

Enzyme-Sensitive Lipid Trigger-Mediated Release of Payload from Liposomes

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Abstract

Esterase enzymes are overexpressed in tumor tissues of certain cancer cell types, and it is this property which makes them interesting candidates as a method of inducing cargo release from liposomes. Esterase-responsive liposomes could be developed that could be exploited to selectively deliver drug cargo to tumor tissues upregulating esterase enzyme. For this purpose, a 1,2-dioleoyl-sn-glycero-3- phosphatidylethanolamine (DOPE) lipid was coupled to a trimethyl lock molecule unit bearing an esterase-sensitive moiety. Upon introduction into an esterase-rich environment, the esterase-sensitive labile moiety would degrade. The activation of the trimethyl lock cyclization is expected to drive the restoration of the non-bilayer properties of DOPE, leading to a shift in lipid aggregate shape that disturbs lipid membrane packing properties and thereby drives release of drug cargo. Trimethyl locked-DOPE (TML-DOPE) lipids were developed and incorporated into liposomes at a 30% inclusion. Liposomes were analyzed by fluorescence release assay, including an incremental addition of esterase enzyme to a liposomal sample and a measure of the release from the liposomes over time after the addition of a discrete volume of esterase enzyme. Dynamic Light Scattering (DLS) experiments were also performed to examine liposome size and uniformity. Our results provide preliminary evidence of content release, and further work will focus on optimizing release conditions.

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List of Abbreviations

ANTS	8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt
CDCl ₃	Deuterated chloroform
CHCl ₃	chloroform
cP	centipoise
CPPs	Cell penetrating proteins
DAG	diacylglycerol
DIEA	N,N-diisopropylethylamine
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DOPE	1,2-dioleoyl-sn-glycero-3- phosphatidylethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DPX	p-Xylene-Bis-Pyridinium Bromide
EDCI	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
EPR	Enhanced permeability and retention effect
equiv.	equivalence
GUV	Giant unilamellar vesicles
h	hours
HCl	Hydrochloric acid
HOBt	1-Hydroxybenzotriazole
KOH	Potassium hydroxide
LUV	Large unilamellar vesicles
M	molar
MeOD	Deuterated methanol
MeOH	Methanol
mg	milligram
MHz	megahertz
mL	milliliter
MLV	Multilamellar vesicle
mM	millimolar
N ₂	Diatomic nitrogen
nm	nanometer
NMR	Nuclear Magnetic Resonance
PP	Packing Parameter
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PHEG	Poly-N(5)-(2-hydroxyethyl) glutamine
PHEG-DODASuc	poly(hydroxyethyl L-glutamine)-N-succinyl-dioctadecylamine
PI	phosphatidylinositol
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine
PS	phosphatidylserine

RES	Reticuloendothelial System
RNA	Ribonucleic acid
sPLA ₂	Secretory phospholipase A ₂
SUV	Small unilamellar vesicles
TBS	Tris-buffered Saline
TLC	Thin-layer Chromatography
T _m	Transitional melting temperature
TML-DOPE	trimethyl locked-1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine
TML-POPE	trimethyl locked-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine
μL	microliter
μmol	micromole
UV	Ultraviolet radiation

Chapter 1: Background

1.1 General Lipid Structure, Properties, and Lipid Polymorphism

Lipids are amphiphilic structures composed of a polar, hydrophilic head and non-polar, hydrophobic tail region. Figure 1 shows the general structure of a lipid. Due to the actions of entropy, lipids spontaneously aggregate into various formations when dispersed into aqueous media. The capacity for lipids to aggregate spontaneously into certain assemblages is described by their packing parameter (PP), shown in Equation 1. The packing parameter of a lipid is defined as the ratio of the volume of the hydrophobic tails (v) versus the area of the hydrophilic head group (a_0) multiplied by the chain length of the hydrophobic tails (l_c).¹

$$\text{Packing Parameter} = \frac{v}{a_0 l_c} \text{ (Equation 1)}$$

The molecular shape of each individual lipid as well as the shape of the assemblages they form in aqueous media are informed by the packing parameter. Lipid molecules exhibit three general shapes depending on PP. Figure 2 shows the general shapes lipid molecules form, along with the shape of aggregates of those lipids. Lipids with a $PP < 1$ have a larger head area compared to that of their tails, and they aggregate into a micelle formation in aqueous media.² Within the micelle, the polar headgroups of the lipids form a barrier to exclude water by the hydrophobic effect. This leaves the core of the micelle a hydrophobic environment comprised of the lipid tails of the aggregating lipids.³ Examples of lipids that tend to form micelles in aqueous media are single-tailed lipids, such as lysolipids.⁴

Lipids for which the area of their hydrophilic heads is smaller than that of their hydrophobic tails, or a $PP > 1$, take up a wedge shape and aggregate into an inverted micelle or reverse hexagonal formation when dispersed in aqueous media.² In inverted or reverse micelle structures,

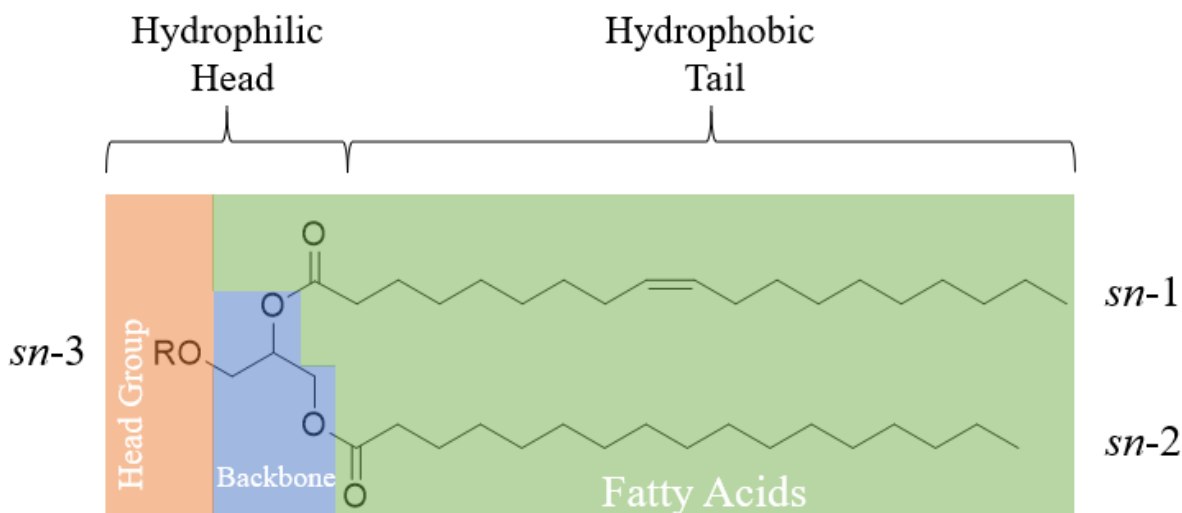
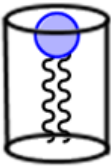
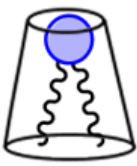
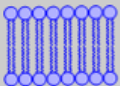
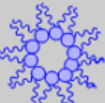


Figure 1: Generic structure of a lipid molecule. The headgroup and backbone make up the polar part of the molecule. The headgroup in natural lipids typically consists of phosphate and a variable headgroup element. The backbone bridges the polar and non-polar parts of the molecule. The fatty acids make up the hydrophobic region of the molecule, which may include fatty acids with a varying number of unsaturated double bonds and chain lengths. The *sn*-1, *sn*-2, *sn*-3 labels refer to the stereospecific numbering of backbone positions in the lipids.⁵

the hydrophobic tail regions form the outer layer of the structure while the hydrophilic head groups form an aqueous core in the inner layer. Individual reverse micelle structures then assemble into a reverse hexagonal phase, wherein the inverted micelle structures self-assemble into a hexagonal lattice formation. The inner aqueous cores of the micelles assemble into along tubes of aqueous media.⁶ An example of lipids that form inverted micellar phases are phosphatidylethanolamines (PE).⁷

Lipids that exhibit a $PP \sim 1$ assume a cylindrical shape and tend to form bilayers. For these lipids, the area of their hydrophilic headgroup is approximately equal to the volume of their hydrophobic tails.² In the lipid bilayer, two layers of hydrophobic tails are sandwiched between two opposing layers of polar heads. The aggregate shape creates two compartments within the liposome. The center of the liposome forms an aqueous core while the layer of hydrophobic tails

(a)

Lipid Shape			
Packing Parameter	$PP < 1$	$PP \sim 1$	$PP > 1$
Spontaneous Curvature	Positive	Zero	Negative
Aggregate Shape	 Micelle	 Bilayer	 Inverted Micelle

(b)

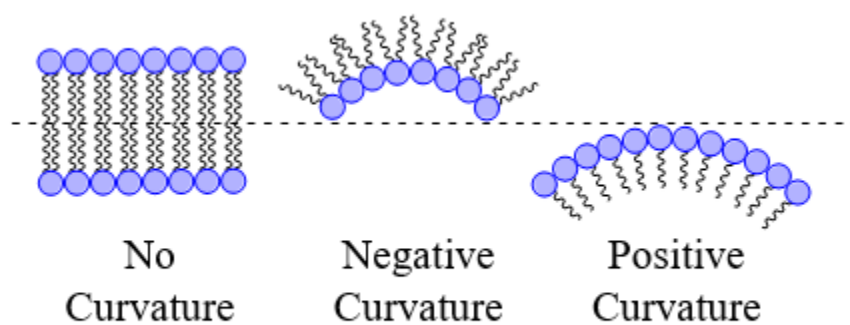


Figure 2: Properties of various lipid shapes dispersed in aqueous media. (a) Properties shown include lipid shape, packing parameter, spontaneous curvature, and aggregate phase. This figure has been modified from a report by Pointer-Keenan, C. D.² (b) Schematic of membrane curvature, showing how the membrane bends under a positive and negative curvature.

creates a nonpolar pocket.⁸ Lipids that self-assemble into bilayer structures are referred to more generally as bilayer lipids, whereas lipid molecules of other shapes are referred to as non-bilayer lipids.⁹ The unique structure of liposomes leads to multiple intriguing consequences for their biological uses, as will be discussed later in this thesis.

As several properties of lipids are intrinsically tied to their shape, changing this shape can have a massive impact on the way the lipid behaves. Derivatization of the headgroup of a lipid, for example, results in a larger overall headgroup, while removing a fatty acid from the hydrophobic region results in a greatly reduced lipid tail volume. In the case of the former, if performed on a non-bilayer forming, wedge-shaped lipid with a small headgroup, the modification may instead cause the lipid to be able to form stable bilayers.^{10, 11} In the case of the latter, if performed on a cylindrical bilayer-forming lipid, this could result in the area of the polar headgroup being relatively larger than that of the diminished hydrophobic region, causing a shift into a cone-shaped lipid.^{12, 13}

1.1.2 Spontaneous Curvature

Lipid molecules, in addition to an intrinsic packing parameter, also possess an intrinsic curvature. Spontaneous curvature is exhibited when lipid molecules have aggregated; the structure of the lipid aggregate is dictated by the spontaneous curvature of the lipids from which it is composed.¹⁴ Spontaneous curvature exists in two directions: positive and negative, which bend the membrane in opposite directions, as shown in Figure 2b.¹⁵ Positive curvatures bend the membrane towards the hydrophobic tails. Negative curvatures bend the membrane towards the headgroups, bringing them closer together.

Cylindrically- shaped bilayer lipids do not impose a spontaneous curvature independently. However, non-bilayer lipids will instill a positive or negative curvature to an encompassing

membrane depending on lipid shape. Lipids that would typically form micelles, or those with large head groups compared to their tails, impose a positive curvature on the incorporating monolayer while lipids with small head group areas impose a negative curvature on a monolayer.

Mixed lipid structures will exhibit a spontaneous curvature based on the average spontaneous curvature of their constituent lipids. Shifts in lipid composition may also result in a change in aggregate shape.¹⁴ The implications of this change will be explored further later in this thesis.

1.1.3 Liposome Phase Transitions

Lipid bilayers exist in two general phases depending on a combination of lipid composition and temperature conditions: the liquid crystalline phase (L_α) and the lamellar gel phase (L_β). These phases are shown in Figure 3. Both phases differ in fluidity, density, and effect on the thickness of the bilayer membrane. The temperature at which the transition between gel and liquid phases occurs is known as the transition temperature (T_m).⁴ In the lamellar gel phase, below the T_m , the acyl chains that make up the hydrophobic tails of the constituent lipids are fully extended into an “all-trans” conformation. This allows for tight packing of the acyl chains leading to an increase in the rigidity of the bilayer membrane and restricts the capacity for individual lipid molecules to diffuse laterally within each membrane leaflet.¹⁶ In the liquid crystalline phase, above the T_m , the lipid tails become disordered within the hydrophobic region of the liposome, which allows for more free lateral movement of lipid molecules within the leaflets leading to a more fluid and permeable membrane.^{4, 16}

The transition temperature of a lipid molecule is dependent on factors including chain length and the degree and placement of unsaturated double bonds within the fatty acid chains.

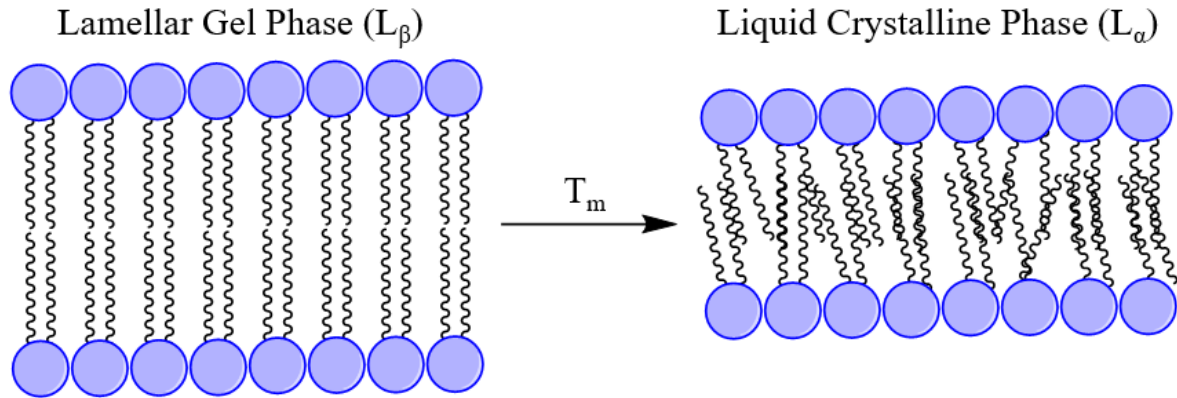


Figure 3: Morphological differences between the two main bilayer phase transitions. In the lamellar gel phase, the lipid tails are ordered and rigid while in the liquid crystalline phase they are less ordered and more fluid, allowing for lateral movement of lipid molecules and a decrease in membrane thickness.¹⁶

Increasing melting temperature is tied to increasing chain length and decreasing melting temperature is linked with unsaturated double bonds within the hydrophobic tails.¹⁷ Double bonds within a fatty acid carbon chain create “kinks” within the membrane, leading to less efficient packing of lipids within the bilayer and results in the membrane exhibiting more fluidity compared to those without unsaturated fatty acid chains.¹⁸

In a bilayer composed of a single lipid type, the transition temperature will reflect that of the constituent lipid within a shallow range. However, natural membranes usually consist of multiple types of lipids with variations in charge, chain length, and degree of unsaturation. This will alter the transition temperature of the bulk membrane as well as the range within which the membrane bilayer will transition completely between gel and liquid crystalline phases.¹⁶ Intermediary phases may also be observed during the transition from the lamellar gel phase to the liquid crystalline phase, such as the Rippled phase.^{4, 17} Self-assembly into a lipid bilayer or liposome occurs only when the lipid molecules may enter the liquid crystalline state.⁴

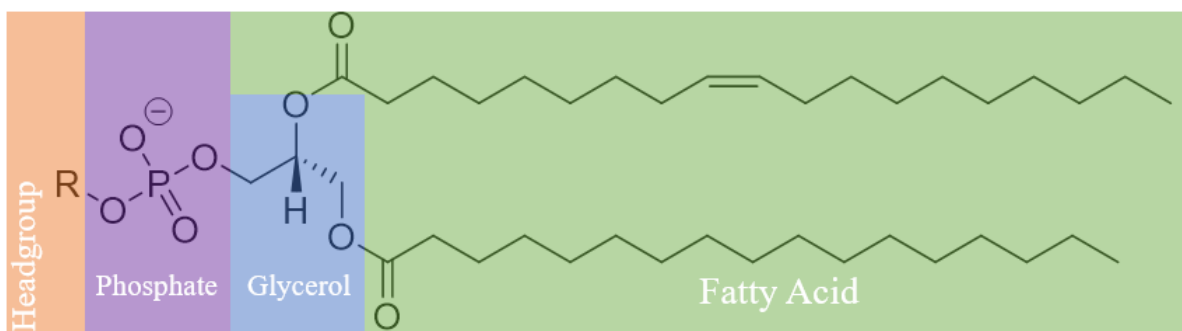
1.2 Biological Significance of Lipids

Lipids play many vital roles in cellular biology including energy storage, cell signaling, and providing the basic structures of cellular membranes. Three major varieties of lipids occur within biological membranes: phospholipids, glycolipids, and cholesterol.¹⁹ Most relevant to further discussion are phospholipids and cholesterol.

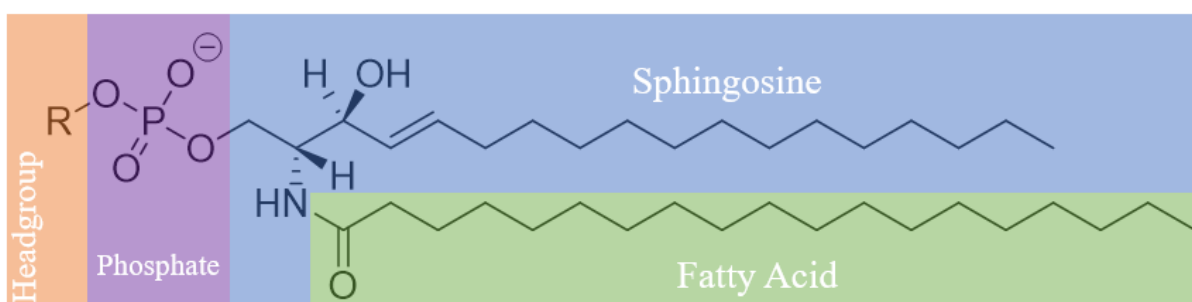
Phospholipids are the most common lipid component of eukaryotic cellular membranes. There are two common backbone structures associated with phospholipids: glycerol and sphingosine. These lead to two different types of phospholipid structures: phosphoglycerides and sphingolipids. Both basic phospholipid structures are shown in Figures 4a and 4b.

Phosphoglycerides are the more abundant of the two and utilize glycerol as the backbone component. From the glycerol backbone two fatty acids and the phosphate with a bonded alcohol group are anchored.¹⁹ Typically, phospholipids contain two fatty acid groups with a variety of chain lengths, though for membrane lipids these tend to range from fourteen to twenty-four carbon units, and these chains may not necessarily be the same length. The fatty acids may also incorporate an unsaturated double bond on one or both chains, and the number of double bonds may vary between chains.¹⁸ These differences alter the phospholipid's ability to pack within the cellular membrane as previously discussed. The alcohol head group attached to the phosphate also serves to differentiate phosphoglycerides by the chemical properties instilled by the head group, including charge. A choline group, shown in Figure 4c, attached to the phosphate will, for example, create phosphatidylcholine (PC), which is a neutral, cylindrically shaped lipid which favors the formation of lipid bilayers. Ethanolamine, also shown in Figure 4c, differs from choline in that it contains a primary amine group as opposed to the quaternary ammonium of the latter. Ethanolamine composes the headgroup of phosphatidylethanolamine (PE), which due to the aforementioned

(a)



(b)



(c)

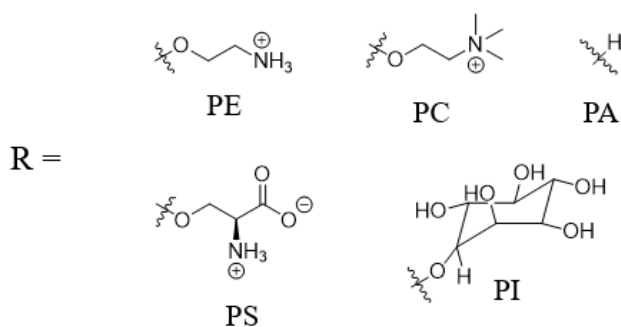


Figure 4: Generic structures and common examples of natural lipids. Includes general structures of (a) phospholipids and (b) sphingolipids. Phospholipids, specifically phosphoglycerides, use glycerol as a backbone whereas sphingolipids use sphingosine. Fatty acids on either molecule will exhibit various chain lengths and number of unsaturated double bonds. (c) Polar headgroups of common natural lipids, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylinositol (PI).

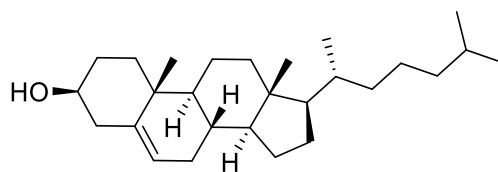
difference in alcohol head group structure instead exhibits non-bilayer forming properties.⁷ Some other common membrane phosphoglycerides are also included in Figure 4c.¹⁸

Sphingolipids incorporate sphingosine as part of their backbone, as shown in Figure 4b. Unlike phospholipids derived from glycerol, sphingosine also includes an unsaturated carbon chain as part of its structure, which carries the consequence of allowing only one fatty acid group to associate with the molecule that is attached to the backbone via an amide bond as opposed to an ester bond found in phosphoglycerides.¹⁹ Sphingomyelin, a sphingolipid, is a common component of cellular membranes.¹⁸

Cholesterol is an amphiphilic lipid that, unlike the previous phospholipids, is a steroid, the structure of which is shown in Figure 5a.¹⁹ Cholesterol serves to support the structure of the membrane bilayer by influencing membrane stability and permeability. The molecules orient in the bilayer parallel to the lipid tails, with the hydroxyl group interacting with the surrounding polar head groups; this orientation within the membrane is shown in figure 5b. The structure partially decreases the mobility of the hydrophobic fatty acid chains, causing the membrane to become less fluid and decreasing permeability. However, in higher concentrations like those found in some cell membranes, the molecule bars entrance into the lamellar gel phase by preventing the lipid tails from forming the tightly packed, ordered structure indicative of that phase. The barring of interaction between lipid molecules when the temperature falls below the transition temperature of the local lipid groups inhibits the phase transition. In most eukaryotic cells, cholesterol is found in large amounts within the plasma membrane while cholesterol is found to be absent in the plasma membranes of prokaryotic cells.¹⁸

Biological cell membranes must maintain a significant degree of flexibility in order to perform certain tasks vital to life in a eukaryotic cell, such as compartmentalization, membrane

(a)



(b)

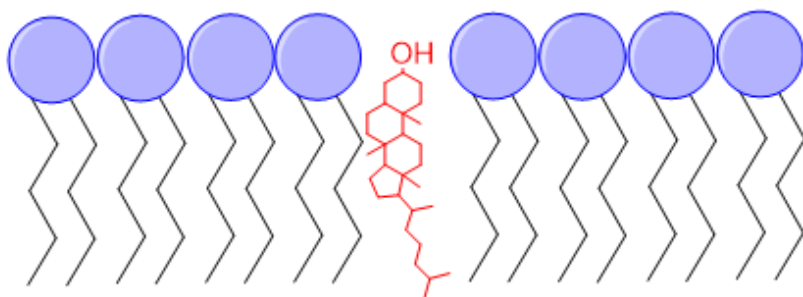


Figure 5: Chemical structure of cholesterol and orientation in the lipid bilayer. (a) Chemical structure of cholesterol. (b) Schematic of the incorporation of cholesterol into the lipid bilayer, with the polar hydroxyl group of the cholesterol associated with the polar headgroups of the lipid molecules.

fusion and fission, and cell signaling.^{18, 20, 21} Some membrane proteins are functional only in the presence of certain phospholipids, and enzymatic activity with the plasma membrane can be inhibited if membrane viscosity is too high.^{18, 19} To this end, cellular membranes are composed of lipids of differing shape, chain length, and functionality. Different biological membranes within a cell vary in lipid composition. In mammalian cells, the four most common phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin. Together, these four phospholipids make up 50-60% of the total lipid within mammalian cells.²² PC is a cylindrically shaped lipid that constitutes a major portion of the plasma membrane as a bulk lipid, its shape favoring bilayer formation.²⁰ Of the common membrane lipids, only PS exhibits an overall charge, specifically a negative charge, at physiological pH. The inner and outer leaflets of the plasma membrane are asymmetrical, with PC and sphingomyelin more

abundant in the outer leaflet and PE and PS more abundant in the inner leaflet. This results in a significant difference in charge between both monolayers, which is important for other cellular functions.^{18, 23} Other phospholipids are minorly present within the membrane, such as phosphatidylinositol (PI) or phosphatidic acid (PA). However, PI plays a key role in protein recruitment for cell signaling and PA is present in abundance within sites of membrane trafficking junctions to provide the large curvatures and fluidity necessary for membrane fusion and fission.^{18, 24} Other lipid structures are present in smaller percentages such as glycolipids and cholesterol.^{18, 19}

While laboratory-made structures such as liposomes do not match the complexity of cellular membranes, principles which govern the fluidity, charge, and functionality of lipids that constitute biological membranes apply to liposomes as well. Liposomal properties vary considerably depending on their constituent lipids. PC is commonly used as a bulk lipid in liposome formulations, and other common membrane lipid components, such as PS, PE, or cholesterol, may also be incorporated as a means of providing a specific charge to the vesicles or tuning membrane fluidity, respectively.²⁵

1.3 Liposomes

Liposomes are spherical synthetic amphiphilic bilayer vesicles consisting of primarily natural non-toxic phospholipids and other lipid membrane additives such as cholesterol. Liposomes vary considerably in size, charge, and functionality depending on their lipid composition and the method by which they were produced. Liposomes are effective for drug delivery applications due to multiple beneficial attributes.²⁶ As liposomes contain separate hydrophobic and hydrophilic regions, this allows for larger diversity in compatible cargo, with hydrophilic compounds encapsulated within the aqueous core while hydrophobic compounds

embed themselves within the hydrophobic lipid tails.²⁷ While compounds are encapsulated within the liposome, they are shielded from degradation within the circulatory system while in transit. As the bulk of liposome composition tends to be composed of naturally occurring phospholipids, liposomes display a low toxicity *in vivo*.^{4, 26} High tunability is also a major advantage of liposomes, as their properties depend heavily on composition and can be tailored to the location and response to specific conditions within a living system.^{4, 28, 29}

1.3.1 Liposome Size and Formation

Liposome size and total number of bilayers (lamellarity) are important factors to control in the preparation of liposomes as these can have a drastic effect on cargo encapsulation and liposome half-life.³⁰ Liposomes are categorized by their size and lamellarity first into multilamellar vesicles containing two or more concentric bilayers (MLVs) and unilamellar vesicles, containing only one bilayer membrane.²⁶ Unilamellar vesicles are further sorted by size into small unilamellar vesicles (SUVs), which are typically less than 100 nm in diameter, large unilamellar vesicles (LUVs), which are between 100-500 nm in diameter, and giant unilamellar vesicles (GUVs), which are over 1 μm in diameter.⁴

Bilayer-prone lipids in aqueous media will spontaneously self-assemble into liposomes, however this does not account for variations in size or lamellarity of the resulting assemblies. Control of these two factors generally falls into the following steps: liberation of lipids from organic solvent, hydration in aqueous media, and purification of the liposomal product.²⁶ A common technique for the preparation of liposomes is the thin-film hydration method. In this technique, set volumes of lipids in organic solvent are dried completely to create a lipid film. The film is then hydrated with the desired aqueous solvent, heated, and agitated.³¹ To encourage the formation of unilamellar vesicles, the film may be subject to freeze-thaw cycles wherein the

liposomes are quickly frozen and then slowly thawed.³² Extrusion of the liposome solution through a filter of the desired final liposome size will produce liposomes of a more uniform size.³³

1.3.2 Challenges of Liposome Circulation and Active and Passive Trafficking of Liposomes *in vivo*

While liposomes are mostly composed of natural lipids and other biomolecules, they are still recognized as foreign agents by the immune system.²⁶ As a result, liposomes face elimination from the circulatory system due to the actions of the Reticuloendothelial System (RES). The RES is composed of tissues from many different organ systems including the lymphatic organs, spleen, liver, lungs, and blood stream, and one of its functions is to eliminate foreign nanoparticle agents from the body.³⁴ Opsonization, a process through which foreign bodies are tagged for elimination by phagocytes, is involved in the elimination of foreign agents from the body.³⁵ Smaller particles are harder to opsonize than larger particles, so they exhibit a longer circulation time.^{26, 34} The same principle applies to liposomes. It has been observed that larger liposomes are cleared much faster from the circulatory system than smaller liposomes.²⁷ In addition, lipid exchange with other lipid-containing structures within the blood such as erythrocytes may lead to the loss of stabilizing lipids with high transition temperatures, leading to cargo loss or liposomal breakdown before arrival at the target site.^{6, 27} Multiple strategies are utilized to improve the circulatory half-life of liposomes within living systems.

Conventional liposomes rely on size and composition alone to stave off RES activity long enough to accumulate in the target tissues (see Figure 6a).^{26, 27} As smaller liposomes are more difficult to target via opsonization, liposomes meant for pharmaceutical use are typically of smaller sizes (200nm or less). Liposomes that include cholesterol are much less susceptible to lipid

exchange and exhibit increased circulation time of incorporating liposomes by several hours. Charge can also play a part in evading both immune effects and lipid exchange as uncharged or uncharged or positively-charged liposomes exhibit increased circulation times.²⁷

Another strategy to enhance liposome circulation is to incorporate surface modifications. A common modification technique is to attach polyethylene glycol (PEG) to the surface of the liposome (see Figure 6b). PEG acts as a steric barrier between the polar surface of the liposome and circulating opsonins, inhibiting recognition and elimination from circulation.^{4, 26, 27} Circulation times of PEGylated liposomes increase dramatically compared to unmodified liposomes, climbing from several minutes to several hours. However, the steric barrier created by PEGylation makes it more difficult for the liposome to interact with any other particle, including the cell surfaces of the target tissues, and this results in the inhibition of membrane-membrane interactions such as fusion that are vital to the therapeutic effects of most liposome delivery strategies. This can be circumvented by designing liposomes that shed the PEG coat before uptake.⁴ Cell penetrating peptides (CPPs) have also been utilized to facilitate the delivery of cargo from PEGylated liposomes (see Figure 6c).³⁶ CPPs are a group of small peptides, about five to thirty amino acids in length, which can pass non-specifically through cell membranes. Therefore, attaching these peptides onto liposomal drug carriers can enhance the passage of the cargo into the cell of interest.³⁷

Longer circulation times also enhance the capacity of the liposomes to localize passively to target tissues by the enhanced circulation and retention (EPR) effect. This describes a phenomenon by which nanoparticles are preferentially accumulated in sites of disease such as tumor sites.³⁸ Tumors and their vasculature grow rapidly and uncontrollably, which leads to poor formation of the tumor vasculature and poor lymphatic drainage. Capillaries propagated by tumor

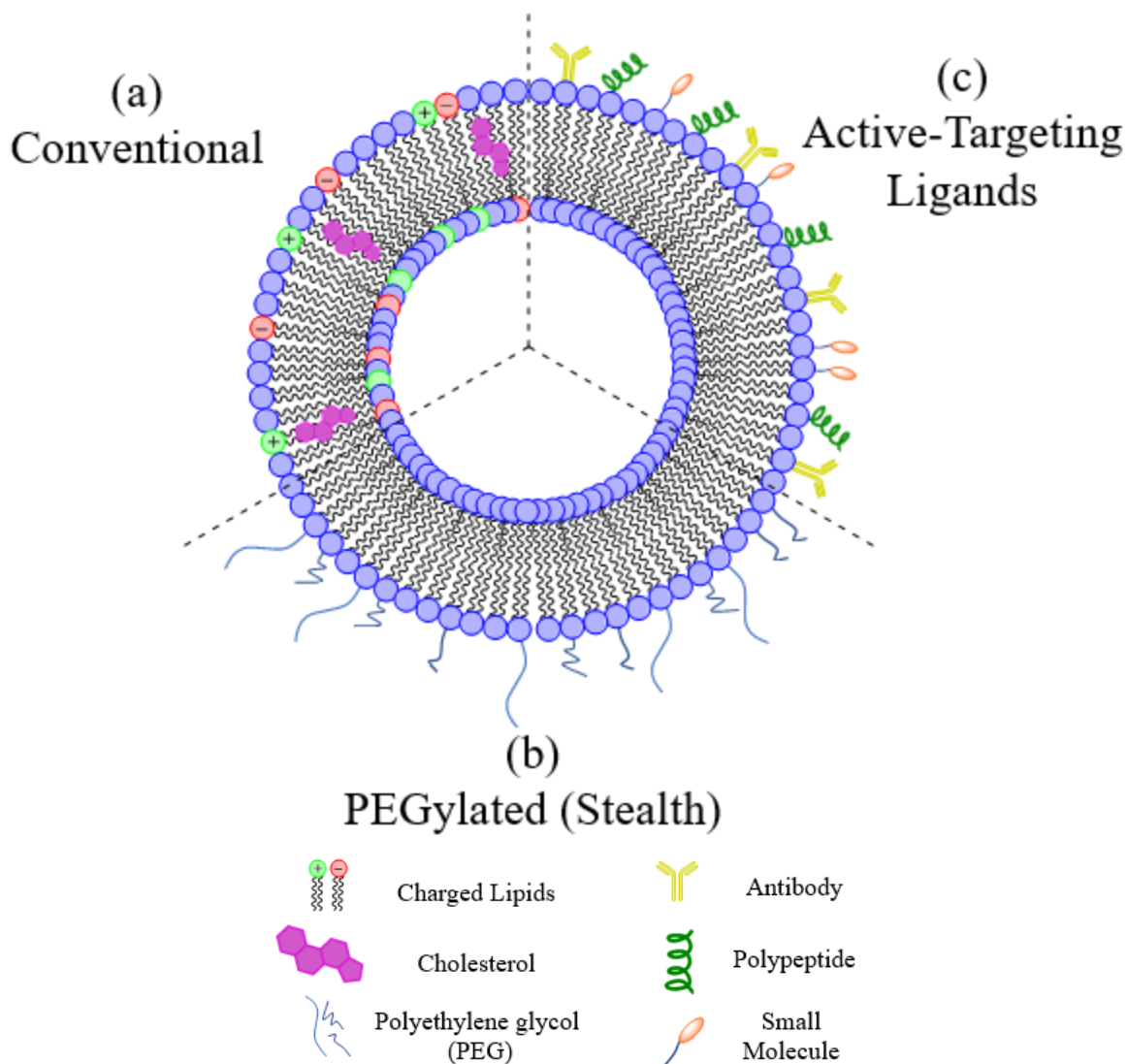


Figure 6: Schematic of three types of surface modifications of liposomes. (a) Conventional liposomes which rely on composition alone. (b) PEGylated liposomes, also known as stealth liposomes, in which the lipid surface is modified with PEG to promote longer circulation times. (c) ligand-targeted liposomes for active targeting of diseased tissues.

tissues are highly permeable as a result, allowing for nanoparticles to more easily pass through the vasculature and enter the surrounding tissues. Lymphatic drainage is also slowed in these tissues, allowing nanoparticles to passively accumulate within the tissues.³⁹

Taking advantage of EPR as a passive targeting strategy has its limitations. The effect cannot be controlled as efficiently as other targeting methods.⁴⁰ Some tumor tissues may not be susceptible to the EPR effect to begin with, and the heterogeneity of tumor tissues has a drastic effect on the diffusion efficiency of the liposomes and drug cargo.⁴¹ The permeability of tumor vasculature may vary throughout the tumor tissues, which may lead to an unequal distribution of the drug. The drug itself may not be able to diffuse efficiently throughout the tissues either, leaving some parts of the tumor tissue with too low of a concentration of the drug to initiate cell death.⁴⁰
⁴² Active targeting strategies seek to alleviate the limitations presented by passive targeting effects.

Active targeting describes the direct targeting of diseased tissues through the action of various conditions that are specific to the disease environment, and this causes the coated liposomes to selectively congregate at the site of disease.⁴³ Active-targeting liposomes may be accomplished by several methods, though generally involves the attachment of a ligand, such as a peptide or antibody, to the bilayer surface. The ligand responds to an antigen either specific to the disease environment or present at the disease environment in a high concentration in order to distinguish from healthy tissues.⁴⁴ Active targeting of liposomes to the diseased tissues of choice also assists in the cellular uptake of liposomes into the aberrant cells.⁴³

Cellular uptake of liposomes and their cargo can occur by a few main pathways including the following: endocytosis followed by endosomal escape, membrane fusion between cell and liposome, and induction of liposomal leakage through interaction with the cell surface.⁴⁵ The pathway utilized by a liposome is highly dependent on its composition. For example, it has been

observed that liposomes composed of pure or significant amounts of PS fuse more readily with the cell surface than liposomes of pure PC.^{45, 46}

Endocytosis is a common pathway to cellular uptake and encompasses two main mechanisms: pinocytosis which describes the uptake of extracellular fluids and small particles or vesicles and phagocytosis for the uptake of larger particles.⁴⁷ Endocytosis may or may not be mediated by receptors or the assistance of clathrin or other molecules to form the endosomal structure. The mechanism of uptake also plays into the trafficking of the internalized materials once within the cell, whether the formed endosome fuses with a lysosome for degradation or stay within endosomes proper in a non-degradative pathway. The path which an internalized liposome is supposed to be destined is dependent on the desired liposomal cargo.⁴⁸ For cargo that must escape the endosome to the cytosol to function, the incorporation of fusogenic lipids such as dioleoyl phosphatidylethanolamine (DOPE) facilitate and promote the interaction of the liposomal and endosomal membranes leading to an exodus from the endosome.⁴⁹

1.4 Triggered Release from Liposomes

Liposomes must be able to accomplish two main things as a delivery vehicle: maintain its structural integrity en route to the target site and deliver encapsulated drug cargo upon arrival to that site. One method to achieve this end is to prompt the liposome to destabilize only when certain conditions are meant, termed triggered release. By developing release mechanisms that trigger only under specific stimuli, a degree of control is achieved over where release of drug cargo occurs within the biological system.^{39, 50}

Release of the drug cargo from the liposome can be achieved by a variety of means such as by inducing a drastic conformational change within the liposome, destroying the liposome, or increasing the permeability of the membrane enough to allow the cargo to leak out of the structure.

Conformational changes induced by altering lipid structure and/or composition is a commonly employed method. In this method, a change in lipid composition or properties is initiated through an intrinsic or extrinsic triggering mechanism. Non-bilayer prone lipids present in a larger percentage within the liposome as a result of the trigger event make contact with the non-bilayer-lipid-rich bilayers of other liposomes forming an aggregate. These aggregates can eventually initiate a full shift from the bilayer formation into another lipid aggregate shape, causing the dissolution of the liposome and release of the encapsulated cargo.^{10, 13}

There are two styles of release trigger that can be adapted into liposomes: a trigger sensitive to a condition specific to the target tissues- an intrinsic factor (passive release)- or one that is applied to the target tissues after the arrival of the liposomes- an extrinsic factor (active release).⁵⁰ These styles of triggered release are shown in Figure 7. Both types of release triggers have been previously utilized in the literature.^{10, 28, 51-56} The microenvironments of sites of disease,

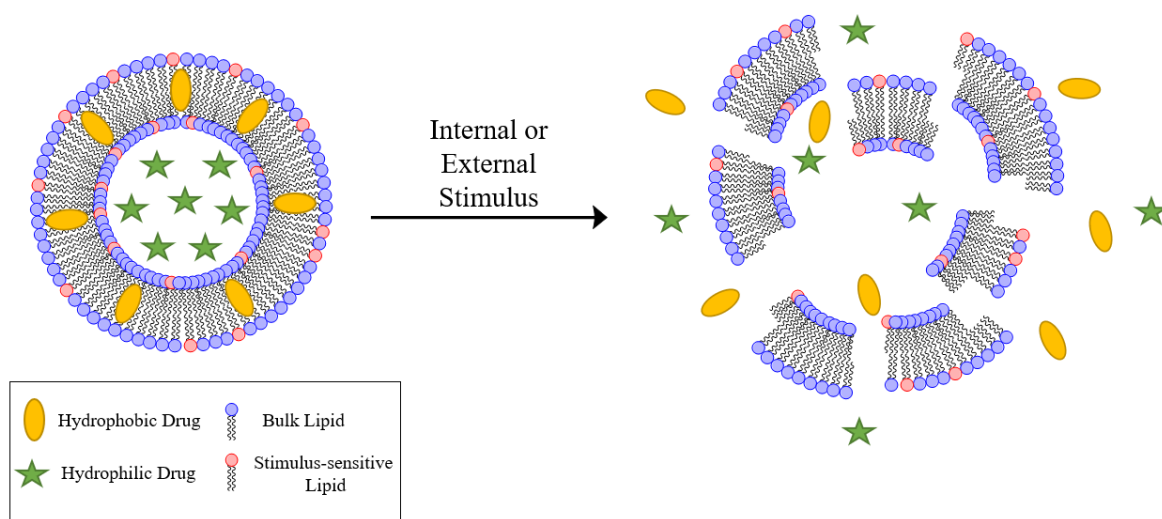


Figure 7: Schematic of stimulus-sensitive lipid-enabled liposome release.

such as cancers, exhibit deviations from healthy tissue microenvironments in many ways that can be exploited as intrinsic release factors. Oxygen levels in solid tumor tissues are lower, or hypoxic, than that of healthy tissues. As a result, the local pH of solid tumor tissues is often slightly more acidic than typical physiological pH.⁵⁰ Certain biomolecules or biologically relevant molecules have been shown to be upregulated in tumor environments and other sites of disease including enzymes, proteins, and metal ions. The types of biomolecules that are upregulated depends heavily on the disease state or cancer type.^{29, 50}

1.4.1 Methods of Triggered Release

As mentioned above, intrinsic release factors seek to exploit the aberrant conditions present at the site of disease to encourage release of drug cargo from liposomes. Liposomes are designed to be sensitive to one or more of these factors. Conditions which have been utilized previous within the literature as viable triggers of cargo release include pH, redox conditions, upregulated biomolecules, and upregulated metal ions.^{10, 29, 50, 56}

Cargo release by an applied extrinsic factor is accomplished by designing the liposome to be sensitive to the desired mode of release so that after the application of the chosen stimulus, the properties of the liposome change to allow for the quick release of the drug cargo. Some extrinsic factors that have been utilized include magnetic field, UV or IR radiation, ultrasound, and heat.^{28, 50, 53-55}

1.4.2 Enzymes as Intrinsic Release Triggers

Enzyme concentration is increased in certain disease microenvironments such as inflammatory diseases, and various cancer types, and these have been utilized as targets for triggered cargo release from liposomes.²⁹ Many classes of enzymes have been utilized in the literature for this purpose, including esterases, proteases, and reductases.^{10, 12, 13, 29}

Cathepsin B is a lysosomal protease that is shown to be upregulated in several cancer types including lung, brain, and colon cancers. In the biological environment cathepsin B participates in the process of protein turnover within lysosomes and accelerates the degradation of the extracellular matrix.^{29, 52, 57} Romberg et. al. utilized this enzyme in the development of fusogenic liposomes made of DOPE and a polymer-lipid conjugate poly(hydroxyethyl L-glutamine)-N-succinyl-dioctadecylamine (PHEG–DODASuc).⁵² PHEG, like PEG, coats the liposome to increase circulation time within the bloodstream. PHEG is prone to degradation by not only cathepsin B but also related proteases papain and pronase E.²⁹ The incorporation of PHEG–DODASuc with DOPE, a typically non-bilayer prone lipid, stabilizes DOPE and allows for the formation of liposomes. After interaction with cathepsin B, papain, or pronase E, the PHEG coating is degraded. The DOPE liposomes then aggregate with other DOPE liposomes and cause a conformational change into the inverted hexagonal phase, causing a release of fluorescent cargo.⁵²

Secretory phospholipase A₂ (sPLA₂) is an esterase enzyme that catalyzes the hydrolysis of the ester group of the fatty acid in the *sn*-2 position of phospholipids, resulting in a free fatty acid and a lysophospholipid.^{12, 13} Lysophospholipids are cone-shaped non-bilayer forming lipids, and after the action of sPLA₂ the increased concentration of non-bilayer lipids in the liposome triggers a conformational change and release of drug cargo.^{4, 12} sPLA₂ is shown to be more active towards lipid aggregates such as liposomes as opposed to free lipids, and this activity is highly dependent on the lipid composition of the liposome. This has led to the careful formulation of liposomes that optimally recruit sPLA₂ to degrade the lipid bilayer. It has been observed that sPLA₂-IIA, a subtype of sPLA₂, shows much higher affinity towards membranes with an overall negative charge compared to zwitterionic membranes.¹² Liposomes containing a high abundance of cholesterol exhibit no interaction with sPLA₂ as it shows no activity for bilayers in the liquid ordered phase,

a membrane phase transition brought on by cholesterol restricting the transition into the gel phase.^{12, 58} One strategy implemented is the use of modified lipids containing the drug of choice appended to the *sn*-2 position of the phospholipid in the creation of a prodrug. The modifications ideally have little effect on the structural integrity of the liposome and serve only to restrict the activity of the drug until after hydrolysis by sPLA₂.¹³

Modification to the lipid headgroup is a common strategy for designing a response-triggered lipid for use in cargo release. In these strategies a small molecule, peptide, or other compound is attached to the lipid headgroup in a manner that is reversible by the induction of a certain stimulus.^{10, 11, 51, 59} Upon reaction with the stimulus of interest, such as esterase enzyme, the modification will be cleaved from the trigger lipid and induce a change in lipid self-assembly properties through an alteration of lipid properties spurred on by the stimulus response.^{10, 51} One such modification reported previously in the literature, the trimethyl lock, is detailed below.

1.5 The Trimethyl Lock

The trimethyl lock describes a mechanism by which an *o*-hydroxydihydrocinnamic acid derivative undergoes a rapid lactonization into a hydrocoumarin. Steric stress between three methyl groups in the molecule is proposed to lead to the rapid lactonization.⁶⁰ The rapid rate observed in this reaction is thought to be due to the relief of steric stress after the formation of the lactone. The generic structure of the trimethyl lock molecule is shown in Figure 8. If the phenolic oxygen is bonded to another functional group, the reaction will be inhibited. Commonly a labile group, R₁, is bonded to the phenolic oxygen so that the trimethyl lock reaction will occur only under specified conditions required to remove the group. The labile group appended to the phenolic oxygen may then act as a trigger for the trimethyl lock mechanism.⁶⁰ Sensitivity to a certain enzyme, such as quinone reductase, has been observed in the literature to act as a suitable trigger.¹⁰

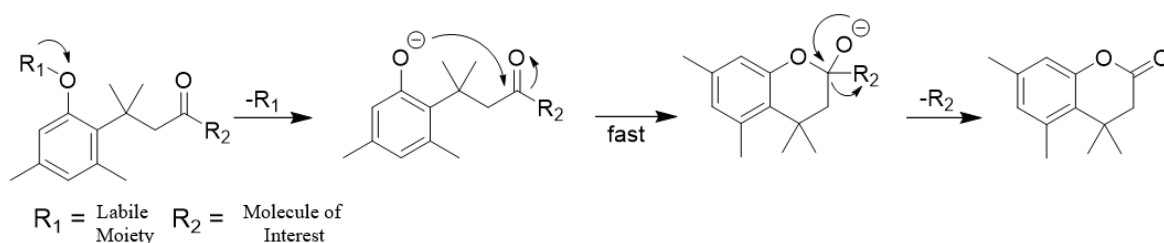
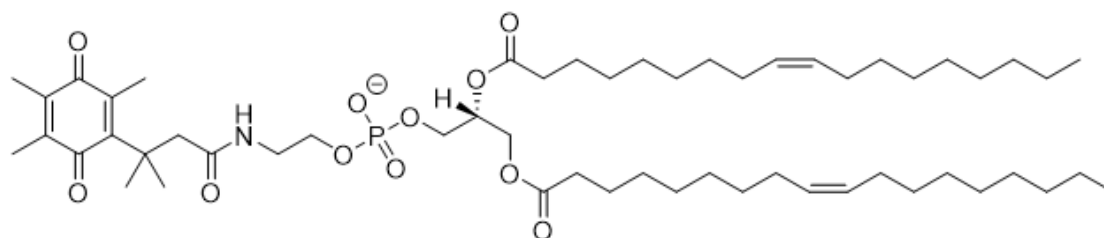


Figure 8: Schematic of the trimethyl lock mechanism of the cleavage of the target molecule R_2 from the trimethyl lock compound. Fast lactonization step noted. R_1 is a labile moiety which is cleaved by the proposed mode of release.

As a result of the lactonization, the group labelled R_2 will be cleaved from the molecule. This property has enabled the release of a variety of R_2 molecules that were initially masked by association with the remainder of the trimethyl lock molecule. This has been utilized as a prodrug strategy during which the drug is coupled to the trimethyl lock molecule typically to be freed through the lactonization process once it has arrived at the target site.⁵¹ The molecule has also been used in the production of latent fluorophores, the trimethyl lock inhibiting fluorescence until a certain condition is met.⁶¹ Most relevant to this thesis is the use of the compound as a means of inducing release from liposomes; an example of the use of this mechanism as a liposome release trigger is explained further.

Ong et. al. utilized the trimethyl lock to induce a conformational change in liposomes after interaction with quinone reductase.¹⁰ The trimethyl lock molecule was appended with a moiety on R_1 to impart sensitivity to quinone reductase, and attached to R_2 was the non-bilayer lipid DOPE. The trimethyl lock molecule was coupled to the head group of the lipid by an amide bond to produce the trigger lipid. Figure 9 shows the structure and schematic of the release of DOPE of the reported quinone reductase sensitive lipid trigger. This caused the area of the polar head group to increase, which altered the shape of the lipid. DOPE is typically a wedge-shaped non-bilayer lipid, but the addition of the head group modification caused the lipid to take on a more cylindrical

(a)



(b)

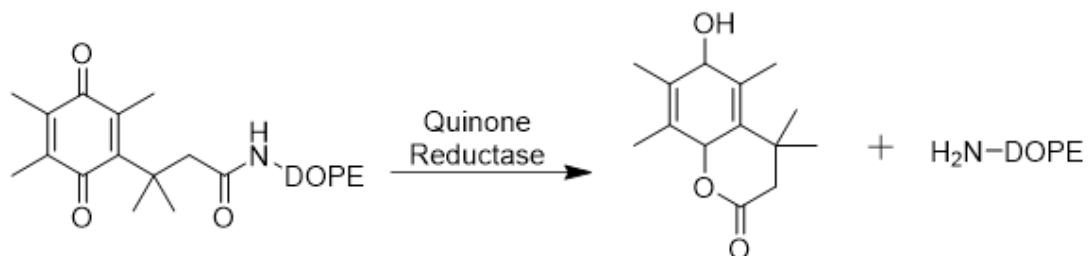


Figure 9: Structure of trimethyl lock trigger lipid and mechanism of release in Ong et al. (a) Structure of trimethyl lock trigger lipid. Note the DOPE lipid is attached to the trimethyl lock molecule by the amine group in DOPE through an amide bond. (b) Schematic of the reaction of the reductase-sensitive lipid upon interaction with enzyme.¹⁰

shape and adopt bilayer-prone properties.^{10, 11, 62} Following a two-electron reduction of the quinone reductase-sensitive moiety on R₁, the trimethyl lock mechanism proceeded, causing the release of the DOPE lipid. DOPE, now unmodified, was exposed and regained its typical non-bilayer forming properties. As more DOPE lipids were exposed, they began to aggregate with the exposed DOPE molecules of other liposomes. This eventually led to a dissolution of the liposome structure into inverted micelles and the release of encapsulated dye.¹⁰

1.6 Summary

While previous research demonstrates the potential of enzymes and esterase enzymes specifically as release triggers, further research is required to evaluate esterase enzyme-enabled drug delivery through liposomes. The trimethyl lock mechanism could be utilized in the development of an esterase enzyme-responsive liposome for drug cargo delivery. This approach is expected to lead to the development of a general strategy for liposome-enabled drug delivery in which the trigger group could be altered to target a variety of enzyme classes after initially probing esterase response. In this thesis, the design, synthesis, and analysis of a trimethyl locked trigger lipid for esterase-responsive liposome drug delivery is described.

Chapter 2: Design, Synthesis, and Analysis of Esterase Sensitive Trimethyl Lock-coupled Phosphatidylethanolamine Lipid for Triggered Release from Liposomes

2.1 Overview of TML-PE Lipid Conjugate Functionality

Esterase represents a diverse class of enzymes that is expressed in a variety of places throughout mammalian cells, such as within the cytosol, endoplasmic reticulum, and the endosomes.⁶³⁻⁶⁵ Esterase enzyme concentration is found to be heightened in select cancer types such as colon cancers, lung cancers, and prostate cancers.⁶⁶⁻⁶⁸ This enzymatic upregulation serves as the basis for the development of an enzyme-sensitive release trigger. Lipid molecules modified with esterase-sensitive groups seek to exploit this overexpression of esterase enzyme in tumor cells to promote the release of drug cargo.

In this project, we seek to design, synthesize, and study an esterase-responsive lipid incorporating a trimethyl lock trigger that could be exploited for the development of general enzyme-responsive therapeutic liposomes. For this purpose, an esterase-sensitive trimethyl lock compound was coupled to the head group of a PE lipid molecule as a trigger for cargo release from liposomes. The acetyl ester moiety appended to the phenolic oxygen of the trimethyl lock molecule was incorporated to act as a substrate for esterase hydrolysis, allowing for the exploitation of the esterase-rich environment in certain cancerous tissues to act as a trigger for cargo release.⁶⁰ Coupling of the ester-capped trimethyl lock to the PE lipid head group, would increase the overall area of the polar head of the lipid, leading to a change in molecular shape. PE lipids are typically wedge-shaped non-bilayer forming lipids, and acylation causes the coupled lipid to exhibit more bilayer forming behavior, allowing for their incorporation into liposomes.^{7, 10, 11} After uptake into

a tumor cell, it is proposed that the acetyl ester moiety will be hydrolyzed by the heightened concentration of esterase enzyme. This triggers the trimethyl lock mechanism, which causes the release of PE lipid, for which non-bilayer forming properties would lead to a destabilization or potential dissolution of the bilayer membrane and release of liposomal cargo. The activation of the trimethyl lock mechanism after interaction with esterase enzyme is shown in Figure 10.

2.2 Research Design

2.2.1 Design and Synthesis of Esterase-Responsive Trimethyl Lock-PE Lipid (TML-PE)

Two trimethyl lock-PE lipid conjugates were synthesized, trimethyl lock-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine (TML-POPE) and trimethyl lock-1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (TML-DOPE). Both structures are shown in Figure 11. The main difference in both molecules is the choice of lipid that will be released. POPE and DOPE differ only in the fatty acid acyl chains present in each lipid. While POPE has one monounsaturated

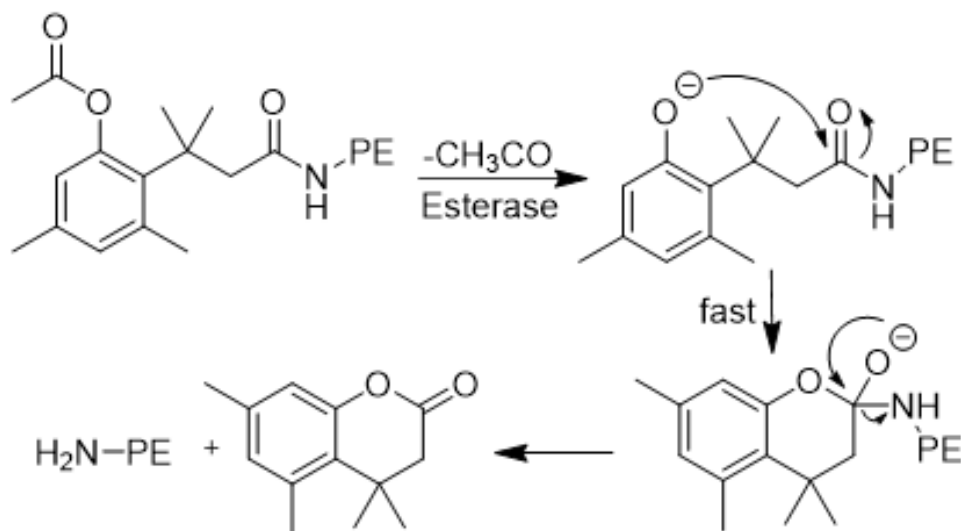


Figure 10: Schematic of the reaction mechanism of the enzyme-responsive TML-lipid after interaction with esterase enzyme.

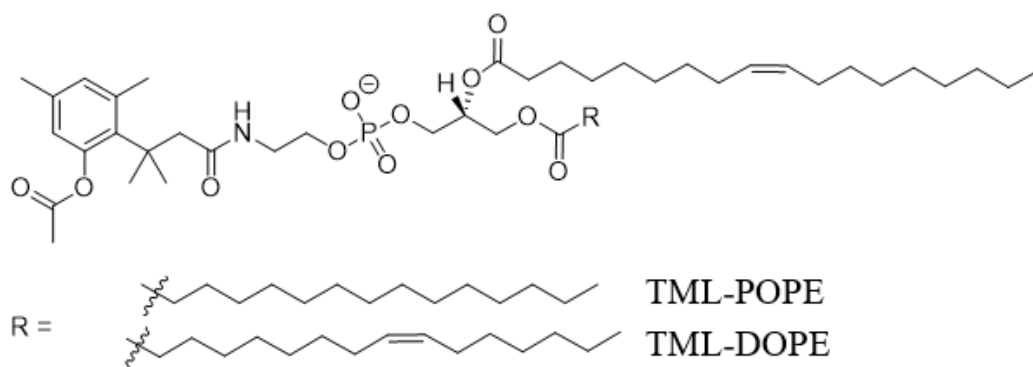


Figure 11: Structures of synthesized trimethyl lock lipids. R differentiates the lipids between trimethyl lock-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (TML-POPE, top) and trimethyl lock-1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (TML-DOPE, bottom).

fatty acid connected to the *sn*-1 position while the other fatty acid contains a saturated, sixteen-carbon long aliphatic chain, DOPE contains two identical eighteen-carbon length monounsaturated fatty acids. The consequence of this difference will be elaborated upon later in this chapter.

All esterase-responsive TML-PE trigger lipids were synthesized using an amide bond coupling reaction, as shown in Figure 12. Commercially available 3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid (abbreviated as trimethyl lock, or TML) was coupled to the terminal amine of the appropriate PE lipid to create an amide bond. After obtaining the crude reaction mixture, the product was purified using flash chromatography with methanol: chloroform (MeOH: CHCl₃). The purified product was analyzed by ¹H NMR, ¹³C NMR, and ³¹P NMR.

2.2.2 Analysis Techniques and Instrumentation

Fluorescent Release Assay

For a method of controlled release to be viable *in vivo*, several conditions must be able to be met by the incorporating liposomes. A liposome formulation must allow the resultant liposome three basic tasks: maintain stability long enough to reach the target site and potentially achieve cellular uptake, encapsulate a therapeutically relevant concentration of the drug of choice, and contain a

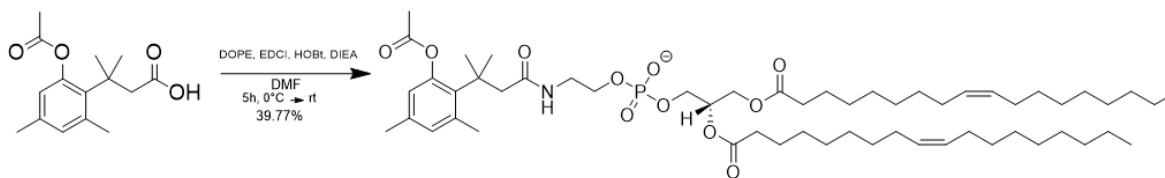


Figure 12: Schematic of amide coupling reaction used to synthesize TML-DOPE.

sufficient concentration of the release compound to allow for the release of cargo.⁶⁹ Of these, the last is most relevant to the initial stages of the project as the efficacy of the proposed release method relies on its ability to successfully unleash the liposomal cargo. Encapsulated drug cargo will be available for therapeutic effect only after the release event has occurred, thus the efficacy of the release event is an important factor.⁷⁰ The proper concentration of the modified lipid is vital, so the liposome is able to release in the presence of the desired trigger but not become active beforehand.

A fluorescent release assay technique was implemented to measure the presence and extent of release from the proposed esterase-sensitive liposomes. In a fluorescence release assay, the liposome is loaded with a fluorescent dye. The release of the dye that is encapsulated within the liposomes by the condition of interest will lead to a detectable change in fluorescence intensity attributable to the release of dye from the liposome. The degree of this change in fluorescence compared to a control indicates the efficacy of the release trigger.

The fluorescent dye used in a fluorescent release assay may be either hydrophobic or hydrophilic. Just as with drug cargo, hydrophilic dyes will be encapsulated within the aqueous core of the liposome while hydrophobic dyes will be incorporated within the hydrophobic bilayer. Three sets of dyes were used over the course of the experiments described in this thesis: Nile Red, Sulforhodamine B, and the dye pair 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt and

p-Xylene-Bis-Pyridinium Bromide (ANTS/DPX). The structures of all fluorescent dyes implemented are shown in Figure 13.

Nile Red is a hydrophobic fluorescent dye compound that is fluorescent only within a hydrophobic environment, exhibiting a high fluorescent intensity while within the lipid bilayer. The dye has very low solubility in aqueous solution, so after the addition of esterase enzyme and initiation of release, Nile Red is expected to precipitate out of the solution, leading to a decrease in fluorescent intensity.^{71, 72}

Sulforhodamine B, unlike Nile Red, is a hydrophilic fluorescent dye.⁷³ While within the liposome, the dye is self-quenched by the high concentration of the dye in the aqueous core, exhibiting a lower fluorescence intensity. Once the fluorescent cargo is released, the dye is no longer self-quenched and fluorescence intensity is expected to increase.⁷⁴

ANTS/DPX is a hydrophilic fluorescent dye/quencher pair. 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) acts as the fluorescent dye while p-Xylene-Bis-Pyridinium Bromide (DPX) is a cationic quencher. ANTS is quenched by DPX through collisional quenching, which occurs only when the compounds are within a very close distance from each other.^{75, 76} This is achievable within the liposome, resulting in a low initial fluorescent intensity. Cargo release dilutes the components away from each other, leading to an increase in fluorescence intensity as ANTS is no longer quenched.⁷⁷

The fluorescent release assays generally took on one of two forms for the purpose of this thesis: incremental addition of esterase enzyme or base and the change in fluorescent intensity over time after the addition of a certain concentration of enzyme. The details of the fluorescence release studies performed on each esterase-responsive lipid will be detailed in their respective sections.

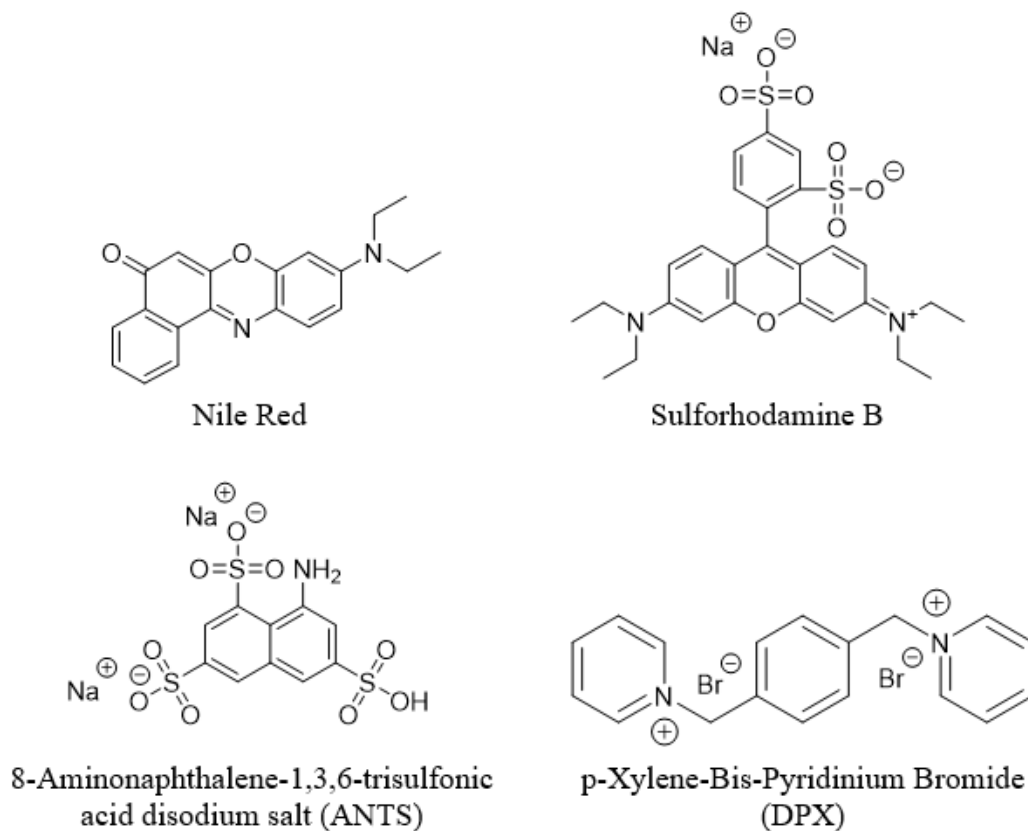


Figure 13: Structures of fluorescent dyes used in fluorescence release studies: Nile Red, Sulforhodamine B, ANTS, and DPX.

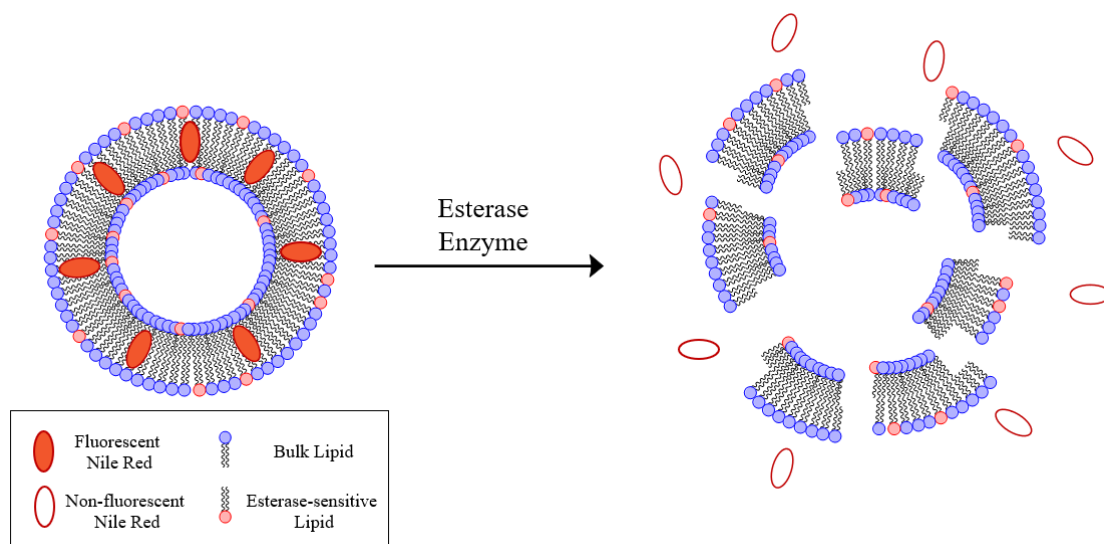


Figure 14: Schematic of fluorescence release with Nile Red. Nile Red is fluorescent only when in a lipophilic environment. Once the liposome interacts with esterase enzyme, the Nile Red will become non-fluorescent and precipitate in the aqueous media due to low solubility.^{71, 72}

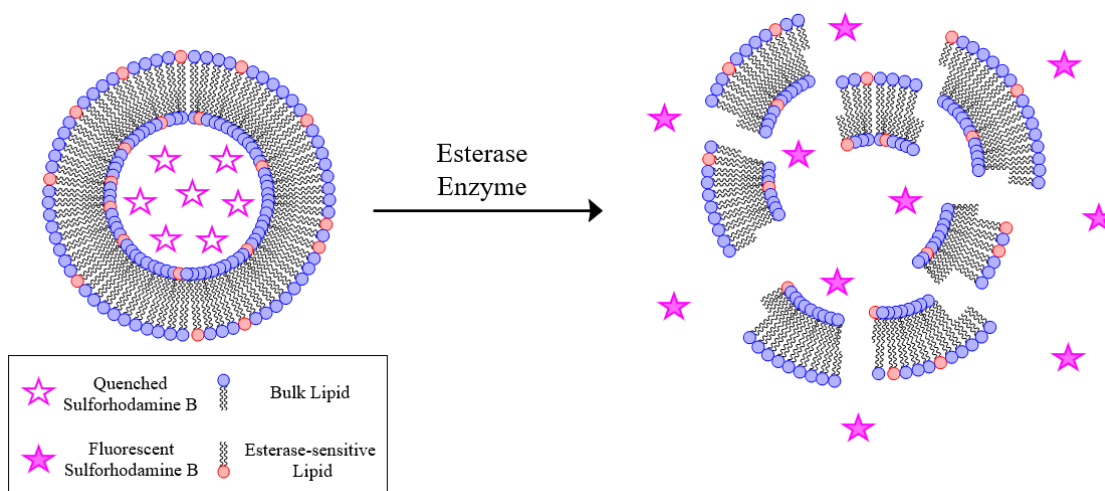


Figure 15: Schematic of fluorescence release with Sulforhodamine B. Sulforhodamine B is self-quenching when in the aqueous core of the liposome. After interaction with esterase enzyme, the Sulforhodamine B will no longer be quenched and fluoresce.⁷⁴

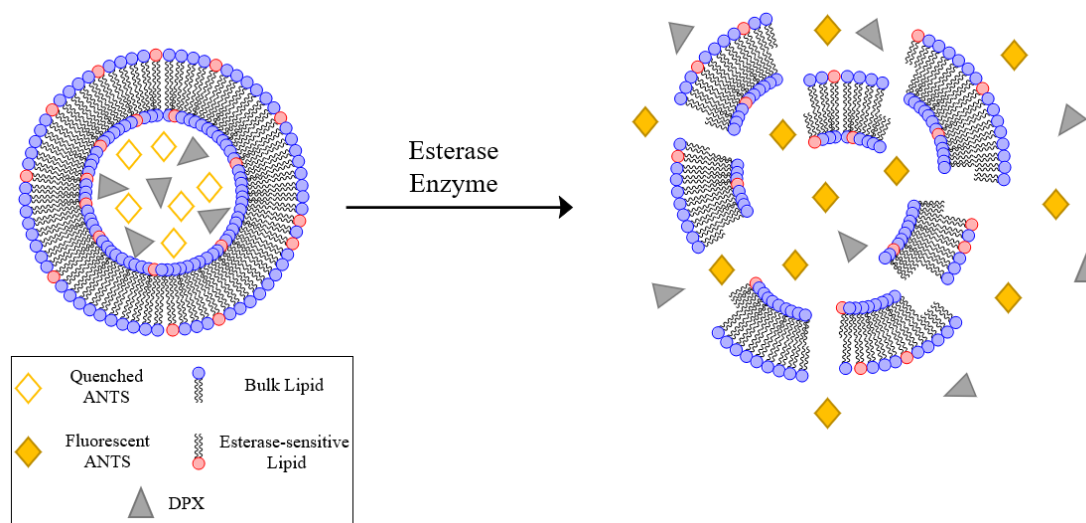


Figure 16: Schematic of fluorescence release with the ANTS/DPX dye pair. DPX acts as a quencher, and ANTS fluorescence is low whilst inside the aqueous core of the liposome. After the release event occurs, ANTS is no longer quenched by DPX and fluoresces.⁷⁵

esterase-sensitive moiety on the trimethyl lock molecule to induce release.⁷⁸ For this reason, base was also used in the initial fluorescence release studies by incremental addition of 0.5 mM potassium hydroxide (KOH) to the liposome sample. However, fluorescent dyes tend to be sensitive to the effects of pH, affecting their fluorescence intensity considerably.⁷⁹⁻⁸¹

Dynamic Light Scattering

Dynamic Light Scattering (DLS) is an analysis technique in which the time-dependent fluctuations of scattered light from the movement of molecules in a solution are measured to determine the size and distribution of micro- and nanoparticles in solution.^{82, 83} Liposomes are extruded to a uniform size during their manufacture. As liposomes are subjected to a fluorescent release assay their particle size may change through aggregation as part of the leakage event, though this is not always observed.⁸⁴ DLS was utilized to determine whether the particle size determined by analysis was similar to the particle size projected by extrusion diameter before

fluorescent release and to investigate the effect of the release event on particle size and size distribution.

2.3 Esterase-sensitive Cargo Release with TML-POPE

2.3.1 TML-POPE Synthesis and Challenges with Synthesis

POPE was initially chosen for the lipid scaffold onto which the esterase responsive TML group would be coupled. TML-POPE was first synthesized successfully by another member of the Best Lab group, Jinchao Lou. This batch of purified product was utilized for initial analyses of TML-POPE-containing liposomes. After depletion of the preliminary stock of TML-POPE, subsequent syntheses were carried out. During these efforts, we encountered issues in the spectral analysis of the ^1H NMR taken following attempts at the purification of the coupled product. The integration for the number of hydrogens in peaks constituting the lipid tails were typically higher than expected values. In addition, the most polar region of the molecule, including the amide bond and phosphate group, returned integrated peaks with lower than expected values for the number of hydrogens in the region.

For the acyl region of the trigger lipid, it was proposed that this occurred due to contamination with unreacted POPE lipid. Due to the chemical similarities between TML-POPE and unmodified lipid, the purification of the coupled lipid proved to be difficult. Multiple solutions were attempted to remedy this. Sequential purifications were performed on separated products. The compound was separated by gravity alone, and the solvent gradient was tuned by increasing the ratio of MeOH. Beginning the separation with a 4% MeOH: CHCl_3 allowed for the most pronounced separation of the free lipid and TML-POPE as indicated by the resultant ^1H NMR spectra (not shown).

For the polar region of the ^1H NMR spectra, in addition to the deviations from the expected hydrogen count integration from the expected values, the region indicated potential contamination by urea byproducts from the coupling reaction. In order to better resolve the polar region, the NMR solvent was altered to include a percentage of deuterated methanol (MeOD). Two ratios of MeOD were used: 20% and 30%. The resolution change between the two percentages of MeOD was minimal, so 20% MeOD was used over 30%. Resolution of the polar region was improved over the use of pure CDCl_3 . TML-POPE was not successfully synthesized and other options for an esterase-responsive lipid were investigated.

2.3.2 Initial Fluorescent Results with TML-POPE Liposomes

Initially, fluorescent assay trials were focused on understanding the amount of TML-POPE required for release. Low percentages of TML-POPE were observed to be ineffective for the release of liposomal cargo when compared to a control. In Figure 17 the y-axis scale is reduced to show a region between 86% and 102% in order to better visualize the minimal change in fluorescence. As this experiment uses Nile Red as the fluorescent dye, the fluorescence should decrease as the concentration of esterase enzyme increases.⁷¹ With an increasing concentration of esterase enzyme, liposomes containing 10% TML-POPE showed little to no release after 0.5 units of total enzyme had been added to the cuvette. This effect was observed in both enzyme addition and base addition trials (not shown).

The ratio of TML-POPE in the liposome formation was increased for subsequent trials. For the trials shown in Figures 18a and 18b, the fluorescent dye used was Sulforhodamine B, and the fluorescent intensity is expected to increase after the addition of the enzyme or strong base.⁷⁴ For liposomes composed of 50% or 70% TML-POPE, the expected increase in fluorescence intensity was observed. These sample exhibited a roughly 20% increase in fluorescence intensity after the

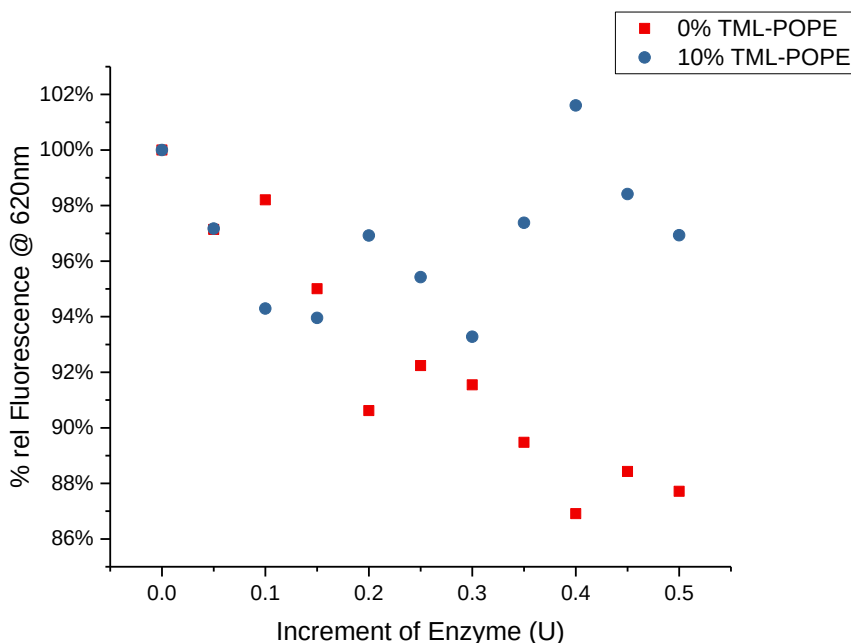


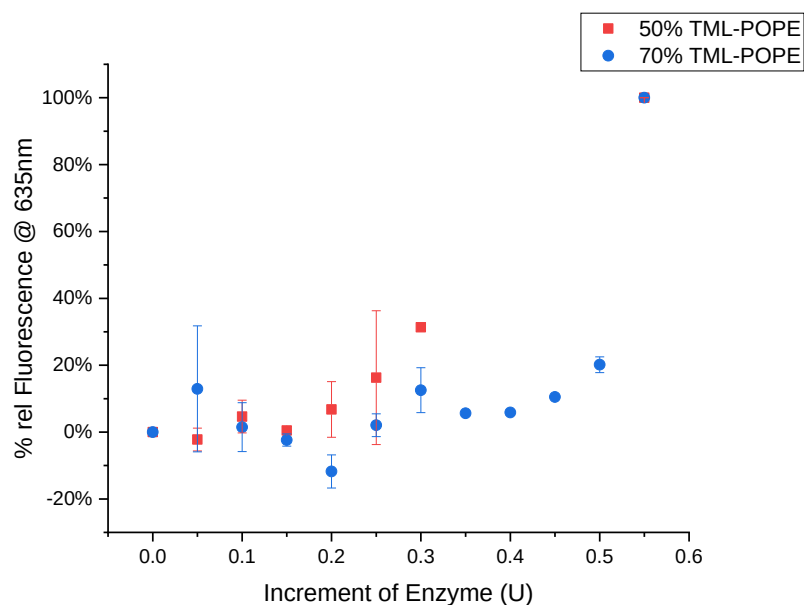
Figure 17: Percent relative fluorescence versus total increment of 50 units/ mL esterase enzyme added of 10% TML-POPE liposomes compared to 0% TML-POPE control. Dye: Nile Red.

addition of either esterase enzyme or strong base.

The liposome formulations thus far have contained only the synthetic lipid, bulk lipid, and fluorescent dye. Natural membranes include non-bilayer components to alter membrane fluidity as well as liposome charge and encourage release of liposomal cargo.⁸⁵ Multiple non-bilayer additives were used for this purpose in the liposome formulations, including: diacylglycerol (DAG), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and phosphatidic acid (PA). These lipid additives were included into liposome formulations to enhance release of cargo from the liposome after the release event and control the properties of the liposomal membrane. The structures for each of the lipid additives are shown in Figure 19.

Diacylglycerols are composed of neutral lipids found in minor amounts within natural cell membranes where they serve as signaling lipids as well as an intermediate in the biosynthesis of

(a)



(b)

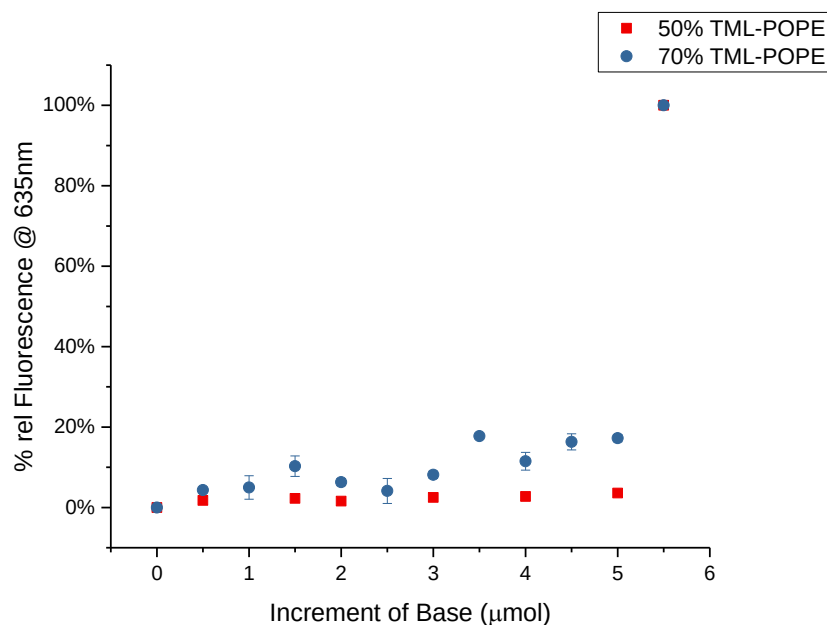


Figure 18: Percent relative fluorescence versus total increment of 50 units/mL esterase enzyme and 0.5M KOH added of 50% and 70% TML-POPE liposomes. (a) Shows 50 units/mL esterase enzyme added for 50% and 70% TML-POPE liposomes (50% TML-POPE: Mean $\pm$ SD, $n = 2$, 70% TML-POPE: Mean $\pm$ SD, $n = 2$) and (b) shows 0.5M KOH added for 50% and 70% TML-POPE liposomes (70% TML-POPE: Mean $\pm$ SD, $n = 2$). The final point on each graph is after the addition of 1 μ L Triton X-100. Dye: Sulforhodamine B.

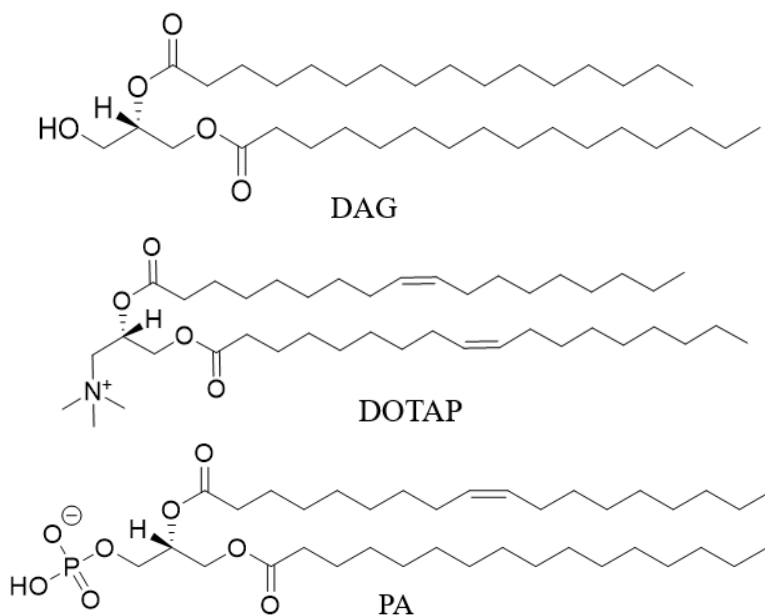


Figure 19: Structures of 1,2-dipalmitoyl-*sn*-glycerol (DAG), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (PA).

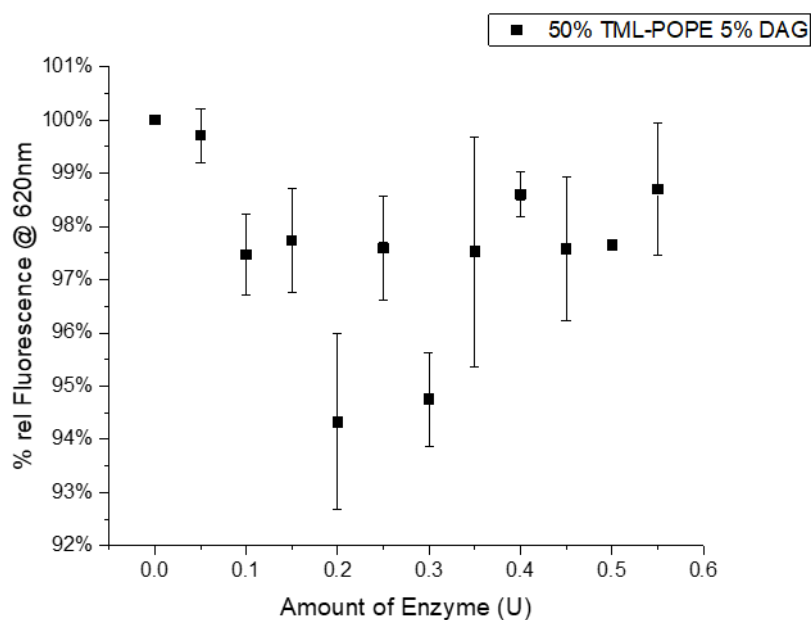
several glycerophospholipids.⁸⁶ They may also facilitate membrane fusion and fission by enhancing the fluidity of the lipid bilayer at membrane trafficking sites.⁸⁷ DAG takes on a wedge shape similar to PE lipids, which imparts a negative curvature on the bilayer membrane.¹⁴

DOTAP is a cationic lipid used in the literature for studies pertaining to the delivery of RNA and DNA components by cationic liposomes. Plasma membranes, as previously mentioned, have a slightly negative electric charge gradient due to heterogeneity among bilayer leaflets.^{18, 23} Addition of charged lipids such as DOTAP to lipid vehicles of this type enhances interactions between the cellular bilayer and liposome. Charged lipids also reduce the aggregation of liposomes with each other due to charge repulsion. DOTAP is often paired up with DOPE and other PE lipids as helper lipids in the formulation of liposomes for DNA transfection. Unlike liposomes for use in drug delivery, which must have some degree of stability to retain drug contents, liposomes for use in transfection are designed to be inherently unstable to better facilitate membrane fusion.⁸⁸

PA is a negatively charged, wedge-shaped phospholipid which, like DAG, plays a key role in the biosynthesis of many glycerophospholipids, cellular signaling, and membrane trafficking. The main difference between DAG and PA beyond structural is the overall negative charge of PA, which allows it to perform other functions beyond those in common with PA such as the regulation of the enzymatic activity and association of enzymes with the membrane. Areas of localized high concentration of PA exhibit a negative curvature which facilitates membrane trafficking with the bilayer.²⁴

DAG was the first non-bilayer lipid component to be added to the liposome formulation. Only a small percentage, 5-10%, of DAG was evaluated for the desired effect, as a high enough concentration of DAG will hinder the formation of liposomes. The limit of DAG inclusion into liposome formulations was quickly determined with the following procedure. Liposomes were formulated with only PC bulk lipid, a variant percentage of DAG, and Nile Red. After the lipid films were hydrated with 1x PBS at pH 8 and agitated as part of the hydration step in liposome production, the Nile Red served as a means for visualizing whether or not the lipid film hydrated properly. Nile Red is insoluble in aqueous solution, and thus if liposomes are unable to form from the lipid film, the Nile Red will not solvate and instead will stay within the lipid film at the bottom of the screw-top vial or break off into a precipitate.⁷¹ From these studies, it was found that no more than 15% DAG could be included in the liposome formulation and still form liposomes. Our liposome release studies with TML-POPE added 5% DAG to encourage release. With the inclusion of 5% DAG, the concentration of TML-POPE required to promote the release of fluorescent cargo was reduced. Figures 20a and 20b display the change in fluorescence of Nile Red liposomes containing 50% TML-POPE and 5% DAG incubated with either esterase enzyme or strong base.

(a)



(b)

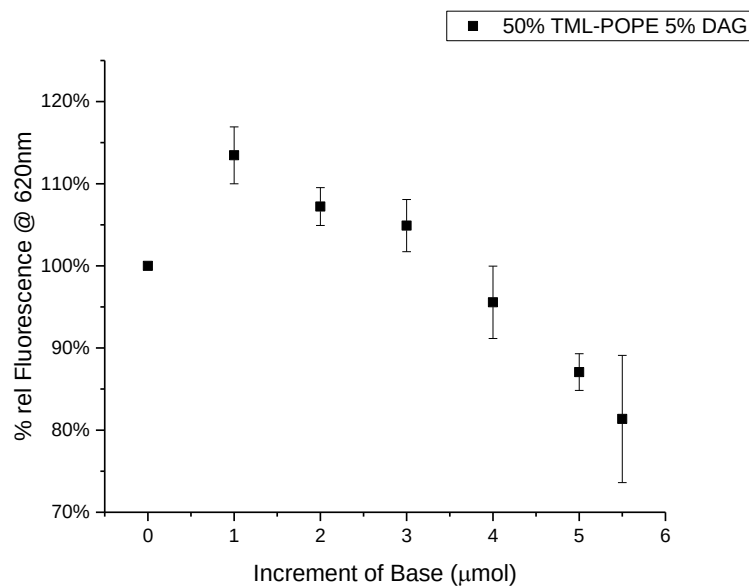


Figure 20: Percent relative fluorescence versus total increment of 50 units/mL esterase enzyme and 0.5M KOH added to 50% TML-POPE 5% DAG liposomes (a) Shows the addition of 50 units/mL esterase enzyme (Mean $\pm$ SD, $n = 2$) and (b) shows the addition of 0.5M KOH (Mean $\pm$ SD, $n = 2$). The final point on each graph is after the addition of 1 μ L Triton X-100. Dye: Nile Red.

However, a decrease in fluorescence intensity with Nile Red was present only within the sample treated with strong base. The y-axis scale of Figure 20a shows only a range from 92% to 101% as the change in fluorescent intensity was shallow in these samples. Large variations in, indicated by the standard deviations of Figure 20a, were also observed. This indicated a poor response to esterase-mediated release for which the liposomes were designed. The experiments performed on TML-POPE as an esterase-responsive release trigger showed the molecule contributed to release only when present in large percentages, and the addition of non-bilayer lipid additives such as DAG were ineffective at enhancing the release observed from the liposomes incorporating the esterase sensitive trigger lipid. The design of the esterase-responsive TML-lipid itself was then put under scrutiny. A change to either the lipid coupled to form the release trigger or a change to the chemical structure of the release trigger could result in a more pronounced release of fluorescent cargo. The former of these approaches was investigated further in this thesis.

2.4 Esterase-sensitive Cargo Release with TML-DOPE

POPE was initially chosen as the coupling lipid as its fatty acid chains matched those on the bulk lipid, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC). This was proposed to allow the fatty acid chains in the designed ester-responsive lipid to solvate well with the bulk lipid and increase the stability of the esterase-responsive trigger lipid while it was embedded within the bilayer. The results discussed above for the TML-POPE trials led to a change regarding the lipid used for the coupling. Other lipids were explored to find a new candidate for lipid coupling.

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), as previously stated, differs from POPE in the fatty acid attached to the *sn*-2 position of the lipid. DOPE is commonly utilized in the literature as both a coupling lipid and as part of the liposome composition in other triggered release applications.^{10, 11, 59, 88} DOPE is recognized for its ability to facilitate membrane fusion, and

this is due to its molecular structure.⁸⁸ Unsaturated double bonds create “kinks” in the fatty acid acyl chains. This affects the lipid molecule’s ability to pack within a bilayer and contributes to its overall molecular shape.¹⁸ Both fatty acid acyl chains of DOPE contain one cis-double bond whereas POPE contains only one unsaturated fatty acid. The double bond on both fatty acids acyl chains of DOPE may cause the lipid tails to spread out more than those of POPE, leading to an increase in the area of its lipid tails.⁸⁹ The effect this difference has on the properties of the lipid is evident in the difference in T_m between liposomes containing DOPE and POPE. DOPE is shown to have a T_m of -16°C while POPE has a T_m of 25°C.⁹⁰ The profound difference in T_m shows DOPE to have a more pronounced effect on the stability fluidity of the lipid bilayer. The saturated fatty acid chain in POPE has also been shown to allow POPE to exhibit more bilayer-prone behavior than DOPE, being found in the cellular membranes of prokaryotes.⁹¹ It is due to these differences that DOPE was investigated further in the design of an enzyme-responsive lipid over POPE.

2.4.1 TML-DOPE Synthesis

DOPE was chosen as an additional candidate for a coupling lipid. TML-DOPE was successfully synthesized using the same amide coupling as described previously. From the TML-POPE synthesis, a few techniques were taken in the purification of TML-DOPE, such as the use of an initial elution with 4% MeOH: CHCl₃ during the separation step. Corresponding NMR spectra and the synthesis details for the amide coupling of TML-DOPE are included at the end of this thesis.

2.4.2 Results and Discussion of TML-DOPE Liposomes

Liposomes with DOTAP as an Additive

DOTAP was observed in the literature used as a liposome additive for enzyme-responsive liposomes.⁵⁹ DOTAP, in Pak et al., was used as a charge counterbalance to the negatively charged

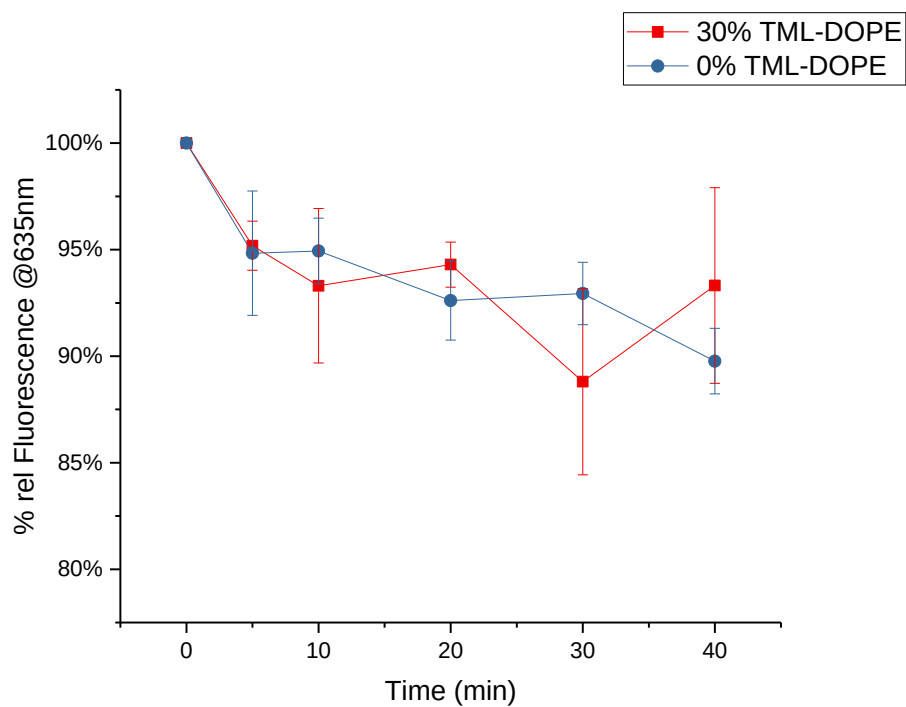
peptide-lipid conjugate on which the paper was focused. Because of this, DOTAP was initially chosen as a liposome additive that was expected to also contribute to the stability of the liposomal membrane along with unmodified DOPE.⁵⁹

Figures 21a and 21b show the results of two types of studies including liposomes containing DOTAP at a 10% inclusion. In Figure 21a, Nile Red was used as the fluorescent dye, and a decrease in fluorescent intensity was expected.^{71, 72} In comparison with the control, little release was observed from the liposomes over a forty-minute period with Nile Red as the fluorescent dye. The y-axis scale of the graph was focused on a range from 80% to 100% to visualize the decrease in relative fluorescent intensity between sample and control.

ANTS/DPX was then used as a fluorescent dye, as seen in Figure 21b. A difference in fluorescent intensity was observed between the sample and control. However, observed in both samples was a rapid release of fluorescent cargo after the addition of esterase enzyme, with the increase in relative fluorescence intensity plateauing after the initial addition.

Unlike other liposomes in this study these liposomes were extruded to a diameter of 100nm to further promote fluidity of the bilayer. When analyzed by DLS (Figure 22a) it was observed that the particle sizes of the 30% TML-POPE liposomes before the addition of esterase enzyme were much larger than expected compared to the extrusion diameter of 100 nm. There are a few potential explanations for this observation. Liposomes could have not been formed from the formulation used, though this is unlikely based on the ANTS/DPX data collected as the lack of liposome formation entirely would have resulted in a decrease in fluorescence intensity from dilution of the sample. Size has a large effect on the stability of a liposome formulation when combined with liposome composition, and smaller liposomes are more susceptible to fusion than those of larger sizes.^{26, 92} The particle size of 100 nm may have been too low and, when combined

(a)



(b)

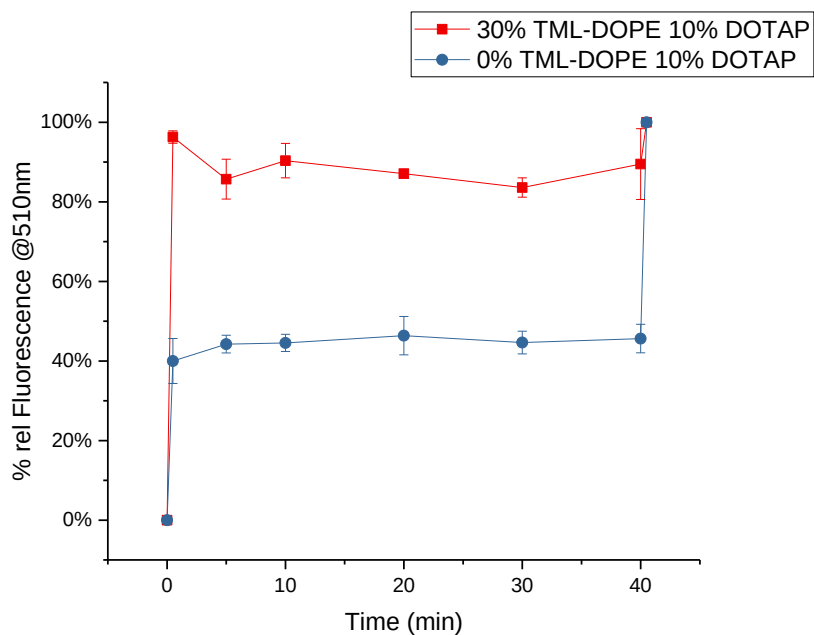


Figure 21: Change in relative fluorescence intensity over time after treatment with esterase enzyme for 30% TML-DOPE 10% DOTAP liposomes. Liposome diameter: 100 nm. Dye: (a) Nile Red (Experimental: Mean $\pm$ SD, n = 3; Control: Mean $\pm$ SD, n = 4) (b) ANTS/DPX (Experimental: Mean $\pm$ SD, n = 4; Control: Mean $\pm$ SD, n = 3) Esterase volume added: 10 μ L.

with the composition of the liposomes led to aggregation and fusion. When liposomes were later extruded to 200 nm as part of a future experiment, shown in Figure 22b, discrepancies in particle size were still observed.

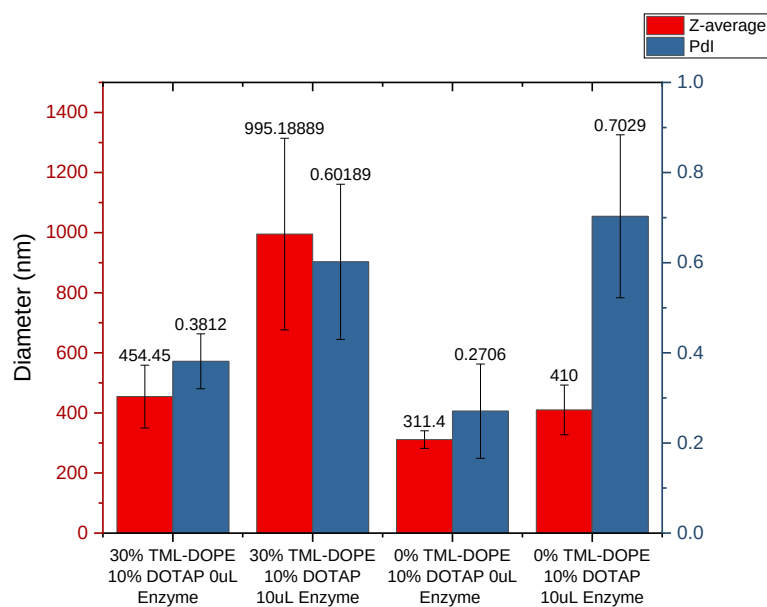
Liposomes with PA as an additive

PA was then investigated as an additive for cargo release. Unlike the DOTAP-containing liposomes, these were extruded to 200 nm to help stave off liposome aggregation and fusion based on size. In comparison to the DOTAP-additive liposomes in Figures 22a and 22b, the PA-additive liposomes exhibited a size closer to that of the extruded diameter based on the DLS data (seen in Figure 23a), which could indicate a more stable liposome formulation compared to the DOTAP-additive liposomes. Figure 23b shows the fluorescent results of the PA-additive liposomes. The PA-additive liposomes exhibited a pattern in terms of increase in fluorescence intensity similar to that observed in the ANTS/DPX results of the DOTAP-additive liposomes shown in Figure 21b. The fluorescence intensity would increase significantly after the addition of 10 μ L esterase enzyme and then this increase would taper or plateau after for the remainder of the analysis time.

One explanation for this pattern was the volume of esterase enzyme added to the sample during fluorescence analysis. This explanation was two-fold. The saturation of the fluorescent sample with esterase enzyme could increase the reaction rate in the cuvette too quickly so the majority of catalytic activity occurs early in the assay. The ANTS/DPX assay is based on the unquenching of ANTS by dilution from DPX.⁷⁷ If there was still enough free dye remaining in the liposome sample after separation, the dilution of the free components by sheer volume could also result in an increase in fluorescent intensity independent from enzymatic activity.

To better understand the effect of the volume of esterase enzyme on the release observed, a variant of the fluorescent assay implemented previously was implemented using variable volumes of enzyme as follows: 0.5 μ L, 1 μ L, 5 μ L, and 10 μ L. In addition, 10 μ L of the aqueous

(a)



(b)

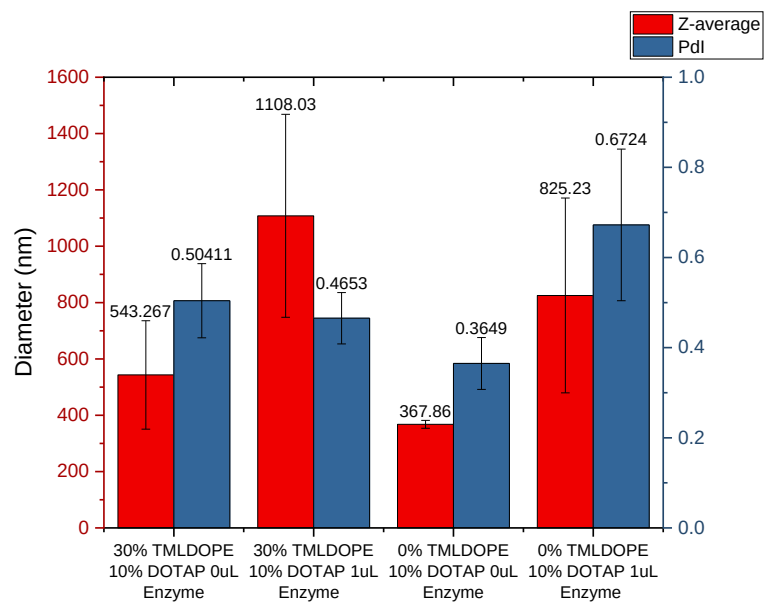
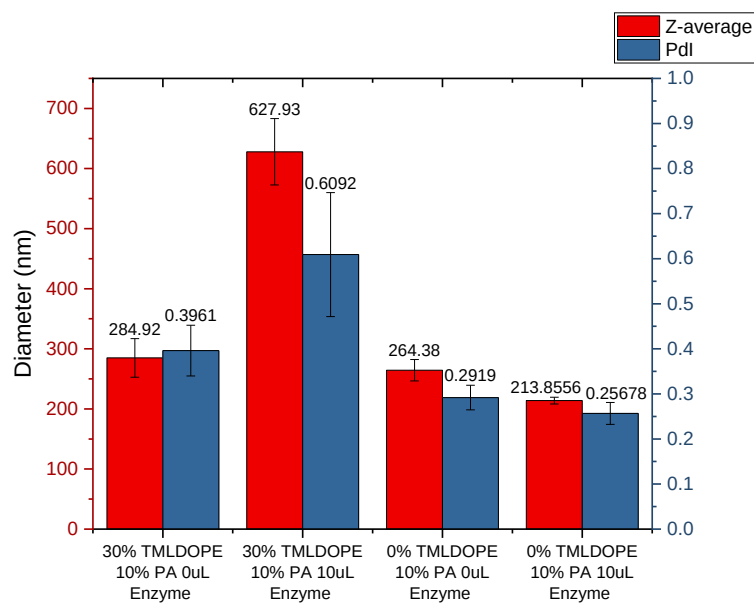


Figure 22: DLS of 30% TML-DOPE 10% DOTAP liposomes plus 0% TML-DOPE control. DLS data taken for liposome samples before and after the addition of esterase enzyme. (a) Extrusion diameter: 100 nm; Volume of esterase enzyme added: 10 μ L. (Mean $\pm$ SD, n = 10) (b) Extrusion diameter: 200 nm; Volume of esterase enzyme added: 1 μ L. (Mean $\pm$ SD, n = 10).

(a)



(b)

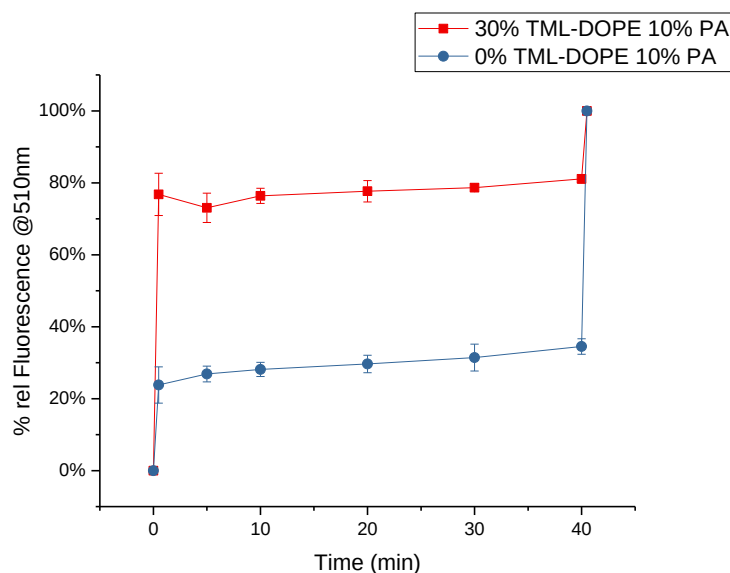


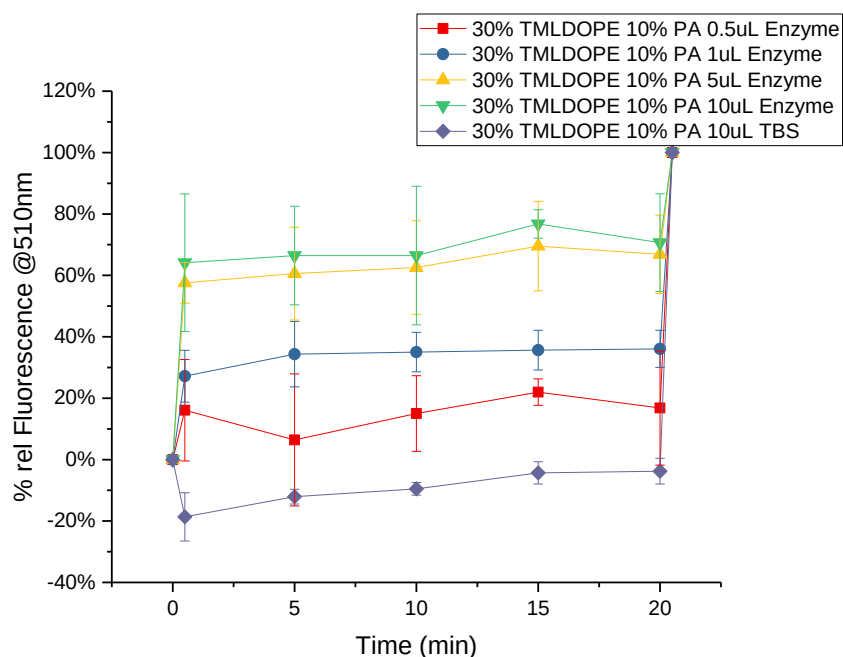
Figure 23: Fluorescence assay and DLS data for 30% TML-DOPE 10% PA liposomes with 0% TML-DOPE control (a) DLS data. DLS data taken for liposome samples before and after the addition of 10 μ L esterase enzyme. (Mean $\pm$ SD, n = 10) (b) Fluorescence assay. 10 μ L of esterase enzyme was added at the “0.5 min” data point. (Experimental: Mean $\pm$ SD, n = 3; Control: Mean $\pm$ SD, n = 3).

buffer (1x TBS at pH 8) was used as a control to determine whether the increase in fluorescent intensity was from dilution alone. The results of these experiments are included in Figure 24a and 24b.

It was apparent in the results that the change in fluorescent intensity caused by dilution alone could be ruled out as liposomes incubated with aqueous buffer instead of esterase enzyme displayed no increase in fluorescent intensity over the analysis time. This was true in both the sample containing the TML-lipid and control. As larger volumes of esterase enzyme were added, the initial fluorescence increase from the addition of enzyme was heightened. As observed in earlier results, the addition of 10 μ L esterase enzyme to either TML-DOPE liposomes or control caused an initial spike then plateau of fluorescence intensity. This pattern was also observed when 5 μ L of enzyme was added. Notable was that the difference in overall change in fluorescence intensity between the 10 μ L and 5 μ L trials was much closer than the 0.5 μ L and 1 μ L trials. This could be indicative of either that esterase enzyme activity became limited by the availability of the TML substrate between these volumes or the liposome solution after the introduction of volumes between 5 μ L and 10 μ L became diluted to the point of negatively affecting the fluorescence intensity.

The 0.5 μ L and 1 μ L volumes of esterase enzyme, unlike the fluorescence patterns observed in 5 μ L and 10 μ L, showed an increase in fluorescent intensity over the analysis time. While an increase in fluorescent intensity was observed in 1 μ L of esterase enzyme added in both sample and control studies, an increase was observed in the control for the 0.5 μ L of esterase enzyme added the same was not observed in the 30% TML-DOPE sample liposomes. From these observations, treatment with 1 μ L of esterase enzyme was investigated further. In addition to the investigation of a 1 μ L volume of esterase enzyme, the analysis time was expanded to 90 minutes

(a)



(b)

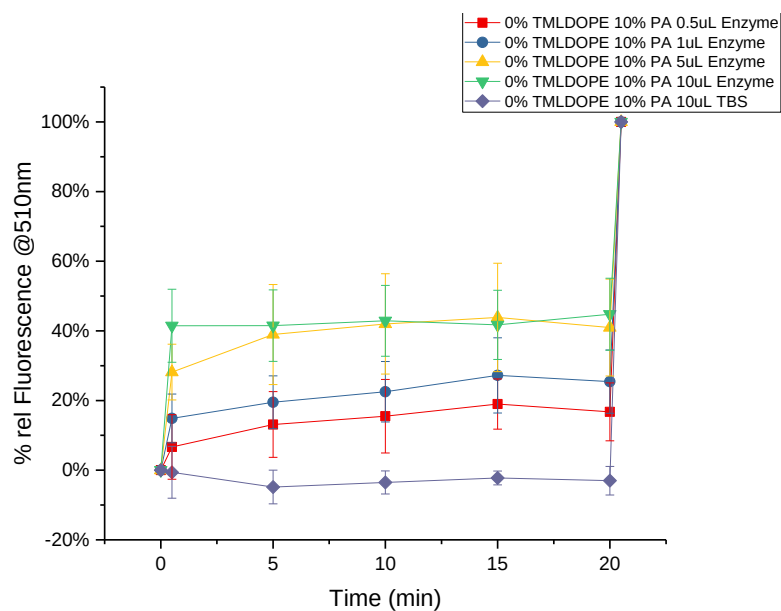


Figure 24: Fluorescence assay of 30% TML-DOPE 10% PA liposomes and control with varying volumes of enzyme added. (a) 30% TML-DOPE 10% PA liposomes (Mean $\pm$ SD, $n = 8$) (b) 0% TML-DOPE 10% PA liposome control. Aliquots of 0.5 μ L, 1 μ L, 5 μ L, and 10 μ L of esterase enzyme were added with 10 μ L 1x TBS at pH 8 added as a control. Trials were performed over a shorter analysis time of 20 minutes for the purpose of time efficiency (Mean $\pm$ SD, $n = 5$).

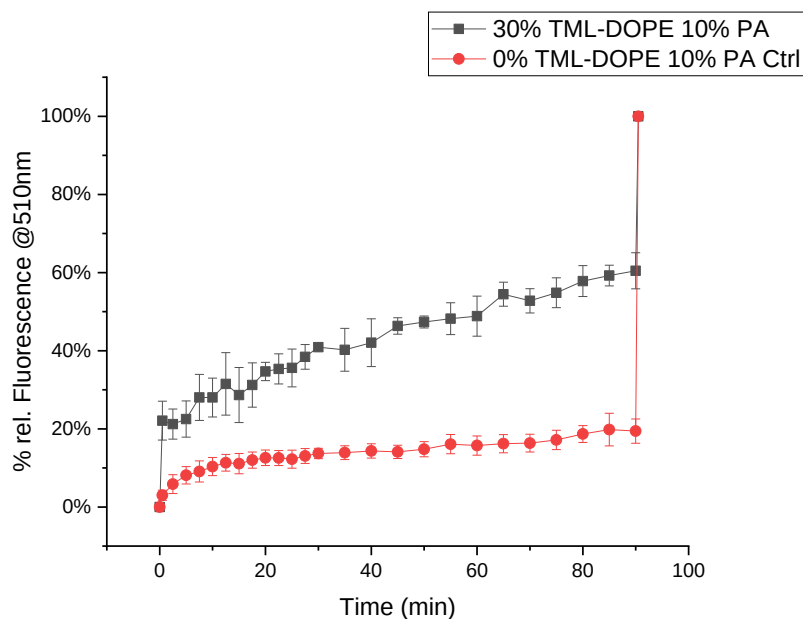
when it was found a more significant increase in fluorescence intensity could be observed beyond the timescale laid out in previous trials. The inclusion of 1 μL of esterase enzyme instead of 10 μL exhibited many effects on the assay. The initial fluorescence increase observed in the 10 μL esterase enzyme trials was largely reduced. This was especially true for the control, which displayed a slope similar to a logarithmic fit. The TML-DOPE containing liposomes showed a more linear-fitting slope in comparison. These results are shown in Figure 25a and 25b. Figure 26 includes DLS results for both experimental and control liposomes including the effect of adding 1 μL TBS to both experimental and control liposomes of 1 μL esterase on size and size distribution.

An inconsistency had become apparent within replicates of these liposomes, which is shown in Figure 25b. While some trials showed a dramatic increase in fluorescence intensity, others exhibited a shallow increase, though both still larger than that of the control. It was initially thought that this phenomenon was caused by a discrepancy with the initial leap in fluorescence intensity between the samples. Figure 27 shows the result of normalizing the data for the initial addition of enzyme instead of the measurement before the addition of enzyme. This proved to reduce a large amount of the deviation between all replicates, though the standard deviation presented between these samples was still higher than those observed in Figure 25. A kinetics experiment has also been investigated to better visualize the initial increase in fluorescence intensity observed in the TML-DOPE liposome fluorescent assays, though preliminary testing with this technique has been unable to visualize this phenomenon (not shown).

2.5 Conclusions and Future Work

The trimethyl lock mechanism has been observed in the literature as a viable means of promoting release of fluorescent cargo from liposomes. Utilization of this technique for the

(a)



(b)

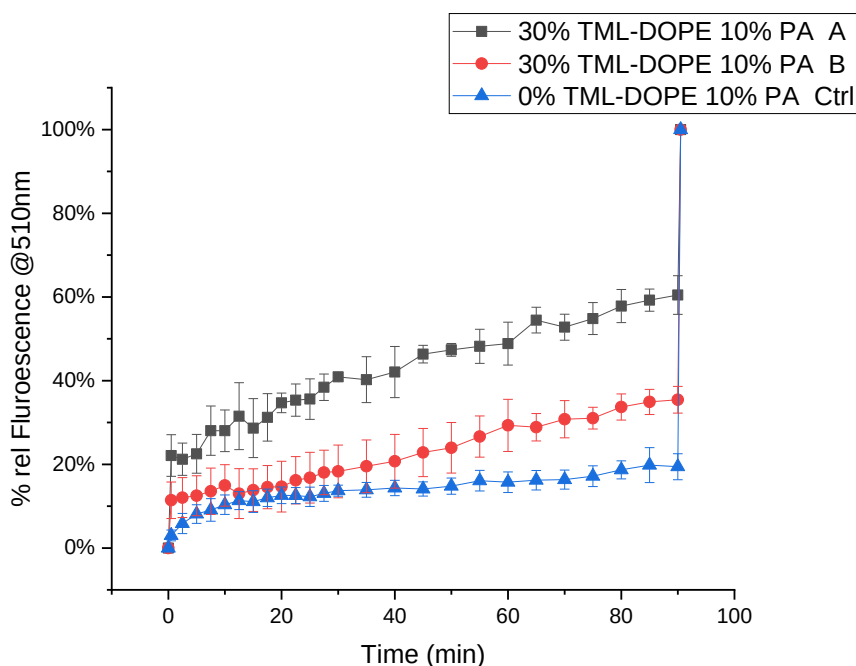


Figure 25: Fluorescence assay of 30% TML-DOPE 10% PA liposomes plus control with 1 μ L added enzyme showing discrepancy in data trend. (a) combined fluorescence assay results showing large increase in fluorescence intensity over control (Experimental: Mean $\pm$ SD, n = 6; Control: Mean $\pm$ SD, n = 4) (b) Both distinct data trends observed, showing a large variation in data. (Experimental A: Mean $\pm$ SD, n = 6; Experimental B: Mean $\pm$ SD, n = 4; Control: Mean $\pm$ SD, n = 4) Addition of 1 μ L TBS was used as a control for both liposome types (not shown).

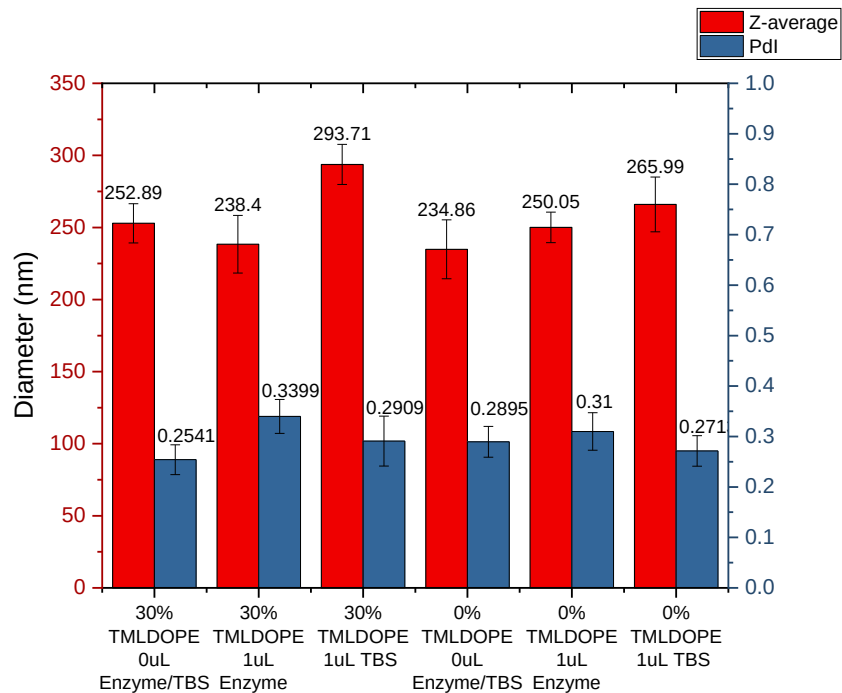


Figure 26: DLS of 30% TML-DOPE 10% PA liposomes plus 0% TML-DOPE control. DLS data taken for liposome samples before and after the addition of 1 μ L esterase enzyme or 1x TBS. Addition of 1 μ L TBS was used as a control for both liposome types (Mean $\pm$ SD, n = 10).

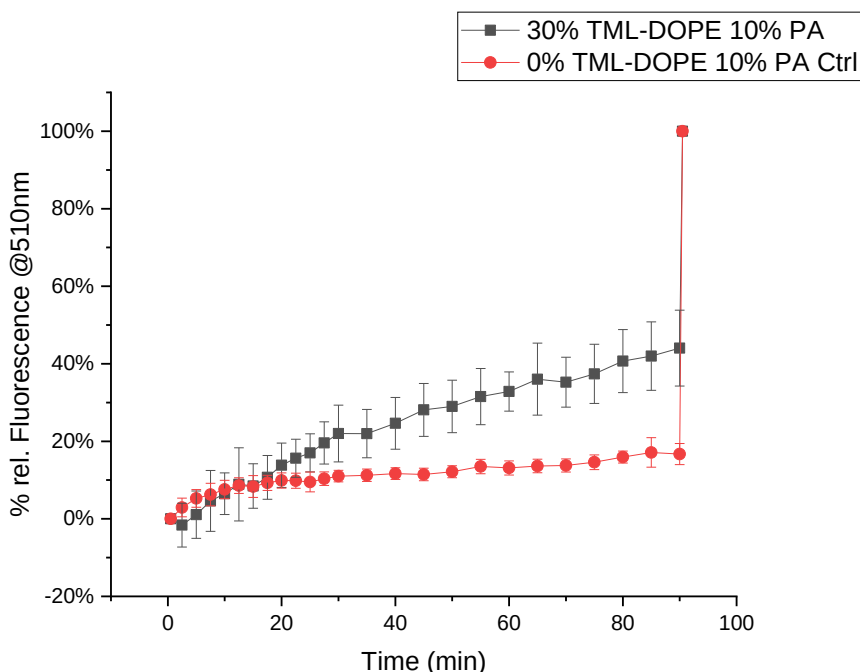


Figure 27: Change in relative fluorescence intensity of ANTS/DPX dye over time of 30% TML-DOPE 10% PA liposomes plus control after addition of 1 μ L of esterase enzyme with data processed by normalizing the data points for the addition of esterase enzyme instead of the point before the addition of enzyme (Experimental: Mean $\pm$ SD, n = 8; Control: Mean $\pm$ SD, n = 4).

purpose of synthesizing enzyme-responsive lipid conjugates for triggered liposome release sensitive to esterase enzyme, an enzyme found to be overexpressed in several specific types of cancers, opens the door to the use of similar techniques for the treatment of such diseases. Fluorescent assays performed on liposomes containing a TML-DOPE couple have exhibited a degree of fluorescent cargo release more pronounced versus control and was shown to be more effective for the purpose of fluorescent cargo release than the TML-POPE containing liposomes for the reasons stated above.

The reliability of the TML-DOPE liposomes in their current formulation is still under investigation. While, as shown above, normalizing the collected fluorescence data to cut out the initial upheaval in fluorescence intensity all data points show a degree of deviation more pronounced than that found in control samples. Additional remedies for this issue have been

proposed. One potential remedy includes the dilution of liposome solutions before analysis. It has been observed in liposome release assays performed by other members of the Best Group that dilution of the liposomes may lead to a more pronounced release.

2.6 Synthesis Details

2.6.1 Materials

3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid was purchased from Sigma Aldrich. All lipids (POPC, POPE, DOPE, DAG, DOTAP, PA) were purchased from Avanti Polar Lipids. 8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS), p-Xylene-Bis-Pyridinium Bromide (DPX), Sulforhodamine B, Nile Red, *N, N*-diisopropylethylamine, and 1-Hydroxybenzotriazole were purchased from Fisher Scientific. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide was purchased from Chem-Impex International. Porcine liver esterase was purchased from Sigma Aldrich and was used as a proof of concept.

2.6.2 Amide coupling of 3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE)

POPE (8.19 mg, 11.41 μmol , 1.0 equiv.) was measured into a 1-dram screw top reaction vial and dried overnight under high vacuum. Commercially available 3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid (4.52 mg, 17.11 μmol , 1.5 equiv.) and 1-Hydroxybenzotriazole (HOBt, 2.096 mg, 13.69 μmol , 1.2 equiv.) were added to the vial containing the POPE lipid film, dissolved in 0.7 mL anhydrous DMF, and stirred on ice for 5 minutes. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI-HCl, 2.624 mg, 13.69 μmol , 1.2 equiv.) and *N, N*-diisopropylethylamine (DIEA, 9.97 μL , 28.52 μmol , 2.5 equiv.) were added to the reaction vial. The vial was flashed with N_2 , sealed, and allowed to stir for 5 hours. The reaction was quenched in 100 mL 1 M HCl and extracted with 5 x 10 mL CHCl_3 . The organic layer was

then washed with 5 x 10 mL brine. The organic layer was reduced to dryness by rotary evaporation. The crude product was purified by column chromatography with a varying gradient of MeOH: CHCl₃. TML-POPE was obtained as a yellow oil (synthesis pathway failed; no spectra included).

2.6.3 Amide coupling of 3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid to 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE)

DOPE (150 μ L, 17.04 mg, 22.9 μ mol, 1.0 equiv.) was measured into a 1-dram screw top reaction vial and dried overnight under high vacuum. Commercially available 3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutyric acid (9.08mg, 34.35 μ mol, 1.5 equiv.) and 1-hydroxybenzotriazole (HOBt, 4.21mg, 27.48 μ mol, 1.5 equiv.) were added to the vial containing the DOPE lipid film, dissolved in 0.7 mL anhydrous DMF, and stirred on ice for 5 minutes. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI-HCl, 5.27 mg, 27.48 μ mol, 1.5 equiv.) and N, N-diisopropylethylamine (DIEA, 9.97 μ L, 57.255 μ mol, 2.5 equiv.) were added to the reaction vial. The vial was flashed with N₂, sealed, and allowed to stir for 5 hours. The reaction was quenched in 100 mL 1 M HCl and extracted with 5 x 10 mL CHCl₃. The organic layer was then washed with 5 x 10 mL brine. The organic layer was reduced to dryness by rotary evaporation. The crude product was purified by column chromatography with a gradient of 4%-20% MeOH: CHCl₃. TML-DOPE was obtained as a dilute yellow oil (9.02 mg, 40% yield). ¹H NMR (500 MHz, 20% MeOD/CDCl₃) δ 0.72 (t, 6H, J = 5 Hz), 1.15 (s, 40H), 1.43 (s, 8H), 1.85 (s, 8H), 2.07 (s, 3H), 2.16 (m, 8H), 2.36 (s, 3H), 2.43 (s, 2H), 3.12 (s, 2H), 3.55 (m, 2H), 3.76 (s, 2H), 4.21 (dd, 1H, J = 10.0, 5.0 Hz), 5.04 (m, 1H), 5.18 (m, 4H), 6.42 (s, 1H), 6.67 (s, 1H), 6.73 (s, 1H) ¹³C NMR (125 MHz; 20% MeOD/CDCl₃) δ 13.86, 21.56, 22.53, 24.74, 25.12, 27.07, 29.02, 29.18, 29.39, 29.61, 31.32, 31.78, 34.06, 39.39, 62.48, 70.26, 123.13, 129.53, 129.91, 132.53, 133.49, 136.50, 138.24, 149.53, 171.26, 173.26, 173.70 ³¹P NMR (300 MHz, 20% MeOD/CDCl₃) δ 1.58.

2.7 Experimental

2.7.1 Experimental procedures with TML-POPE

Liposome Formation

Specific volumes of liposomal components suspended in chloroform were measured into a 1-dram screw-top vial and dried under nitrogen flow. The lipid components were dried completely under high vacuum for two hours. Lipid films were hydrated depending on the fluorescent dye used. Lipid films to use hydrophobic dye were hydrated with Milli-Q deionized water or 1x PBS at pH 8. Lipid films to use hydrophilic dye were hydrated with 20 mM Sulforhodamine B in 1x PBS in pH 8. Hydrated lipids were then heated in a warm water bath set to 50°C for thirty minutes and agitated via a vortex mixture every five minutes. The samples were then subjected to a freeze-thaw cycle during which the liposomes were agitated, frozen completely in a bath of acetone and dry ice, and thawed in a warm water bath set to 50°C. This process was repeated ten times. Liposomes were extruded to a uniform size of 200 nm over 15 passes with a hand extruder. If the liposomes were produced using a hydrophilic dye, the liposomes were subjected to gel filtration with Sephadex G-50 to separate the liposomes from free dye. To ensure the correct fraction was chosen for fluorescence analysis, before fluorescence assay trials were to begin, the fluorescence of a 100 μ L aliquot of a liposome fraction was measured, then a 1 μ L aliquot of 10% Triton-X was added to the solution and the fluorescence was measured again. The liposome fraction that showed an increase in total fluorescence was the fraction that contained the liposomes. Liposomes were formulated to a concentration of 2 mM. Liposome formulations are included in Table 1.

Fluorescent Release Assay

To a quartz microcuvette was added a 100 μ L aliquot of extruded and purified liposome solution. A 1 μ L aliquot of 50 units/mL esterase enzyme or 0.5 M KOH was added to the microcuvette. A

Table 1: List of liposome formulations utilized for which data is shown including the concentrations of stock solutions for TML-POPE liposomes.

	Egg PC	TML-POPE	DAG	Nile Red
Concentration of Stock Solutions	32.0 mM	5.0 mM	3.5 mM	10.0 mM
Percentage/Volume	90%/ 8.4 μ L	10%/ 6.0 μ L	-*	10%/ 3.0 μ L
	50%/ 4.7 μ L	50%/ 30 μ L	-	-**
	30%/ 2.8 μ L	70%/ 42 μ L	-	-
	40%/ 3.8 μ L	50%/ 30 μ L	5%/ 4.3 μ L	5%/ 1.5 μ L

* This formulation did not include diacylglycerol

**This formulation used 20mM Sulforhodamine B as a fluorescent dye instead

fluorescence measurement was performed on the solution immediately after the addition of the 1 μ L aliquot and after a five-minute rest at room temperature. This process was repeated until 10 μ L total enzyme or base was added. In the case of hydrophilic dyes, a 1 μ L aliquot of 10% Triton-X was added to the cuvette to stimulate a near complete release of dye contents from the liposomes, and this data point was then normalized to 100% release for the purpose of determining relative fluorescence intensity of the sample. Instrumental conditions for fluorescent release assays are reported in Table 2.

2.7.2 Experimental procedures with TML-DOPE

Liposome Formulation

The procedure for liposome formulation was similar to the procedure used for the TML-DOPE liposomes with the following key differences. Lipid films were hydrated with 1x TBS at pH 8 or 12.5 mM ANTS/ 45 mM DPX in 1x TBS at pH 8, depending on whether the resultant liposomes were to use hydrophobic dye or hydrophilic dye respectively. Hydrated lipids were heated in a warm water bath set to 60°C for one hour and agitated via a vortex mixture every five minutes.

Table 2: Instrumental conditions for fluorescence release assays performed on TML-POPE liposomes.

	Nile Red	Sulforhodamine B
Excitation Wavelength	552 nm	550 nm
Emission Wavelength	620 nm	635 nm
Emission Wavelength Range	560-680 nm	560-660 nm
Excitation Slit	10 nm	7.5 nm
Emission Slit	5 nm	5 nm
Name of Instrument	PerkinElmer LS 55 Fluorescence Spectrometer*	

*This instrument was used for the fluorescence release assays of TML-POPE but became unavailable before testing of TML-DOPE liposomes would begin. Fluorescent release assays of TML-DOPE liposomes were instead performed on a Cary Eclipse Fluorescence Spectrophotometer.

Liposomes were extruded to a uniform size of 100 nm or 200 nm over 21 passes with a hand extruder. A new procedure was added to the fraction-determining step. In order to quickly investigate for the presence of free dye, liposome solution was dabbed onto a silica aluminum-backed TLC plate and placed under a UV lamp. The free dye components were UV active and would show up as a spot on the plate.⁹³ The fraction that contained the purified liposomes would often be the fraction before the appearance of dye under UV. The same test of fluorescence intensity by fluorometer was used to ensure the fraction which contained the liposomes without free dye was chosen. Liposome formulations are included in Table 3.

Fluorescence Release Assay

measurements. At the end of analysis time a 1 μ L aliquot of 10% Triton-X 100 was added to the sample to encourage a near complete release from the liposomes. The measurement including Triton-X 100 was normalized to 100% release for the purpose of determining relative fluorescence intensity. Instrumental conditions are reported in Table 4.

Dynamic Light Scattering

A 50 μ L aliquot of liposomal sample was inserted into a plastic microcuvette for analysis. For each liposome sample DLS data was collected at three stages: before the addition of enzyme, after the

addition of a specified volume of enzyme, and after the addition of 1 μL 10% Triton-X 100.

Instrumental conditions are reported in Table 5.

Table 3: List of liposome formulations utilized for which data are shown including the concentrations of stock solutions for TML-DOPE liposomes.

	POPC	TML-DOPE	DOPE	DOTAP*	PA*	Nile Red**
Concentration	33.0 mM	5.0 mM	10.0 mM	10 mM	3.5 mM	10 mM
Percentage/ Volume	10%/ 0.9 μL	30%/ 18.0 μL	50%/ 15.0 μL	10%/ 3.0 μL	10%/ 8.5 μL	5%/ 1.5 μL

*Either DOTAP or PA would be used in one formulation.

**Nile Red was added to the formulation only when it was used as the fluorescent dye.

Table 4: Instrumental conditions for fluorescence release assays performed on TML-DOPE liposomes.

	Nile Red	ANTS/DPX
Excitation Wavelength	552 nm	350 nm
Emission Wavelength	635 nm	510 nm
Emission Wavelength Range	570-750 nm	420-650 nm
Excitation Slit	5 nm	20 nm
Emission Slit	10 nm	10 nm
Instrument Name	Cary Eclipse Fluorescence Spectrophotometer	

Table 5: Instrumental conditions used for reported DLS data.

Material Refractive Index	1.470
Solvent Refractive Index	1.311
Solvent Viscosity	1.0076 cP
Temperature	20°C
Number of Runs	10
Number of Measurements per Run	5
Instrument Name	Zetasizer Nano ZS

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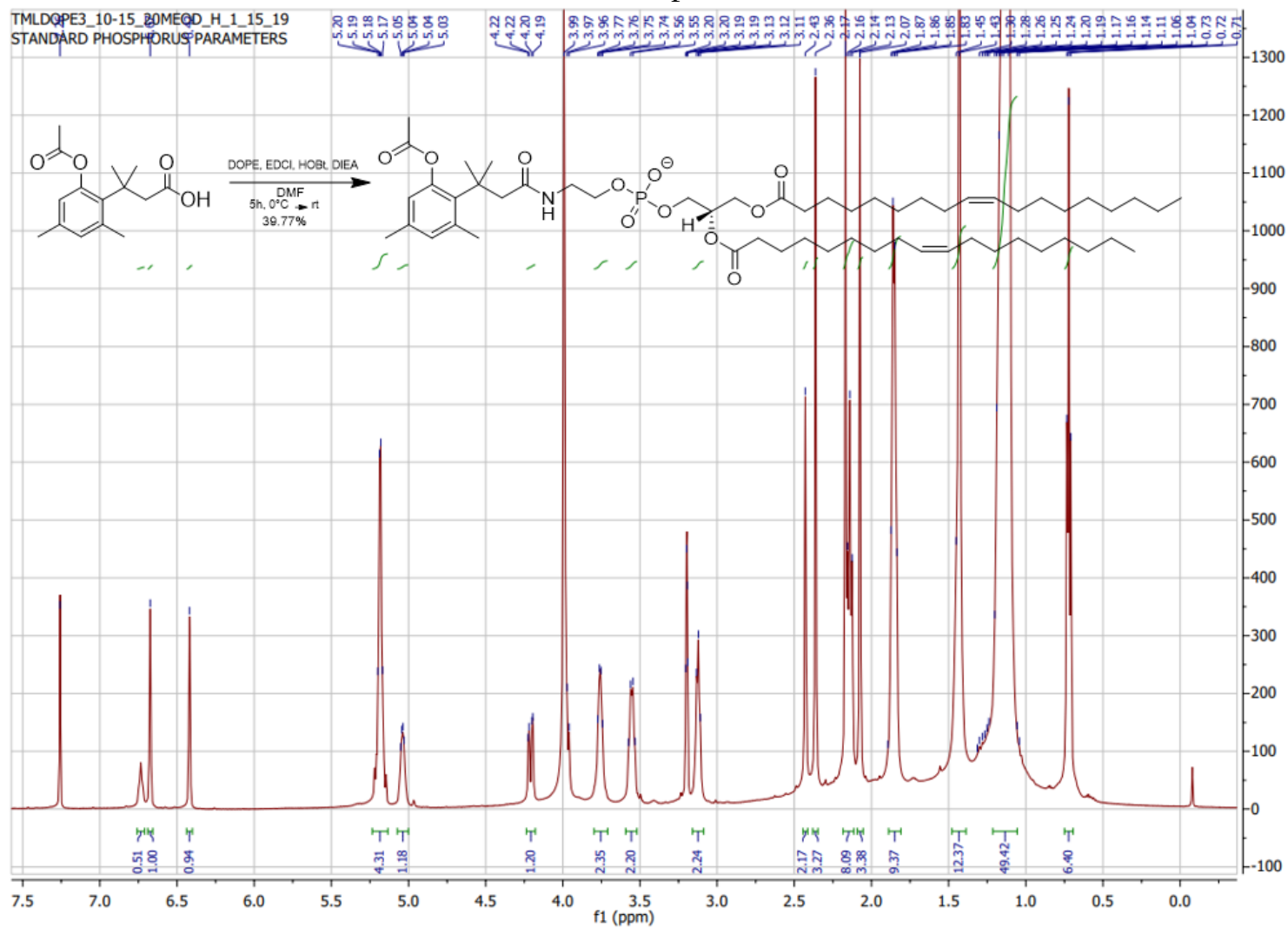
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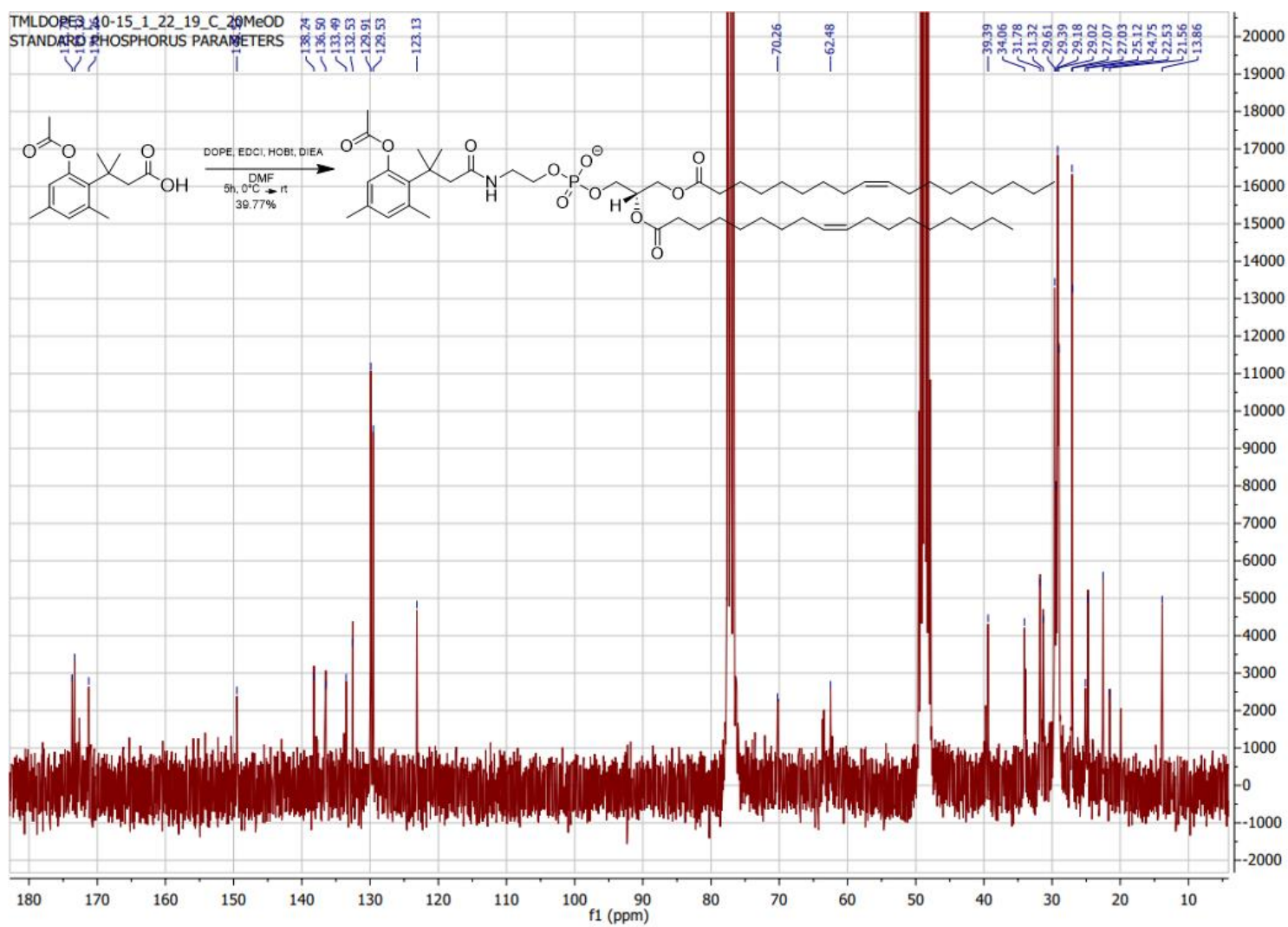
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Appendix

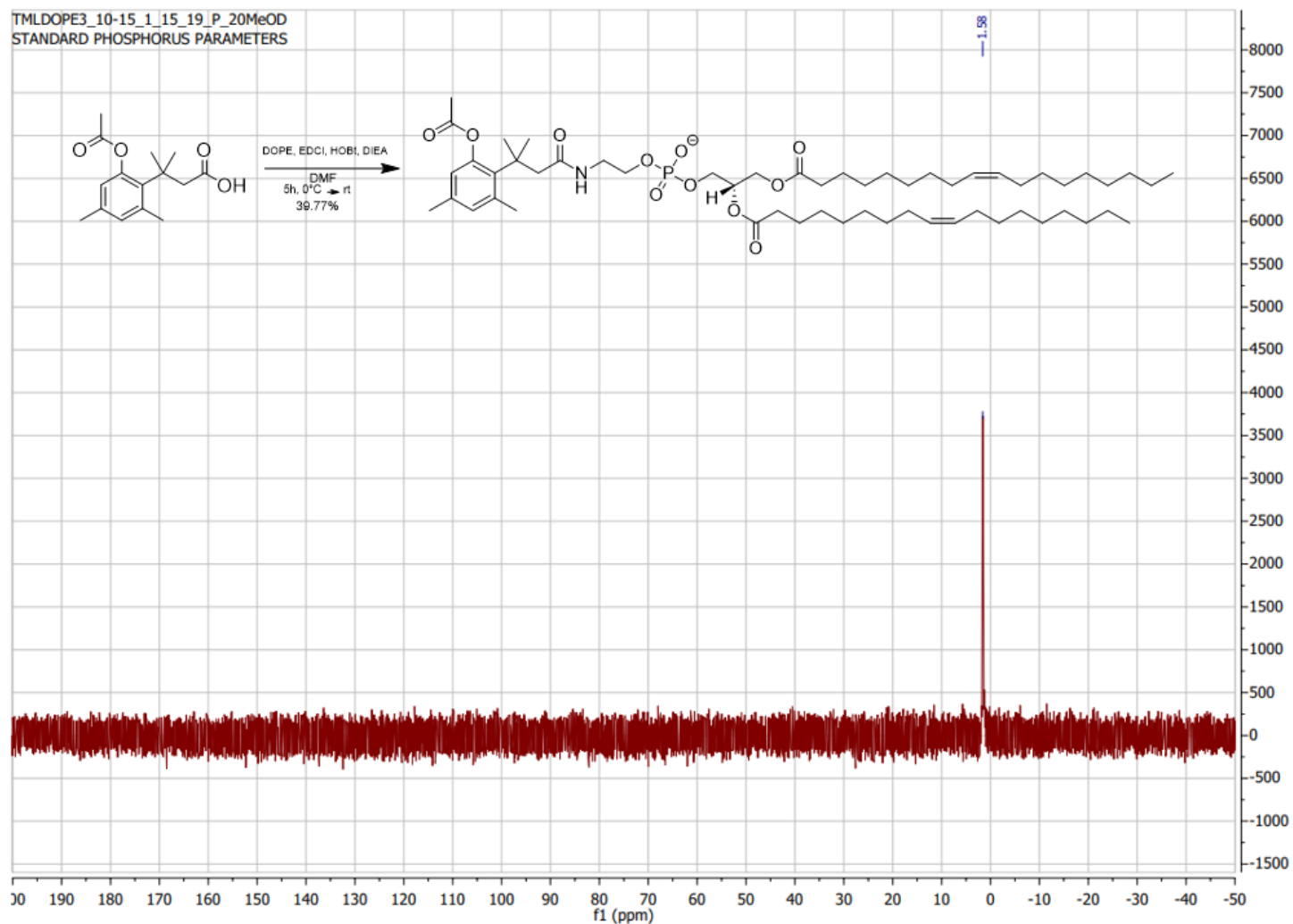
NMR Spectra



Spectrum 1: ^1H NMR (500 MHz, 20% MeOD/ CDCl_3) of TML-DOPE.



Spectrum 2: ^{13}C NMR (500 MHz, 20% MeOD/ CDCl_3) of TML-DOPE.



Spectrum 3: ^{35}P NMR (300 MHz, 20% MeOD/ CDCl_3) of TML-DOPE.

Vita

Sara Barker was born in Worcester, Massachusetts in 1995. In pursuit of an undergraduate degree, Sara attended Millsaps College and received a B.S. in Biochemistry with a minor in Environmental Science. While pursuing an undergraduate degree at Millsaps College, Sara fulfilled the requirements of the Honors Program to graduate with honors in chemistry. Sara went on to pursue a graduate degree at the university of Tennessee and under the guidance of Dr. Michael Best and contributed to the development of esterase-responsive liposomes.