Title
Overcoming cellular and tissue barriers to improve liposomal drug delivery

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Overcoming cellular and tissue barriers to improve liposomal drug delivery

by

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DISSERTATION

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DOCTOR OF PHILOSOPHY

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GRADUATE DIVISION

of the

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AND
Dedication

For my Mother for her sacrifice, never ending love, and motivation.

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Abstract

Forty years of liposome research have demonstrated that the anti-tumor efficacy of liposomal therapies is, in part, driven by three parameters: 1) liposome formulation and lipid biophysics, 2) accumulation and distribution in the tumor, and 3) release of the payload at the site of interest. This thesis outlines three studies that improve on each of these delivery steps. In the first study, we engineer a novel class of zwitterlipids with an inverted headgroup architecture that have remarkable biophysical properties and may be useful for drug delivery applications. After intravenous administration, liposomes accumulate in the tumor by the enhanced permeability and retention effect. However, the tumor stroma often limits liposome efficacy by preventing distribution into the tumor. In the second study, we demonstrate that depletion of hyaluronan in the tumor stroma improves the distribution and efficacy of Doxil® in murine 4T1 tumors. Once a liposome has distributed to the therapeutic site, it must release its payload over the correct timescale. Few facile methods exist to quantify the release of liposome therapeutics \textit{in vivo}. In the third study, we outline and validate a simple, robust, and quantitative method for tracking the rate and extent of release of liposome contents \textit{in vivo}. This tool should facilitate a better understanding of the pharmacodynamics of liposome-encapsulated drugs in animals. This work highlights aspects of liposome behavior that have prevented successful clinical translation and proposes alternative approaches to improve liposome drug delivery.
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Abbreviations

Tumor Biology
EPR effect – Enhanced permeability and retention effect
ECM – Extracellular matrix
IFP – Interstitial fluid pressure
HA – Hyaluronan
RHAMM – Hyaluronan-mediated motility receptor
HAS2 – Hyaluronan synthase 2
HAS3 – Hyaluronan synthase 3
RES – Reticuloendothelial system

Lipids
PC – Phosphatidylcholine
PE – Phosphatidylethanolamine
T_m – Phase transition temperature
HSPC – L-α-phosphatidylcholine hydrogenated
POPC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
DPPG – 1,2-dipalmitoyl-sn-glycero-3-phospho-(1’-rac-glycerol)
DiD – 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine
PEG – Poly(ethylene glycol)
DSPE-PEG – 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
ABC effect – Accelerated blood clearance effect
CPL – Liposomes composed of conventional phospholipids and cholesterol
C_{12} PC – 1,2-dilauroyl-sn-glycero-3-phosphocholine
C_{14} PC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine
C_{16} PC – 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
C_{18} PC – L-α-phosphatidylcholine hydrogenated
C_{20} PC – 1,2-diarachidoyl-sn-glycero-3-phosphocholine
C_{22} PC – 1,2-dibehenoyl-sn-glycero-3-phosphocholine
C_{18:1} PC – 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
DOPC – 1,2-dioleoyl-sn-glycero-3-phosphocholine
SML – sterol-modified lipids
DCHEMSPC – 1,2-dicholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{12} SML – 1-lauroyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{14} SML – 1-myristoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{16} SML – 1-palmitoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{18} SML – 1-stearoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{20} SML – 1-rachidoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{22} SML – 1-behenoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
C_{18:1} SML – 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine
Methylumbelliferone and derivatives and liposomal agents
MU – 4-methylumbelliferone
MU-P – Methylumbelliferyl phosphate
L-MU-P – Liposome encapsulated MU-P
MU-S – Methylumbelliferyl sulfate
MU-G – Methylumbelliferyl glucuronide
CF – carboxyfluorescein

Chemicals and reagents
DCM – Dichloromethane
DMAP – Dimethylaminopyridine
DCC – N,N’-Dicyclohexanecarbodiimide
MTT – (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ITS – Insulin, transferrin, and sodium selenite
CMC – Carboxymethylcellulose
PBS – Phosphate buffered saline
BSA – Bovine serum albumin
FITC – Fluorescein isothiocyanate
DPH – 1,6-diphenyl-1,3,5-hexatriene
EDTA – Ethylenediaminetetraacetic acid

Assays
DSC – Differential Scanning Calorimetry
HPFC – High performance flash chromatography
MALDI-TOF – Matrix-assisted laser desorption/ionization – time of flight
TLC – Thin-layer chromatography
ELISA – Enzyme-linked immunosorbent assay
RT-PCR – Reverse transcriptase polymerase chain reaction
DNA – Deoxyribonucleic acid
cDNA – Complementary DNA
IACUC – Institutional Animal Care and Use Committee
HPLC – High-performance liquid chromatography
FRET – Fluorescence resonance energy transfer

Proteins
SIRP α – Signal regulatory protein-α
B-actin – Beta actin
GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
yCD – Yeast cytosine deaminase
mKate – Monomeric Katushka

Units and measures
bp – base pair
g – gravity
g – gram
mg – miligram
μg – microgram
M – molar
mM – milimolar
μM – micromolar
nM – nanomolar
mm – milimeter
μm – micrometer
nm – nanometer
L – liter
mL – mililiter
μL - microliter
IC50 - inhibitory concentration for 50% inhibition
KD – equilibrium dissociation constant
pKa – Acid dissociation constant
t1/2 – Half-life
AUC – Area under curve
Chapter 1: Designer lipids for drug delivery – from heads to tails

1.1 Introduction

Lipid vesicles, or liposomes, are micro- or nano-structures formed from a bilayer of lipid surrounding an aqueous core. In the past 30 years they have been widely used to modify the pharmacokinetics, biodistribution, and cellular trafficking of drugs, nucleic acids, and proteins. Liposomal therapeutics have had preclinical and commercial success with more than 46,000 publications, 850 patents, and 13 clinically approved liposome agents with greater than $750 million in revenue in 2011. These drugs continue to advance through the clinic, and the results from a number of pivotal phase III trials, including those from Merrimack Pharmaceuticals (NCT01494506) and Celator Pharmaceuticals (NCT01696084), will be available in the next 18 months.

Liposomes can be tailored to deliver a range of cargo using a diverse toolbox of lipids with well-characterized biophysical behavior. Lipids in this toolbox can be naturally occurring or rationally designed using a variety of hydrophilic headgroups, linkers, and hydrophobic moieties. Selecting the appropriate combination of lipids and the method of assembly gives the drug delivery scientist control over liposome macrostructures, biophysical characteristics, and subsequent in vivo behavior.

At the most fundamental level, the properties of a liposome depend upon the subtle physicochemical interactions among the various lipid species in its composition. A wealth of research has focused on the design, synthesis and characterization of naturally occurring and synthetic lipids. Individual lipids can be combined to form a myriad of superstructures
including bilayers, and bilayer properties can be tuned to modulate drug release and membrane stability **(Figure 1.1A,B)**. In a simplified bilayer model, acyl chain length dictates bilayer thickness and $T_m$, acyl chain saturation controls bilayer fluidity, and headgroup interactions impact inter- and intra-lipid molecular forces **(Figure 1.1B)**. Liposome behavior can be adjusted by incorporating synthetic lipids such as lipid prodrugs, fusogenic lipids and functionalizable lipids into the bilayer **(Figure 1.1C)**. As a result, there has been 50 years of synthetic efforts to develop novel lipids with properties that improve delivery while maintaining low cytotoxicity and immunogenicity. A number of databases classify lipids by structure⁴, organize information related to lipid $T_m$ and phase preferences

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**Figure 1.1:** *Modulating liposome behavior.* Liposome behavior can be controlled across a number of length scales: (A) engineering of individual lipids (B) modification of bilayer biophysics and (C) inclusion of lipids that direct macroscopic liposome behavior and interactions.
into phase diagrams\(^2\), or detail methods for liposome characterization (cyberlipid.org; lipidmaps.org). This abundance of information provides the drug delivery scientist with accessible resources to guide the development of lipids for drug delivery.

As a starting point, nature has provided a variety of lipids that have evolved to satisfy diverse structural and functional purposes. Phospholipids with neutral, zwitterionic, or anionic headgroups, such as: PC, PE, and sphingomyelin are the primary components of cell membranes and are essential for membrane stability and intracellular trafficking. Glycerides are neutral lipids that serve as energy sources and signaling molecules in mammalian cells. Naturally occurring anionic lipids, including phosphatidylglycerol, phosphatidylinositol, cardiolipin, phosphatidic acid, and phosphatidylserine are also found in mammalian cell membranes, and play a critical role in cellular signaling, lipid-protein interactions and membrane trafficking\(^3\)-\(^7\). These naturally occurring lipids are components of FDA approved therapeutics such as Doxil\(^\circledR\), AmBisome\(^\circledR\), and DepoCyt\(^\circledR\). Half a century of characterization of the physicochemical properties of these lipids allows the lipid engineer to build from a wealth of structure-function relationships to design systems with control over stability and payload release.

1.1.1 Synthetic lipids for drug delivery

There are three key steps in liposomal drug delivery that can be improved with synthetic lipids: 1) extended circulation of the liposome after intravenous administration, 2) directed lipid headgroup interactions and cell targeting and 3) controlled payload release (Figure 1.3). Synthetic lipids can be formulated in liposomes alongside naturally occurring lipids to serve these structural or functional roles.
After administration, liposomes circulate in the bloodstream and accumulate in tumors by the EPR effect. Increasing the circulation half-life of the liposomes allows a higher fraction of the dose to transit to the tumor and increases the probability that liposomes will extravasate into the tumor parenchyma. Functionalizing lipid headgroups with polymers, proteins, or peptides, can extend liposome circulation time by reducing liposome adhesion to RES cells and preventing destabilizing interactions with serum proteins (Figure 1.2).

The next step in delivery involves delivering payload to specific cell types by attachment of targeting ligands. While cell targeting is not a requisite liposome characteristic, a number of next-generation delivery systems look to take advantage of targeting particular cell types. To that end, engineering lipids with chemistries that allow for facile attachment of proteins, sugars, or other targeting moieties are of particular interest. In addition to targeting, lipids with specific and programmable interactions in the headgroup can direct macrostructure formation and
membrane biophysics. Such synthetic lipids can be used to probe the intra- and inter-
molecular forces governing superstructure formation and function.

In the final delivery step, liposomes must release their cargo in the appropriate
tissue or cellular compartment over the correct timescale. There are a number of ways to
control contents release from liposomes: first is to change the liposome formulation,
second is to make the cargo more hydrophilic such that it loads into the aqueous
compartment, and third is to use remote loading to cause the agent to accumulate inside
the liposome\(^9\). These techniques are the subject of a number of excellent reviews and will
not be discussed in this article\(^10\). An additional method to control liposome contents
release relies on including triggerable lipids that are sensitive to environmental stimuli (pH,
shear stress, oxidative environment, etc.) in the liposome formulation. Such lipids allow for
burst delivery in the correct cellular compartment and have been of particular interest in
siRNA delivery, where engineered pH-dependent fusogenic lipids allow for delivery to the
cytosol. These lipids have also been extensively reviewed\(^11,12\). Herein we focus on a number
of alternatives to these triggerable systems to control liposome functionalization and
payload release, including lipid prodrugs, lipids with inverted headgroup architectures, and
lipids with covalently attached hydrophobic structural or drug moieties (Figure 1.2).

\[ \text{Figure 1.3: Three key steps in liposome drug delivery.} \]
\[ \text{After administration, liposomes circulate and accumulate at the disease site. Directing liposome headgroup interactions allows for targeting to the appropriate cellular or tissue compartment. Payload release can be controlled by modulating membrane thickness, fluidity and interfacial charge orientation.} \]
While naturally occurring lipids are the workhorses of liposomal systems, clinical advances would not have been possible without the development of a number of synthetic lipids. In this review we focus on synthetic lipids developed over the past 10 years to probe certain aspects of liposome behavior or to improve liposome \textit{in vivo} stability and therapeutic activity. We concentrate on lipids for drug delivery and highlight selected successes and failures of such lipids in animal studies or in the clinic. Nucleic acid delivery systems have recently been reviewed in detail\textsuperscript{11,12}.

\textbf{1.2 Lipids that extend circulation}

\textbf{1.2.1 Polymer headgroup lipids}

Conventional liposomes, those without steric stabilization, are cleared from circulation by the phagocytic cells of the RES and accumulate mostly within the spleen and liver\textsuperscript{13}. Liposomes modified on the surface with a hydrophilic polymer are known as sterically stabilized liposomes. At a certain surface density, the polymer extends from the surface of the liposome in a brush or mushroom configuration and can decrease protein adsorption to the lipid membrane, conceal surface charge, reduce liposome adhesion to cell surfaces, and, as a consequence of these factors, extend circulation time. This extended circulation improves the probability that a liposomal drug will accumulate at the desired location that increases the exposure of rapidly eliminated drugs. Increasing circulation time is also valuable for passive targeting strategies that depend on the EPR effect\textsuperscript{14}, in which liposomes preferentially escape the poorly organized tumor vasculature and accumulate near the tumor blood vessels. Steric stabilization further reduces the fraction of the drug that distributes to the liver, spleen, and bone marrow.
The first sterically-stabilized liposomes incorporated glycolipids such as GM1 ganglioside, cerebroside sulfate, or phosphatidylinositol$^{15,16}$. A significant breakthrough in the design of SLs came in the late 1980’s with the attachment of PEG to the liposome surface. The ability of PEG to increase circulation half-life of proteins and other biomaterials was well documented, and it was discovered that similar effects were achievable by including PEGylated lipids into liposome formulations$^{17,18}$. Doxil®, a liposomal doxorubicin formulation incorporating DSPE-PEG is the only FDA approved PEGylated liposomal therapeutic$^{19,20}$; its development was recently reviewed$^{21}$.

In the early work on PEG proteins, it was assumed that PEG was immunologically inert$^{17}$. However, it is now recognized that under certain circumstances PEG can induce an immune response. Importantly, PEGylated drugs and liposomes can initiate an immune response known as the ABC effect$^{22}$. In this effect, PEGylated materials lose their ability to evade uptake by immune cells following initial dosing. Subsequent doses of PEGylated liposomes are rapidly cleared from circulation and accumulate largely in the liver and spleen$^{22,23}$. This clearance is caused by the production of anti-PEG IgM upon the first injection, which can then label subsequent doses of PEGylated liposomes for removal from circulation$^{24}$.

Other synthetic polymer-modified lipids also increase circulation half-life by providing a hydrophilic steric coat (Figure 1.4). HPMA (poly[N-(2-hydroxypropyl)methacrylamide])$^{25-27}$, PVP (poly(vinylpyrrolidone))$^{28,29}$, PMOX (poly(2-methyl-2-oxazoline))$^{30,31}$, PAcM (poly(N-acryloyl morpholine))$^{32,33}$, PAA (poly(acrylamide))$^{34}$, PG (poly(glycerol))$^{35,36}$, PVA (poly(vinylalcohol))$^{37-39}$, pNIPAM (poly(n-isopropylacrylamide))$^{40}$, and pAAs(poly(amino acids))$^{41-44}$ all increase the
circulation half-life of liposomes in vivo. However, inconsistent experimental procedures make it difficult to directly compare these polymers. While only PMOX coated liposomes demonstrated similar circulation times to PEG, a number of favorable properties could make these alternative polymers a better choice for certain drug delivery applications. In particular, polymers with lower viscosities than PEG and the ability to avoid the ABC effect may be advantageous for the delivery of protein, peptide, and other macromolecular therapeutics.

1.2.2 Alternatives to polymer headgroups for extending circulation

Polymer coatings for liposomes are limited by their high intrinsic viscosity and their induction of the IgM-mediated ABC effect. They can also hinder liposome uptake into diseased cells and fail to stop absorption of serum proteins that promote clearance such as IgG. Approaches that avoid these limitations have been promising in preclinical studies. Masking nanoparticles with markers of “self” is also emerging as an alternative approach to extending liposome circulation. Red blood cell surface proteins have been investigated as nanoparticle coatings that allow for evasion of the RES. Hu and colleagues coated PLGA nanoparticles by extruding them along with disrupted erythrocyte membranes. These nanoparticles, coated in an erythrocyte membrane, had extended circulation compared to nanoparticles coated with PEG or unmodified nanoparticles in mice. In a more defined approach, Rodriguez and colleagues computationally designed a 21 amino acid peptide mimetic of CD47, a marker of self on erythrocytes that impedes phagocytosis by signaling
through phagocyte receptor (SIRP-α)⁴⁶. Nanoparticles opsonized with IgG were coated with peptide or with a PEG brush. While PEG had no mitigating impact on macrophage uptake of the opsonized particles in vitro and in vivo, the peptide prolonged nanoparticle circulation four fold. Further, nanoparticles coated with CD47 showed a longer circulation time than those coated with PEG. To our knowledge, this peptide has yet to be tested in a liposomal system. A number of additional factors, including shape, surface chemistry, and mechanical properties are under investigation to improve the in vivo properties of nanoparticles⁴⁶.

Going forward, it will be important to perform controlled studies comparing these alternatives to PEG and other polymers for extended circulation in vivo. Such studies should place a heavy emphasis on understanding the immunogenicity of these systems.

1.3 Lipids for directed headgroup interactions

Lipid headgroups can act as points for liposome functionalization for cargo, polymer coatings, or targeting ligands. These functionalization points often are covalent coupling moieties that allow for the attachment of ligands that alter pharmacokinetics or biodistribution, such as cell or tissue targeting groups, membrane-active peptides, or polymer coatings. In addition, headgroups contribute to lipid-lipid interactions, and influence the biophysical characteristics and macromolecular behavior of a liposome. Modifications to the headgroup allow for the control of specific molecular interactions such as H-bonding, pi-stacking, and electrostatics. Nucleosides/nucleotides, peptides, and headgroups that can undergo a charge reversal have been extensively investigated as tools for nucleic acid and drug delivery and have been recently reviewed⁴⁹. Inorganic lipid headgroups are also being investigated⁵⁰.
1.3.1 Lipids with a nucleic acid headgroup

Nucleolipids are lipids with a nucleic acid headgroup, and are unique because they can interact with nucleic acids via hydrogen bonding and pi stacking, as well as through electrostatics. They are an excellent model system to study the hierarchy of molecular forces between lipid headgroups and how those forces contribute to superstructure formation. Further, these headgroups are biologically relevant, as they complex with single-stranded nucleic acids via base pairing and are useful in nucleic acid and drug delivery and as lipid prodrugs. Advancements in nucleolipid structures and their applications in transfection have recently been reviewed. In this chapter, we focus on engineered lipids with a monomeric nucleoside or nucleotide headgroup with a particular emphasis on understanding the forces governing headgroup interactions with nucleic acids.

The deoxyribose sugar is the scaffold for nucleolipid construction (Figure 1.5A). In general, a nucleobase (A, T, C, G or U) is conjugated to the 1’ position by a beta-glycosidic linkage. This base allows for selective interactions with other nucleolipids, single-stranded nucleic acids, or drugs via electrostatics, hydrogen bonding, and pi-stacking. The 2’ and 3’ positions of the ribose are generally functionalized to hydrophobic domains via an ester or ether linker. Finally, anionic, cationic, zwitterionic and non-ionic groups have been conjugated to the 5’ position in order to alter the behavior and distinct self-assembly properties of these lipids.

A few studies isolate the role of H-bonding and electrostatics in nucleolipid headgroup interactions with nucleic acids by engineering lipids that lack the functional groups required for such interactions. Ceballos and colleagues synthesized nucleolipids with 3-nitropyrrrole, 5-nitroindole or 4-nitroimidazole headgroups, universal bases known to form complementary base pairs with all four natural bases. The headgroups are
further functionalized with a quaternized amine at the 5’ position and two oleyl chains to allow for lipid self-assembly (Figure 1.5B-D). These bases lack hydrogen-bonding capabilities, and are limited to interactions via pi-stacking interactions and electrostatics. These forces were sufficient to complex siRNA and knock down GAPDH protein levels in a number of cell lines. However, the hierarchy between pi-stacking and electrostatic interactions remains unclear in these lipids. To our knowledge, this is the first example of protein knockdown using nucleolipids transfecting siRNA. Interestingly, the authors increased the nucleolipid affinity for siRNA by altering the stereochemistry of the base at the 1’ position. This increase in affinity correlates with an increase in transfection efficiency, and this relationship was corroborated by density functional theory modeling, a quantum mechanical method to investigate the electronic structure of a molecule. Defining such quantitative structure-function relationships is useful in designing amphiphilic molecules.

Since hydrogen-bonding and pi-stacking interactions are weaker than electrostatic interactions via cationic groups with DNA, it is difficult to decouple their role in directing nucleolipid-DNA complexes. To understand the function of these forces, Banchelli et al. synthesized nucleolipids with a formal negative charge. Two octanoyl chains were conjugated to a nucleobase at the 5’ position via an anionic phosphate linkage and an adenosine nucleotide was linked to the 1’ position (Figure 1.5E). The hydrogen bonding and pi-stacking interactions of these nucleolipid overcame the repulsive force between the negative charge in the headgroup and the negative charge on nucleic acids in order to complex polyuridylic acid. In a similar vein, Khiati and co-workers synthesized anionic nucleotide-lipids to decouple cationic interactions from H-bonding and pi-stacking (Figure
1.5F$^{60}$. Again, the forces of H-bonding and pi-stacking dominated the anionic repulsive forces between the nucleolipid and nucleic acid to complex and transfect eGFP into HEK cells \textit{in vitro}$^{60}$. These studies show that electrostatic interactions are not necessary to complex and transfect nucleic acids \textit{in vitro}, as H-bonding and pi-stacking forces in a polyvalent system are sufficient.

\textbf{Figure 1.5: Nucleolipid headgroup structures.} Predicted forces governing interactions with nucleic acids are shown.

The selective intermolecular binding properties of nucleolipids have been incorporated into other rationally designed lipids. In a series of papers, Ma and colleagues designed synthetic multivalent hydrogen-bonding lipids with a melamine or cyanuric acid headgroup that replace the sugar-phosphate backbone of DNA with a phospholipid$^{61,62}$. These lipids formed large unilamellar vesicles and demonstrated complementary base pairing interactions in membrane mixing and surface plasmon resonance studies (Figure
Polidori and colleagues extensively investigated the role of lipid shape on macromolecular structure formation of synthetic lipids with a H-bonding tris(hydroxymethyl) aminomethane (tris) moiety linked with an aminoglycerol group. Increasing the length of the hydrophobic tails increased the T_m of the lipids and altered the length of the tubules they formed, their stability, and their ability to stably entrap CF. Substitution of an ester linkage for a carbamate linkage allowed for the formation of unstable vesicles rather than stable tubes, highlighting the lipid linkage as an important parameter in lipid design and behavior. Godeau and co-workers synthesized H-bonding glycosyl-nucleoside lipids as low-molecular-weight hydrogelators for nucleic acid delivery. Lipids were first conjugated to nucleobases using click chemistry and subsequently clicked with glycosyl groups (Figure 1.5I) to achieve reversible nanofibers, hollow nanotubes, and hydrogels capable of transfecting cultured cells in serum. This chemistry allows for a universal scaffold for facile lipid functionalization and engineering. Two lipid parameters dictated the formation of gels in solution: 1) H-bonding between headgroups and 2) saturation of acyl chains. H-bonding headgroups (30 fold) and saturated acyl chains (25 fold) dramatically lowered the concentration necessary for gelation.

While many publications have sought to simplify the synthesis, characterize the biophysics, and assess the in vitro behavior of nucleolipids and H-bonding lipids, little is published on their in vivo activity. Special attention, however, must be paid to the immunogenicity of such lipids. Nucleolipid headgroups may act as haptens, especially when delivering known TLR agonists such as double-stranded DNA or single-stranded RNA. Such studies should be prioritized in order to understand the potential of these lipid systems for drug delivery.
1.3.2 Headgroups for attachment of targeting ligands

In addition to engineered lipid headgroups that rely on intrinsic molecular interactions such as H-bonding and pi-stacking, a myriad of synthetic lipid headgroups have been developed that contain specific chemistries that permit controlled covalent attachment of targeting ligands or functional groups. In particular, lipids with maleimide, avidin, ether, ester, thiol, carboxylic acid, and hydrazine moieties in the headgroup have been extensively reviewed\textsuperscript{67,68}. The maleimide lipid is most commonly used as a functionalization point of lipids because of the reactivity of the group with a free thiol; the maleimide is typically separated from the lipid headgroup by a spacer that reduces steric hindrance in the coupling reaction. This is particularly useful, as a thiol containing ligand, such as a single chain antibody fragment, can be coupled to preformed liposomes containing a small mole fraction of maleimide lipid\textsuperscript{69}.

These covalent attachment approaches are broadly applicable but can require complex chemistry and can partially inactivate the proteins\textsuperscript{70}. Further, the number and location of attachment sites on the ligand dictate the orientation of ligand attachment. For example, a maleimide group can form a thioether bond with any solvent exposed cysteine on a protein.

Several attempts to engineer lipid headgroups for facile non-covalent attachment have been pursued with limited success. Protein A, which interacts specifically with the Fc region of IgG, can be adsorbed to the surface of polymer nanoparticles. Addition of IgG to the nanoparticles results in uniform orientation of the antibodies and nearly 100% attachment efficiency. However, this system is limited by its stability \textit{in vivo}, as the adsorbed protein is displaced by proteins in serum\textsuperscript{71}. 


A number of groups have linked His-tagged proteins to liposomes using nickel chelating moieties\textsuperscript{72-76}. His-tags are popular motifs that can be easily engineered into proteins and act as handles for protein purification or binding by nickel chelation. This interaction is reversible by stripping chelated nickel from NTA\textsuperscript{77}. In an effort to develop a general attachment approach for liposomes, van Broekhoven and Altin\textsuperscript{78,79} engineered trivalent nitrilotriacetic acid (tris-NTA) lipids with nanomolar affinities for polyhistidine tagged (His-tag) proteins. Our group provided an alternative synthetic route to the tris-NTA lipid\textsuperscript{80,81}. Increasing the valency of the chelating moiety in the lipid headgroup increased the affinity for His-tagged proteins (Figure 1.6). yCD and mKate, a far red-fluorescent protein, maintained their activity while attached to the liposomes via the tris-NTA lipid. These proteins were stably attached to the liposome in fetal calf serum and mouse plasma. However, liposome attachment via tris-NTA lipids did not enhance the circulation time of proteins \textit{in vivo}, likely due to competing interactions from plasma proteins and other histidine motifs. While the \textit{in vivo} applications of these chelating lipids are limited, they have shown utility in subcutaneous vaccine delivery\textsuperscript{82} and they can effectively be used to simplify rapid screening of lipid-protein conjugates \textit{in vitro} to identify binding and internalizing antibodies for use in targeted drug delivery\textsuperscript{83}.
1.4 Lipids that direct membrane biophysics and payload release

Ideally, liposomes are designed to be stable in circulation until they reach the target site. Upon interaction with the appropriate compartment in target cells, the payload should be rapidly released, particularly if cytotoxicity is the objective. Hydrophilic liposome payload can be encapsulated in the aqueous core of the liposome or substituted for polar liposome headgroups in the bilayer as a lipid prodrug. In both scenarios, destabilization of the membrane and release of the therapeutic are critical steps in delivery.

As such, a keen understanding of membrane biophysics and stability is integral to tuning drug release. The stability of the bilayer is driven by its $T_m$, phase, and composition. At the $T_m$, the bilayer undergoes a gel to liquid phase transition causing lipid-packing defects that increase bilayer permeability. Lipid components can dictate the $T_m$ and other bilayer physical properties. Generally, lipids with long saturated acyl chains have higher $T_m$ than those with shorter or unsaturated chains. The inter- and intramolecular interactions of lipid headgroups further control $T_m$ and membrane permeability. Regardless of the lipid composition, cholesterol can be included in bilayers to eliminate the phase transition and encourage a stable gel-like phase. In addition to intrinsic liposome parameters, microenvironmental factors such as redox state, pH, temperature, and enzyme activity have a profound impact on stability and can be used to trigger drug release\textsuperscript{84,85}.

1.4.1 Covalent attachment of hydrophobic moieties

Sterols are important components of natural membranes that play a critical role in regulating membrane fluidity. Cholesterol is the most common sterol in mammalian membranes and is the preferred sterol in several FDA approved liposome therapies including DaunoXome®️️️, Myocet®, Depocyt®, Marqibo®, and Doxil®️️️. While cholesterol or cholesterol esters do not form bilayer structures on their own, their amphipathic nature
allows for their inclusion in liposome bilayers. Incorporation of cholesterol into liposomes at 30 mol % eliminates the phase transition of diacylphospholipids\textsuperscript{87,88}, reduces membrane permeability\textsuperscript{89,90}, and forces the bilayer into a stable gel-like state. Because of their stability, liposomes of this composition are widely used in the formulation of chemotherapeutic drugs\textsuperscript{91}. Below 30 mol %, cholesterol does not pack uniformly in diacylphospholipids and fails to completely eliminate their phase transition\textsuperscript{85}. Above 50 mol %, cholesterol phase separates in the membrane and can form crystals\textsuperscript{92}. As such, changes in cholesterol composition can have profound effects on liposome formation, membrane stability, and permeability. \textit{In vivo}, free cholesterol can rapidly transfer from liposomes into biomembranes and lipoproteins\textsuperscript{93-95}. This constant flux of cholesterol destabilizes the liposomes and promotes contents release while in circulation.

In order to prevent such transfer of cholesterol, our group synthesized a family of SMLs by covalently attaching cholesterol to the glycerol backbone of PC (\textit{Figure 1.7}). An early example of such a cholesterol containing phospholipid was used to investigate the role of cholesterol binding on ATPase activity in a lipid micelles but had not been demonstrated to form liposomes\textsuperscript{96}. We showed that SMLs readily formed liposomes by themselves or when mixed with diacyl PC lipids. By anchoring cholesterol in the membrane, we reduced cholesterol transfer and increased \textit{in vivo} liposome stability. SMLs are easily synthesized, commercially available, and recapitulate

\textbf{Figure 1.7: Structure of SMLs.}
the biophysical properties of liposomes incorporating cholesterol\textsuperscript{97-99}. Compared to liposomes formulated with free cholesterol, SMLs eliminate the phase transition of diacylphospholipids\textsuperscript{98}, exhibit similar permeability to entrapped hydrophilic molecules\textsuperscript{98,99} and demonstrate similar membrane fluidity\textsuperscript{99}. SMLs maintain these properties while preventing cholesterol transfer from the bilayer\textsuperscript{98}.

\textit{In vivo}, SML liposomes encapsulating doxorubicin demonstrate comparable efficacy to Doxil\textsuperscript{®} in a C-26 colon carcinoma model\textsuperscript{99}. Interestingly, we found that at acyl chain lengths of C\textsubscript{16} and C\textsubscript{18}, SML liposomes are more stable in circulation than liposomes containing free cholesterol. Further, we found that these SML liposomes had improved uptake into and slower clearance from the liver and spleen compared to traditional liposomes\textsuperscript{99}. These studies highlight the stability of SML systems \textit{in vivo} and their potential utility as drug carrier systems.

In addition to cholesterol, hydrophobic moieties for therapy and diagnostics can be covalently coupled to lipids to prevent loss in circulation. Liposomal formulations of porphyrins, hydrophobic and photosensitive agents with applications in photodynamic therapy, have been used to improve their solubility. These nanoparticle systems are limited by the amount of porphyrin that can be included in the formulation (15 mol \%) and by transfer of porphyrin out of the bilayer \textit{in vivo}\textsuperscript{100,101}. Anchoring of pyropheophorbide, a chlorophyll-derived porphyrin analogue, to lyso PC prevented bilayer transfer of the porphyrin and enhanced its self-quenching in liposomes\textsuperscript{100}. Lipid systems composed of these engineered lipids along with PEG-DSPE and cholesterol were termed “porphysomes”. These nanoparticles were safe at high doses in mice, demonstrated favorable pharmacokinetics, and could be loaded with hydrophilic payload. After accumulation in
tumors, these theranostic particles could be imaged for diagnostic purposes or irradiated for photothermal therapy\textsuperscript{100}. This interesting technology has been extended to applications in triggerable systems and acoustic imaging\textsuperscript{102,103}.

### 1.4.2 Triggerable lipids

As previously described, liposomes are designed to optimize stability and time in circulation in order to drive a high dose of payload to the target site. However, this stability in circulation must be balanced with the release of payload in diseased tissue. Incomplete release of drug at the site of action can limit the clinical success of therapeutics, as evidenced by the failure of liposomal cisplatin in the clinic\textsuperscript{104}. To improve release, researchers have developed a number of triggerable liposome systems. The trigger can be an external cue (heat, light, ultrasound) or intrinsic to the disease site (pH, redox environment, enzymes). These systems have been thoroughly reviewed\textsuperscript{105,106}, but have been disappointing in practice\textsuperscript{105}. This is especially true for systems dependent on external triggers, since only primary tumors, and not nascent metastasis, can be targeted via such cues. ThermoDox\textsuperscript{®} (Celsion Corporation) is a prime example of the failure of these systems in the clinic. This heat sensitive liposome formulation incorporates lysolipid that promotes liposome degradation at mildly elevated temperatures\textsuperscript{107} and is administered alongside radiofrequency ablation or ultrasound. However, incorporation of lysolipids can destabilize the liposome in circulation\textsuperscript{108} and may lead to contents release before the liposome reaches the tumor site. ThermoDox\textsuperscript{®} recently failed to meet its primary endpoint in a Phase III study in patients with hepatocellular carcinoma (Celsion Corporation press release January 31, 2013).
1.4.3 Lipid Prodrugs

Incorporation of small molecule drugs or prodrugs into self-assembling, amphiphilic molecules can improve their biodistribution, pharmacokinetics, safety profile, trafficking and stability. In particular, lipid prodrugs can improve the characteristics of small molecule drugs that are not well suited for traditional liposomal encapsulation and delivery due to their molecular properties. In principal, a hydrophilic drug is conjugated to a hydrophobic lipid tail such that it is substituted for a polar lipid headgroup; this amphiphilic lipid prodrug can then be incorporated into a liposome. The long history of these prodrugs have been reviewed. However, a number of recent clinical advances and setbacks suggest that the clinical on the development of these drugs will be challenging. In this section we review recent developments in lipid prodrugs of doxorubicin, gemcitabine, cytarabine (Ara-C), mitomycin C (MMC), and paclitaxel. We focus our discussion on clinical and in vivo preclinical developments.

Doxorubicin (dox), a topoisomerase II inhibitor, is the chemotherapeutic most often studied in nanoparticle formulations because of the thorough understanding of its pharmacology and the success of Doxil® in the clinic. Systemic doses of dox lead to myelosuppression, gastrointestinal toxicity and cardiotoxicity. These issues make

**Figure 1.8: Prodrug structures.** Selected structures of prodrugs that have advanced into the clinic.
doxorubicin a prime candidate for nanoparticle formulation, and a number of dox prodrugs have had preclinical success. The most promising dox derivative is not a lipid prodrug, but an albumin binding dox derivative: Aldoxorubicin® (CytRX) (Figure 1.8). Binding of doxorubicin to albumin improves the pharmacokinetics and efficacy of the free drug and has shown promise in early clinical trials. A number of lipid prodrugs of dox are in preclinical testing. A docosahexanoic acid (DHA) dox conjugate proved more efficacious than free dox in L1210 leukemia and B16 melanoma models. Further, Duhem and colleagues conjugated dox to tocopherol succinate via an amide linkage which self assembled into 250 nm macrostructures when stabilized with PEG lipid. These nanoparticles have improved efficacy over free dox in CT26 tumors in vivo. While these dox derivatives are promising, future experiments should be carried out to benchmark these dox-prodrug systems to Doxil®.

MMC is an alkylating chemotherapeutic agent limited by toxicities such as leukopenia, thrombocytopenia, and mucous membrane toxicity. A number of lipid prodrugs have been developed to improve the therapeutic index of MMC. In particular, Gabizon and colleagues have developed an MMC prodrug in which the drug is conjugated to a 1,2-distearoyl glycerol lipid via a cleavable dithiobenzyl linker (Figure 1.8). In this system, the MMC acts as the polar lipid headgroup and the prodrug is easily incorporated into liposome membranes. This prodrug liposome system has demonstrated impressive efficacy in a range of preclinical models, and is now advancing in a phase I dose escalation study (Promitil®, LipoMedix, NCT01705002).

Nucleoside analog chemotherapeutics, including gemcitabine and Ara-C, are a major class of chemotherapeutics. Small structural differences have a profound impact on the
activity of these molecules: Ara-C is primarily used to treat hematological tumors while gemcitabine, which is structurally similar except for two fluorine atoms, is used against solid tumors. Both drugs are limited by short circulation times and can be rapidly deactivated by deamination reactions in vivo. As such, a wealth of research has been focused on lipid prodrug modifications of these compounds to prolong circulation of the active form. Gemcitabine lipid prodrugs have shown promise in preclinical studies and are comprehensively reviewed\textsuperscript{115}. Modification of Ara-C at the 5’ position with an elaidic acid chain (Elacytarabine\textsuperscript{®}, Aqualis ASA) circumvented drug resistance in vitro and resulted in an improvement in in vivo anti-tumor efficacy as compared to unmodified Ara-C (Figure 1.8)\textsuperscript{116,117}. However, Elacytarabine\textsuperscript{®} demonstrated no advantage in efficacy over the control arm in two phase III studies in acute myeloid leukemia and pancreatic cancer (Aqualis ASA press release November 12, 2012 and April 1, 2013). While the lipid prodrugs improved pharmacokinetic and biodistribution of Ara-C, these changes did not translate into a survival advantage in patients. Another prodrug, CP-4126, is a 5’ elaidic ester of gemcitabine that has advanced into the clinic (Figure 1.8)\textsuperscript{118}. Orally administered CP-4126 was poorly absorbed and subject to metabolism before systemic exposure\textsuperscript{118}. As such, future work with this compound will focus on intravenous administration. These studies highlight the difficulties and unknowns in moving lipidated prodrugs into the clinic.

Similar to the nucleoside analog prodrugs, paclitaxel lipid prodrugs have had disappointing clinical outcomes. Paclitaxel is highly hydrophobic and its encapsulation in liposomes is problematic. Modification of paclitaxel at the 2’ hydroxyl with DHA (Taxoprexin\textsuperscript{®}, Protarga Inc.) improved anti-cancer efficacy by extending circulation \textsuperscript{119}. However, these improvements in pharmacokinetics did not translate into improvements in
efficacy, as Taxoprexin® had only modest activity in gastric and esophageal adenocarcinoma\textsuperscript{120}. Further, a phase III trial in metastatic melanoma showed no survival advantage of Taxoprexin® versus dacarbazine\textsuperscript{121}. Squalenoylation of paclitaxel and gemcitabine may represent a new class of prodrugs for clinical evaluation\textsuperscript{122-124} but their promise must be viewed in the light of the challenges experience by the other lipid prodrugs reviewed above.
1.5 Conclusions

The large variation of lipid structures outlined in this review can be combined to form liposomes with multifunctional capabilities including increased serum stability, extended circulation, ligand targeting, optimized drug loading, and triggered release. These lipids have been vital tools for probing lipid membrane biophysics, especially for understanding the role of inter- and intra-molecular interactions on liposome superstructure formation and behavior. Such studies have led to a wealth of well-characterized lipid structure function relationships that are useful to those looking to synthesize novel molecules. For this reason, systemic manipulation of lipid headgroups, linkers and hydrophobic domains to further our understanding of lipid biophysics remains a worthwhile and productive area of research.

While a number of novel lipids have been synthesized and published for applications in drug delivery, their pharmacokinetics, pharmacodynamics and immunogenicity are rarely thoroughly characterized\(^\text{125}\). Looking forward, such studies should be prioritized in order to realize the translational potential of these systems. Special attention should be paid to commonly overlooked areas such as lipid immunogenicity, and extreme care should be taken to ensure that liposome preparations administered \textit{in vivo} are endotoxin free to avoid confounding immunological factors.

The setbacks of Lipoplatin®, Thermodox®, Elacytarabine®, and Taxoprexin® in the clinic provide insight into the critical parameters in drug delivery to solid tumors. While in circulation, liposome payload should be stably encapsulated in order to maximize accumulation in the tumor. However, once the liposome extravasates from the vasculature into the tumor, it must quickly release its payload uniformly throughout the tumor. The
complex process of liposome delivery then involves three parameters: 1) stability in circulation, 2) distribution in the tumor, and 3) payload release over the appropriate timescale. Future work should focus on the balance between these parameters in order to optimize formulations for drug delivery. As has been written elsewhere\textsuperscript{126}, the incorporation of engineered lipids into a delivery system must take into account the higher costs, complexity of manufacturing, and complicated intellectual property of the multi-component systems. In order to justify the added costs of these systems, liposomes, like other nanomedicines, must offer significant clinical advantages in both safety and efficacy.
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Chapter 2: Synthesis and Characterization of Betaine-like Diacyl Lipids: Zwitterionic Lipids with the Cationic Group at the Bilayer Interface

2.1 Introduction

An increasingly diverse toolbox of lipid headgroups, linkers and hydrophobic domains is being populated by the systematic study of natural lipid components and surfactants. This lipid collection has elucidated the influence of headgroup properties such as hydrogen bonding capability, size, and charge on superstructure formation and molecular interactions\textsuperscript{1-2}. For instance, zwitterionic surfactants interact with ions with higher affinity than do surfactants with a nonionic headgroup\textsuperscript{3}. However, there are a number of lipid headgroup architectures that have not been well characterized. In particular, modifications to the phosphocholine headgroup have been investigated less than other classes of lipids, despite their important role in biological membranes. As a result, less is known about the effects of zwitterionic headgroup structure and charge orientation on superstructure formation and lipid interactions.

Naturally occurring zwitterionic headgroups are oriented with an anion proximal to, and a cation distal to the membrane interface. Inverting this charge orientation in single chain surfactants by placing the cation proximal to the interface has yielded insight into the charge interactions of zwitterionic micelles\textsuperscript{4}. Zwitterionic sulfobetaine micelles and lecithin liposomes can specifically interact with anions; this interaction follows the Hofmeister series and Pearson’s hard—soft classification\textsuperscript{5-7}. “Soft” bases with low charge
densities and high polarizability, such as ClO₄⁻, bind these micelles more effectively than “hard” bases with high charge densities and low polarizability, such as F⁻.

In a series of recent studies, our group has systematically examined modifications to the PC headgroup. We set out to understand the role of 1) the relative location of charged moieties in the headgroup and 2) the type of charged moiety on lipid behavior. To that end, we synthesized a library of lipids with an inverted architecture: the cation is proximal to the membrane while the anion extends into the aqueous space. This family includes phosphates (CP), ethyl phosphates (CPe), sulfonates (SB) and sulfates (CS) as the anionic moiety (Figure 2.1). SB, CP and CPe lipids form liposomes when formulated with cholesterol. This new class of inverse zwitterionic (IZ) lipids has distinct biophysical properties from traditional PC lipids including elevated Tₘ and limited interactions with divalent cations. Most importantly, these studies have highlighted the pivotal role of the charge at the bilayer interface in drug permeability and ionic interactions of liposomes. While single chain surfactants with an inverted headgroup architecture have been studied, these studies extend the understanding of headgroup charge inversion in lipid systems⁴.

Herein, we expand this work by synthesizing a class of inverse zwitterionic lipids that have an anionic acetic acid group extending into the

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Figure 2.1: Structures of IZ lipids.
aqueous phase and a cationic quaternized amine adjacent to the bilayer interface (Figure 2.1). These Acetate Quaternized amine (AQ) lipid vesicles bind to anions and have interesting phase transition temperatures. To our knowledge, this is the first example of the synthesis of diacyl AQ headgroup lipids and of the characterization of the supramolecular assemblies and biophysical properties of such lipids.
2.2 Materials and Methods

2.2.1 Materials

DMPC, DPPC, DSPC, and cholesterol were obtained from Avanti Polar Lipids. Trifluoroacetic acid was obtained from AK Scientific; all other solvents were obtained from VWR Scientific. PD-10 sephadex columns were obtained from GE Healthcare. All other reagents were purchased from Sigma Aldrich. Buffers were prepared using Milli-Q deionized water. GraceResolv Silica thin layer chromatography plates were obtained from Grace Davison Discovery Sciences.

2.2.2 Instruments

NMR measurements were taken on a Bruker 300 MHz Avance system and analyzed using TopSpin software. Chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard. $^{13}$C NMR measurements were composed of 10,000 scans while $^1$H measurements were composed of 32 scans. $^{13}$C experiments were performed in CDCl$_3$ with 10% MEOD. $^1$H experiments were performed in CDCl$_3$ or deuterated DMSO. MALDI-TOF measurements were taken on PerSeptive Biosystems Voyager-DE from Applied Biosystems. HPFC was carried out using a Grace Reveleris Flash System with pre-packed silica gel columns. Zeta potential and size measurements were carried out using a Nano-ZS Dynamic Light Scattering Instrument from Malvern. DSC measurements were obtained using a high-temperature MC-DSC 4100 calorimeter from Calorimetry Sciences Corp. Fluorescence measurements were made on a FLUOstar plate reader from BMG Labtech with excitation at 485 nm and emission at 518 nm.
2.2.3 Synthesis

The AQ lipid library was synthesized from a 3-(dimethylamino)-1,2-propanediol core (2) via a three-step synthesis (Figure 2.2). The synthesis is straightforward and provides a high yield of the title compounds.

2.2.3.1 Alkyl acid conjugation (i)

One molar equivalent (6.3 mmol, 0.75 g) of 3-(dimethylamino)-1,2-propanediol was solubilized in 10 mL DCM. Next, 2.2 molar equivalents of the alkyl acid chain (myristic acid (3a), palmitic acid (3b), or stearic acid (3c)) were added and the reaction was diluted to a final concentration of 0.1 M with DCM. Next, 0.5 molar equivalents of DMAP and 2.5 equivalents of DCC were added to the reaction. The reaction proceeded overnight at room temperature and was monitored by thin layer chromatography (solvent: 10-20% methanol in DCM, visualized by bromocresol green and iodine vapor) and MALDI-TOF. If necessary, DCC was added to drive the reaction toward completion. In order to purify the product (3a-c), the remaining DCC was converted to DCU by addition of 2 mL glacial acetic acid. The produced DCU was removed by vacuum filtration through 55 mm filter paper. To remove residual DCU, the reaction was diluted with 50 mL DCM, washed twice with 10 mL 1 M HCl. The DCM solution was dried over Na$_2$SO$_4$. Solvent was removed by rotary evaporation and then by vacuum. The product was purified using HPFC (0-10% methanol in chloroform); this step had 70-80% yield. The compound structure was confirmed using TLC, MALDI-TOF, and $^1$H NMR.

2.2.3.2 Amine quaternization with tert-butyl bromoacetate (ii)

One molar equivalent (2 g) of 2a-c was solubilized in 30 mL DCM by stirring at room temperature. Next, 3 molar equivalents of Hunig’s base were added to the reaction. Two molar equivalents of tert-butyl bromoacetate were gradually dripped into the reaction in
order to avoid excessive heating. The reaction proceeded overnight at 40°C in an oil bath and was monitored by TLC (10-20% methanol in DCM) and MALDI-TOF. An additional 0.5 equivalents of tert-butyl bromoacetate and Hunig's base were added to drive the reaction to near completion. The reaction mixture was diluted with 50 mL DCM, washed twice with 10 mL 1 M HCl to remove residual Hunig’s base and tert-butyl bromoacetate, and dried over Na₂SO₄. The quaternized product was purified by precipitation at 0°C in DCM. Solvent was removed by rotary evaporation and vacuum; this step had 90% yield. Formation (4a-c) was confirmed by TLC, MALDI-TOF, and ¹H NMR.

2.2.3.3 Deprotection of tert-butyl ester (iii)

4a-c was solubilized in 20 mL DCM and 10 mL TFA. 1 mL of triisopropylsilane was added as a scavenger of carbocations. The reaction was allowed to proceed for 1 hour at room temperature and was monitored by TLC (10-20% methanol, visualized by bromocresol green and iodine vapor) and MALDI-TOF. The reaction was diluted with 50 mL DCM, washed twice with 10 mL 1 M NaHCO₃ to remove residual TFA, and dried over Na₂SO₄. 2-((2,3-bis(myristoyloxy)propyl)dimethylammonio)acetate (1a), 2-((2,3-bis(palmitoyloxy)propyl)dimethylammonio)acetate (1b), and 2-((2,3-bis(stearoyloxy)propyl)dimethylammonio)acetate (1c) were formed in 50-70% overall yield. Product was confirmed by TLC, MALDI-TOF, ¹H NMR, ¹³C NMR and C, H, N elemental analysis.

2.2.4 Structural confirmation of reaction products

3a: ¹H NMR (CDCl₃:MeOD ~10:1): δ 0.88 (t, 6H); δ 1.26 (m, 40H); δ 1.64 (p, 4H); δ 2.3-2.6 (m, 4H); δ 2.78 (s, 6H); δ 3.1-3.3 (m, 2H); δ 4.15 (m, 1H); δ 4.45 (m, 1H); δ 5.47 (p,1H). MALDI-TOF calculated: 539.49, observed: 541.40.
3b: $^1$H NMR (CDCl$_3$:MeOD ~10:1): δ 0.88 (m, 6H); δ 1.26 (m, 48H); δ 1.64 (m, 4H); δ 2.3-2.6 (m, 4H); δ 2.78 (m, 6H); δ 3.1-3.3 (m, 2H); δ 4.15 (m, 1H); δ 4.45 (m, 1H); δ 5.45 (m, 1H). MALDI-TOF calculated: 595.55, observed 598.37.

3c: $^1$H NMR (CDCl$_3$:MeOD ~10:1): δ 0.88 (m, 6H); δ 1.26 (m, 56H); δ 1.64 (m, 4H); δ 2.3-2.6 (m, 4H); δ 2.78 (s, 6H); δ 3.1-3.3 (m, 2H); δ 4.15 (m, 1H); δ 4.45 (m, 1H); δ 5.45 (p, 1H). MALDI-TOF calculated: 651.62, observed 654.11.

4a: $^1$H NMR (CDCl$_3$:MeOD ~10:1): δ 0.88 (t, 6H); δ 1.26 (m, 40H); δ 1.52 (s, 9H); δ 1.64 (m, 4H); δ 2.35 (m, 4H); δ 3.78-3.95 (m, 6H); δ 4.05-4.25 (m, 2H); δ 4.5-4.8 (m, 4H); δ 5.58 (m, 1H). MALDI-TOF calculated: 654.57, observed 655.59.

4b: $^1$H NMR (CDCl$_3$:MeOD ~10:1): δ 0.88 (t, 6H); δ 1.26 (m, 48H); δ 1.52 (s, 9H); δ 1.64 (m, 4H); δ 2.4 (m, 4H); δ 3.78 (m, 6H); δ 4.05-4.25 (m, 2H); δ 4.5-4.9 (m, 4H); δ 5.6 (m, 1H). MALDI-TOF calculated: 710.63, observed 713.49.

4c: $^1$H NMR (CDCl$_3$:MeOD ~10:1): δ 0.88 (m, 6H); δ 1.26 (m, 56H); δ 1.52 (s, 9H); δ 1.64 (m, 4H); δ 2.4 (m, 4H); δ 3.78 (m, 6H); δ 4.05-4.25 (m, 2H); δ 4.5-4.9 (m, 4H); δ 5.6 (m, 1H). MALDI-TOF calculated: 766.69, observed 768.86.

1a (DMAQ): $^1$H NMR (CDCl$_3$:MeOD ~10:1): δ 0.88 (m, 6H); δ 1.26 (m, 40H); δ 1.64 (m, 4H); δ 2.35 (m, 4H); δ 3.35 (m, 6H); δ 4.05-4.25 (m, 2H); δ 4.0-4.4 (m, 6H); δ 5.55 (m, 1H) (Supplementary Figure 1). $^{13}$C NMR (CDCl$_3$): δ -0.02, δ 14.10, δ 22.68, δ 24.65, δ 24.74, δ 29.09, δ 29.11, δ 29.25, δ 29.29, δ 29.35, δ 29.56, δ 29.50, δ 29.65, δ 29.68, δ 31.91, δ 33.86, δ 34.20, δ 51.87, δ 52.53, δ 63.26, δ 63.47, δ 65.66, δ 66.61, δ 164.53, δ 172.79, δ 173.09 (Supplementary Figure 2). MALDI-TOF calculated: 597.50, observed: 599.72. Elemental analysis (% expected, % observed): C (70.3, 70.09), H (11.29, 11.05), N (2.34, 2.36).
1b (DPAQ): $^1$H NMR (DMSO): δ 0.88 (t, 6H); δ 1.26 (m, 48H); δ 1.55 (m, 4H); δ 2.35 (m, 4H); δ 3.35 (m, 6H); δ 3.8-4.1 (m, 4H); δ 4.2-4.4 (m, 4H); δ 5.55 (m, 1H) (Supplementary Figure 3). $^{13}$C NMR (CDCl$_3$): δ -0.11, δ 14.02, δ 22.63, δ 24.63, δ 29.29, δ 29.62, δ 31.87, δ 33.78, δ 34.09, δ 51.79, δ 52.26, δ 63.20, δ 63.53, δ 165.80, δ 173.39 (Supplementary Figure 4). MALDI-TOF calculated: 653.56, observed: 656.51. Elemental analysis (% expected, % observed): C (71.62, 71.28), H (11.56, 12.08), N (2.14, 2.17).

1c (DSAQ): $^1$H NMR (DMSO): δ 0.88 (t, 6H); δ 1.26 (m, 56H); δ 1.55 (m, 4H); δ 2.35 (m, 4H); δ 3.35 (m, 6H); δ 3.8-4.1 (m, 4H); δ 4.2-4.4 (m, 4H); δ 5.55 (m, 1H). (Supplementary Figure 5). $^{13}$C NMR (CDCl$_3$): δ 3.75, δ 17.89, δ 26.53, δ 28.53, δ 33.20, δ 33.54, δ 35.78, δ 37.70, δ 37.99, δ 55.71, δ 56.07, δ 57.16, δ 67.18, δ 69.49, δ 169.98, δ 177.01, δ 177.44 (Supplementary Figure 6). MALDI-TOF calculated: 709.62, observed: 712.66. Elemental analysis (% expected, % observed): C (72.73, 72.33), H (11.78, 11.28), N (1.97, 2.00).

2.2.5 Elemental analysis
Five-ten mg of dry lipid was submitted to Microanalytical Laboratory at the University of California Berkeley for elemental analysis determinations.

2.2.6 Transmission electron microscopy (TEM)
TEM images were obtained at the University of California Berkeley Robert D. Ogg Electron Microscope Laboratory. Thin films of DPAQ:Chol formulations were rehydrated in 50 mM HEPES 150 mM NaCl to a final lipid concentration of 20 mM. Preparations were sonicated for 7 minutes at 80°C. Ten µL of liposome solutions were added to glow discharged copper grids with 400 mesh and Formvar/carbon coatings from Structure Probe, Inc. Liposomes were allowed to adsorb on grids for two minutes. Grids were
washed three times with distilled water. Liposomes were negatively stained with 1% uranyl acetate and imaged using an FEI Tecnai 12 TEM.

2.2.7 Liposome formation in buffers with various anions

Thin lipid films were hydrated in a range of buffers to a final concentration of 20 mM. Preparations were heated at 80 °C for 10 minutes and then sonicated for 7 minutes at 80 °C. After allowing samples to stabilize at room temperature, zeta potential and diameter were measured on a Malvern Nano-ZS. Zeta potential was fit using the Smoluchowski model, while Mark-Houwink parameters were used to determine diameter. All samples were run in triplicate.

2.2.8 Effect of [Ca²⁺] on vesicle zeta potential and size

Liposomes were prepared as outlined above. Liposome suspensions were spiked into a solution containing between 0 and 10 mM CaCl₂ and made isotonic with NaCl. Zeta potential and size measurements were obtained using the Malvern Nanosizer. All samples were run in triplicate.

2.2.9 CF release from DMAQ and DMPC vesicles

The carboxyfluorescein encapsulating protocol was adapted from Weinstein et al. Ten µmol lipid film was rehydrated in 10 mM Tris, 100 mM CF, pH 7.4 to a final concentration of 20 mM lipid. Each sample was heated at 80°C for 10 minutes and subsequently sonicated at 80°C for 10 minutes. Liposomes were then extruded 13 times through a 100 nm polycarbonate membrane. Free CF was removed by size exclusion chromatography with a PD-10 sephadex column. Two concentrations of liposomes (5 or 20 µL) were incubated in 200 µL of 105 mM NaCl, 10 mM HEPES pH 7.4 with or without 30% (v/v) fetal bovine serum, 0.02% sodium azide at 37°C for one week. Leakage was
measured with a FLUOstar plate reader with excitation at 485 nm and emission at 518 nm. Percent leakage values were obtained by normalization to the fluorescence of the samples after lysis of liposomes using 0.5% C₁₂E₁₀. Leakage measurements were run in triplicate.

2.2.10 DSC
DSC experiments were based upon a previously described protocol⁹. DSC was performed with three reusable Hastelloy sample ampoules and a reference ampoule. Data were collected over a range of 5-90°C at 1°C/min with the relevant buffer as the reference. The CpCalc 2.1 software package was used to convert the raw data into a molar heat capacity. The data was then exported and plotted in Matlab. Liposomes for DSC measurements were prepared as outlined above and loaded into the ampule using a glass syringe (250 µL per sample). Samples were scanned through a heat-cool-heat cycle and data was collected from the second heating cycle.

2.2.11 Cell viability assay
C26 murine colon carcinoma cells were seeded at 10⁴ cells per well in 100 µL media in a cell culture treated 96-well plate (Costar). After 24 hours, liposomes were added in 100 µL at varying concentrations. Two lipid formulations with similar transition temperatures were investigated: DPAQ liposomes: DPAQ:Cholesterol:DPPG (60:40:5 mol ratio) and DSPC liposomes: DSPC:Cholesterol:DSPG (60:40:5: mol ratio). Cells were incubated with liposomes for 72 hours. Cells were washed with PBS without calcium and magnesium and fresh media was added. Ten µL of 12 mM MTT was added to each well and cells were incubated for 4 hours at 37°C. All but 25 µL of the media was removed and 50 µL of DMSO was added to each well. After a 10-minute incubation at 37°C, absorbance was read at 540 nm using a OptiMax Microplate Reader from Molecular Devices.
2.2.12 Liposome distribution and pharmacokinetics in vivo

All animal experiments were performed in compliance with the NIH guidelines for animal research under a protocol approved by the Committee on Animal Research at the University of California, San Francisco. Thirty μmol of total lipid was rehydrated in Hepes buffered saline. For liposome stability and liposome fluorescence, DPPG and DiD were incorporated in liposome formulations, respectively. Two lipid formulations with similar transition temperatures were investigated: DPAQ liposomes: DPAQ:Cholesterol:DPPG:DiD (60:40:5:0.2 mol ratio) and DSPC liposomes: DSPC:Cholesterol:DSPG:DiD (60:40:5:0.2 mol ratio). Lipid formulations with cholesterol showed no transition temperature. To prepare liposomes, the lipids were sonicated at 80°C for 20 minutes and extruded 9-15 times through 100 nm polycarbonate membranes. Samples were then dialyzed overnight against Hepes buffered saline (10 mM Hepes, 50 mM NaCl) and filtered through a 200-micron membrane. Two hundred μL of each liposome preparation was injected into the tail vein of BALB/C mice tumored with C26 murine carcinoma cells. Relative lipid concentrations were measured using the DiD signal. The mice were divided into two cohorts. Cohort 1 (n=3 mice per formulation) was bled at 10 minutes, 30 minutes, and 300 minutes post injection. Cohort 2 (n=3 mice per formulation) was bled at 90 minutes, 24 hours and 48 hours post injection. Both cohorts were sacrificed after the last bleeding. Standard DiD curves were constructed for the relevant organs and the plasma by spiking known quantities of DiD into organ and plasma samples. DiD concentration was then measured according to these curves. In order to calculate pharmacokinetic parameters, data was fit to a two-compartment model using MATLAB.
2.3 Results

2.3.1 Synthesis

A straightforward three-step synthetic route was employed to synthesize the AQ lipids (Figure 2.2). The starting compound, 3-(dimethylamino)-1,2-propanediol (2), was linked to myristic acid (C_{14}), palmitic acid (C_{16}), or stearic acid (C_{18}) by DCC coupling (i). After purification via HPFC, the disubstituted product (3a-c) was obtained in high yield (70-80%). Next, the amine was quaternized by S_{N}2 addition of tert-butyl bromoacetate (4a-c, ii). Finally, the carboxylic acid was deprotected under acidic conditions to give the final product (1a-c, iii). Column purification was not necessary after the second or third step, as the quaternized compounds readily precipitated out of dichloromethane (DCM).

The yields for the last two synthetic steps were effectively quantitative and the overall yield for the synthesis was 50-70% from starting material. The lipids were named using the following acronym: Di-Myristoyl/Palmitoyl/Stearoyl Acetate Quaternized-amine (DMAQ, DPFaq, and DSAQ respectively) (Figure 2.3A). Lipid products were confirmed by MALD-TOF mass spectrometry, ^1H NMR, ^13C NMR and C, H, N elemental analysis (Materials and methods, 2.2.4; Figures A1-6).

![Chemical structure](image)

**Figure 2.2**: Synthesis of AQ lipids. (i) Dichloromethane, 4-Dimethylaminopyridine, N,N'-Dicyclohexylcarbodiimide, RT, 16 hours (ii) Dichloromethane, N,N'-Diisopropylethylamine, 40 °C, 16 hours (iii) Dichloromethane, Trifluoroacetic acid, RT, 1 hour.
2.3.2 Vesicle formation and characterization

The ability of AQ lipids to form superstructures was investigated. Single component AQ lipid formulations formed large aggregated vesicles and planar lipid bilayer sheets after heating and sonication as visualized by negative stain TEM (Figure 2.2B, left).

![Figure 2.2: AQ lipid structure and vesicle formation. (A) The structures of the synthesized AQ lipids are shown. (B) TEM images of (right) DMAQ lipids after heating and sonication and (left) of DPAQ:Chol liposomes. (C) Size and zeta potential measurements for AQ:Chol liposomes. Formulations composed of 60% AQ lipids and 40% cholesterol form stable vesicles with neutral zeta potential. Directly after sonication, optically clear suspensions were observed. After 5 minutes at room temperature, these dispersions reorganized into viscous hydrogels or large lipid aggregates. These formulations did not form stable vesicles regardless of saturated chain length or the pH and salt concentration of the hydration buffer. However, incorporation of]
40 mole percent cholesterol in the formulation allowed for the formation of vesicles. Small diameter uniform vesicular-like structures are observed by TEM (Figure 2.3B, right). The TEM images corroborated the size distribution data obtained by DLS (Figure 2.3C). DMAQ formulations including more than 50% molar DPPC formed vesicles at a temperature above the phase transition temperature. However, when the temperature was reduced below the \( T_m \) of DMAQ, the vesicles aggregated and their contents released (data not shown).

2.3.3 Anions bind to DMAQ liposomes according to the Hofmeister series

Zwitterionic sulfobetaine liposomes\(^{10}\) bind to anions in aqueous solutions to a greater extent than do phosphatidylcholines. To investigate if this binding preference is a general phenomenon associated with the inverted headgroup, we measured the zeta potential of DMAQ:Chol liposomes in the presence of various anions and compared it to the zeta potential of DMPC:Chol liposomes measured under the same conditions. DMAQ:Chol liposomes demonstrate more negative zeta potentials than DMPC:Chol liposomes for each anion investigated (Figure 2.4A). This result indicates that anions bind DMAQ:Chol liposomes more effectively than DMPC:Chol liposomes. DMAQ:Chol liposomes sequester anions with high polarizability (\( \text{ClO}_4^-\), \( \text{I}^-\)) more than those with low polarizability (\( \text{Cl}^-\)) (Figure 2.4A), and this preference parallels the Hofmeister series\(^{11}\). Fluoride, which is less polarizable than \( \text{Cl}^-\) in the Hofmeister series, demonstrated similar interactions with AQ:Chol liposomes as did \( \text{Cl}^-\). DMPC:Chol liposomes also bind ions according to the Hofmeister series; however, the magnitude of the change in zeta potential is reduced as compared to DMAQ:Chol liposomes (Figure 2.4A).
**Figure 2.4:** Ionic interactions and stability of AQ vesicles. (A) Zeta potential measurements of anionic interactions of DMPC:Chol and DMAQ:Chol vesicles. Anions with low charge density decrease the zeta potential more than those with a high charge density. (B) Calcium interactions of DMAQ:Chol and DMPC:Chol liposomes. Both formulations become increasingly positive in the presence of increasing \([\text{Ca}^{2+}]\). DMAQ:Chol vesicles remain negatively charged at 10mM \([\text{Ca}^{2+}]\) while DMPC:Chol liposomes adopt a positive charge. (C) DMAQ:Chol vesicles show comparable stability to DMPC:Chol vesicles at 37 °C in 30% serum over one week. For (A)-(C), samples were run in triplicate and error bars represent +/- standard deviation.

**2.3.4 Interaction of calcium with AQ liposomes**

We investigated the interaction of calcium with the DMAQ:Chol and DMPC:Chol liposome compositions using the zeta potential as a surrogate for direct calcium binding (Figure 2.4B). The surface potential of the DMPC:Chol vesicles becomes more positive as the Ca\(^{2+}\) concentration increases. While the zeta potential for the DMAQ:Chol vesicles increases along with Ca\(^{2+}\) concentration, the rate of change is less than observed in
DMPC:Chol vesicles and the surface potential remains negative at 10 mM [Ca\textsuperscript{2+}] (Figure 2.4B). Conversely, DMPC:Chol vesicles adopt a positive charge at 10 mM [Ca\textsuperscript{2+}].

In vivo, divalent cations are known to interact with lipid headgroups to induce aggregation and fusion of vesicles\textsuperscript{12}. As such, we measured the impact of increasing Ca\textsuperscript{2+} concentrations on the aggregation of DMAQ:Chol and DMPC:Chol vesicles. In the presence of 10 mM Ca\textsuperscript{2+} neither formulation showed aggregation (data not shown).

2.3.5 Permeability of AQ liposome to an encapsulated water-soluble anion

The charge at the membrane interface is believed to influence the permeation of charged molecules through the membrane\textsuperscript{13}. Naturally occurring phospholipids have an anionic phosphate group positioned at the membrane interface, while the AQ lipids have a cationic group adjacent to the interface. To better understand the role of this inverted orientation on the permeability of the membrane to water soluble compounds, we measured the leakage rate of CF, a water soluble anion, from DMAQ:Chol and DMPC:Chol liposomes (Figure 2.4C)\textsuperscript{8}. In the absence of serum both DMPC:Chol and DMAQ:Chol liposomes showed minimal leakage of CF at 37°C. In the presence of serum, DMPC:Chol liposomes exhibited slightly more leakage than did the DMAQ:Chol vesicle compositions.

2.3.6 Phase transition temperatures of lipid dispersions prepared from AQ lipids

The phase transition temperature of a lipid formulation can lend insight into the intermolecular forces governing lipid headgroup and hydrophobic domain interactions. The phase transition temperatures for DMAQ, DPAQ, and DSAQ lipids were investigated by DSC; all three lipids demonstrated transition temperatures significantly greater than analogous di-substituted saturated chain PC lipids but similar to analogous PE lipids (Figure 2.5A).
In the presence of anions with low charge density (ClO$_4^-$, I$^-$), DMAQ lipids exhibited a downward shift in T$_m$ (Figure 2.5B). A shift in the T$_m$ was not observed in the presence of large cations, such as tert-butyl ammonium or in the presence of F$^-$ (data not shown). DPAQ and DSAQ lipids exhibit no such shift in transition temperatures in the presence of various anions (Figure 2.5C,D). DPAQ lipids exhibit a pre-transition at low salt concentrations that is eliminated at increased ionic strength (Figure 2.5C).

Figure 2.5: AQ lipids show elevated transition temperatures. (A) AQ transition temperatures are plotted alongside those for PC and PE lipids. (DMAQ Tm 48.8 °C, DPAQ Tm 58.3 °C, DSAQ Tm 65.5 °C). (B-D) Thermograms of DMAQ, DPAQ, and DSAQ lipid dispersions in various salts.
2.3.7 *in vitro* cytotoxicity of liposomes composed of AQ lipids

To determine if the AQ lipids are tolerated when applied to cells in culture, we compared the viability of C26 colon carcinoma cells exposed to DPAQ:Chol or DSPC:Chol liposomes using an MTT assay. At concentrations that are commonly used in vitro or obtained after in vivo administration, both formulations were well tolerated by cells and demonstrated little toxicity (*Figure 2.6A*). At millimolar concentrations, the DPAQ liposomes showed a two fold greater reduction in cell viability in culture than did the DSPC liposomes.

![Graph A: Fraction viable cells vs. [Lipid] (M)](image1)

![Graph B: % Injected dose vs. Time (hours)](image2)

![Graph C: % Injected dose vs. Organ] (image3)

![Graph D: Pharmacokinetic parameters of DPAQ and DSPC liposomes](image4)

**Figure 2.6:** Cytotoxicity, pharmacokinetics and biodistribution of AQ lipid vesicles. (A) Viability of C26 colon carcinoma cells in the presence of DPAQ:Chol or DSPC:Chol liposomes. Samples were run in triplicate. Error bar represent +/- standard error of the mean. (B) Pharmacokinetics and (C) biodistribution of DPAQ and DSPC liposomes. Experiments were run with n=3 mice per group. (D) Pharmacokinetic parameters of DPAQ and DSPC liposomes. Error bars represent +/- standard deviation.
2.3.8 *in vivo* properties of AQ lipids

After confirming the *in vitro* stability of liposomes prepared from the AQ lipids and the low *in vitro* cytotoxicity, we investigated their biodistribution and pharmacokinetic parameters in BALB/C mice bearing subcutaneous C26 tumors. To measure the *in vivo* distribution of AQ liposomes we incorporated DiD into the lipid composition as a fluorescent lipid marker. Two lipid formulations were compared: DSPC:Chol:DSPG:DiD (60:40:5:0.2 mol ratio) and DPAQ:Chol:DPPG:DiD (60:40:5:0.2 mol ratio). Small diameter vesicles were prepared form both lipid compositions (DPAQ: 87 nm, DSPC: 78 nm). It is worth noting that neither formulation contained DSPE-PEG. The pharmacokinetic data indicates that the DPAQ liposomes demonstrate an extensive alpha phase and a terminal half-life of 9.5 hours (*Figure 2.6B, D*). The DSPC have a shorter distribution phase and a terminal half-life of 12.7 hours. Both formulations accumulate in the liver and spleen, with the AQ composition having a greater uptake into the liver and spleen at 48 hours post-administration than the DSPC liposomes (*Figure 2.6C*). Liposome uptake into the tumor was greater for the DSPC liposome composition than for the AQ composition (*Figure 2.6C*).
2.4 Discussion

We describe the synthesis of a new class of inverse-zwitterionic lipids with betaine-like headgroups. These lipids form vesicles in formulations that contain cholesterol and interact with anions with a greater affinity than do liposomes prepared from naturally occurring PC lipids. In the absence of cholesterol, AQ lipids form a turbid dispersion of lipid aggregates. TEM reveals that these formulations form structures resembling planar bilayer sheets. AQ:Chol formulations form a clear solution of small lipid vesicles. Cholesterol will act as a spacer between adjacent AQ lipids and may interfere with extra static interactions between adjacent AQ lipids. It may also allow for a greater membrane curvature necessary to transition from a planar bilayer to spherical vesicles.

The charge at the bilayer interface dictates the ion interactions of the liposome membrane. We hypothesize that these anions intercalate into the interfacial region of bilayer and disrupt lipid packing, which leads to decreased transition temperatures. The bilayer packing disruption may be relatively more pronounced for DMAQ than for DPAQ or DSAQ lipids because of its shorter acyl chains. Alternatively, the anion binding may disrupt hydrogen bonding or electrostatic interactions among adjacent lipids. It is thought that PE lipids show greater transition temperatures than PC lipids with matching hydrophobic tails because of the hydrogen-bonding capabilities of PE headgroups\textsuperscript{14}. As such, the increased $T_m$ of AQ lipids may be due to an altered headgroup conformation that reduces surface area and increases headgroup packing as compared to PC lipids.
A number of the biophysical properties observed for AQ lipids extend to other lipids in the IZ class. Liposomes composed of AQ and SB lipids interact with anions according to the Hofmeister series (Figure 2.7A), a classification of ions according to their ability to salt in or salt out proteins. Ions with high charge densities (F⁻, Cl⁻) are strongly hydrated, while ions with low charge densities (I⁻, ClO₄⁻) are weakly hydrated. Anions with low charge density interact with the bilayer of AQ and SB membranes more than those with high charge density. Further, all anions interact with AQ and SB membranes to a greater extent than PC membranes (Figure 2.7A). We believe that these interactions are directed by the positive charge at the membrane interface.

![Figure 2.7: Biophysical properties of IZ lipids.](image)

(A) IZ lipids (AQ, SB) preferentially interact with ions according to the Hofmeister series. (B) IZ lipids have elevated Tₘ compared to PC lipids.

While the position of the positive charge enhances interactions with anions, it reduces interactions with cations. Divalent cations, especially Ca²⁺, are of particular interest because they are ubiquitous in biological systems and can interact with lipid headgroups to cause membrane destabilization via aggregation, fusion, or alteration of surface charge. CP, CPe, AQ and SB liposomes do not aggregate in the presence of physiological levels of Ca²⁺, and IZ liposomes interact with Ca²⁺ less than PC liposomes. Reduced divalent cation interactions make IZ liposomes interesting
candidates to investigate as *in vivo* delivery systems. In addition to ions found in biological environments, the charge at the membrane interface can influence the permeation of entrapped liposome cargo. CPe liposomes are more permeable to negatively charged contents and relatively less permeable to neutral contents than PC liposomes\(^\text{16}\). In this way, IZ lipids may be useful to tune release properties of certain entrapped liposome drugs.

The inverted headgroup architectures also alter inter- and intra- headgroup interactions. The \(T_m\) is a measure of the intramolecular forces driving hydrophilic interactions between lipid headgroups and hydrophobic and van der Waals interactions between lipid tails. Stronger interactions in these two lipid regions lead to elevated \(T_m\). PE lipids have strong headgroup interactions because of their H-bonding capability, and as such, have higher \(T_m\) than non H-bonding PC lipids. Similarly, lipids with long saturated acyl chains have stronger hydrophobic interactions and thus higher \(T_m\) than those with shorter or unsaturated chains. IZ lipids have very high transition temperatures characteristic of PE lipids rather than PC lipids (*Figure 2.7B*). To our knowledge, CS lipids have the highest transition of any lipid, natural or synthetic (unpublished data). Interestingly, these \(T_m\) are dependent on the ionic character of the surrounding solution. For SB, CS and certain chain lengths of AQ, anion interactions with the membrane according to the Hofmeister series shift the transition from PE-like to PC-like. This shift is likely driven by inter- or intra- headgroup interactions that are disrupted when anions interact with the bilayer surface.

IZ lipids have potential as triggerable *in vivo* delivery systems. AQ liposomes have similar pharmacokinetics and biodistribution to PC liposomes, but have dramatically different biophysical properties that may be exploited for thermo-responsive systems\(^\text{17}\).
Further, CP and CS lipids may be useful in enzyme-triggered systems. The CP lipid headgroup can be cleaved by alkaline phosphatase, yielding a lipid with a net positive charge that may destabilize the membrane and release trapped cargo (unpublished data). Such triggerable systems may be useful in drug delivery to organs enriched in phosphatase or sulfatase activity.
2.6 References

Chapter 3: A robust and quantitative method for tracking liposome contents after intravenous administration

3.1 Introduction

A quantitative understanding of where and when liposome encapsulated agents are released in vivo is critical in the rational design of liposomes for drug therapy. While liposomes may accumulate at the target site, only drug released from the liposome, “cellular available” drug, has biological activity. Drug trapped in the lipid bilayer has little therapeutic consequence as illustrated by the poor performance of liposome-encapsulated cis-platinum in humans\textsuperscript{1}. Liposomal therapeutics should be optimized to release their payload over a timescale defined by the pharmacology of the payload and the biology of the therapeutic target. While there are several methods to quantify liposome pharmacokinetics and assess accumulation of liposomes and payload in target tissues, few approaches can differentiate the signals of the encapsulated and released payload. This paucity of experimental approaches to measure cellular availability has limited the optimization of liposomes for drug delivery.

Microscopic, radioactive, magnetic resonance, and fluorescent tracers have been the principal tools for tracking liposomes in vivo\textsuperscript{2}. Microscopy studies have demonstrated the cellular compartmentalization of liposomes and established the RES as a mediator of liposome clearance\textsuperscript{3}. Encapsulated radioactive tracers or iodinated lipid markers have confirmed that the liver, spleen, bone marrow, and tumor are the primary sites of liposome accumulation in vivo\textsuperscript{4-8}. However, these studies have provided little insight into the release of liposomal payloads in tissues. Results that rely on bilayer embedded or encapsulated
fluorescent tracers such as carbocyanine dyes\textsuperscript{9} or FRET pairs\textsuperscript{10}, can be confounded due to exchange of the probe into lipoproteins and cell membranes\textsuperscript{11}. Encapsulation of self-quenching fluorescent compounds, such as CF\textsuperscript{12} and doxorubicin\textsuperscript{13-15}, or fluorophore-quencher pairs\textsuperscript{16} is useful for measuring entrapped and released contents in plasma samples, but physical and chemical tissue homogenization steps that disrupt the lipid bilayer limit the ability of these probes to report on the cellular availability.

A small number of studies have focused on decoupling the signals of entrapped and released liposome contents in tissues\textsuperscript{17-20}. Laginha and colleagues approximated the fraction of leaked doxorubicin by measuring doxorubicin in tumor nuclei and assuming that all released drug is bound to DNA\textsuperscript{18}. However, this approach is specific for the \textit{in vivo} disposition of doxorubicin crystallized in the liposome and reliant on the drug’s interactions with DNA. The Baldeschwieler group used perturbed angular correlation spectroscopy to quantify entrapped and released $^{111}\text{In}$\textsuperscript{20}. While safe and broadly applicable, this method is limited by its sensitivity. Previously, our group quantified the cellular availability of liposomal contents using a dual radiolabeled reporter system: $[^{51}\text{Cr}]$EDTA and $[^{22}\text{Na}]$\textsuperscript{21}. While $[^{22}\text{Na}]$ is exported by the cell, $[^{51}\text{Cr}]$ is not, and the ratio of the two components measures the liposome cellular availability. While promising, this method has proven to be too complicated for widespread use. Taken together, these studies show that there is a need for quantitative methods to distinguish between entrapped and released liposomal contents in tissues.

We developed a broadly applicable and sensitive method for tracking liposome cellular availability \textit{in vivo} in which MU-P, a water soluble profluorophore of MU is encapsulated in liposomes (Figure 3.1)\textsuperscript{22}. Release of this compound from liposomes \textit{in vivo}
results in its rapid dephosphorylation to form MU (Figure 3.2A); MU, MU metabolites and MU-P can then be quantified by fluorescence or by HPLC. This method allows researchers to obtain a new level of granularity when investigating liposome biodistribution.

We use this method to determine if restricting the transfer of cholesterol out of the liposome bilayer reduces the release of liposome contents in tissues. We used liposomes composed of SML, in which cholesterol is covalently attached to the phosphoglycerol backbone in place of an acyl chain. We compare pharmacokinetics, biodistribution, and cellular availability of SML to that of liposomes composed of conventional phospholipids and cholesterol (CPL) and find that while SML and CPL have similar pharmacokinetic profiles; MU-P encapsulated in SML has greater accumulation and longer persistence in the liver and spleen. This indicates that at certain chain lengths SML release contents slower than CPL.
3.2 Materials and Methods

3.2.1 Lipids

C_{12} PC, C_{14} PC, C_{16} PC, C_{18} PC, C_{20} PC, C_{22} PC, C_{18:1}PC, DOPC, DCHEMSPC, C_{12} SML, C_{14}SML, C_{16}SML, C_{18}SML, C_{20}SML, C_{22}SML, C_{18:1} SML, DSPE-PEG, and cholesterol were purchased from Avanti Polar Lipids, kindly donated by Dr. Zhaohua Huang, or synthesized as previously described \(^{23,24}\).

3.2.2 Materials and instrumentation

MU, MU-P, MU-S, MU-G and 7-hydroxycoumarin were obtained from Sigma. Solvents were removed under reduced pressure using a rotary evaporator. Average liposome diameter and zeta potential measurements were determined using the Zetasizer Nano ZS (Malvern Instruments). Fluorescence spectroscopy was measured on a FluoroLog-3 spectrofluorimeter (Horiba Jobin Yvon) equipped with a temperature-controlled stage (LFI-3751) or using a Tecan Infinite 4300 (Tecan Group Ltd). Data acquisition was done through FluorEssence software (Horiba Scientific). High-pressure liquid chromatography (HPLC) was performed on an Agilent 1100 HPLC (Agilent).

3.2.3 Serum conversion of MU prodrugs

MU-P or MU-S were dissolved in phosphate buffered saline (2.7mM KCl, 1.5mM KH\(_2\)PO\(_4\), 136.9mM NaCl, 8.9mM Na\(_2\)HPO\(_4\), pH 7.4; PBS) and incubated with 50% mouse serum at 37°C. Prodrug conversion to MU was monitored by measuring MU fluorescence every 5 minutes over 12 hours. Data was fit to a Michaelis-Menten model using GraphPad Prism.

3.2.4 Liposomes for contents leakage

Lipids were dissolved in chloroform, dried to form a thin film, and placed under high vacuum overnight. Lipid mixtures included Diacyl:Chol:PEG (55:40:5), Diacyl:SML:PEG.
(55:40:5), SML:PEG (95:5), and DiChems:Diacyl:PEG (20:75:5) with acyl chain lengths of C_{12}-C_{22}. The films were re-hydrated with 1 mL CF (50 mM) or 1 mL MU-P (300 mM) in Hepes buffered saline (10 mM Hepes, 140 mM NaCl, pH 7.4; HBS) at 60°C and vortexed to obtain a lipid concentration of 5 mM. The liposomes were sonicated at 60°C until opalescent (~10 min) and extruded through 200 nm and 100 nm polycarbonate membranes at 60°C. Liposomes were purified on a Sephadex G-25 size exclusion column. In order to quantify CF release from liposomes, CF fluorescence (excitation 492 nm, emission 517 nm) was measured before and after disrupting the lipid membrane with C_{12}E_{10} surfactant. MU-P fluorescence was measured by diluting liposomes into HBS containing calf intestinal phosphatase (New England BioLabs). MU-P outside the liposomes is converted to MU, while MU-P inside the liposomes remains phosphorylated. The fluorescence of MU was measured (excitation 360, emission 449) before and after liposome lysis. The liposome samples were incubated at 37°C in the presence or absence of 30% fetal bovine serum (FBS) for 30 days. Sodium azide (0.05%) was added to limit bacterial growth.

### 3.2.5 Liposomes for animal studies

Liposomes that were used in animals were prepared by an ethanol injection method followed by extrusion. Lipids dissolved in chloroform were dried to form a thin film and placed under high vacuum overnight. Lipids were dissolved in 200 µL ethanol, heated to 50°C and injected into a stirring 1.8 mL solution of 300 mM MU-P in HBS. After 20 minutes, the liposomes were extruded through 200 nm and 100 nm polycarbonate membranes and dialyzed against HBS with frequent buffer exchange. Before injection into animals, liposomes were separated from non-encapsulated MU-P on a Sephadex G-25 size exclusion column and filtered through 0.2 micron sterile filters into sterile polystyrene tubes.
3.2.6 Fluorescent anisotropy

Lipids were dissolved in chloroform, dried to form a thin film, and placed under high vacuum overnight. The films were hydrated with PBS at 65°C and vortexed to obtain a lipid concentration of 5 mM. The liposomes were sonicated at 65°C until opalescent and then extruded through 200 nm and 100 nm polycarbonate membranes at 65°C. Liposomes were diluted 8-fold with PBS and 6 µl of DPH (Molecular Probes) in tetrahydrofuran (0.15 mg/ml) was added to the sample. To allow the DPH to integrate into the bilayer, the mixture was incubated at 65°C for 1 hour. Anisotropy measurements were obtained as previously described 25. Briefly, DPH was excited at 350 nm and the fluorescence detected at 430 nm. With mixing, the liposomes were heated or cooled in 5°C increments from 5–62°C, with a 5–10 minute equilibration between transitions.

3.2.7 Biodistribution and pharmacokinetics studies

All animal experiments were performed in compliance with the NIH guidelines for animal research under a protocol approved by the Committee on Animal Research at the University of California, San Francisco. CD-1 mice were injected via the tail vein with 20 mg/kg MU-P equivalents in a volume of 0.2 mL. For pharmacokinetic studies, blood was collected 10, 20, 60, 180, 1440 and 2880 minutes (3 mice per group) after injection and centrifuged for 10 min at 15,000 g. Serum was collected in a tube with a 1:100 dilution of Phosphatase Inhibitor Cocktail 2 (Sigma). Ten µL of serum was added to 2 mL of HBS along with 10 µL of a 15% C12E10 solution. A standard curve was made by titrating MU-P loaded liposomes into the serum from an untreated mouse. MU-P (excitation 320 nm, emission 385 nm), MU (excitation 360 nm, emission 449 nm), and DiD (excitation 644 nm, emission 664 nm) were measured from each serum sample. The data was fit to a two-compartment model using GraphPad Prism. For biodistribution studies, tissues were isolated and flash
frozen 3 and 48 hours after injection.

3.2.8 Biodistribution of MU-P and metabolites by HPLC

Tissues were thawed at room temperature and homogenized by bead beating in a homogenization solution (50% Methanol, 0.1% TFA, 1:100 dilution of Phosphatase Inhibitor Cocktail 2, 1 μg/mL 7-hydroxycoumarin as an internal standard). Homogenized tissues were frozen overnight and sedimented by centrifugation (15,000 g, 15 minutes). The supernatant was passed through a 0.22 μm filter (Fisherbrand, 13mm). Forty μL of the filtered tissue homogenate was injected on a C8 column (ZORBAX Eclipse XDB-C8 5-Micron) with the following solvent system: Mobile Phase A: 0.1% TFA, Mobile Phase B: 0.1% TFA/MeOH at 1.0 mL/min over a gradient of 10% B to 70% B over 10 minutes with a 5 minute re-equilibration at 40°C and detection at 320 nm. Biodistribution data was extrapolated by fitting peak areas to standard curves of MU, MU-P, and MU-G. All data was normalized to the internal standard.
3.3 Results

3.3.1 MU-P reporter system

MU derivatives are commonly used reagents for determining enzymatic activity of phosphatases (MU-P) and sulfatases (MU-S) by fluorescence (Figure 3.1)\textsuperscript{22,26}. The MU profluorophores, MU-P (excitation 320, emission 385) and MU-S (excitation 334, emission 370), have shifted fluorescent spectra from MU (excitation 360, emission 449) that allows for independent quantification. We used these common reporter systems to develop an assay to measure liposome contents release (Figure 3.2A).

We characterized MU-P and MU-S stability in serum in order to choose the optimal profluorophore for encapsulation in liposomes (Figure 3.2B,C). The kinetics of MU-P conversion to MU (K\textsubscript{m}: 0.47 mM, V\textsubscript{max}: 10.2 nmol/min) were significantly greater than the kinetics of MU-S to MU (K\textsubscript{m}: 16.8 mM, V\textsubscript{max}: 0.32 nmol/min). This may be due to higher levels of phosphatases than sulfatases in serum or due to a difference in enzyme affinity for MU-P and MU-S. We hypothesized that if the lipid bilayer was disrupted, the profluorophore would be released into the tissue environment and rapidly converted to MU. As such, we chose MU-P as the tracer molecule because of its rapid conversion to MU in serum. We further assume that MU-P in the

\textbf{Figure 3.1: Relevant structures.} Structures of 4-methylumbelliferone (MU), MU metabolite 4-methylumbelliferyl glucuronide (MU-G), MU prodrugs 4-methylumbelliferyl phosphate (MU-P) and 4-methylumbelliferyl sulfate (MU-S), carboxyfluorescein (CF), and HPLC standard 7-hydroxycoumarin (Std).
liposome is protected from phosphatase activity. Distinct quantification of MU-P and MU therefore gives a reading of entrapped (MU-P) and released (MU) contents.

**Figure 3.2: MU-P reporter system.** (A) MU-P is encapsulated in liposomes. Upon leakage from liposomes, MU-P is rapidly converted to MU by endogenous phosphatases. (B,C) Michaelis-Menten kinetics of MU-P and MU-S conversion in mouse serum.

### 3.3.2 Effects of acyl chain length, saturation, and liposome composition on membrane stability

CF ([Figure 3.1](#)) is widely used to measure contents release from liposomes\(^{12}\), as CF fluorescence is quenched while concentrated in the liposome, but easily quantifiable after release from the liposome. Using the CF reporter system as a benchmark, we first sought to validate our reporter system *in vitro* by establishing a relationship between liposome formulation and stability. CF and MU-P were passively encapsulated into a library of liposome formulations ([Figure 3.3C](#)) and the release of the reporter molecules was
monitored by fluorescence in the presence (Figure 3.3) and absence (Figure 3.4) of serum.

Figure 3.3: Effects of acyl chain length, saturation, and liposome composition on membrane stability in serum. CF (A) or MU-P (B) was passively encapsulated in (C) a range of CPL (○) or SML (△). Molar ratios of liposome formulations are given. Liposomes were incubated in HBS containing 30% serum. The percent of MU-P leaked from liposomes after 14 days is shown.
MU-P is more stably encapsulated in liposomes than CF (Figure 3.3): likely due to the dual negative charge on the phosphate (pKa 7.8) that prevents the drug from crossing the hydrophobic core of the lipid bilayer. As expected, formulations composed of lipids with long and saturated acyl chains are more stable than those composed of lipids with short or unsaturated acyl chains (Figure 3.3; Figure 3.4; Table 3.1).

**Figure 3.4:** Effects of acyl chain length, saturation, and liposome composition on membrane stability. CF (A) or MU-P (B) was passively encapsulated in a panel of liposomes. Liposomes were incubated in HBS. The % of MU-P leaked from liposomes after 14 days is shown.

SML (△) encapsulating CF are most stable if they contain lipids with intermediate acyl chain length (C₁₆ and C₁₈), and are less stable at both lower and higher chain lengths or when the SML acyl chain is unsaturated (Figure 3.3A,B; Figure 3.4). This “U” shaped stability pattern indicates an optimal SML bilayer packing conformation at intermediate chain lengths, with less stable packing conformations at both higher and lower chain lengths. In contrast, CPL (○) exhibit binary stability: they are highly unstable at chain lengths below C₁₆ and show minimal leakage at chain lengths above C₁₆ (Figure 3.3A,B). This dramatic change in stability is due to tighter membrane packing because of increased hydrophobic interactions at higher chain length. While SML appear more stable than CPL at low chain lengths (C₁₂ and C₁₄), they are still relatively unstable, releasing more than 60%
of their contents over 14 days. Formulations incorporating DCHEMSPC as a substitute for cholesterol (□) and liposomes containing a mix of di-substituted saturated acyl chains and SML (◇) follow a similar pattern to CPL: they are unstable at low chain lengths but stable at high chain lengths (Figure 3.5).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MU-P Avg Leakage</th>
<th>Std</th>
<th>CF Avg Leakage</th>
<th>Std</th>
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<td>0.6</td>
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<td>101.0</td>
<td>1.0</td>
</tr>
<tr>
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<td>0.6</td>
<td>101.0</td>
<td>1.7</td>
</tr>
<tr>
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<td>42.7</td>
<td>0.6</td>
</tr>
<tr>
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<td>0.6</td>
<td>91.3</td>
<td>10.6</td>
</tr>
<tr>
<td>DOPC:DCHEMSPC</td>
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<td>1.9</td>
<td>102.0</td>
<td>2.6</td>
</tr>
<tr>
<td>DOPC:DCHEMSPC Serum</td>
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<td>0.6</td>
<td>98.7</td>
<td>2.3</td>
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<td>99.0</td>
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<tr>
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<tr>
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<td>100.0</td>
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<tr>
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<td>0.6</td>
<td>99.0</td>
<td>1.7</td>
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Table 3.1: In vitro leakage of liposomes formulated with unsaturated lipids. The average and standard deviation of the total percent leakage of CF or MU-P after 14 days in HBS or HBS containing serum from liposomes is summarized.

In contrast to CF, MU-P is stably encapsulated in both SML (△) and CPL (○) across chain lengths (Figure 3.3A,B). This stability highlights the utility of MU-P as a probe to measure contents release from highly unstable liposome formulations that could not be measured using CF (Figure 3.3A,B, lower left quadrant). Liposomes composed of a
mixture of SML and diacyl lipids were highly unstable if they contained acyl chains shorter than C_{18} (Figure 3.5). This reduction in stability may be indicative of heterogeneous packing of the SML and diacyl lipids in the membrane.
Figure 3.5: Effects of acyl chain length, saturation, and liposome composition on membrane stability. CF (A,B) or MU-P (C,D) was passively encapsulated in a panel of Diacyl:SML (◇) or Diacyl:DiCHEMS (□) liposomes. Liposomes were incubated in (A,C) HBS or (B,D) HBS containing serum. The % of MU-P leaked from liposomes after 14 days is shown.
3.3.3 SML and CPL demonstrate comparable membrane fluidity

To further characterize the membranes of liposomes composed of SML, we measured membrane fluidity using fluorescence anisotropy. A fluorescent probe was incorporated into the liposome membrane and fluidity was deduced by measuring the motility of a fluorophore in the bilayer by anisotropy\textsuperscript{28}. Membranes with higher anisotropy values are more rigid while those with lower anisotropy values are more fluid. The SML and the control formulation (C\textsubscript{16} PC:cholesterol) showed similar anisotropy values over a range of temperatures (Figure 3.6A). As expected, membrane fluidity increases as a function of temperature.

\textbf{Figure 3.6: SML and CPL demonstrate comparable membrane fluidity.} Fluorescence anisotropy measurements for SML lipids are compared alongside C16 CPL. (B) Sigmoidal anisotropy plots of pure PC lipids.

Anisotropy for a typical PC lipid will follow a sigmoidal pattern: high anisotropy values at low temperatures are indicative of the rigid, crystalline state of the lipids. As temperature increases, the lipids undergo a phase transition to a fluid state, indicated by a sharp change in anisotropy (Figure 3.6B). Addition of cholesterol into a membrane eliminates the lipid’s natural phase transition and forces the bilayer into a permanent gel-like state that is neither fluid nor rigid. This state is crucial to the maintenance of bilayer stability. The loss in phase transition is reflected on the anisotropy plots (Figure 3.6). SML
exhibit no phase transition, which indicates that the SML bilayers are also in a gel-like state. As such, while conjugation of cholesterol to the lipid backbone influences contents release rates, it does not significantly modify the bilayer structure.

3.3.4 Evaluation of SML and CPL stability in circulation by fluorescence

We compared the pharmacokinetics of SML and CPL after intravenous injection via the tail vein in mice. MU-P was passively encapsulated into liposomes and a far-red fluorescent lipid, DilC_{18} (DiD), was incorporated into the lipid membrane. This permitted independent tracking of the liposome and its contents. After encapsulation of MU-P, all liposomes studied had similar sizes and zeta potentials (Table 3.2). Serum was collected from mice directly into a tube with phosphatase inhibitor. The liposome contents (MU-P), released contents (MU), and lipid (DiD) were measured in serum samples by quantifying fluorescence after injection (Figure 3.7).
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Diameter (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
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<td>0.12</td>
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<tr>
<td>C18 SML</td>
<td>103.5</td>
<td>0.20</td>
<td>-2.87</td>
<td>0.40</td>
</tr>
<tr>
<td>C20 SML</td>
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<td>0.27</td>
<td>-3.00</td>
<td>0.45</td>
</tr>
<tr>
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<td>0.20</td>
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<td>0.32</td>
</tr>
<tr>
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<td>0.04</td>
<td>-2.20</td>
<td>0.22</td>
</tr>
<tr>
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<td>0.10</td>
<td>-2.00</td>
<td>0.15</td>
</tr>
<tr>
<td>C18:1 SML</td>
<td>114.6</td>
<td>0.08</td>
<td>-1.13</td>
<td>0.23</td>
</tr>
<tr>
<td>C18:1 CPL</td>
<td>132.8</td>
<td>0.06</td>
<td>-2.23</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Table 3.2: Diameter and zeta potential of the liposome formulations used in the pharmacokinetic and biodistribution studies.

After injection of MU-P containing liposomes, MU could not be detected by fluorescence. After MU-P is released from the liposomes, it is rapidly converted to MU by serum phosphatases and is rapidly eliminated. We assume that the MU-P signal measured in serum was entrapped in liposomes, as free MU-P is also quickly cleared from circulation ($t_{1/2}$: 17 minutes). The MU-P and DiD serum concentrations over time were fit to a two-compartment model (Figure 3.7, Table 3.2), and the ratio of the total exposure of MU-P to the total exposure of lipid ($\text{AUC}_{\text{MU-P}}/\text{AUC}_{\text{DiD}}$) quantifies how stably the liposomes retain their contents in circulation (Figure 3.7I).

SML and CPL with acyl chain lengths of C$_{16}$ and C$_{18}$ formed the most stable liposomes (Figure 3.7A-D), as their $\text{AUC}_{\text{MU-P}}/\text{AUC}_{\text{DiD}}$ ratios approach 1. In sharp contrast, liposomes with C$_{20}$ acyl chain lengths or with unsaturated fatty acid tails were relatively unstable in circulation, as demonstrated by the rapid loss of contents signal after injection (Figure 3.7E-H). C$_{20}$ CPL were more stable than C$_{20}$ SML. C$_{20}$ lipids form liposomes with a thicker bilayer than lipids with shorter acyl chains. In this bilayer context, it may be advantageous for cholesterol to be in a free state to adopt its optimal packing...
conformation. Constraining the cholesterol in the C20 SML by covalently attaching it to the headgroup may contribute to the relative instability of the SML.

Taken together, these results highlight the increased serum stability of SML as compared to CPL and validate the MU-P/MU fluorescent reporter system as a method to track liposomes in circulation.
Figure 3.7: Evaluation of SML and CPL stability in circulation by fluorescence. (A-H) MU-P, MU and DiD signals were measured in serum samples by fluorescence. The MU-P and DiD data is fit to a two-compartment model. MU was undetectable in serum at all time points. (I) The ratio of \( \frac{AUC_{MU}}{AUC_{DiD}} \) for each formulation is shown.
3.3.5 SML alter contents delivery to the liver and spleen

Most current methods for measuring tissue biodistribution of liposomes fail to differentiate between entrapped and released contents, as the liposome membrane is often compromised during tissue homogenization. The MU-P reporter system allows for simultaneous and independent quantification of encapsulated (MU-P) and free (MU) drug by fluorescence. To increase the sensitivity of this method, we developed an HPLC approach for quantification of MU, MU-P, and MU-G, the major MU metabolite, in homogenized tissues (Figure 3.1, Figure 3.8A). An internal standard, 7-hydroxycoumarin (Std), was included to determine the tissue extraction efficiency and monitor the resolution and accuracy of the column. MU, MU-P, MU-G and Std were all readily quantifiable by HPLC in liver, spleen and kidney tissues at both 3 and 48 hours post injection of MU-P loaded liposomes with a detection limit of ~100 ng/mL (Figure 3.8A, Figure 3.9). To further validate this approach, we compare HPLC and fluorescence measurements of MU and MU-P in serum samples and determine that they provide equivalent results (Figure 3.10).

The encapsulated contents primarily distribute to the liver and spleen, a finding in accordance with the extensive literature on liposome biodistribution over the past 40 years. The MU-P signal was higher in the liver than the spleen, while the inverse was true for MU: indicating faster liposome degradation and higher cellular availability in the spleen. As expected, the MU-G metabolite was the most abundant species in the kidneys (Figure 3.9)29.
Figure 3.8: SML alter delivery to the liver and spleen. (A) HPLC trace from the liver, spleen, and kidney of a mouse injected with liposomal MU-P. Percent of injected dose in the (B) liver and (C) spleen at 3 hours (left) and 48 hours (right) after administration. MU, MU-P and MU-G levels are shown and sum to the total amount of MU equivalents in the tissue.
Figure 3.9: SML and CPL delivery to the kidneys.

Figure 3.10: Fluorescence and HPLC measurements give similar results. C18 CPL (left) and C18 SML (right) liposome pharmacokinetics were fit to a two-compartment model. Lipid signal was measured by fluorescence. MU-P was measured by both fluorescence and by HPLC (dashed line) from serum samples.

The biodistribution results corroborate with the results from the pharmacokinetic study, as the most stable formulations in circulation show the highest drug accumulation in tissue. Importantly, tissue accumulation correlated with total MU-P signal in circulation rather than total lipid signal in circulation, which indicates that the stability of the liposome dictates tissue delivery. The C_{16} SML offers improved tissue uptake over the C_{16} CPL at both 3 and 48 hours after injection. While the C_{18} SML and C_{18} CPL demonstrate similar delivery at 3 hours (Figure 3.8B,C), contents delivered in the C_{18} SML persist in the tissue longer than contents delivered in the C_{18} CPL (Figure 3.8B,C). This trend suggests that in addition to being more stable in circulation, the C_{16} and C_{18} SML demonstrate improved
persistence in the liver and spleen as compared to CPL formulations. In stark contrast, the $C_{20}$ CPL demonstrate higher delivery than the $C_{20}$ SML, which again mirrors the stability of these formulations in circulation. Both SML and CPL lipids with unsaturated chains show relatively less delivery to both the liver and spleen, but SML provide better delivery than CPL (Figure 3.8B,C).
3.4 Discussion

We have developed a robust and quantitative method for measuring a critical aspect of liposome disposition in vivo: cellular availability. Drug release from liposomes over the wrong timescale or in the wrong cellular location can limit efficacy. Allen and colleagues demonstrate that premature doxorubicin release from unstable liposomes in circulation increases toxicity and decreases efficacy\textsuperscript{30}. A large database is now published on the role of stability on circulation of a variety of liposome encapsulated drugs and their efficacy\textsuperscript{30-33}. As mentioned earlier, the failure of liposome encapsulated cisplatin in the clinic has been attributed to incomplete release of the drug from the carrier at the tumor site\textsuperscript{34}; we think that both the chemistry of the drug and the stability of the liposome contribute to this limitation. The cellular availability of MU-P adds new information concerning the release of contents from the liposome that can be incorporated into pharmacodynamic models to help explain and predict the activity of other liposomal agents.

This chapter details a method to trace liposomes in vivo, and it enables distinct quantification of entrapped and released liposome contents in tissues. While other methods for quantification of liposome contents release exist, their utility is restricted by their complexity\textsuperscript{21}, sensitivity\textsuperscript{20}, or narrow applicability\textsuperscript{18}. In designing this approach, we searched for a tracer compound that would meet the following criteria: (1) safe at high doses, (2) highly fluorescent, (3) fast excretion when released into extracellular fluids, and (4) undergoes a conformational change when released from the liposome. MU-P fulfills all four of these criteria. MU is extremely safe both in mice and humans\textsuperscript{29}. In mice, the maximum tolerated dose for MU-P administered intravenously was greater than 500 mg/kg (data not shown). MU-P released from the liposome is either cleared from
circulation or rapidly converted to MU. When tissue samples are isolated and homogenized in the presence of a phosphatase inhibitor, the MU-P that is detected is entrapped in the liposome.

We use this method to explore how liposome composition influences the cellular availability of MU-P after intravenous administration of MU-P liposomes. We find that liposomes composed of SML, in which the cholesterol is covalently anchored in the bilayer, release MU-P more slowly than traditional liposomes containing free cholesterol. This result suggests that SML release contents over a longer timescale and may be useful in the delivery of water soluble drugs that require a sustained duration to exhibit a maximal therapeutic effect. This sustained release can be tuned by adjusting the length of the acyl chain at the 1 position of the SML. Interestingly, SML and CPL show similar pharmacokinetic profiles but different biodistribution and degradation patterns in tissues. This result suggests that cholesterol transfer is an important component of liposome degradation in the liver and spleen.

Our approach has broad applicability in vitro and in vivo. The in vitro leakage experiments clearly demonstrate that MU-P is stably encapsulated in a range of lipid formulations. In contrast to CF, MU-P can be used to probe the stability of formulations with short saturated or unsaturated chains. More important, however, is the in vivo utility of this method. We show that it is possible to continuously monitor the fraction of intact liposomes in a given tissue; this is a measurement not easily determined using other approaches.
3.5 References


5930 (1989).

Chapter 4: Improving the distribution of Doxil® in the tumor matrix by depletion of tumor hyaluronan

4.1 Introduction

Nanoparticle therapeutics, especially liposomes, have been investigated as anti-cancer drug carriers for four decades with a number of clinical and commercial successes\(^1\). Encapsulating a drug in a liposome extends its circulation time and improves its biodistribution. Successful delivery of liposomes into a tumor depends on: 1) prolonged circulation in the bloodstream, 2) extravasation from the tumor vasculature, and 3) distribution into the tumor parenchyma. The large diameter of nanoparticles allows for their selective accumulation at the tumor site by the EPR effect\(^2,3\). However, the diameter of liposomes is a double-edged sword: while a boon for drug loading, pharmacokinetics, and biodistribution, it limits distribution in the tumor\(^2,4\). As a result, despite improved pharmacokinetics, liposome therapies have offered limited improvements in efficacy over the un-encapsulated drug\(^3\).

Liposome distribution away from the vasculature is limited by both the steric barrier of the tumor ECM and the elevated tumor IFP\(^5\). A myriad of molecular approaches have been used to improve the tumor distribution of macromolecular therapeutics including: tumor penetrating peptides\(^6-8\), hyperthermia\(^9,10\), degradable nanoparticles\(^11\), enzymes and small molecules that modify the tumor ECM\(^12-16\), vascular priming\(^17\), and vascular normalization\(^18,19\). The effectiveness of these approaches depends on the pharmacology of the drug and the biology of the tumor. While certain tumors may exhibit a pronounced EPR effect, others do not\(^20\); further, while a subset of tumors have an ECM rich
in collagen, others have an ECM rich in HA. As such, each tumor type presents a different physical barrier to delivery, and this heterogeneity calls for a number of potential approaches to improve tumor distribution.

The tumor ECM is composed of a network of collagen and HA that interact with other hydrated proteoglycans. The matrix contributes to the high IFP by entrapping water and prevents penetration of liposomes into the tumor core\textsuperscript{21}. HA is an intractable barrier to liposome drug delivery and hence a potential therapeutic target. A high molecular weight glycosaminoglycan, HA is enriched in breast, colorectal, gastric, glioma, lung and ovarian tumors\textsuperscript{22-26}. Nearly 90\% of pancreatic adenocarcinomas are characterized by abundant accumulation of HA\textsuperscript{27,28}. HA is involved in tumor progression; high levels correlate with the motility, invasiveness, and metastatic potential of tumor cells\textsuperscript{29,30}. HA directs cell motility by interacting with cell receptors CD44 and RHAMM and can shield tumor cells from host immune cells\textsuperscript{31}. For the above reasons, strategies to remove HA or block its synthesis could improve drug delivery into solid tumors.

Given its abundance across tumor types and role in metastasis, HA has been the target of various therapeutic interventions. Systemic administration of PEGPH20 (Halozyme Therapeutics), a PEGylated human hyaluronidase, transiently depleted HA, decreased tumor IFP, and enhanced drug activity in prostate and pancreatic tumor models\textsuperscript{32,33}. Further, PEGPH20 improved the uptake and distribution of liposomal doxorubicin in osteosarcoma xenograft models\textsuperscript{34}. PEGPH20 is currently being investigated in combination with gemcitabine in clinical trials for pancreatic cancer (NCT01453153).

A small molecule, MU, has also been used to deplete HA and reduce metastasis in solid tumors\textsuperscript{35-39} and has been extensively evaluated \textit{in vitro}\textsuperscript{35,40-42}. MU is a safe, well
characterized, and clinically approved choloretic and antiplasmodic agent\textsuperscript{43-47}. MU inhibits HA synthesis by down-regulating HAS2 and HAS3 and by depleting the cellular pool of UDP-glucuronic acid, one of the building blocks of HA \textbf{(Figure 4.1A)}\textsuperscript{35,48}. However, MU is limited by its low potency (daily oral doses of 450mg/kg)\textsuperscript{35} and low water solubility.

In this study, we circumvent these limitations of MU by encapsulating a water-soluble phosphorylated prodrug of MU (MU-P) in a liposome (L-MU-P). We demonstrate that L-MU-P is a more potent inhibitor of HA synthesis than oral MU in the 4T1 murine mammary carcinoma model. We further demonstrate that, under certain conditions, HA depletion improves the tumor distribution of liposomes, which results in enhanced anti-tumor efficacy.
4.2 Materials and Methods

4.2.1 Lipids
HSPC, POPC, DSPE-PEG, and cholesterol were purchased from Avanti Polar Lipids. DiD (excitation 644 nm, emission 664 nm) was obtained from Life Technologies.

4.2.2 Antibodies, dyes and tracer molecules
MU (excitation 360 nm, emission 449 nm), MU-P (excitation 320 nm, emission 385 nm), MU-S, MU-G, and 7-hydroxycoumarin were obtained from Sigma. Purified rat anti-mouse CD31 (clone MEC 13.3) was purchased from BD Pharmingen. Secondary FITC-conjugated donkey anti-rat IgG (712-095-153) was purchased from Jackson ImmunoResearch Laboratories. Hoechst 3342 nuclear stain was purchased from Thermo Scientific.

4.2.3 Instrumentation
Solvents were removed under reduced pressure using a rotary evaporator. Fluorescence spectroscopy was measured on a FluoroLog-3 spectrofluorimeter (Horiba Jobin Yvon) equipped with a temperature-controlled stage (LFI-3751) or using a Tecan Infinite 4300 (Tecan Group Ltd). Data acquisition was done through FluorEssence software (Horiba Scientific). High-pressure liquid chromatography (HPLC) was performed on an Agilent 1100 HPLC (Agilent).

4.2.4 MU-P hydrolysis in serum
MU-P or MU-S were dissolved in phosphate buffered saline (2.7mM KCl, 1.5mM KH2PO4, 136.9mM NaCl, 8.9mM Na2HPO4, pH 7.4; PBS) and incubated with 10-50% human, mouse, chicken, goat, rabbit, porcine, or fetal bovine serum at 37°C. All serum was purchased from Gibco. Prodrug conversion to MU was monitored by measuring MU
fluorescence (excitation 360, emission 449) every 5 minutes over 12 hours. Data was fit to a Michaelis-Menten model using GraphPad Prism.

4.2.5 Quantification of HA in cell culture

4T1 murine breast carcinoma cells were seeded in 96 well cell culture plates (Corning Costar) at 5,000 cells per well. Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum. MU was solubilized in dimethyl sulfoxide (DMSO), diluted into RPMI 1640 media supplemented with insulin, transferrin, and sodium selenite (ITS, Sigma), and added to cells. MU-P was solubilized directly into RPMI-ITS and added to cells. Twenty-four hours after plating, cells were washed 3x with phosphate buffered saline (2.7mM KCl, 1.5mM KH2PO4, 136.9mM NaCl, 8.9mM Na2HPO4, pH 7.4; PBS). MU and MU-P were added to cells after washing with or without Phosphatase Inhibitor Cocktail 2 (Sigma). Forty-eight hours later, 50 µL of media was removed and frozen. Fifteen µL of alamarBlue (Life Technologies) was added to cell media and incubated for 3 hours. Cell number was quantified by measuring alamarBlue fluorescence at 585 nm after excitation at 570 nm using a Tecan Infinite 4300 (Tecan Group Ltd).

HA levels in culture media were quantified by using the HA DuoSet ELISA assay (R&D Systems). Frozen media samples were thawed and diluted 1:100 in Reagent Diluent (R&D Systems). The ELISA was run according to the manufacturer’s specifications. Total HA in culture media was normalized to cell number using the alamarBlue assay and to DMSO control wells.

4.2.6 Liposome preparation

Liposomes that were used in animals were prepared by an ethanol injection method followed by extrusion. Dry lipid powders were weighed out and solubilized in 2 mL of
ethanol. After warming to 65°C, lipids were rapidly injected into a heated 18 mL solution of 300 mM MU-P or Hepes buffered saline (HBS; 10mM Hepes, 140mM NaCl, pH 7.4) for control and DiD labeled liposomes. Two liposome formulations were administered in the HA depletion and survival studies: POPC:Cholesterol:DSPE-PEG (55:40:5) and HSPC:Cholesterol:DSPE-PEG (55:40:5). In the liposome distribution study, POPC:Cholesterol:DSPE-PEG:DiD (55:40:5:0.2) was used. The solution was serially extruded through polycarbonate membranes using a high-pressure lipid extruder (Northern Lipids) heated to 65°C: 4x through a 200 nm membrane, 4x through a 100 nm membrane, and 2x through an 80 nm membrane. Lipids were then dialyzed against HBS with frequent buffer exchange. Before use, liposomes are sterilized by filtration through a 0.2 µm membrane. MU-P loading was measured by lysing liposomes and fitting MU-P fluorescence to a standard curve. Average liposome diameter and zeta potential measurements were determined using the Zetasizer Nano ZS (Malvern Instruments). Zeta potential was fit using the Smoluchowski model, while Mark–Houwink parameters were used to determine diameter. All samples were run in triplicate. Release of MU-P from liposomes was quantified as previously described49.

4.2.7 Cation exchange of MU-P

Diammonium MU-P was generated by making a 500 mM stock solution of the MU-P disodium salt. An AG 50W-X8 cation exchange resin in the H⁺ form was converted to the ammonium form using 3 column volumes of a 1 M ammonium chloride solution. The column was washed with 3 column volumes of deionized water and the MU-P sample was added. The MU-P sample was eluted with 1M ammonium chloride. MU-P was measured in elution fractions by fluorescence in 0.2M sodium carbonate buffer pH 9.0. The
diammonium MU-P solution was adjusted to 250mM [MU-P] and the pH was adjusted to pH 5.4.

4.2.8 Active loading of doxorubicin into MU-P loaded liposomes

Active loading of doxorubicin was adapted from Huang et al\textsuperscript{50}. A 20 µmol lipid preparation of HSPC:Chol:DSPE-PEG (55:40:5) was dried down to a thin film by rotary evaporation and placed under high vacuum overnight. The lipid film was hydrated with a 250 mM solution of ammonium MU-P at 60°C. Liposomes were sonicated under argon and serially extruded through 200nm, 100nm and 80nm polycarbonate membranes. Liposomes were then dialyzed against 10% sucrose at 4°C with frequent buffer exchange. Two µmol of doxorubicin in a 10% sucrose solution was added to the liposomes and the solution was incubated for 1 hr at 60°C in the dark with intermittent vortexing. The sample was allowed to cool to room temperature and then mixed with Dowex 50Wx4 resin to remove the free doxorubicin. The sample was rotated for 1 hr in the dark at room temperature. Finally, the sample was passed through a column and the doxorubicin liposomes were collected. Doxorubicin encapsulation efficiency was measured using fluorescence of liposomes diluted into 90% isopropyl alcohol 0.075M HCl. Fluorescence was plotted against a standard curve to determine concentration.

4.2.9 Transmission electron microscopy (TEM)

TEM images were obtained at the University of California Berkeley Robert D. Ogg Electron Microscope Laboratory. Ten µL of liposome solutions were added to glow discharged copper grids with 400 mesh and Formvar/carbon coatings from Structure Probe, Inc. (West Chester, PA). Liposomes were allowed to adsorb on grids for 2 min. grids were washed three times with distilled water. Liposomes were negatively stained with 1%
uranyl acetate and excess stain was removed with by a single wash with distilled water. Grids were imaged using an FEI Tecnai 12 TEM (FEI, Hillsboro, OR).

4.2.10 HA depletion in tumors
All animal experiments were performed in compliance with the NIH guidelines for animal research under a protocol approved by the Committee on Animal Research at the University of California, San Francisco. Mice were tumored by orthotopic injection of 300,000 4T1 cells into the mammary fat pad in a volume of 50 µL. Mice were treated with MU or L-MU-P to reduce tumor HA levels. MU was dosed daily by oral gavage at 400 mg/kg 5 days a week. MU was mixed in a 1% CMC solution in a volume of 1 mL per dose. L-MU-P was dosed intravenously at a dose of 18 mg/kg MU-P in a volume of 0.2 mL. L-MU-P was administered in 1-4 doses on days 14, 17, 20, and 24. Forty-eight hours after the final dose, tumors were isolated, homogenized in lysis buffer (0.1% Triton X-100 in PBS), and then rotated in lysis buffer for 48 hrs at 4°C. Homogenates were centrifuged at 6500g for 1 hr and then 13000g for 1 hr and the supernatants were collected. Samples were diluted 1:500 before adding to the ELISA plate. The ELISA was run according to the manufacturer's specifications. Total tumor HA was normalized to the weight of the tumor sample.

4.2.11 Immunofluorescence
Organs were isolated from mice and snap frozen. Organs were embedded in O.C.T. compound (Tissue-Tek) and sectioned into 20 µm sections and stored at -20°C. Sections were fixed acetone for 5 minutes at -20°C and washed 3x with PBS. Sections were blocked with blocking buffer (1% goat serum, 1% BSA, PBS) for 2 hours at room temperature. Anti-CD31 antibody was added to sections at a 1:100 dilution in blocking buffer for 1 hour at room temperature. After 3 washes in PBS, secondary antibody was added at a 1:400
dilution in blocking buffer for 40 minutes at room temperature. After another 3 washes in PBS, sections were incubated with Hoechst nuclear stain at a 1:1000 dilution in blocking buffer for 20 minutes at room temperature. After 3 washes in PBS, sections were mounted using ProLong Gold anti-fade reagent (Life Technologies). Sections were imaged 24 hours after mounting.

4.2.12 Immunohistochemistry

Organs were isolated from mice and immediately placed in 70% ethanol, 5% glacial acetic acid, 4% formalin solution for 48 hours\textsuperscript{52}. Organs were embedded in paraffin, cut into 20 µm sections, and hydrated by serial washes through Clear-Rite (Thermo Scientific), 100% ethanol, 50% ethanol, 10% ethanol and deionized water. Hydrated sections were washed three times in deionized water and placed in 3% acetic acid solution for 3 minutes. Slides were incubated in 1% Alcian Blue solution pH 2.5 for 30 minutes at room temperature. Sections were rinsed with tap water for 5 minutes and deionized water for 5 minutes and then incubated with Nuclear Fast Red (Sigma) for 5 minutes. Sections were rinsed for 5 minutes in deionized water and then dehydrated and mounted with Permount (Fisher Scientific). Sections were imaged 24 hours after mounting. Control sections were incubated with hyaluronidase from bovine testes (Sigma H3884) for 1 h at 37°C before the incubation with acetic acid.

4.2.13 RT-PCR

mRNA was isolated from cells in culture or tissue samples using Trizol reagent according to the manufacturers protocol (Life Technologies). The SuperScript III First-Strand Synthesis System for RT-PCR was used to convert mRNA to cDNA. Oligo(dT)\textsubscript{20}
primers were used for first strand synthesis of 5 µg total RNA. Primer sets are shown below and were purchased from Integrated DNA Technologies:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Optimal concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAS1 F</td>
<td>GAGAACAAGACGGAGAAGAGAG</td>
<td>900</td>
</tr>
<tr>
<td>HAS1 R</td>
<td>AGGATGAGCAGGGCAAAAG</td>
<td>900</td>
</tr>
<tr>
<td>HAS2 F</td>
<td>CTATGCTTGCACCTGCCTC</td>
<td>300</td>
</tr>
<tr>
<td>HAS2 R</td>
<td>AAAGCCATCCAGTATCTCAG</td>
<td>300</td>
</tr>
<tr>
<td>HAS3 F</td>
<td>GAAGCCGAACAGTATAGCCTG</td>
<td>300</td>
</tr>
<tr>
<td>HAS3 R</td>
<td>CAGCCATGAAACTAGAACAAC</td>
<td>900</td>
</tr>
<tr>
<td>CD44 F</td>
<td>CAACACCTCCCACCTAGACAC</td>
<td>300</td>
</tr>
<tr>
<td>CD44 R</td>
<td>CTGTAGCGAGTACCACACAG</td>
<td>900</td>
</tr>
</tbody>
</table>

Table 4.1: Primer sets and optimal concentrations for qPCR studies.

Fifty ng of cDNA was combined with both forward and reverse primer at the above concentrations along with FastStart Universal SYBR Green Master (Roche). The reaction was run on a Stratagene Mx3005P RT-PCR machine with the following program: 1 cycle (95°C for 10 s), 70 cycles (95°C for 15 s, 60°C for 60 s), 1 cycle (95°C for 60 s, 55°C for 30 s, 95°C for 30 s). Primer dimer sets were removed by analysis of the dissociation curves. C_T values for each gene were determined from amplification curves and delta C_T values were computed against a Beta-actin control. Murine Beta-actin primers were purchased from MCLAB.

4.2.14 Liposome distribution studies

Liposome distribution in tumors was measured by tracking liposomes labeled with DiD in 4T1 tumors. Mice were dosed with 4 doses of 18 mg/kg equivalents of 0.2 mL of L-
MU-P or PBS by intravenous tail vein injection on days 9, 13, 16, and 19 after tumoring. On day 20, mice were dosed with empty liposomes labeled with DiD. On day 21, mice were sacrificed and tumors were isolated and snap frozen for immunofluorescence.

4.2.15 Tumor imaging and analysis
Tumor sections were imaged on a Nikon Ti-E microscope and processed using NIS Elements 4.20 (Nikon) and Fiji. Three-channel images were captured on the microscope: nuclear stain Hoechst3342, CD31 stain FITC; and lipid membrane dye DiD. Imaging and exposure settings remained consistent through all experiments. Tumors were imaged at 20x and large composite images were stitched together using the NIS Elements software. When collecting large images, the microscope was refocused on every second image and exposure times and microscope settings remained constant across all samples. Each channel was saved in a separate TIFF file and loaded in Fiji. Background was subtracted from images using a Rolling Ball background subtraction with a radius of 50. Then an absolute background subtraction of a pixel value of 1500 was applied. To quantify the % positive area for DiD, the tumor region of interest (ROI) was selected and the % area was measured using the “Measure %Area” function. To further quantify the extent of colocalization between CD31 blood vessels and DiD liposomes, Mander's colocalization analysis was performed across the entire tumor and the extent of colocalization between the DiD and CD31 signal is reported.

4.2.16 Survival studies
Mice were dosed with 4 doses of 18 mg/kg equivalents of L-MU-P or PBS (0.2 mL) by intravenous tail vein injection on days 4, 8, 11, and 14 after tumoring. On day 15, mice were given a single intravenous dose of Doxil® at 6 mg/kg or 10 mg/kg or PBS. Mice were
then monitored daily until removal from the study by IACUC guidelines. Tumor dimensions were measured with digital calipers and tumor volume was determined by taking two perpendicular measurements. Kaplan Meier plots were generated using GraphPad Prism.

4.2.17 HPLC tissue analysis of MU-P and doxorubicin

Tissues were analyzed for MU-P and metabolites as previously reported. Briefly, tumors were homogenized in the presence of Phosphatase Inhibitor Cocktail 2 (Sigma), MU-P and metabolites were extracted into the homogenization solution (50% Methanol, 0.1% TFA, 1:100 dilution of, 1 µg/mL 7-hydroxycoumarin as an internal standard). After centrifugation, samples were injected on a C8 column (ZORBAX Eclipse XDB-C8 5-Micron). To measure doxorubicin levels, tumors were homogenized and doxorubicin was extracted using acidified methanol (1% acetic acid). Samples were vortexed and then placed at -80 °C for 1 hour. Samples were then centrifuged for 10 minutes and the supernatant was analyzed by HPLC.

4.2.18 Biodistribution and Pharmacokinetics Studies

Twenty mg/kg MU-P equivalents in a volume of 0.2 mL were injected via tail vein into CD-1 mice. For pharmacokinetic studies, blood was collected 10, 20, 60, 180, 1440 and 2880 minutes (3 mice per group) after injection and centrifuged for 10 min at 15,000 g. Serum was collected in a tube with a 1:100 dilution of Phosphatase Inhibitor Cocktail 2 (Sigma). Ten µL of serum was added to 2 mL of HBS along with 10 µL of a 15% C12E10 solution. A standard curve was made by titrating MU-P loaded liposomes into the serum from an untreated mouse. MU-P were measured from each serum sample. The data was fit to a two-compartment model using GraphPad Prism. For biodistribution studies, tissues were isolated and flash frozen 3 and 48 hours after injection.
4.3 Results

4.3.1 MU-P depletes HA in culture media

MU-P is rapidly dephosphorylated over the course of minutes to form MU in serum (Figure 4.1B) and in a number of tissue homogenates\(^{49}\). This conversion is slower in heat-inactivated serum, indicating a loss in endemic serum phosphatases (data not shown). HA-rich 4T1 murine mammary carcinoma cells\(^{32}\) and HA-poor C26 murine colon carcinoma cells were used as model systems to test MU-P mediated HA depletion. MU and MU-P reduced HA levels in culture media of 4T1 cells in a dose-dependent manner (Figure 4.1C). Blocking MU-P dephosphorylation by including a phosphatase inhibitor in the media decreased the activity of HA synthesis inhibition of MU-P. Conversely, MU and MU-P had little effect on the low levels of HA found in C26 culture media (Figure 4.2A). Measurements of HA levels were normalized to cell number in order to account for cell cytotoxicity of MU and MU-P at high concentration.
**Figure 4.2**: *MU and MU-P do not reduce HA levels in C26 tumors. (A) MU and MU-P do not reduce HA levels in C26 cells in culture (n=3). (B) L-MU-P does not reduce HA levels in C26 subcutaneous tumors (n=4).

### 4.3.2 Characterization of liposomal MU-P

MU-P was passively encapsulated in a range of liposome formulations and its rate of release from each formulation was quantified using a simple *in vitro* assay. Two MU-P encapsulating formulations, *HSPC* and *POPC*, were selected for further study (Figure 4.3A). These liposome formulations had similar diameter, zeta-potential, and encapsulation efficiency (Figure 4.3A,B). However, in serum the formulations demonstrated different leakage rates of MU-P. While MU-P is stably encapsulated in the *HSPC* formulation, it slowly leaks from the *POPC* formulation at 37°C in 30% fetal bovine serum (Figure 4.3C). This difference in leakage rates is mirrored *in vivo*. While both formulations dramatically extended the circulation time of MU-P compared to the free drug, MU-P leaked faster from *POPC* liposomes than from *HSPC* liposomes in circulation (Figure 4.3D). We hypothesized that the rapid yet steady release profile of MU-P from *POPC* liposomes would lead to a sustained reduction of HA at the tumor site.
Liposomal MU-P depletes HA in 4T1 tumors

To investigate the activity of L-MU-P as an inhibitor of HA synthesis, POPC MU-P was evaluated in the 4T1 tumor model. L-MU-P was administered intravenously while MU was given by oral gavage (450 mg/kg/day) (Figure 4.4A). At all dose levels, L-MU-P reduced tumor HA to a greater extent than oral MU as determined by an HA ELISA assay (Figure 4.4B). This result was corroborated by histochemistry, as Alcian Blue staining of HA is reduced in a dose dependent fashion (Figure 4.4C). In contrast, L-MU-P had little effect on

Figure 4.3: Characterization of MU-P liposomes. (A) TEM images of POPC:Cholesterol and HSPC:Cholesterol liposomes encapsulating MU-P (top) and summary of liposome characteristics including polydispersity index (PDI), diameter, and surface potential (table, n=3). Scale bar is 50 µm. (B) MU-P is passively encapsulated in both liposomes to a similar extent (n=3), but (C) leaks more quickly from POPC liposomes in the presence of serum at 37°C. (D) Both HSPC and POPC MU-P liposomes have extended circulation times compared to free MU-P.
HA levels in HA-low C26 tumors (Figure 4.2B). L-MU-P had no effect on 4T1 tumor growth or metastatic progression (Figure 4.5A,B).

Figure 4.4: L-MU-P depletes HA in 4T1 tumors. (A) Mice with orthotopic 4T1 tumors were given 1-4 doses of L-MU-P according to the given schedule or oral MU daily. A single dose of doxorubicin was administered to measure liposome uptake into tumors (n=10). (B) L-MU-P reduced HA levels in a dose dependent manner as measured by an HA-specific ELISA assay or (C) Alcian Blue staining. (D) MU-P and MU accumulate in the tumor in a dose dependent manner, while (E) Doxil® accumulation is not affected by HA levels. Statistical analyses were performed with (D) Student’s t test (B) or ANOVA and are relative to oral MU. Error bars denote mean ± standard deviation.
In order to understand the exposure of the tumor to MU-P, we measured the “cellular availability” of L-MU-P liposomes in 4T1 tumors. This term describes the fraction of drug released from the liposome and available for therapeutic activity\(^49\). The presence of both MU and MU-P in the tumor 48 hours after administration indicates the persistence of intact liposomes, and the presence of MU supports the hypothesis of sustained MU-P release from liposomes to inhibit HA synthesis (Figure 4.4D).

We also measured the effect of HA depletion on Doxil® accumulation in the tumor. Interestingly, HA depletion by L-MU-P did not lead to an increase in Doxil® accumulation in the tumor (Figure 4.4E), indicating that HA depletion by L-MU-P does not enhance the EPR effect and improve tumor uptake of nanoparticle therapeutics.

Figure 4.5: L-MU-P does not slow tumor growth as monotherapy or improve the efficacy of doxorubicin. (A) L-MU-P alone does not slow tumor growth or (B) improve overall survival of mice with 4T1 tumors (n=4). (C,D) L-MU-P does not improve the efficacy of free doxorubicin (n=6).
4.3.4 MU-P in vivo activity is not due to down-regulation of HAS2

While MU may inhibit HA synthesis by depleting cellular glucuronic acid, it may also do so by reducing levels of HAS2\(^\text{35,48}\). HAS2 is an established oncogene and is over-expressed in a number of murine and human mammary tumors\(^\text{37}\). We found that HAS2 is the most prevalent HA synthase in 4T1 tumors both in vitro and in vivo (Figure 4.6A,C). Further, in vitro, MU selectively reduced HAS2 expression in vitro while having little effect on CD44 and HAS3 expression levels (Figure 4.6B). In contrast, in vivo, L-MU-P had little effect on HAS2 levels, which may be due to an insufficient dose of MU in the tumor (Figure 4.6D).

![Figure 4.6: L-MU-P modulates gene expression in vitro, but not in vivo. Gene expression levels were examined by RT-PCR. (A) HAS2 is the most abundant HA-synthase in cell culture. (B) MU down regulates expression of HAS2 in vitro (n=3). (C) HAS2 is the most abundant HA-synthase in 4T1 orthotopic tumors. (D) L-MU-P has no effect on expression of HAS2, HAS3, or CD44 in vivo (n=10).]
4.3.5 HA depletion improves liposome distribution in 4T1 tumors

After confirming that L-MU-P reduced HA levels in 4T1 tumors, we examined if HA depletion altered the distribution of liposomes. To that end, we injected DiD labeled empty liposomes intravenously in mice with 4T1 tumors, removed the tumors 24 hours later, prepared sections of the tumors, and quantified the liposome distribution by microscopy (Figure 4.7; Figure 4.8). The tumor distribution of labeled liposomes was quantified across the entire tumor using two metrics: 1) the degree of colocalization between the liposome DiD signal and endothelial cell marker CD31 and 2) total tumor area positive for liposome signal (Figure 4.7D,E). Both metrics are described in further detail in Materials and Methods, and images of entire tumor sections are presented in Figure 4.8.

Liposomes enter the tumor by escaping the porous vasculature via the EPR effect and then permeate into the tumor parenchyma by diffusion. This distribution away from endothelial cells can be quantified by computing the colocalization of the liposome signal (DiD) and vasculature signal (CD31) across the entire tumor. If the liposomes do not leave the vasculature, the two signals will be perfectly colocalized and the fraction of non-colocalized liposomes will be low. In contrast, if the liposomes distribute away from the vasculature, the fraction of non-colocalized liposomes will be high. An alternate approach to computing liposome distribution is to examine the entire tumor area for liposome signal. Tumors with a greater fraction of area positive for liposomes may have a greater exposure to the liposome encapsulated drug as it is released in the tumor.

In 4T1 tumors, liposomes are confined to the area immediately surrounding the vasculature (Figure 4.7B, Figure 4.8A,B) and exhibit a low fraction of non-colocalized liposomes (Figure 4.7D). After HA depletion, DiD labeled liposomes distributed away from the vasculature (Figure 4.7C, 4.8C,D), and this distribution is reflected by a higher fraction
of non-colocalized liposomes (Figure 4.7D). Similarly, the fraction of total tumor area positive for liposomes significantly increased after HA depletion (Figure 4.7E). These results demonstrate that HA depletion by L-MU-P increases the distribution of liposomes in HA-rich tumors.

Figure 4.7: HA depletion improves liposome distribution in 4T1 tumors. (A) Orthotopic 4T1 tumors were treated with 4 doses of L-MU-P followed by a single dose of fluorescently labeled liposomes (n=4). (B) Liposome penetration in control tumors is less pronounced than in (C) tumors with reduced HA. (D) Liposome distribution quantified by colocalization analysis across the entire tumor as well as by (E) percent area analysis across the entire tumor. Statistical analyses were performed with Student’s t-test. Error bars denote mean ± standard deviation.
Figure 4.8: Liposome distribution after HA depletion. Representative 4T1 tumor sections showing liposome distribution throughout the tumor in (A,B) untreated or (C,D) L-MU-P treated tumors.
4.3.6 Improved liposome distribution improves efficacy of Doxil®

Based on this improved tumor distribution, we hypothesized that HA depletion would increase the efficacy of Doxil®, and we next investigated if L-MU-P given with Doxil® or with non-encapsulated doxorubicin altered the anti-tumor effect of the drug. HA depletion using a four-dose regime of L-MU-P was followed by a single dose of Doxil® (Figure 4.9A). The combination of L-MU-P and Doxil® had greater anti-tumor efficacy than Doxil® monotherapy. The combination therapy slowed tumor growth and improved overall survival (Figure 4.9B,C), but did not improve Doxil® uptake into 4T1 tumors (Figure 4.4E). The efficacy of higher doses of Doxil® (10 mg/kg and 20 mg/kg) were not improved by HA depletion (Figure 4.10), suggesting that there is an optimal dose window of Doxil® where efficacy is improved by increased penetration. In contrast to Doxil®, L-MU-P did not improve the efficacy of free doxorubicin (Figure 4.5C,D). This result is not surprising, as the distribution of small molecule drugs is less hindered than that of nanoparticles.
Figure 4.10: L-MU-P does not improve the efficacy of 10 mg/kg or 20 mg/kg doses of Doxil® (n=10).
4.4 Discussion

HA is recognized as an important target in oncology because of its role in tumor progression and abundance across a range of solid tumors\textsuperscript{57}. Therapies that target HA may be widely applicable and may slow tumor progression as a monotherapy or improve the effect of another therapeutic in a combination therapy\textsuperscript{32,33}. In this study, we build on previous work that validates MU as an inhibitor of HA synthesis to demonstrate that 1) encapsulation of a prodrug of MU (MU-P) in a liposome (L-MU-P) effectively depletes tumor HA, 2) HA depletion improves liposome distribution, and 3) improved distribution improves Doxil® efficacy and extends overall survival.

In comparison to other methods to deplete HA in tumors, such as oral MU or PEGPH20, our studies implicate increased tumor distribution of Doxil® as the mechanism for improved efficacy rather than improved Doxil® uptake, MU cytotoxicity, or altered HA synthase gene expression. In contrast to PEGPH20 treatment, treatment with L-MU-P does not alter the EPR effect and enhance tumor uptake of nanoparticles\textsuperscript{58}. PEGPH20 depletes HA over the course of hours, but L-MU-P depletes HA over the course of days. This difference in kinetics may allow the tumor vasculature to adapt to changes in the microenvironment, which may prevent improved uptake of liposomes into the tumor. For example, PEGPH20 may transiently reduce the IFP gradient across the wall of tumor blood vessels and increase extravasation of therapeutics into the tumor\textsuperscript{57}. Further, PEGPH20 slows tumor growth as a monotherapy\textsuperscript{32}, although this may be partially driven by the tumor collapsing on itself without the presence of the HA matrix.

At the doses administered in this study, L-MU-P does not have an anti-tumor effect as a monotherapy (Figure 4.5) and may not catastrophically alter the tumor
microenvironment in the same way as PEGPH20. L-MU-P has no effect on expression of HAS2 and HAS3 in the tumor although MU has previously been reported to slow tumor progression and affect HAS2 expression levels. However, these in vitro studies used high doses of MU that may not be achievable in the tumor. As such, increasing the dose of L-MU-P may have the additional benefit of altering gene expression.

In this study, we demonstrate that L-MU-P is a useful tool for increasing the efficacy of liposome therapeutics (Figure 4.9). In addition to liposomes, L-MU-P may be used to improve the distribution of other macromolecular therapeutics including antibodies, proteins and cell-based therapies. However, the low potency of MU and its derivatives still limit their clinical use. Future work should be focused on developing more potent and specific inhibitors of HAS2 and HAS3 as combination therapies to augment distribution and efficacy of macromolecular therapeutics in HA rich tumors.
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Chapter 5: Conclusion

5.1 Summary of findings

For four decades, liposomes composed of both naturally occurring and synthetic lipids have been investigated as delivery vehicles for low molecular weight and macromolecular drugs. These studies paved the way for the clinical and commercial success of a number of liposomal drugs each of which requires a tailored formulation; one liposome size does not fit all drugs. Instead, the physicochemical properties of the liposome must be matched to the pharmacology of the drug. An extensive biophysical literature demonstrates that varying lipid composition can influence the size, membrane stability, and \textit{in vivo} interactions of a liposome. In turn, these properties dictate the macroscopic behavior of the liposome in the tumor including its drug release rate, penetration away from the vasculature, and subsequent anti-tumor efficacy. In this thesis, we study three critical aspects of liposome drug delivery: 1) lipid architecture and biophysics, 2) liposome payload release and 3) liposome distribution in solid tumors.

In Chapter 1, we describe synthetic lipid headgroups, linkers and hydrophobic domains that can provide the drug delivery scientist control over the intermolecular forces, phase preference, and macroscopic behavior of liposomes. These synthetic lipids further our understanding of the biophysical attributes of the liposome, enable environmentally triggered behavior, promote targeted drug delivery, allow for functionalization of preformed liposomes or improve \textit{in vitro} and \textit{in vivo} stability. We further highlight the immune reactivity of novel synthetic headgroups as a key design consideration. For instance it was originally thought that synthetic PEGylated lipids were immunologically
inert; however, it's been observed that under certain conditions PEGylated lipids induce humoral immunity. Such immune activation may be a limitation to the use of other engineered lipid headgroups for drug delivery. In addition to the potential immunogenicity of engineered lipids, further investigations of liposomal systems in vivo should pay particular attention to the location and dynamics of payload release.

Chapter 2 describes the synthesis and characterization of a series of zwitterionic, acetate-terminated, quaternized amine diacyl lipids (AQ). These lipids have an inverted headgroup orientation as compared to naturally occurring phosphatidylcholine (PC) lipids; the cationic group is anchored at the membrane interface, while the anionic group extends into the aqueous phase. AQ lipids preferentially interact with highly polarizable anions (ClO$_4^-$) over less polarizable ions (Cl$^-$), in accord with the Hofmeister series, as measured by the change in zeta potential of AQ liposomes. Conversely, AQ lipids have a weaker association with calcium than do PC lipids. The transition temperatures of the AQ lipids are similar to the $T_m$ observed with PE lipids of the same chain length. AQ lipids form large lipid sheets after heating and sonication; however, in the presence of cholesterol these lipids form stable liposomes that encapsulate CF. The AQ:Cholesterol liposomes retain their contents in the presence of serum at 37°C, and when injected intravenously into mice, their organ biodistribution is similar to that observed with PC:Chol liposomes. AQ lipids demonstrate that modulating the headgroup charge orientation significantly alters the biophysical properties of liposomes. For the drug carrier field, these new materials provide a non-phosphate containing zwitterlipid for the production of lipid vesicles.

Chapter 3 introduces a method for tracking the rate and extent of delivery of liposome contents in vivo based on encapsulation of MU-P, a profluorophore of 4- MU. MU-
P is rapidly dephosphorylated by endogenous phosphatases in vivo to form MU after leakage from the liposome. The change in fluorescence spectra when MU-P is converted to MU allows for quantification of entrapped (MU-P) and released (MU) liposome contents by fluorescence or by a sensitive high performance liquid chromatography assay. We define the “cellular availability” of an agent encapsulated in a liposome as the ratio of the amount of released agent in the tissue to the total amount of agent in the tissue; this parameter quantifies the fraction of drug available for therapy. The advantage of this method over existing technologies is the ability to decouple the signals of entrapped and released liposome contents. We validate this method by tracking the circulation and tissue distribution of MU-P loaded liposomes after intravenous administration. We use this assay to compare the cellular availability of liposomes composed of engineered phosphocholine lipids with covalently attached cholesterol, SMLs, to liposomes composed of conventional phospholipids and cholesterol. The SML liposomes have similar pharmacokinetic and biodistribution patterns as conventional phospholipid-cholesterol liposomes but a slower rate of contents delivery into the tissue. Thus, MU-P enables the tracking of the rate and extent of liposome contents release in tissues and should facilitate a better understanding of the pharmacodynamics of liposome-encapsulated drugs in animals.

Liposomes improve the pharmacokinetics and safety of rapidly cleared drugs, but have not yet improved the clinical efficacy compared to the non-encapsulated drug. This inability to improve efficacy may be partially due to the non-uniform distribution of liposomes in solid tumors. The tumor extra-cellular matrix is a barrier to distribution and includes the high molecular weight glycosaminoglycan, HA. Strategies to remove HA or block its synthesis may improve drug delivery into solid tumors. Orally administered
methylumbelliferone (MU) is an inhibitor of HA synthesis, but it is limited by low potency and limited solubility. Chapter 4 describes a novel approach to depleting tumor HA by encapsulating a water-soluble phosphorylated prodrug of MU (MU-P) in a liposome (L-MU-P). L-MU-P is a more potent inhibitor of HA synthesis than oral MU in the 4T1 murine mammary carcinoma model using both a quantitative ELISA and histochemistry. HA depletion improves the tumor distribution of liposomes computed using Mander's colocalization analysis of liposomes with the tumor vasculature. Hyaluronan depletion also increases the fraction of the tumor area positive for liposomes. This improved distribution extends the overall survival of mice treated with Doxil®.
5.2 Outlook

Liposomes have proved to be exceptionally useful and practical delivery tools in the last half-century. Looking forward, there are a number of aspects of liposome drug delivery to solid tumors that should be considered. First, as highlighted in this thesis, the time and place of drug release is paramount in drug delivery. Liposome systems are moving towards complexity: targeting ligands, complicated functionalization chemistries, and synthetic lipids increase the cost of liposome therapeutics. However, the true utility of these technologies may not be realized unless the drug is appropriately released. Solving the problem of drug release in the right compartment may prove more fruitful than increasing the complexity of lipid systems. Second, special attention should be given to the cellular or tissue compartment of liposome drug release. Improving penetration away from the vasculature into the tumor parenchyma has been a useful approach in mouse models, but methods to increase tumor penetration have not yet reached the clinic. HA is a particularly compelling target, but research must be focused on developing more potent and specific inhibitors of HA synthesis. Cell specific delivery is another important parameter. In the absence of a targeting ligand, liposomes traffic to the cells of the RES system. Such cells, especially macrophages, are intimately involved in tumor progression. Future work with liposomes should take advantage of the natural tropism of liposomes for macrophages to target and reprogram malignant tumor macrophages.
Appendix

Figure A1: $^1$H NMR spectra of DMAQ in CDCl$_3$. 

CDCl$_3$
Figure A2: $^{13}$C NMR spectra of DMAQ in CDCl$_3$. 
Figure A3: $^1$H NMR spectra of DPAQ in DMSO.
Figure A4: $^{13}$C NMR spectra of DPAQ in CDCl3.
Figure A5: $^1$H NMR spectra of DSAQ in DMSO.
Figure A6: $^{13}$C NMR spectra of DSAQ in CDCl$_3$. 
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