DSPC formulations were also tested in an ex vivo setting, where a GFP-mouse bone marrow cells were isolated and treated with the formulations over the course of 72 h. This ex vivo experiment confirmed the observation made for the dendritic cells. No significant difference in gene silencing was observed for the 10 and 40 mol% DSPC LNPs. The cellular uptake also was very similar between the 10 and 40 mol% DSPC formulations. These results, put together with the observations made in the cancer cell line (H1299-dGFP), indicated that LNPs have a relatively high tolerance for helper lipid variation in an in vitro setting. This might at first seem
contradicting to a previous in vitro study on helper lipid affect on siRNA-LNP gene silencing which suggested that replacement of DSPC with other helper lipids rendered the LNP inactive (Leung 2014).

The most significant trend observed was the inability of POPC to function as an effective helper lipid. DOPE has been previously shown to be inefficient as helper lipid for gene silencing in siRNA LNP systems (Leung 2014). However, the findings presented in this chapter contradicted previous results showing that DOPE-LNPs are in fact capable of efficient in vitro gene silencing at least at the normal 10 mol% helper lipid content. This contradiction is perhaps explained by the different cationic lipid used in the previous reports. DLin-KC2-DMA used in the mentioned studies is an overall less optimized cationic lipid compared to DLin-MC3-DMA used in this chapter and it is likely that the presence of a highly optimized cationic lipid can help desensitize the LNP to some level of helper lipid variation. DOPE inferiority becomes more evident at high helper lipid content however (Figure 3.2), rendering the 40 mol% DOPE-LNP less efficacious in gene silencing compared to 40 mol% DSPC. This most likely indicates that there is still a limit to helper lipid variation-tolerance even for the more efficient cationic lipids, and also does align with the previous studies proving that DOPE is overall not a suitable helper lipid for siRNA-LNP.

Sphingomyelin (ESM) proved to be as efficient as DSPC for in vitro gene silencing. Unlike the DOPE and POPC formulations, at both 10 and 40 mol% helper lipid concentrations GFP knockdown was comparable between DSPC and ESM. However, unlike DSPC-LNP, ESM-LNP uptake did not decrease at 40 mol% helper lipid content. This suggested that perhaps ESM could be a more efficacious helper lipid for siRNA-LNP. Therefore, the 10 and 40 mol% ESM-LNP were tested in vivo in a hepatic gene silencing assay along with 10 and 40 mol% DSPC-
LNP. 10 mol% ESM-LNP was as efficient as the 10 mol% DSPC-LNP in Factor VII gene silencing, however both ESM and DSPC formulations were incapable of efficient Factor VII gene silencing at 40 mol%. This result was surprising because ESM had an improved cellular uptake at 40 mol% but the in vivo silencing did not improve at 40 mol% helper lipid by ESM replacement for DSPC.

Since the 40 mol% helper lipid formulation is relatively efficient for in vitro gene silencing but not for in vivo hepatic gene silencing, it was hypothesized that high helper lipid content might be more efficient for extra-hepatic gene silencing. Thus the 40 mol% DSPC was tested in a GFP expressing mouse and GFP knockdown was assessed in liver and bone marrow. The results indicated no change in protein levels in bone marrow and about 30% reduction in GFP mRNA levels for both 10 and 40 DSPC mol% formulations. However, statistic analysis showed these comparisons to be non-significant. Consistent with previous results of the Factor VII study the 40 mol% formulation was less efficacious at hepatic gene silencing. Gene silencing was, however, overall more effective in the GFP mouse experiment, most likely due to the high dose of injection (15 vs 0.3 mg/kg siRNA injected for siGFP vs siFac VII).

Overall it was observed that hepatic silencing was greatly impacted by high DSPC levels while bone marrow was not. Bone marrow GFP silencing was still quite inefficient even at 10 mg/kg siRNA. The absence of GFP protein level change could potentially be due to the low turn-over rate of GFP protein (unlike in the H1299-dGFP cells, the mouse model GFP was not destabalized which leads to potentially longer turn-over rate for the protein). However, the mRNA levels were still quite high despite the very high administered dose of siRNA and did not differ drastically from the GFP protein levels. Moreover, the GFP expression in the liver does
not discriminate among different cells which may vary in terms of GFP expression as well as with regard to uptake and cytosolic presence of the siRNA. A study that correlates better the delivery and efficacy in different cellular components of the liver may help explain the lack of the LNP-siRNA efficacy in the hepatocytes when 40% helper lipid LNPs are used to deliver the siRNA. These results indicate that simply by changing LNP helper lipid concentration, LNP efficacy in the liver can be drastically affected. This modification however, does not significantly impact bone marrow gene silencing, suggesting that perhaps by fine-tuning LNP composition—specifically the helper lipid type or concentration—extra-hepatic delivery can be improved.

3.5 Discussion and future directions

DSPC has perhaps not received a lot of attention in the context of cationic lipid-nucleic acid LNPs. The differential gene silencing effect of in liver and bone marrow for the 10 and 40 mol% DSPC suggests that this lipid might be able to assist with the extra-hepatic gene delivery via LNPs. However, the inferior hepatic gene silencing in the high helper lipid-content LNP will need to be further assessed by allowing a longer incubation time in vivo. It is possible that high DSPC LNPs are just slower at releasing their cargo and need more time to show equal hepatic gene silencing as observed for 10 mol% DSPC LNP. Another interesting study would be to further investigate higher mol% ESM particles for both hepatic and extra-hepatic gene silencing. The in vitro uptake of these particles was not impaired unlike high DSPC particles but in the FVII assay they did not perform as well as the 10 mol% helper lipid LNPs. The high ESM particles can potentially also benefit from longer incubation and also need to be tested at higher doses for both hepatic and extra-hepatic knockdown. Further investigations into high DSPC and
ESM LNPs can bring the field closer to better understanding of the role of helper lipids in siRNA-LNP gene silencing and open new doors into tissue targeted gene delivery.
**Chapter 4. Summarizing Discussion**

Small molecule drugs have been traditionally entrapped in lipid nanoparticles by means of establishing a proton gradient across lipid bilayer membranes. This method has been successful for amphipathic weak bases, such as doxorubicin into liposomes with acidic interior (Mayer et al. 1986). Weak bases are capable of crossing the lipid bilayer in their deprotonated state, typically at neutral pH. The acidic interior of the liposome protonates and, therefore, locks the drug inside. Similar principles also apply for another common method that uses ammonium sulfate to establish the transmembrane pH gradient (Bolotin et al. 1994). As effective as this remote loading method can be for weak base drug molecules, weak acids cannot be as easily entrapped using this method. A more modern and well-known entrapment method is the cationic lipid loading of negatively charged nucleic acids, such as plasmid and siRNA through microfluidic mixing. In this method the cationic lipid, bearing a positive charge at acidic pH, interacts with the nucleic acid molecules during the microfluidic mixing process, spontaneously forming nanoparticles. This method, however, has been mostly optimized for larger nucleic acid molecules, such as siRNA (Belliveau et al. 2012; Leung et al. 2012) and plasmid (J. A. Kulkarni et al. 2016). Microfluidic mixing has also been leveraged to entrap hydrophobic small molecule drugs in emulsion-like lipid nanoparticles (Zhigaltsev et al. 2012, 2015). Nevertheless, the encapsulation of small negatively charged cargo has not been yet been explored using this method.

The studies described in the second chapter of this thesis aimed to look into the possibility of using the microfluidic mixing technology to encapsulate small negatively charged cargo using cationic lipids. Of the multiple small molecules tested in this chapter, those with larger negative size and/or molecular weight were most efficiently entrapped in cationic lipid
LNPs. It was also found that permanently charged cationic lipids were more efficacious in entrapping the charged small molecules.

Since the traditional remote loading was not sufficient for the retention of our weak acid small molecule drug (EP4-A and its analogs), and the hydrophobic prodrug form was highly unstable, a bisphosphonate conjugate was tested for encapsulation with cationic lipids via microfluidic mixing. The idea of the bisphosphonate conjugate encapsulation came from studies that were performed to investigate small charged molecule encapsulation into cationic LNP. In Chapter 2, it was attempted to encapsulate a variety of small negatively charged molecules to determine the size cut-off for efficient encapsulation in cationic LNP. siRNA molecules encapsulated using this method usually have a molecular weight of ~16000 g/mol with about 50 negative charges. The molecules tested in Chapter 2 are at maximum of 4000 g/mol and 13 negative charges (Table 1.2). Regardless of the cationic lipid used in the LNP, it was observed that larger molecules or those with a denser negative charge were more easily entrapped. Going back to the structure of the LNP proposed by Leung et al. 2012, the cationic lipid forms inverted micelle structures around the siRNA molecules forming complexes that come together to form the nanoparticle.

One hypothesis regarding the small cargo encapsulation is that smaller molecules can escape the LNP lipid complexes more easily due to their small size and, therefore, are more difficult to entrap. This small molecule escape can be partially compensated for by having a larger negative size on smaller molecules. As observed in Chapter 2, while AMP could not get entrapped in the conditions tested, addition of one phosphate group helped, and as the phosphates increased—all the way to five groups—a gradual increase in encapsulation efficiency was observed. A similar trend was also observed for the single stranded poly-adenosine
oligonucleotides. With a string of 5 adenines about 30-50% encapsulation was observed, but this was significantly improved for the 7-mer poly-A and again, a gradual increase of encapsulation efficiency was observed for longer strings. While A5pA and the 5-mer poly-A oligonucleotide have the same number of phosphate groups—and despite the larger size of the 5-mer poly-A—A5pA is more readily entrapped. This indicates that both charge and size play a role in encapsulation of small cargo when it comes to ionic interaction-based drug loading. Smaller molecules with higher charge density can form stronger interactions with the positively charged lipid interactions and therefore, can be potentially easier to encapsulate in cationic LNP systems.

Another important observation from Chapter 2 was that the permanently charged cationic lipid DOTMA was more efficient in entrapping small charged cargo compared to the ionizable lipid DODMA. The smaller/less charged molecules might require either an entirely permanently charged cationic lipid LNP or, at the very least, a combination of permanently charged and ionizable lipids. For example, dexamethasone 21-phosphate could not be encapsulated at all when the LNP was composed entirely of ionizable lipid. But when permanently charged and ionizable lipids were mixed at a 1:1 molar ratio to form the cationic component of the LNP, the encapsulation was rescued, to about 65%. This format can be used to entrap other difficult-to-encapsulate small molecule drugs. However, it is important to note that—as it was observed in this chapter—every small molecule is unique and might require optimization on the drug molecule and the LNP composition details.

The role and significance of cationic lipids in LNPs has been extensively studied before (Leung et al. 2015; Maurer et al. 2001; Heyes et al. 2005; Jayaraman et al. 2012; Maier et al. 2013; Semple et al. 2010). The type and amount of polyethylene glycol has also been studied and shown to have a significant impact on LNP size and function (Chen et al. 2016; Tam et al. 2000;
Monck et al. 2000). While the importance of tailoring the LNP composition to the cargo of choice and the significance of the cationic lipid choice on siRNA-LNPs do not go unnoticed, there is still work to be done to better understand the role of helper lipids in efficacy of LNPs as drug carriers. Chapter 3 looks at the effect of DSPC titration and replacement both in vitro and in vivo. It was previously reported that the absence of DSPC or its replacement by DOPE can worsen the gene silencing potency of siRNA-LNPs (Leung 2014). When titrated in LNP systems with DLin-MC3-DMA as cationic lipid, however, it was observed that the loss of DSPC did not greatly impact the in vivo hepatic gene silencing of FVII. While the lack of DSPC showed no impact, its presence at high molar ratio (40%) dampened hepatic FVII gene silencing. The same trend was observed for ESM as a DSPC replacement. Even though the in vitro LNP uptake with 40% ESM was not as greatly impacted as it was for the 40% DSPC, both formulations behaved similarly in vivo and failed at hepatic gene silencing, suggesting that perhaps the hepatic in vivo uptake of the two does not significantly differ.

Microscopy imaging of the 40% DSPC LNPs revealed atypical hybrid multilamellar structures. This is in contrast to what is currently known as the solid core (electron-dense) structure for the gold standard siRNA-LNP formulations (Leung et al. 2012), and can potentially explain the lower efficiency of the high-DSPC LNPs in hepatic gene silencing. The morphology observed here resembles that of LNPs formulated with high nucleic acid/cationic lipid ratios (1.5:1 or higher) (Kulkarni et al. 2018), however the 40% DSPC LNPs discussed in this thesis contain the more clinically relevant N/P ratio of 3:1 where there is an excess of cationic lipid compared to the amount of nucleic acid present. More work needs to be done on these systems to determine how this morphology affects the potency of the particle and if it is responsible for lack of hepatic gene silencing potency. It is also of interest that gene knockdown in bone marrow was
not as dramatically impacted by the increase in DSPC content as it was in the liver, suggesting that through in-depth optimization, the LNPs can be tailored a lot more specifically to the tissue/organ of target.

The findings in this thesis reveal a new format of drug encapsulation for small molecule drugs and highlights the importance of particle optimization based on the drug and tissue of target. These findings point to new possibilities for developing nanoparticle therapeutics.
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