Photo-Switchable Control of Membrane Properties of Liposomes and Biochemical Processes

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I am submitting herewith a dissertation written by Andrew Michael Bayer entitled "Photo-Switchable Control of Membrane Properties of Liposomes and Biochemical Processes." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Chemistry.

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Photo-Switchable Control of Membrane Properties of Liposomes and Biochemical Processes

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Andrew Michael Bayer
December 2014
Acknowledgments

This journey through graduate school has been education yet challenging, and I could not have accomplished it on my own. For their major role in the furtherance of my academic career, I would like to thank my committee. First, thank you to my advisor, Dr. Michael Best for his constant advice and guidance. I can remember coming to this department and feeling excited every time I heard him discuss his research. It aligned with my interests perfectly, I am grateful that he accepted me into his research family and has continued to push me over the years. I want to thank Dr. Baker, who has always been a great advisor, teacher, storyteller, and encourager. His vast knowledge has been a vital instrument in my success. To Dr. Bin Zhao, I want to thank him for his involvement in my academic career and for his valuable time. To Dr. Engin Serpersu, I thank him for his insightful advice. Biology is not my primary area of expertise, but his questions and insights have helped me to clarify many concepts, as well as always providing helpful suggestions. And finally to Dr. Francisco Barrerra, I want to thank you for stepping in at the last minute when I needed another committee member. Your joining of this university has been fortunate for the Best laboratory, and your input has been essential to my learning process.

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Furthermore, to my labmates, graduate and undergraduate, past and present, you have inspired me to work harder and smarter, giving me invaluable advice, have been a source for companionship and humor in the work place, challenged me to be better than what I am, and have been there when I needed someone to help. Never undervalue your importance to each other. If you are starting your first semester or your last, never stop learning, from each other and from the vast work already accomplished in scientific research. Remember that graduate school is just the beginning, not the end.
Use your time wisely. Remember to stop every once in a while and step back to get a bigger picture of your own work, of your lab’s work, of your field’s work, of where you want to be in life, and of what your purpose and direction should be. You will make mistakes, but as I am sure Thomas Edison would agree with his many ways of not making a light bulb, failing is one method of discovery and learning.

And also to my family I am grateful. They have always supported me, prodded me, and been there to encourage me in any way they could offer. Thank you for your love, prayers, and support.

Thank you everyone. I wish you the very best.
Abstract

Liposomes are promising agents for drug delivery. They have the ability to encapsulate therapeutic drugs, resulting in decreased toxicity and prolonged circulation time. However, many obstacles to achieving broad utility in liposomal drug delivery still exist, including the ability to control release of therapeutic drugs and modulate surface reactivity. A primary focus of this dissertation involves the development of synthetic photocleavable lipids for controlled release from membranes.

Phosphatidylcholine (PC) is a natural lipid that comprises the majority of structural membranes in eukaryotes. It contributes heavily to the formation of lipid bilayers in cell membranes, and modifications to the bilayer can induce membrane transitions and changes in permeability. As such, a PC analogue has been developed with a photocleavable 2-nitrobenzyl acyl chain. This lipid (NB-PC) was synthesized in nine steps from 4-(aminomethyl)benzoic acid and lyso-phosphatidylcholine (LPC). This system is designed such that ultraviolet light degrades the fatty acid tail, changing the properties of the liposomes they form and releasing entrapped hydrophobic molecules. This occurred in about half an hour, as determined by a fluorescence assay involving the release of the dye Nile red. Phosphatidylethanolamine (PE), cholesterol, and polyethylene glycol (PEG) were incorporated as additives to examine the versatility of release from liposomes with varying membrane properties. It was found that release remained robust regardless of lipid content. Furthermore, another photocleavable lipid was developed containing an extended conjugated system, 2-nitrobiphenethyl, to enhance photocleavage efficiency and enable two-photon release. This lipid, NBP-PC, was synthesized in seven steps, and UV irradiation reached maximal release within five minutes.

This dissertation also describes molecules that have been synthesized or are in progress for other projects. A nitrobenzyl-protected diacylglycerol has been synthesized, which is suitable for in situ binding studies with DAG-binding proteins, such as protein kinase C (PKC). Also synthesized are a biotin–
azide linker for anchoring molecules onto streptavidin-coated surfaces and various azobenzene derivatives for studying chiral isomerization.
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<td>(Boc)$_2$O</td>
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<td>tetrakis(triphenylphosphine) palladium(0)</td>
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<td>DIEA</td>
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<tr>
<td>DSPE</td>
<td>distearoylphosphatidylethanolamin</td>
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DSPS  distearoylphosphatidylserine
EANBP  2-(4’-[bis[2-(2-methoxyethoxy)ethyl]amino]-4-nitro-[1,1′-biphenyl]-3-yl)propan-1-ol
EPR    enhanced permeability and retention effect
Et$_3$N  triethylamine
Fab’    fragment antigen binding
GFP    green fluorescent protein
HCl    hydrochloric acid
IR     infrared
K$_2$CO$_3$  potassium carbonate
KI     potassium iodide
LPC    lyso-phosphatidylcholine
LPG    light protecting group
m      meta
MeOH   methanol
MPS    mononuclear phagocyte system
MRI    magnetic resonance imagine
MS     mass spectrometry
Na$_2$CO$_3$  sodium carbonate
Na$_2$S$_2$O$_3$  sodium thiosulfate
NaNO$_2$  sodium nitrite
NaOAc  sodium acetate
NaOH   sodium hydroxide
NB-DAG nitrobenzyl diacylglycerol
NB-PC   2-nitrobenzyl phosphatidylcholine
NBP-PC  nitrobiphenyl phosphatidylcholine
NMR    nuclear magnetic resonance
NPPOC  2-(2-nitrophenyl)propoxy)carbonyl
Ns     nosyl
$o$ ortho
$\alpha$-NB ortho-nitrobenzyl
$p$ para
PA phosphatidic acid
PC phosphatidylcholine
PE phosphatidylethanolamine
PEG polyethylene glycol
PEMB 3-(2-propyl)-4′-trisethoxy(methoxy)-4-nitrophenyl
PG phosphatidylglycerol
PIP phosphatidylinositol
PIP5K phosphatidylinositol 4-phosphate 5-kinase
PIPn phosphatidylinositol polyphosphate
PMBCI para-methoxybenzyl chloride
PS phosphatidylserine
RNA ribonucleic acid
scFv single chain fragment variables
SM sphingomyelin
sPLA$_2$ secretory phospholipase A$_2$
TAG triacylglycerol
TAM tumor associated macrophage
TBAF tetrabuylammonium fluoride
TBDPSCI tert-butyldiphenylsilyl chloride
tBuOH tert-butanol
tBuOK potassium tert-butoxide
Tc/Tm transition temperature
TEA triethylamine
TFA trifluoroacetal
TFA trifluoroacetic acid
<table>
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<td>TFAA</td>
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</tr>
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<td>THF</td>
<td>tetrahydrofuran</td>
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<td>TLC</td>
<td>thin layer chromatography</td>
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<td>TPP</td>
<td>2,3,5-trichloroacetyl chloride</td>
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<tr>
<td>TsOH</td>
<td>p-toluenesulfonic acid</td>
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<td>UV</td>
<td>ultraviolet</td>
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Chapter 1: Introduction to Drug Delivery

1.1 Diversity of Chemistry in Biological Systems

Biological systems contain a wealth of chemical variety. Within mammalian systems, these compounds and structures are organized in four main categories: proteins, carbohydrates, nucleic acids, and lipids. In each case, smaller units can aggregate or polymerize to form larger structures. (Figure 1.1) Proteins are three-dimensional biopolymers consisting of amino acid subunits, and they are responsible for enacting most biological functions that occur. Carbohydrates, on the other hand, are composed of sugar molecules that serve as important building blocks for bodily tissues as well as sources of energy. Nucleic acids are structures that form RNA and DNA, which are the genomic blueprints from which all proteins are made. Finally, lipids compose biomolecules that encompass a wide variety of structures. They are largely amphiphilic molecules in which the organic and water soluble regions self-assemble to form larger structures to become soluble. Lipid surfaces are the site for many biochemical reactions.  

Lipids serve three main roles. The primary function is the formation of the plasma membrane and organelle membranes, thus acting to separate for biological reactions. However, lipids are not static structures, as they are additionally responsible for the storage of energy, especially through the hydrolysis of triacylglycerols (TAGs), structures that act as reservoirs for fats. Furthermore, lipids serve as secondary messengers for signal transduction thereby controlling important biological events. Protein–lipid binding interactions are critical in many biological pathways. There are a variety of classes of lipids in which lipids activate protein function, including triacylglycerols, sterols, phospholipids, and sphingolipids.

1.2 Lipid Structures and Roles

Phospholipids comprise a class of lipids that are related by their amphipathic properties, having a glycerol backbone with a hydrophilic polar headgroup at the sn-3 position and hydrophobic nonpolar fatty acid chains esterified to the hydroxyl groups at the sn-1 and sn-2 positions of glycerol (Figure 1.2).
Larger biological entities, such as proteins, carbohydrates, nucleic acids, and membranes are composed of smaller chemical building blocks.
The headgroup consists of the hydroxyl group of the glycerol backbone, often coupled to a phosphate group, and the tail portion is composed of one or more fatty acids. Phospholipids commonly contain a mixture of saturated and unsaturated lipid tails. Unsaturated tails tend to cause lipids to have more freedom of motion in the membrane, whereas saturated tails lead to more rigidity.

All phospholipids are derivatives of diacylglycerol, which contains only a hydroxyl group at the headgroup, yet is important for binding certain proteins responsible in growth pathways, such as protein kinase C. Attaching a phosphate group to DAG produces phosphatidic acid (PA), which is also an important signaling lipid. An even more diverse structural class of lipids is the phosphatidylinositol polyphosphates (PIP$_n$s), which are vital to many biological pathways and can be phosphorylated at any combination of the 3, 4, and 5 positions of the myo-inositol ring on the headgroup. While all the previous are signaling lipids, responsible for protein localization, bulk lipids comprise the vast majority of cellular membranes. The major bulk lipid is phosphatidylcholine (PC), but since the headgroups differ among different lipids, this results in variation among lipids, affecting the shapes of the lipids as well as their overall function. (Figure 1.3)

The major membrane lipid PC contains a phosphocholine group, which possesses a neutral charge at physiological pH due to the positively charged quaternary amine and negatively charged phosphate. Phosphatidylethanolamine (PE), on the other hand, contains an unmethylated nitrogen. Due to this, the headgroup occupies a smaller area, and as a result, it disfavors bilayers, instead favoring a negative curvature at higher concentrations within the membrane. This is useful for transient processes such as membrane fusion. Phosphatidylserine (PS) is similar to PE, but with an additional carboxylate group, resulting in an overall negative charge. It is a lipid known for binding certain proteins as well as acting as a signaling marker for cell death. The PIP$_n$s
Figure 1.2: Structure of a typical phosphatidylcholine lipid

The sn-3 position of glycerol is conjugated to a headgroup, in this case phosphocholine. The sn-1 and sn-2 positions contain either a fatty acid tail with unconjugated double bonds (unsaturated) or without any double bonds (saturated).

Figure 1.3: Examples of the main phospholipids.

Phosphatidylcholine (PC) comprises the majority of cellular membranes. Phosphatidylethanolamine (PE) is important in fusion events. The phosphatidylinositol polyphosphates (PIPₙₕ) have multiple phosphorylation patterns. Phosphatidylserine (PS) is prominent in apoptosis. Phosphatidylglycerol (PG) is found in the lining of the lungs.
are interesting lipids because they are capable of multivalent protein binding, and exhibit different biological properties depending on which combination of the 3, 4, or 5 hydroxyl groups is phosphorylated.\textsuperscript{14} PA participates in fission and fusion, and it also is a signaling lipid for various proteins, such as phosphatidylinositol 4-phosphate 5-kinase (PIP5K).\textsuperscript{14} Phosphatidylglycerol (PG) can be found in the lining of the lungs.\textsuperscript{15} On their own, phospholipids will self-assemble in solution to form structures made of lipid monolayers. In each monolayer, the polar headgroups are exposed to the aqueous environment, and the nonpolar tails are arranged so that they avoid the aqueous solvent by aggregating with other tails in the membrane. The precise membrane structures that are favored for each lipid varies and will be discussed later.

Another class of lipids is the sphingolipids \textit{(Figure 1.4)}. These have ceramide (Cer) as their base, which contains a hydroxyl group as the headgroup. The sphingolipids commonly contain two saturated or mostly saturated chains. This leads to a structure that is overall taller and more rigid than that of phosphocholine.\textsuperscript{16} The type main types of sphingolipids are sphingomyelin, which has a phosphocholine headgroup, and glycosphingolipids, which contain at least one sugar conjugated to the headgroup.\textsuperscript{6}

A third class of lipids is the sterols, which are biomolecules containing tetracyclic rings. The main sterol essential to membranes is cholesterol \textit{(Figure 1.4)}. It stabilizes to membrane bilayers by adding fluidity and abolishing the transition temperature at 30 mole percent cholesterol in the membrane.\textsuperscript{17} The transition temperature is the temperature at which liposomes transition to a stiff phase where lipids have low mobility to a phase where lipids have high mobility and can mix more freely to become more homogeneous. By abolishing the transition temperature, this results in bilayers that have the stiffness of a solid phase, but with the mobility of a liquid phase.\textsuperscript{6} No more than 50% cholesterol should be included, or else it will precipitate from the membrane. One of the key benefits of cholesterol is its ability to rigidify the liposomal structure, minimizing leakage in studies.\textsuperscript{17}
Figure 1.4: Ceramide lipids, the sterol cholesterol, and a triacylglyceride.

Ceramide, sphingomyelin, and glycosphingolipids are all types of sphingolipids. Cholesterol is a sterol essential for membrane support. Triacylglycerides are important for energy storage.
An additional class of lipids consists of the storage lipids, or fats. These include triglycerides (Figure 1.4), sterol esters, and wax esters. They serve as energy stores and building blocks for the formation of cellular membranes in the form of fat reservoirs. Energy is released upon hydrolysis of their ester bonds. Defects in the production of these types of neutral lipids lead to diseases such as diabetes and obesity.

1.3 Types of Delivery Vesicles

As pharmaceutical research has progressed, a need became evident to develop methods for delivering drugs with selectivity, since potent molecules that are toxic to diseased cells are often also toxic to healthy tissues. Since A. D. Bingham discovered in the 1960s that lipids suspended in aqueous dispersions formed bilayer structures called liposomes, they have been considered to be a promising drug delivery system. One benefit is that liposomes can reduce toxicity by encapsulating the cytotoxic chemical for release at the location of the diseased tissue or simply for a more gradual release in the bloodstream. Liposomes are also versatile in that polar drugs can be carried in the internal aqueous environment and nonpolar drugs can be carried in the liposomal tail region. Finally, lipids are biocompatible and can avoid immune responses as they are natural biomolecules.

There are many issues, such as poor localization, specific release, off-site effects, and elimination by the immune system that occur with unencapsulated or unconjugated drugs and can be ameliorated by drug carriers. There is of course the liposome, which already has some drug formulations on the market, such as DaunoXome (danorubicin) and Myocet (doxorubicin). Carbon nanotubes are polycyclic structures that have high surface areas for attachment of drugs, proteins, or other molecules. Cyclodextrins, another drug delivery system, are made by polymerizing glycopyranose units and are promising for their low toxicity. Dendrimers are a specific type of polymer that have regular branching with a large surface area for functionalization (Figure 1.5).
Figure 1.5: Types of drug delivery carriers.

Liposomes can encapsulate hydrophobic drugs in the membrane or hydrophilic drugs in the aqueous core. Micelles are only capable of hydrophobic entrapment. Polymerosomes, like ones made of sugars, can also be used in drug delivery. Dendrimers, a type of center-grown polymer, are yet another avenue. Also nanoshells and polymeric micelles have been used.
1.4 Packing Parameters

In solution, lipids and charged amphiphilic surfactants can form self-assembled structures. Most importantly, these self-assembled structures can vary depending on conditions,\textsuperscript{11a} including thermodynamic and entropic properties. Common structures lipids can form are spherical micelles, cylindrical micelles, spherical bilayers, or planar bilayers.\textsuperscript{11a} Micelles are single layer vessels that have hydrophilic heads facing outwards and hydrophobic tails facing inwards. Bilayers, on the other hand, have hydrophilic heads pointed toward both the outside and inside, with the two layers of tails facing each other. This creates an aqueous core, as opposed to the oil-soluble centers of micelles. Other structures are also possible, including reverse micelles, which have head and tails pointing in the opposite direction of micelles.

The packing parameter $\rho$ is used to describe the structural shape that leads to micelles or bilayers, and it is defined by $\frac{v}{a\ell}$ where $v$ is the volume, $a$ is the cross sectional area of the headgroup, and $\ell$ is the length of the tail. When $\rho$ is less than one third, a spherical micelle is formed. As the tail shortens to the point that $\rho$ is between one half and one third, rodlike micelles are formed in the inverted hexagonal structure, favoring reverse micelles. Further on, a spherical bilayer is formed when $\rho$ is between one half and one, with more flexible bilayers generated at values closer to one half. The spherical bilayer unfolds to a planar bilayer when $\rho$ is one (Figure 1.6).\textsuperscript{11,26}

The choice of lipid used is important for forming the desired assembly. Lipid bilayers can be used as carriers in the bloodstream, while at the same time encapsulating either hydrophilic or hydrophobic drugs. For a vesicle to be formed, the surface tension must be high and the stretching ability of the material low. This can occur for two tailed lipids, or even for lipids that have tails of different lengths. Lipids such as PC favor spontaneous aggregation in aqueous dispersions into spherical bilayers, or liposomes, due its lack of net charge and cylindrical shape.\textsuperscript{27}
Packing parameters help determine the optimal structure that will form based on the lipid structure. For example, lyso-phosphatidylcholine (LPC) has a large headgroup compared to the tail, so the resultant shape is conical, and this favors micelles. Phosphatidylethanolamine (PE), with a non-methylated headgroup, has a small headgroup compared to the tails, so it will have an inverted cone shape, forming reverse micelles in organic solution or more complicated inverse hexagonal structures in aqueous solution. Phosphatidylcholine (PC) forms bilayers due to the cylindrical shape it adopts.

Figure 1.6: Packing parameters of vesicle formation
The one-tailed lysolipids, such as LPC, favors micelle formation. Additionally, phospholipids like PE with smaller effective headgroups form reverse micelles as an inverted hexagonal structures in aqueous solution, forming rod-like clusters. Lipids such as LPC, however, encourage the formation of micelles, because of the smaller effective tail volume.\textsuperscript{28} For drug delivery, it is desirable to be able to incorporate different types of lipids. Thus, it is ideal to develop delivery methods that are effective with a wide range of lipids with different properties.

1.5 Liposomes and Considerations

One of most essential components to proper liposome formation is purity.\textsuperscript{29} Lipids can be assayed for purity by thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC). There are several ways in which the impurities can be displayed, such as isomerization. For example, the natural 1,2-diacylglycerol (DAG) has an equilibrium with its 1,3 isomer, whereby the ester linkage on the \textit{sn}-2 position migrates to the \textit{sn}-3 position.\textsuperscript{30} The TLC will show this by the appearance of a spot slightly higher on the TLC plate than the 1,2-isomer. Another way is by oxidative hydrolysis of lipids to lysolipids. The main method of decomposition, however, is by oxidation of saturated lipid tails to peroxides. A good precaution against oxidation is storing the lipids under an inert gas, under nitrogen or argon, and storing the samples at -20° C or colder.\textsuperscript{29}

Another feature of importance when considering to the choice of lipids in a liposome is the transition temperature ($T_c$ or $T_m$). Below this temperature liposomes are rigid and lipid diffusion is low. Above this temperature liposomes are in the gel phase, and lipids are more fluid with higher mixing diffusion. For initial formation of liposomes, a heat above the transition temperature should be applied in order to obtain homogeneous mixtures of lipids within liposomes populations. For example, the $T_m$ of dioleyl phosphatidylcholine (DOPC) liposomes is -22° C, while liposomes consisting entirely of distearoyl phosphatidylcholine (DSPC) have transition temperatures as high as 58° C.\textsuperscript{31} (Table 1.1) This
Table 1.1: List of typical phospholipids and transition temperatures

Different types of phospholipids are designated by their chain length and saturation. For example, DOPC contains 18-carbon fatty acid chains with one double bond between C9 and C10. The transition temperature, which marks the division between the crystal and gel phase, rises with increasing length and lowers with decreasing saturation. The type of headgroup also impacts the transition temperature, as seen by comparing DOPC, DOPS, and DOPE. The designation is read as the carbon length, followed by the degree of saturation in the type of fatty acid chains. For example, DOPC has 18-carbon chains with one π bond in each tail. If multiple π bonds exist, they are unconjugated in natural lipids.

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<tbody>
<tr>
<td>Dioleoyl phosphatidylcholine (DOPC)</td>
<td>18:1</td>
<td>DOPC</td>
<td>-17</td>
</tr>
<tr>
<td>Dioleoyl phosphatidylethanolamine (DOPE)</td>
<td>18:1</td>
<td>DOPE</td>
<td>-16</td>
</tr>
<tr>
<td>Dioleoyl phosphatidylserine (DOPS)</td>
<td>18:1</td>
<td>DOPS</td>
<td>-11</td>
</tr>
<tr>
<td>Dilauroyl phosphatidylcholine (DLPC)</td>
<td>12:0</td>
<td>DLPC</td>
<td>-2</td>
</tr>
<tr>
<td>Dimyristoyl phosphatidylcholine (DMPC)</td>
<td>14:0</td>
<td>DMPC</td>
<td>24</td>
</tr>
<tr>
<td>Dipalmitoyl phosphatidylcholine (DPPC)</td>
<td>16:0</td>
<td>DPPC</td>
<td>41</td>
</tr>
<tr>
<td>Distearoyl phosphatidylcholine (DSPC)</td>
<td>18:0</td>
<td>DSPC</td>
<td>55</td>
</tr>
<tr>
<td>Distearoyl phosphatidylserine (DSPS)</td>
<td>18:0</td>
<td>DSPS</td>
<td>68</td>
</tr>
<tr>
<td>Distearoyl phosphatidylethanolamine (DSPE)</td>
<td>18:0</td>
<td>DSPE</td>
<td>74</td>
</tr>
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demonstrates that as the lipid becomes more unsaturated, the transition temperature lowers. On the other hand, the lengthening of the chains will raise the $T_m$.

**1.6 Liposomal Properties**

Typical liposomes contain first and foremost some amount of PC, because it is the major component in cellular membranes and forms stable bilayers. The fatty acid tails of the lipids within liposomes can potentially modulate membrane surface interactions via the formation of macrodomains or microdomains. For example, Elegbede et al.\textsuperscript{32} found in studies that release of carboxyfluorescein (CBF) from liposomes driven by the MMR-9 enzyme was more efficient with palmitoyl–oleyl phosphatidylcholine (POPC) than DOPC liposomes, and DSPC liposomes showed little or no release of encapsulated dye. McGregor et al. and Takahashi et al. demonstrated that liposomes containing lipids with longer fatty acid chains exhibiting a greater degree of transfection across membranes.\textsuperscript{33} It is theorized that triggered release properties of liposomes can be optimized by fatty acid composition. Nadler et al. reported that binding of diacylglycerol (DAG) to a green fluorescent protein (GFP) was enhanced with a stearyl–arachidonyl DAG compound and a stearyl–linoenyl DAG, while little or no binding was observed with a palmitoyl–oleyl or dioctanoyl DAG.\textsuperscript{34} Besides PC lipids, PE lipids are at times included in small percentages, again with different lengths and degrees of saturation. This serves as a destabilizing agent for phase change events such as cell fusion.\textsuperscript{35} Cholesterol is known to stabilize lipid bilayers against temperature effects, dampening the permeability of ions and small molecules.\textsuperscript{36}

Another important consideration is the size of the liposome, which can vary from under 20 nm in diameter to over 1 μm upon formation.\textsuperscript{31} Liposomes classified by size can be either small unilamellar vesicles (SUV) if they are 50 nm or less or large unilamellar vesicles (LUV) if they are about 100 nm. They can also be giant unilamellar liposomes (GUV) if they are over a micron in size. The lamelarity refers to how many bilayers are present in the liposome.\textsuperscript{15} Typically unilamellar liposomes at sizes of 200 nm are utilized for drug delivery in order to penetrate tumor fenestrae that occur to uncontrolled growth.\textsuperscript{37}
sizes larger than 100 nm and without further modifications, the liposomes begin to favor multiple bilayers. These are termed multilamellar vesicles (MLVs).\textsuperscript{15,17}

Both the size and lamelarity of liposomes can be controlled by the method of preparation. The main liposome preparation techniques are mechanical, such as hydration, sonication, freeze–thaws, and extrusion. Hydration is the method in which an organic suspension of lipids is dried and then rehydrated in an aqueous medium, vortexing them to give an aqueous suspension.\textsuperscript{15,38} This is one of the first techniques used, notably by Alec Bangham.\textsuperscript{39} Sonication is a method of using an ultrasonic probe in the aqueous suspension until the liposomes are all of uniform size. ULVs are favored by a cycle of cold and warm baths, also known as freeze–thaws. Furthermore, a liposome extruder, which passes the liposomes through a polycarbonate membrane with preset pores, enhances the uniformity of size distributions.\textsuperscript{15,40}

There are also a wide range of instrumental techniques for characterization of liposomes.\textsuperscript{15} Dynamic light scattering (DLS) measures the distribution of particle sizes via the angles of diffraction of light from different particles in solution. Transmission electron microscopy (TEM) magnifies the liposomes for visual confirmation of sizes, and it also shows the shapes and forms of liposomes. Tracking the $^{31}$P signal with a lanthanide shifting agents can give an idea of the ratio of outer labeled phosphates to inner ones, verifying if liposomes are unilamellar.\textsuperscript{41} Surface potential, which relates to the overall charge of the inner and outer leaflet of the liposomes, can be tracked by zeta potential measurements. Furthermore, there are assays for phospholipid concentration, osmolarity, and even animal toxicity to characterize liposomes for various purposes.

Liposome stability and permeability is an important issue in liposome applications. In general, liposomal size stays constant for approximately 48 hours. The permeability increases with decreasing acyl chain length and decreasing saturation. An incorporation of at least 33\% cholesterol helps prevent
permeability dramatically.\textsuperscript{29} Certain lipids can furthermore only be incorporated below a certain threshold. For example, at room temperature and under physiological pH the lipid PE can only be incorporated up to 70\% with PC before the self-assembly shifts to the inverted hexagonal structure.\textsuperscript{42}

1.7 Types of Liposomes

There are four main types of liposomes used for drug delivery: conventional, cationic, stealth, and targeted (\textbf{Figure 1.7}).\textsuperscript{43} The conventional liposomes have no protection and thus have low survival times in circulation, being quickly uptaken by the reticuloendothelial system (RES) in mammalian systems. A second type of liposome is a cationic liposome, which is used to complex with negatively charged nucleotide drugs and for cell membrane transfection.\textsuperscript{44} Long-circulating liposomes (or stealth liposomes) have polyethylene glycol (PEG) chains conjugated on the liposomes surface. This shields the liposomes from proteins of the RES. It also prolongs the circulation time of liposomes dramatically.\textsuperscript{43, 45} A popular method to attach the PEG chains is to conjugate them to the amine headgroup of a PE lipid. Finally, there are immunoliposomes, which have antibodies conjugated to the liposome for target-specific recognition, enabling precise delivery to diseased cells.\textsuperscript{43, 46}

1.8 Antibodies and Their Use in Liposomal Systems

Antibodies are proteins of the immune system bred to specific antigens, like a lock and key. Being such, they have been used for site-specific delivery of drugs. Antibodies can even be affixed to liposomes for the purpose of targeted delivery. These are called immunoliposomes, and they demonstrate desirable features for drug-specific delivery, such as inhibition of tumor growth factors.\textsuperscript{47} Antibodies are best used to target sites where antigens are overexpressed so the healthy cells that produce normal levels of those same antigens will not be targeted.\textsuperscript{48} Antibodies are utilized for high binding and specificity, and with liposomes their binding does not change significantly when attached to a liposomal surface.\textsuperscript{48}
Conventional liposomes can be used to encapsulate drugs for delivery purposes. Cationic liposomes are used for transfection of membranes. Stealth liposomes have polyethylene glycol (PEG) stranded grafted on the surface to evade recognition by the immune system. Targeting liposomes contain antibodies grafted onto the surface for specific targets.
Though immunoliposomes can recognize targets, whole antibodies are easily recognized by the MPS as foreign particles and are subsequently taken up by the liver and spleen for destruction, especially if the liposome contains negatively charged headgroups such as phosphatidylycerol (PG) or phosphatidylserine (PS). For this reason, smaller portions of the antibodies are used, such as Fab’ or scFv fragments. Fragment antigen binding, or Fab’, is developed by pepsin digestion of the antibody, leaving the Y-shaped end of the antibody. Single-chain fragment variables, scFv fragments, on the other hand, consist of the smallest portion of the antibody that contains the whole binding site, containing the heavy and light flexible chains of the antibody termini connected by a string of amino acid linkers. Furthermore, adding a coating of 5—10% PEG_{2000} or PEG_{5000} on immunoliposomes tends to improve the durability and delivery of these structures. Antibody applications include targeting of macrophages that promote tumor growth and targeting neovascular growth regions. An example is tumor associated macrophages (TAMs), which encourage growth of tumor cells upon uptake of immunoliposomes containing anionic lipids such as PG and PS, after which the drugs are released and the macrophage is killed. Peptide coated and manosylated liposomes can also aid in uptake. Antibodies are thus an essential part of modern drug delivery systems.

1.9 Drug Delivery Obstacles

As pointed out earlier, many drugs that are intended for certain diseased areas can be toxic to normal cells. Thus side effects are common if certain potent drugs are not localized to solely the diseased area. An example is doxorubicin, which is toxic to the heart. A further complication of potent drugs with the use of a carrier is fast clearance by the immune system. Additionally, many drugs are hydrophobic, so they will precipitate in the bloodstream. Finally, many current drug carriers do not exhibit efficient release of encapsulated molecules.
Figure 1.8: Types of immunoliposomes.

Type A contains antibodies alone on the surface of the liposome. Type B has PEG chains and antibodies both on the surface. Type C, which contains the optimal strategy, has antibodies conjugated onto the distal end of the polyethylene glycol (PEG) liposomes.
The use of delivery vessels solubilizes nonpolar particles in the hydrophobic region, and the toxicity is subsequently decreased until released at the target site with a greater concentration at the site of interest.\textsuperscript{53} Drug delivery vessels should be large enough to not be filtered by the kidneys, but also small enough to advantageously utilize the larger fenestrae of the cancer tumor, which can range between 600 to 800 nm in size.\textsuperscript{37}

Several drugs have already been approved on the market as liposomal formulations for various diseases.\textsuperscript{54} Among them are doxorubicin (for Kaposi’s sarcoma, ovarian cancer, breast cancer, and myeloma), danorubicin (for Kaposi’s sarcoma and leukemia), and cytarabine (for certain leukemia and lymphoma cases). Liposomes can cause certain negative effects on certain patients, such as skin hypersensitivity and respiratory problems.\textsuperscript{55} These effects are more pronounced in formulations where PEG chains are introduced. The effects of skin conditions are due to liposomal tendency to deposit onto the skin while still holding encapsulated drugs in tumorous tissue as well as in the skin. However, the side effects decrease substantially after the first round of treatments.

The optimization of drug carriers also involves many difficult tasks. If the lipids do not form a tight seal, the encapsulated drug will leak before the liposomes reach the target site. It is also important to protect the liposomes from recognition by the mononuclear phagocytic system (MPS), which includes various structures of the immune system, such as more simple monocytes and professional phagocytes.\textsuperscript{56} Additionally, disease localization could be enhanced if the liposomes passively aggregate at the diseased tissues. This is achieved by grafting PEG strands on the liposomal surface. Finally, liposomes that are very stable within the body have a tendency to have slow release times. This can be circumvented by using a dependable triggering mechanism, such as a system that targets acidic regions or introduces an external stimulus. All of these issues must be addressed in liposomal design if drug delivery is to be successful.
1.10 Types of Internal-Stimulated Release

Various types of stimuli have been used in conjunction with drug delivery systems. Stimuli-responsive materials are beneficial for enhancing delivery to targeted diseased area. One reason for this is that passive diffusion, also termed Fickian diffusion, progresses at the same rate for both diseased and healthy site. Therefore distribution necessitates selective triggering in order to provide a focused distribution. In terms of tumor targeting, PEGylation addresses this issue by shielding liposome from opsonin-targeting of the immune system, thus prolonging circulation time to enhance the probability of tumor penetration via the EPR effect. However, to further enhance this effect, or to induce treatment equivalent to that of the free drug of the target, stimuli-induced release is beneficial. Different approaches are available, including redox chemistry, enzymatic approaches, acidic release, and magnetism.

One approach, redox effects, was used by Ong et al. with their liposomal system that included a quinone–PE lipid (Figure 1.9). Cancer cells overexpress the quinone reductase, so under these reducing conditions, quinone moiety will be reduced, and a cyclization will occur that liberates the bilayer-disfavoring PE lipid. The liposomes will then undergo phase transition to micelles. A more common route is the incorporation of disulfide bonds for the reductive cleavage of upregulated glutathione in cancer cells. However, targeting specific redox release can be difficult, as illustrated by the drug Celltech, which failed to produce therapeutic results after it was approved by the Food and Drug Administration.

Enzymatic approaches have also been employed to target overexpressed enzymes at diseased sites. For example, since secretory phospholipase A2 is more active at tumor and inflammation sites, Andresen et al. developed lipids with a PE–PEG headgroup and an ether and ester tail. In a liposome formulation, the ester bond would be enzymatically cleaved and the cytotoxic ether lysolipid prodrug would be released. This system is especially prone to hydrolytic cleavage since polymer coatings and
liposome formulations are more prone to sPLA₂ activity than lipid drugs alone. Because PEG coating may slow passive release, enzyme-cleavable PEG attachments have also been synthesized, as with the polyaminoacid coatings developed by Romberg et al.⁶⁰ The targeting via enzymes overall requires an intimate knowledge of the surface chemistry occurring at the diseased site.

Release using heat has been utilized by taking advantage of dilation of blood vessels, including tumor vessels, that occur at lightly elevated temperature,⁶¹ in the range of 34 to 39 °C. Temperatures below this range do not increase tumor permeability, and higher temperatures lead to damage in the form of hemorrhage and stasis.⁶² Furthermore, liposome formulations have been developed with a Tₐ around 44° C, such as the commercially available formulation ThermoDox (Celsion). These liposomes are prone to a drastic leakage in the absence of cholesterol. Also, ultrasound⁶³ has also been used in conjunction with heat to visualize the release of particles, as in the case of Ranjan et al.⁶⁴ to gain a better understanding of thermal application to solid tumors. Additionally, heat can be used to cause an indirect release of entrapped compounds, as with solutions that contain ammonium bicarbonate and release carbon dioxide as a byproduct of mild hypothermia, thus perturbing the membrane.⁶⁵

Furthermore, since tumors are typically slightly more acidic than the surrounding tissue, there are numerous examples of acid-sensitive targeting examples. Acid-cleavable systems needs to enact a drastic change with a slight pH change, since healthy tissues have a pH around 7.4 and tumor sites have a pH of 6.5 to 7. One strategy is to develop acid-cleavable bonds, such as hydrazone bonds. Kale et al.⁶⁶ developed an acid-cleavable lipid conjugate that effectively hydrolyzes at a pH of 5.5. Another type of acid-sensitive strategy involves the protonation of certain residues. For example, tumor necrosis factor alpha (TNFα) can be released by a protonation of amino acid residues, as shown by Deng. et al.⁶⁷ Protonation of PE conjugated to PEG or poly(ethylene imine) leads to an increase in membrane transfection efficiency.
It should also be pointed out that these are only a few types of release. Others such as ultrasound-mediated, magnetic, electric, glucose-mediated, and light also exist. Among these, light is of special interest because it operates independently of internal cues or bodily mechanisms. This will be the focus of our work and will be discussed in further detail in chapters two and three.
There are a variety of approaches for the passive triggered release of encapsulated cargo from liposomal containers. The first example is suited for release of phosphatidylethanolamine in areas overexpressing quinone reductases. Enzymatic approaches can be used to deliver ether prodrugs via ester hydrolysis by secretory phospholipase enzyme A2. Acidic linkages, such as hydrozones, can be cleaved under slightly acidic conditions. Finally, liposome formulations with lysolipids can tune a thermal release at a slightly increased temperature slightly above body temperature.
Chapter 2 Phosphatidylcholine Analog Lipid (NB-PC) with Photocleavable 2-Nitrobenzyl sn-2 Acyl Chain

2.1 Introduction

The previous examples of redox, heat, and enzymes in chapter one depend on the local environment. In the case of tumors, these types of release take advantage of the local environment, such as overexpressed enzymes or inducing larger vasculature. However, these measures do not allow for external spatiotemporal control over release. One promising method that has been explored is the use of light as an external stimulus, since it does not depend on the local environment, or on inducing a change in the local environment. This penetration is an important feature in light-controlled therapy.

Light is fundamentally a form of energy. A lower the wavelength corresponds to a higher the energy of the light is used, according to Plank’s law.

\[
E = \frac{hc}{\lambda} \quad \text{or} \quad E = hv \quad \text{(Eq. 2.1)}
\]

In this equation, \(E\) is the energy of a photon, \(c\) is the speed of light, \(v\) is the frequency, and \(\lambda\) is the wavelength. Light can give access to excited states that are not easily accessible otherwise. This is especially important in terms of bond breaking, because light irradiation excites electrons into antibonding orbitals. In respect to clinical trials, light penetration into the skin barrier depends on a number of factors, including wavelength, duration, power, and skin pigment. For someone with fair skin, a UV beam of 350 nm can penetrate 6 \(\mu m\) into the skin, while a beam of 700 nm can penetrate 750 \(\mu m\).

Light is an appealing source of release because it has easily controllable properties, such as wavelength, intensity, duration, and localization. Light is used for many applications, including cross-linking in protein–lipid binding and photoaffinity labeling. It can also be utilized for the activation or deactivation of certain molecules by decomposing photochemically-responsive moieties. For this
purpose, light protecting groups (LPGs) have been synthesized. One of the more studied LPGs is the 2-nitrobenzyl group.\textsuperscript{71} Other groups include 7-nitroindoline, coumarin-4-ylmethyl, \(p\)-hydroxyphenacyl, and nitrophenethyl (Figure 2.1).\textsuperscript{72}

### 2.2 The \textit{o}-Nitrobenzyl Protecting Group

With photo-release, chemical or biological substrates can be protected and then activated by stable photolabile protecting groups, or cages.\textsuperscript{72} One of the first literature examples of caging is the protection of ATP in 1978 by Kaplan and co-workers\textsuperscript{73} in order to study the mechanism of the sodium and potassium ion pump. (Figure 2.2). The caged substrate (2.1) was irradiated to form the nitroso byproduct (2.2) and ATP (2.3). Other substrates that can be protected range from biological components, including nucleotides and peptides, to organic and inorganic substrates, such as calcium ions, phosphates, and carboxylates.\textsuperscript{71} Lipids have also been added to the list of protected substrates in the past few years.\textsuperscript{74} These include diacylglycerol,\textsuperscript{75} phosphatidic acid,\textsuperscript{76} and phosphatidylinositol-(3,4,5)-trisphosphate.\textsuperscript{77} It would be preferable to obtain a photo-release in the near IR region, because this area of light limits damage to nucleic acids and proteins in the body. At present, however, photo-release of molecules tends to occur in the violet or near ultraviolet portion of the spectrum.\textsuperscript{19}

Many other substrates have been successfully protected and released by the \textit{o}-nitrobenzyl (\textit{o}-NB) moiety, such as serotonin,\textsuperscript{78} calcium,\textsuperscript{79} and choline.\textsuperscript{80} The mechanism of release for the \textit{o}-NB chromophore is well studied, and literature has provided ideas for increasing its usefulness for drug delivery by shifting the wavelength to the near infrared (IR) region, where techniques such as two-photon excitation (2PE) are useful\textsuperscript{81} and will be discussed in chapter 3.

The manner by which the \textit{o}-NB group releases its substrate and becomes a nitroso byproduct occurs by a chemical decomposition via a Norrish type II mechanism (Figure 2.3).\textsuperscript{82} The whole process begins with excitation of 2.4, leading to a radical intermediate by electron promotion into the \(\pi^*\) orbital
Used for caging a substrate until it is cleaved by photolysis. The 2-nitrobenzyl group is one of the longer known and better understood LPGs.
Figure 2.2: Light-induced cleavage of ATP from the o-nitrobenzyl group.

Caged ATP (2.1) is liberated by UV light, giving the nitroso byproduct (2.2) and ATP (2.3).
to 2.5. From the singlet or triplet state, the radical on the oxygen abstracts hydrogen from the nearby benzylic position, generating a resonance-stabilized benzylic radical (2.6). From the Z-nitronic acid resonance intermediate (2.7), deprotonation occurs to the rate-determining aci-nitro (2.8) intermediate, which is then deprotonated to form the E-nitronic acid (2.9), where the outer nitro oxygen is protonated. Internal cyclization subsequently occurs, forming an oxazole intermediate (2.10). Fragmentation of this intermediate proceeds through intermediate 2.11 before expulsion of the leaving group to arrive at the nitroso aldehyde (2.12). When the enthalpy of expulsion of the leaving group is less favorable or there is a sufficiently low pH, 2.11 is the product.\textsuperscript{71, 78}

2.3 Photocleavable Liposomes

Several liposomes have been formulated with photoreactive groups, especially the nitrobenzyl group (Figure 2.4). For examples, Zhang and coworkers\textsuperscript{42} formulated liposomes containing 50% PE and 50% of an o-NB-protected PE analogue (2.13). The o-NB lipids were stable for liposome bilayer formation, just like PC. Under UV light, the o-NB groups were removed and the vesicles transitioned to the inverse hexagonal phase, which is the favored structure for 100% PE structures. This approach has the advantage of a very short synthesis, as a chloroformate version of the o-NB group can be purchased to react with commercially available PE lipids. This system, however, still needs to be tested in biological systems to validate if the nitrobenzyl is as biocompatible as a choline headgroup.

In another example, Nagasaki et al.\textsuperscript{83} developed a cationic amphiphile with a nitrobenzyl group in the middle to enhance the transfection of pGL3 into COS-1 cells. Chandra et al.\textsuperscript{84} developed a single chain amphiphile (2.14) that demonstrated release of fluorescein when formulated with PC lipids, while Subramaniam\textsuperscript{85} devised a similar compound that instead include a photocleavable coumarin moiety instead (2.15). All of these previous examples demonstrated that effectiveness of dye release are influenced by the type of tails in the PC lipids. On the other hand, these compounds cannot be used without aid of PC lipids, because both compounds are single-stranded amphiphiles that favor micelles.
Figure 2.3: Photocleavage of the o-nitrobenzyl system

Irradiation of the conjugated nitrobenzyl compound (2.4) yields radical excitation of the N-O π bond upon irradiation to diradical 2.5. Abstraction of the benzylic hydrogen gives 2.6, which is in resonance with Z-nitronic acid 2.7. Next, a proton transfer via aci-nitro intermediate 2.8 yields E-nitronic acid 2.9. Cyclization leads to oxazole 2.10. Deprotonation of this intermediate leads to nitroso 2.11, and expulsion of the leaving group gives 2.12.
Zhang et al.\textsuperscript{42} developed a liposomal system of 50\% of 2.13 and 50\% PC for release by a PE-driven phase transition. Chandra et al.\textsuperscript{34b} utilized 5\% of 2.14 for carboxyfluorescein (CBF) release from liposomes. This amphiphile was best for UV release, while 2.15 is a coumarin amphiphile meant for two-photon release. A photo-induced based silica molecule was used with the isomerizable azobenzene for mechanical release of the dye Nile red from the membrane.
Dithiane lipids have also been used for liposome disruption under UV light by Wan and co-workers. They retain the phosphocholine headgroup, but this lipid has only been used at percentages up to 20% in liposomes. Finally, azobenzene molecules have been used as an impeller mechanism by Liang et al. with to release Nile red via trans–cis isomerism. These lipids produce cerasomes or silica-modified liposomes, but the effect of silica on living systems is still uncertain. Photocrosslinking has also been used in the literature. Spratt et al. utilized PC lipids with polyunsaturated lipid tails so the conjugated π bonds cross-link upon irradiation and form tight domains, forming space and causing the entrapped dye to escape in the process.

2.4 Discussion of Synthesis of NB-PC

For photocleavable liposomes that could be biocompatible, we sought to design and synthesize a PC analogue (Figure 2.5) bearing a photocleavable moiety in the fatty tail (2.17, NB-PC). With the o-nitrobenzyl group, photocleavage would shorten the lipid chain (2.18), also producing aldehyde 2.19 as a byproduct. This would alter the membrane properties, and stimulate release. Furthermore, the free nitrogen could also potentially cyclize with the nearby ester to release lyso-phosphatidylcholine (LPC, 2.20), which does not favor bilayer formation on its own, as well as succinamide (2.21). This would cause liposome disruption and subsequent cargo release.

A primary benefit of this system is that 2.17, also referred as NB-PC, bears a remarkable resemblance to the natural PC. Because the headgroup is intact and the tails have the same ester linkages, NB-PC should bear the same or similar biocompatibility and stability to natural PC in the bloodstream of living systems. Additionally, because the overall structure is similar to PC, which forms stable membrane bilayers, the percent of NB-PC in liposomes that result in stable membranes should be much more flexible than prior photocleavable analogues. Furthermore, it was expected that the o-NB groups in the membrane could lead to a higher stability in the absence of light, due to π-π stacking. Finally, as discussed previously, after irradiation of NB-PC, the photocleaved shortened tail of 2.18.
A. Photocleavage of 2.17 (also known as NB-PC) leads to shortening of the tail to 2.18 and aldehyde 2.19. Cyclization could also occur to 2.20, or LPC, and 2.20. B. The decomposition of NB-PC lipids in the bilayer leads to reorganization/decomposition and simultaneous release of encapsulated molecules.

Figure 2.5: Proposed Photocleavage of NB-PC in liposomes.

A. Photocleavage of 2.17 (also known as NB-PC) leads to shortening of the tail to 2.18 and aldehyde 2.19. Cyclization could also occur to 2.20, or LPC, and 2.20. B. The decomposition of NB-PC lipids in the bilayer leads to reorganization/decomposition and simultaneous release of encapsulated molecules.
(along with aldehyde 2.19) could induce some membrane defects. Furthermore, if cyclization occurs, this would cause subsequent destabilization, because the formed LPC (2.20) will revert to the micellar phase, as with the previous example of Zhang et al.42 Thus the NB-PC system is a widely tunable and biocompatible system for stable liposome delivery and release.

The synthesis of NB-PC (Figure 2.6) began with commercially available 4-(aminomethyl)benzoic acid 2.22 (Chem Impex International). The first several steps to 2.25 were carried out according to a literature precedent.34b By first utilizing the reaction of 2.22 with trifluoroacetic anhydride (TFAA), the benzyl amine was protected with an acid-stable trifluoroacetamide protecting group to produce 2.23. Next, nitration was carried out with a sulfuric–nitric acid mixture. The methylamine serves as an ortho/para direction group, and the carboxylate group acts as a meta directing group, so nitration is reinforced for the positions ortho to the aminoalkyl group in the product (2.24).

After nitration, the trifluoroacetyl (TFA) protecting group was removed and a tert-butyl carbamate (Boc) group was introduced for 2.25, both under basic conditions. As a side note, the pH of the solution upon removal of the TFA group (at least a pH of 8) must be sufficiently basic for the deprotection to be successful. Therefore it is very important that after nitration, the product, precipitated from ice, be thoroughly washed with water or extracted from a large quantity of water with ethyl acetate in order to ensure removal of residual nitric or sulfuric acid. Upon addition of the tert-butyl anhydride for the Boc protection, it is important to allow venting for at least the first 10 minutes for evolution of carbon dioxide, which is one of the byproducts of both Boc protection and deprotection.

The Boc-protected product could in most cases be obtained after reaction completion (as indicated by disappearance of the ninhydrin spot over the course of time) by a double extraction, first washing the carboxylate with diethyl ether, then acidifying to the carboxylic acid and extracting with the more polar organic solvent ethyl acetate. Any residual ethyl acetate peaks in the NMR spectra can be
Protection of the amine of 2.22 led to compound 2.23. After this, nitration was carried out to produce 2.24. Protecting group exchange led to base stable 2.25. Elongation with hexylamine led to 2.26. Next deprotection in acid and chain extension via ring opening of succinic anhydride produced 2.27. Coupling of this compound to lyso-phosphatidylcholine (LPC, 2.20) resulted in cleavable lipid 2.17.
diminished or removed by concentrating from dichloromethane several times. The next reaction was carried out according to a previous procedure, but with a shorter alkyl amine for coupling than the one used in the literature. Hexylamine was coupled to the carboxylic acid with hydroxybenzotriazole (HOBt) and N,N,N′,N′′-tremamethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate (HBTU), which provided a modest result for 2.26. Attempts for coupling with dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) proved less fruitful. However, the diminished yield may be due to unconverted starting material. Also, using the same procedure, the Boc-protected octadecylamine chain was also produced. This was performed to vary the hydrophobicity of the lipid in case the hexylamine chain of the original design was too short. After adding the hexylamine chain, the Boc group was removed with trifluoroacetic acid (TFA) to give the free amine. This compound was basified through extraction from a hydroxide solution and carried to the next step, the reaction with succinic anhydride to furnish a carboxylic acid (2.27). This product, which is the full modified fatty acid tail, may utilize workup by either column chromatography or recrystallization from diethyl ether to obtain product.

The final step involved coupling of the synthetic fatty acid 2.27 to commercially available lyso-phosphatidylcholine (LPC) from Avanti Polar Lipids with dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP). Crushed glass from test tubes was included to increase the surface area of reaction, since lipids tend to like to reaction on glass surfaces. Sonication was also used to speed up the formation of 2.17. Both the glass and sonication were used according to the example of Rosseto et al.89 Unreacted fatty acid 2.27 was collected from column chromatography when possible.

Other routes for the synthesis of 2.17 were also attempted (Figure 2.7). Route A entailed the synthesis of the glycerolipid backbone after which the lipid headgroup could be introduced. This began with (S)-glycerol acetonide (2.28). Using the Williamson ether synthesis, para-methoxybenzyl chloride (PMB-Cl) was used to protect the free alcohol, producing 2.29. Catalytic acid produced the diol 2.30 in
Figure 2.7: Alternative methods to photocleavable PC analogues.

83% over two steps. Protection of the primary alcohol with tert-butylidiphenylsilyl afforded 2.31. Further work would include adding an ether tail via 2.32 to produce 2.33, and deprotection with tetrabutylammonium fluoride to produce 2.34. Fatty acid 2.27 would then be coupled to 2.34 to produce 2.35, followed by PMB deprotection with 2,3-dichloro-2,2-dicyanohydroquinone (DDQ) to 2.36, and phosphoramidite chemistry with 2.37 to produce analog 2.38.

Another method was attempted, first condensing glycerol (2.39) and benzaldehyde (2.40) with catalytic acid to form dioxane 2.41. An ether or ester tail could then be conjugated to the sn-2 hydroxyl group. For example, an 18-carbon sulfonate (2.42) was used to produce 2.43. Then the remaining alcohols were deprotected in methanolic acid to afford 2.44. Mono-acylation with 2.27 would then give the racemic 2.36, and as before, phosphoramidite chemistry would afford racemic 2.38. Lastly, a route with glycerophosphocholine (2.45) was attempted, where the photocleavable fatty acid tails can be acylated directly from 2.27 with 2.46 2.27, which contains two o-NB tails (Figure 2.8 and Figure 2.9).

2.5 Discussion of NB-PC Studies
To test the photocleavage and encapsulation of these produced liposomes, a suitable dye assay needed to be chosen. The best method for this approach was very similar to the example of cerasomes described by Liang et al., which used Nile red. Nile red is a hydrophobic dye that favors lipophilic environments, such as the bilayer membrane. It fluoresces in the membrane, and the fluorescence is abolished upon precipitation in aqueous solution.

The dye 5(6)-carboxyfluorescein (CBF), which was initially utilized for the kinetic assay, is hydrophilic and favors encapsulation in the aqueous liposome interior. In this assay, a 50 mM CBF solution was utilized, and it also contained 150 mM sodium chloride to mimic physiological osmolarity and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), buffered at a pH of 7.40. This CBF solution was hydrated with the liposomes, and later free CBF was separated from liposome-
The fluorescence of Nile red is diminished as it is expelled from the membrane over the course of 90 minutes.
Figure 2.9: Plot of decreased fluorescence of Nile red over time.

The decrease of the fluorescence of Nile red in response to irradiation-induced expulsion produces a curve similar to that shown above, which can be fit to an exponential decay equation of form Equation 2.1.
The dye 5(6)-carboxyfluorescein (CBF), which was initially utilized for the kinetic assay, is hydrophilic and favors encapsulation in the aqueous liposome interior. In this assay, a 50 mM CBF solution was utilized, and it also contained 150 mM sodium chloride to mimic physiological osmolarity and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), buffered at a pH of 7.40. This CBF solution was hydrated with the liposomes, and later free CBF was separated from liposome-encapsulated CBF by a Sephadex G-50 size exclusion column loaded in a 50 mL syringe and packed with a solution of 150 mM sodium chloride and 20 mM HEPES. The design of this assay was to first encapsulate quenched CBF in the liposomes at 50 mM. Upon release from the liposomes with the detergent sodium dodecylsulfate (SDS), the detergent Triton X, or irradiation with 350 nm light, a drastic increase in fluorescence would occur. This assay, however, was not useful, because it was experimentally determined that photobleaching occurred regardless of liposome composition. This was exemplified by an overall decrease in fluorescence. This was attributed to the absorbance of CBF at 492 nm being too close to the irradiation wavelength applied to the sample at 350 nm. The deciding experiment was an irradiation of CBF alone, which led to a decrease in fluorescence due to photobleaching, rather than a steady signal. It is also likely that release from the aqueous interior was not effective, leading to minimal signal change.

For the Nile red assay, each lipid used was diluted with 500 μL of chloroform. Enough solution was aliquoted into a fresh vial to reach 5 mM of total lipids in a 500 μL solution. Nile red solution was added based on a 250 μM calculation in 500 μL. The lipids and dye were dried under nitrogen and vacuum in the same vial and after a few hours rehydrated with 500 μL of deionized water from a MilliQ system. For specific examples see the experimental section. Liposomes were prepared per the normal procedure, which include heating at 60° C for 1 hour, with intermittent vortexing, then 10 freeze–thaw cycles, and finally 21 passes through a 200 nm extruder. After dilution to a usable range in the fluorimeter, three milliliters were used in two different samples, and the fluorescence of Nile red was
monitored as a function of time using a Perkin–Elmer fluorimeter. After every five minutes of irradiation, the fluorescence was scanned \((\lambda_{\text{ex}} = 553 \text{ nm}, \lambda_{\text{em}} = 614 \text{ nm})\). If the Nile red was released from the membrane, the maximum fluorescence decreased. This decrease is caused by precipitation of Nile red from the membrane bilayer into the aqueous solution. As an example, the data of the fluorescence of 5 mM of NB-PC only with Nile red encapsulated in the membrane was recorded. (Figure 2.8). The maximum fluorescence was subsequently plotted as a function of irradiation time with 350 nm light over the course of 90 minutes (Figure 2.9). The data was fitted to an equation for exponential decay.

\[
Y = y_0 + Ae^{-kt},
\]  
(Eq. 2.1)

In the above equation, \(Y\) is the % of initial fluorescence, \(y_0\) and \(A\) are fit parameters, and \(k\) is the first order constant. The half-life \(t_{1/2}\) can then be calculated by the equation:

\[
t_{1/2} = \frac{0.693}{k}.
\]  
(Eq. 2.2)

In the first study, we evaluated the release from liposomes composed of 100% NB-PC. Several controls were also performed. Irradiation of the 100% NB-PC sample led to an 80% decrease in fluorescence over approximately 60 minutes (Figure 2.8 to Figure 2.10 and Table 2.1). On the other hand, when liposomes composed of 100% NB-PC were kept in the dark or when the natural lipid DOPC comprised 100% of the liposomes either in the light or dark, there was minimal release. These results demonstrate that NB-PC is indeed responsive to UV irradiation and useful for the release of hydrophobic small molecules from the membrane. Also, liposomes composed of the NB-PC lipid are stable in the dark. Data fitted to Equation 2.1 and calculated according to Equation 2.2 are tabulated in Table 2.1.

Secondly, the effect of percentage of NB-PC in the liposome was tested, ranging from 10% to 50% (Figure 2.11 and Table 2.2). Additionally, 50% DOPE was included and the remainder of the lipids was DOPC. The importance of including DOPE is that it will not favor bilayer membranes on its own or in
Figure 2.10: Irradiation of 100% NB-PC and 100% DOPC and dark controls

Under UV light, NB-PC liposomes exhibited a decrease in fluorescence due to expulsion of Nile red from the membrane. Controls were performed without light, without photocleavable lipids (DOPC only), or both. Only minimal decrease in fluorescence from passive leakage occurred.

Table 2.1: Irradiation of 100% NB-PC and 100% DOPC, along with dark controls

Data from Figure 2.10 was fitted to Equation 2.1 and tabulated using Equation 2.2

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<th>% DOPC</th>
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<th>A</th>
<th>k (min$^{-1}$)</th>
<th>k (s$^{-1}$)</th>
<th>$R^2$</th>
<th>$t_{1/2}$ (s)</th>
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Figure 2.11: Variation of NB-PC in liposomes with 50% DOPE and remainder DOPC

NB-PC is utilized at different percentages within the liposome, from 10% to 100%. An increasing percentage of NB-PC leads to greater release of Nile red. Thus this liposome system is tunable across all percentages.

Table 2.2: Regression data for variation of NB-PC in liposomes with 50% DOPE and remainder DOPC

The data for Figure 2.11 is tabulated using Equation 2.1 and Equation 2.2.

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<th>% DOPE</th>
<th>% DOPC</th>
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<th>A</th>
<th>(k) (\text{min}^{-1})</th>
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<td>23.9</td>
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high percentages with PC, and may therefore enhance release. In this case, it was found that increasing percentage of NB-PC led to increased release of Nile red from the membrane. Thus NB-PC is tunable over a wide range of concentrations. Again, NB-PC only exhibited release of Nile red under UV light. 100% NB-PC was superimposed in Figure 2.11 for comparison.

The effect of PE addition to liposomes was tested to see if more PE conferred greater liposome instability, since PE is a non-bilayer forming lipid and promotes negative curvature (Figure 2.12 and Table 2.3). Again, NB-PC only exhibited release of Nile red under UV light, and studies using 100% NB-PC were superimposed for comparison. It was thought that the percentage of PE enhances liposome destabilization and release. Therefore, by increasing the percentage of PE, an enhanced release may be observed, or liposomes may not form. For this experiment, 50% NB-PC was used, and 0 to 50% PE was tested, with DOPC as the remaining lipid up to 100%. The curve for 100% NB-PC was superimposed again. The results illustrated that PE has little effect on liposomal release from NB-PC liposomes for the percentages used. Additionally, no effect was found from samples kept in the dark.

Next, the effect of cholesterol on liposomal release was tested, to determine if the formulation was more stable with added cholesterol (Figure 2.13 and Table 2.4). Cholesterol favors bilayer formation, so a higher amount of cholesterol may display greater stability in the dark, which would be evident by a diminished release in the dark or under irradiation. Cholesterol percentages ranging from 0% to 50% were used with 50% NB-PC, with DOPC as the remainder lipid content. 100% NB-PC was superimposed for comparison. No clear trend was again observed, showing that NP-PC is compatible with the use of cholesterol. This further demonstrates that cholesterol has little effect on release from NB-PC liposomes. It is beneficial that this system tolerates a wide range of lipids with different properties, so the liposome used for delivery can be easily tuned for optimal release.

Lastly, since liposomes can have longer circulation times with the inclusion of polyethylene
Figure 2.12: Effect of DOPE on liposomal release and dark controls

DOPE lipids, which are non-bilayer forming, were incorporated into liposomes from 10% to 50%. Additionally, 50% NB-PC was incorporated for all samples. The difference was minimal among all samples.

Table 2.3: Effect of DOPE on liposomal release and dark controls

Data was tabulated from Figure 2.12 using Equation 2.1 and Equation 2.2.

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<th>% DOPC</th>
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<th>$k$ (min$^{-1}$)</th>
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Figure 2.13: The effect of cholesterol on liposomal release

Cholesterol, which stabilizes bilayer formation, were introduced into 50% NB-PC liposomes, from 10% to 50%. No significant difference was observed for all samples.

Table 2.4: Regression data for the effect of cholesterol on liposomal release

Data from Figure 2.13 was tabulated using Equation 2.1 and Equation 2.2.

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glycol (PEG) chains, we decided to study liposomes including 15% of PEG–PE lipids, which is on the higher order of PEG used in liposome delivery formulations. We compared this data (Figure 2.14 and Table 2.5) to 100% NB-PC and found little difference. Thus, NB-PC liposomes are tunable over a wide range of different lipid compositions and properties. They can tolerate lipids that stabilize bilayers, lipids that destabilize bilayers, and PEG lipid conjugates for protection from the immune system.

Dynamic light scattering spectra were also taken of different liposome samples, before and after photocleavage, to visualize any size changes. Representative samples shown in the appendix include those composed of 100% NB-PC both kept in the dark and irradiated at 350 nm for 90 minutes (Spectra 2.30 and Spectra 2.31). A slight size increase was seen in the average diameter, which could be random fluctuation, or it could infer that the liposomal size is increasing, for example due to a fusion event.

2.6 Conclusion
A photocleavable analogue of phosphatidylcholine has been synthesized in eight steps. This 2-nitrobenzyl-containing lipid forms liposomes over all percentages. It undergoes structural modification with ultraviolet irradiation, releasing entrapped Nile red into the aqueous solution in response to a change in the morphology of the lipids in the membranes. This photodisruptable liposome is widely tunable, tolerating at least 50% of the bilayer disfavoring lipid PE, 50% of the bilayer favoring cholesterol, and also polyethylene glycol-conjugates up to at minimum 15%. Thus the PEG addresses drug delivery issue of extending circulation time and shielding from the immune system, while this remote light-triggerable system addresses the issue of efficient release. Future studies can study the impact of antibodies affixed onto the end of the PEG chains for targeting to cells to further validate the usefulness of this system.

2.7 Materials and Methods
Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. Palmitoyl–lysophosphatidylcholine and DSPE–PEG(2000) amine were purchased from
Figure 2.14: Effect of polyethylene glycol on liposomal release

For drug delivery purposes, PEGylated PE lipids were incorporated into NB-PC liposomes at 15% of the total lipid. Results were similar to 100% NB-PC liposomes.

Table 2.5: Regression data from effect of PEG on liposomal release

Data from Figure 2.14 was tabulated using Equation 2.1 and 2-2.

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<th>% NB-PC</th>
<th>% PEG-PE</th>
<th>$y_0$</th>
<th>A</th>
<th>k (min$^{-1}$)</th>
<th>k (s$^{-1}$)</th>
<th>$R^2$</th>
<th>$t_{1/2}$ (s)</th>
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</table>
Avanti Polar Lipids, Inc. (Alabaster, AL) and (4-methylamino)benzoic acid was purchased from ChemImpex International (Wood Dale, IL). Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Varian Mercury 300 MHz or Varian NMR 500 MHz spectrometers. Mass spectra were obtained with a JEOL AccuTof DART or a JEOL AccuTOF ESI spectrometer with high resolution capabilities. Optical rotations were measured with a Perkin-Elmer 241 Polarimeter using the sodium D line. Ultrapure water was purified via a Millipore water system (≥ 18 MΩ·cm) triple water purification system. 4-((Tert-butoxycarbonyl)amino)methyl)-3-nitrobenzoic acid was synthesized from (4-methylamino)benzoic acid according to a prior literature procedure.\textsuperscript{74} Detection of Nile red emission decreases attributed to release was performed using a Perkin-Elmer LS55 fluorescence spectrometer. Samples were irradiated with a Rayonet Preparative Type RS photoreactor while suspended in a cuvette with Pyrex as a filter.

4-((2,2,2-Trifluoroacetamido)methyl)benzoic acid. **Compound 2.23.**

![Chemical structure](image)

Commercially available \( p \)-aminomethylbenzoic acid (2.22, 2.03 g, 13.5 mmol) was slowly added to 6 mL of trifluoroacetic anhydride, cooled to 0°C. After addition, the reaction was allowed to warm slowly to room temperature, at which point the reaction became homogeneous. After two hours, ice cold water was added, which caused precipitation of the crude product. This solid was rinsed with ice cold water and filtered. The crude was then extracted using ethyl acetate in three portions (3 x 100 mL) from water. The combined organic layer was dried with magnesium sulfate and filtered, and then concentrated using rotary evaporation. The product was redissolved with dichloromethane (3 x 100 mL)
and reconcentrated, then dried under vacuum to give 3.04 g of product 2.23 as a white solid (91%), and the characterizations matched previous literature.\textsuperscript{74}

\( ^1H \) NMR (500 MHz, DMSO-\( d_6 \)) \( \delta: 12.93 \) (s, 1H), 10.08 (t, \( J=6.1 \) Hz, 1H), 7.93 (d, \( J=8.3 \) Hz, 2H), 7.39 (d, \( J=8.3 \) Hz, 2H), 4.46 (d, \( J=6.1 \) Hz, 2H). \( ^13C \) NMR (126 MHz, DMSO-\( d_6 \)) \( \delta: 172.22, 147.61, 135.02, 134.75, 134.08, 132.56, 122.31, 47.54.\n
3-Nitro-4-((2,2,2-trifluoroacetamido)methyl)benzoic acid. Compound 2.24.

\[
\begin{array}{c}
\text{O} \quad \text{O} \\
\text{CF}_3 \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{O} \\
\text{O} \\
\text{OH} \\
\text{H}_2\text{SO}_4 \quad \text{HNO}_3 \\
\text{89\%} \\
\text{2.23} \\
\text{2.24}
\end{array}
\]

To 50 mL of concentrated sulfuric acid was added 2.23 (2.96 g, 12.0 mmol) at 0° C. A mixture of 10 mL of 1:1 sulfuric acid to nitric acid was added dropwise via addition funnel. After completion of addition, the reaction was stirred for two hours and then poured into ice, causing precipitation. The resultant solid was filtered and rinsed with ice cold water on a filter. The crude was then extracted using ethyl acetate (3 x 100 mL) from water. The combined organic layer was dried with magnesium sulfate and filtered, and then concentrated using rotary evaporation. The product was redissolved with dichloromethane (3 x 100 mL) and concentrated, then dried under vacuum to give 3.12 g of product 2.24 as a slightly yellow solid (89%), with characterizations matching literature data.\textsuperscript{74}

\( ^1H \) NMR (500 MHz, DMSO-\( d_6 \)) \( \delta: 10.12 \) (t, \( J=5.6 \) Hz, 1H), 8.54-8.41 (m, 1H), 8.25 (dd, \( J=7.9 \), 1.9 Hz, 1H), 7.63 (dd, \( J=8.2 \), 3.1 Hz, 1H), 4.77 (d, \( J=5.2 \) Hz, 2H). \( ^13C \) NMR (126 MHz, DMSO-\( d_6 \)) \( \delta: 166.97, 158.35, 149.56, 147.40, 138.00, 135.22, 133.09, 131.74, 41.82.\n
4-(((tert-Butoxycarbonyl)amino)methyl)-3-nitrobenzoic acid. Compound 2.25.
To 2.24 (1.04 g, 3.56 mmol) was added potassium carbonate (1.24 g, 8.97 mmol) and 10 mL of a 1:1 mixture of methanol–water. The pH was tested via litmus paper and was found to be a pH of at least 8. After stirring overnight, the solvent was concentrated on a rotary evaporator. The resultant crude was dissolved in 24 mL of a 1:1 mixture of dioxane–water. Then di-tert-butyl dicarbonate (1.18 g, 5.41 mmol, 1.5 eq) was added, dissolved in 10 mL of the solvent, and the reaction was stirred for three hours. The solvent was again concentrated on a rotary evaporator. The crude was then diluted with 100 mL of water and washed with diethyl ether (3 x 100 mL). Afterwards, 10% citric acid was added dropwise until a pH of 3 was achieved, and the aqueous portion was extracted with ethyl acetate three times. The combined organic layers were then dried with magnesium sulfate, filtered, concentrated with rotary evaporation and dried under vacuum to 981 mg of product 2.25 as a yellow solid (93%) over two steps, and characterizations matched literature procedures. 

$^1$H NMR (300 MHz, CDCl$_3$) δ: 8.73 (s, 1H), 8.29 (d, t=7.8 Hz, 1H), 7.75 (d, J=8.0 Hz, 1H), 5.36 (s, 1H), 4.65 (d, J=6.5 Hz, 2H), 1.44 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ: 166.97, 158.35, 149.56, 147.40, 138.00, 135.72, 133.09, 131.74, 127.06, 41.82.

**tert-Butyl (4-(hexylcarbamoyl)-2-nitrobenzyl)carbamate. Compound 2.26.**

to 2.25 (1.67 g, 5.64 mmol) of compound, dissolved in 350 mL of chloroform, was added
diisopropylethylamine (2.95 mL, 16.9 mmol), hydroxybenzotriazole (0.229 g, 1.69 mmol), and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (2.78 g, 7.33 mmol). After 30 minutes, hexylamine (2.24 mL, 16.9 mmol) was added. The reaction was then allowed to stir overnight, after which it was washed with 100 mL of water, and the aqueous portion was extracted with chloroform (2 x 100 mL). The organic layers were then combined and washed with saturated sodium chloride, dried with magnesium sulfate, filtered and concentrated by rotary evaporation. Column chromatography using gradient elution with 25–50% ethyl acetate/hexanes gave 1.13 g of orange-yellow product 2.26 (53%). $R_f = 0.18$ (25% ethyl acetate/hexanes).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 8.33 (d, $J = 1.8$ Hz, 1H), 7.92 (dd, $J = 8.0$, 1.9 Hz, 1H), 7.54 (d, $J = 8.1$ Hz, 1H), 7.01 (t, $J = 5.9$ Hz, 1H), 5.55 (t, $J = 6.5$ Hz, 1H), 4.53 (d, $J = 6.5$ Hz, 2H), 3.40 (q, $J = 7.0$ Hz, 2H), 1.58 (m, 2H), 1.40 (s, 9H), 1.35 – 1.22 (m, 6H), 0.86 (t, $J = 6.8$ Hz, 3H). $^{13}$C NMR (300 MHz, CDCl$_3$) $\delta$: 165.01, 156.07, 147.69, 137.61, 135.15, 132.17, 130.89, 123.68, 80.16, 42.26, 40.51, 31.56, 29.51, 28.40, 26.75, 22.62, 14.10. HRMS-DART: [M-H] calcd for C$_{19}$H$_{29}$N$_3$O$_5$, 378.2034; found 378.2018.

tert-Butyl (4-(octadecylcarbamoyl)-2-nitrobenzyl)carbamate. Compound 2.47.

![Chemical structure of tert-Butyl (4-(octadecylcarbamoyl)-2-nitrobenzyl)carbamate](image)

To 1.12 g (3.78 mmol) of compound 2.26, dissolved in 40 mL of chloroform, was added diisopropylethylamine (DIEA, 3.30 mL, 18.9 mmol), hydroxybenzotriazole (HOBt, 509 mg, 3.77 mmol), and O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU, 1.43 g, 3.78 mmol.) After 30 minutes, octadecylamine (2.03 g, 7.53 mmol) was added. The reaction was then
allowed to stir overnight, after which 100 mL water was added, and it was extracted with chloroform (2 x 100 mL). The organic layers were then combined and washed with saturated sodium chloride, dried with magnesium sulfate, filtered and concentrated by rotary evaporation. Column chromatography using gradient elution with 10–25% ethyl acetate/hexanes gave 3.56 g of orange-yellow product 2.47 (53%). Rf = 0.26 (25% ethyl acetate-hexanes).

\(^1\)H NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) 8.40 (s, 1H), 7.99 (s, 2H), 7.67 (s, 1H), 6.49 (s, 1H), 5.00 (dd, \(J = 402.9, 6.4\) Hz, 1H), 3.45 (s, 2H), 1.72 – 1.57 (m, 2H), 1.44 (s, 9H), 1.36 – 1.14 (m, 28H), 0.88 (t, \(J = 7.0\) Hz, 3H).

4-((4-(Hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid. Compound 2.27.

To compound 2.26 (1.19 g, 4.01 mmol), which was placed in a 50 mL round-bottom flask and chilled in an ice bath, was added 15 mL of a 20 v/v% solution of trifluoroacetic acid in dichloromethane. After one hour of stirring, starting material was no longer detected via TLC, and the solution was then concentrated and dried under high vacuum. To the resulting residue was then added acetonitrile (40 mL), dry potassium carbonate (1.67 g, 12.0 mmol), and succinic anhydride (0.442 g, 4.42 mmol), and the mixture was allowed to stir at room temperature. After 24 hours, 10% citric acid (50 mL) was added, and the reaction was extracted with ethyl acetate (3 x 50 mL). The organic layers were then combined and washed with saturated sodium chloride, dried with magnesium sulfate, filtered, and concentrated using rotary evaporation. Column chromatography with gradient elution of 50–100% ethyl acetate/hexanes.
including 0.2% acetic acid gave product 2.27 as a light yellow solid (893 mg, 59% yield). Rf=0.36 (10% methanol-dichloromethane).

\[ ^1H \text{ NMR (300 MHz, CD}_3\text{OD): } \delta 8.45 \text{ (d, } J = 1.7 \text{ Hz, 1H), 8.04 \text{ (dd, } J = 8.1, 1.6 \text{ Hz, 1H), 7.68 \text{ (d, } J = 8.1 \text{ Hz, 1H), 4.68 \text{ (s, 2H), 3.35 \text{ (t, } J = 7.2 \text{ Hz, 2H), 2.64 - 2.50 \text{ (m, 4H), 1.67-1.51 \text{ (m, 2H), 1.44 - 1.24 \text{ (m, 6H), 0.89 \text{ (t, 6.6 Hz, 3H).}} \]

\[ ^13\text{C NMR (300 MHz, CD}_3\text{OD): } \delta: 176.20, 175.10, 173.45, 149.33, 138.38, 135.85, 135.85, 131.05, 124.67, 74.12, 43.82, 41.22, 32.64, 30.29, 29.79, 27.74, 23.61, 14.36. \]


1-Palmitoyl-2-(4-((4-hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanylo-sn-glycero-3-phosphocholine (NB-PC). Compound 2.17.

In a six dram vial capped with a rubber septum, compound 2.27 (211 mg, 0.556 mmol) was combined with dicyclohexylcarbodiimide (DCC, 0.86 mL, 0.556 mmol) and N,N-dimethylaminopyridine (DMAP, 0.068 g, 0.556 mmol) in 4Å-molecular sieve-dried ethanol-free chloroform (2 mL) under argon, along with crushed glass, similar to a method described by Rosseto and Hadju.\textsuperscript{29} After 30 min, palmitoyl-lysophosphatidylcholine (2.20, LPC, 69 mg, 0.138 mmol) was added in one portion and an argon atmosphere was then reestablished. After 6.5 hours of sonication, Dowex 50Wx8 residue was added and the mixture was sonicated for 30 min before filtration through a fritted filter. After filtration and concentration, normal phase column chromatography was carried out through elution with 15%
methanol-dichloromethane containing 0.2% acetic acid to remove any unreacted acid, followed by 65:25:4 chloroform-methanol-water to remove the product. Residual water was removed by repeatedly concentrating with acetonitrile. The solution was then concentrated to provide a slightly yellow lipid-like substance 2.17 (65%). R\textsubscript{f} = 0.32 (65:25:4 chloroform-methanol-water).

\(^1\)H NMR (500 MHz, 60% CDCl\textsubscript{3}-CD\textsubscript{3}OD) δ: 8.58 (s, 1H), 8.51 (s, 1H), 8.47 – 8.40 (m, 1H), 8.11 (d, J = 8.0 Hz, 1H), 7.67 (d, J = 7.8 Hz, 1H), 5.27 – 5.20 (m, 1H), 4.73 (s, 2H), 4.36 (d, J = 11.3 Hz, 1H), 4.27 – 4.15 (m, 3H), 4.08 – 3.97 (m, 2H), 3.58 (s, 2H), 3.45 – 3.37 (m, 2H), 3.27 – 3.16 (m, 9H), 2.80 – 2.53 (m, 4H), 2.32 (t, J = 7.0 Hz, 2H), 1.63 (m, 4H), 1.43 – 1.22 (m, 30H), 0.95 – 0.85 (m, 6H). \(^{13}\)C NMR (500 MHz, 60% CDCl\textsubscript{3}-CD\textsubscript{3}OD) δ: 174.45, 173.55, 172.81, 166.31, 148.43, 137.34, 135.37, 132.33, 130.35, 124.44, 71.53, 66.78, 64.23, 62.72, 59.48, 54.34, 41.16, 40.86, 34.34, 32.30, 31.93, 30.73, 30.68, 30.05, 30.02, 30.01, 29.99, 29.91, 29.87, 29.72, 29.66, 29.50, 27.11, 25.21, 22.94, 14.22. \(^{31}\)P NMR (500 MHz, 60% CDCl\textsubscript{3}-CD\textsubscript{3}OD) δ: -1.35, referenced to triphenyl phosphate at -17.70 ppm. AccuTOF DART-ESI: [M+Na]\textsuperscript+ calcd for C\textsubscript{42}H\textsubscript{73}N\textsubscript{4}O\textsubscript{12}P, 879.4866; found 879.4869. [\(\alpha\)]\textsubscript{D}\textsuperscript{22.5} +4.3° (c = 2.9, CHCl\textsubscript{3}).

\((\text{S})\)-4-(((4-methoxybenzyl)oxy)methyl)-2,2-dimethyl-1,3-dioxolane. Compound 2.49.

\[
\begin{align*}
\text{HO} & \quad \text{NaH, DMP} \\
\text{2.48} & \quad \text{PMBCI} \\
\longrightarrow & \quad \text{PMBCI} \\
\text{2.49} & \quad \text{PMBCI}
\end{align*}
\]

Sodium hydride (439 mg, 60%, in mineral oil, 12.2 mmol) in 40 mL dimethylformamide was placed in a flame-dried 100 mL flask. To this was added compound 2.28 (1.00 mL, 8.10 mmol) in 20 mL of dimethylformamide dropwise at 0° C. After one hour, \(\text{para}\)-methoxybenzyl chloride (1.65 mL, 12.2 mmol) was added. After stirring the reaction overnight, it was quenched with methanol. The reaction was then diluted with 10 mL of water and extracted with dichloromethane (3 x 50 mL). It was then dried with magnesium sulfate and filtered. After concentrating via rotary evaporation, the crude was purified.
with 25% ethyl acetate/hexanes to give a mixture of products. This crude 2.29 was concentrated and used directly for the next reaction.

**(R)-3-((4-methoxybenzyl)oxy)propane-1,2-diol. Compound 2.30.**

Crude compound 2.29 (2.08 g, 8.01 mmol based on prior step) was dissolved in 65 mL methanol and para-toluenesulfonic acid (169 mg, 0.891 mmol) was added. After two days, sodium bicarbonate (150 mg, 1.78 mmol) was added to quench the reaction, and the solution was concentrated with rotary evaporation. Column chromatography with ethyl acetate then gave the product as a 1.41 g of clear oil 2.30 that solidified over time (83%, 2 steps). Characterization matched prior literature. 91

\[ ^1H \text{NMR (300 MHz, CDCl}_3\] \( \delta \): 7.32 – 7.13 (m, 2H), 6.84 (d, \( J = 8.6 \text{ Hz}, 2H)\), 4.42 (s, 2H), 3.84 (s, 2H), 3.75 (s, 3H), 3.68 – 3.48 (m, 3H), 3.48 – 3.38 (m, 1H). \[ ^13C \text{NMR (75 MHz, CDCl}_3\] \( \delta \): 159.25, 129.87, 129.43, 113.80, 73.05, 71.27, 70.87, 63.94, 55.20.

**(S)-1-((tert-butyldiphenylsilyl)oxy)-3-((4-methoxybenzyl)oxy)propan-2-ol. Compound 2.31.**

To compound 2.30 (127 mg, 0.600 mmol) dissolved in 10 mL anhydrous dimethylformamide was added tert-butyldiphenylsilyl chloride (0.190 mL, 0.720 mmol) and imidazole (102 mg, 1.50 mmol). After 18 hours, the reaction solvent was removed, and the crude product was diluted with 15 mL of water, and extracted with dichloromethane (3 x 20 mL). The crude was then dried with magnesium sulfate, filtered, and concentrated via rotary evaporation. Column chromatography with 25–50% ethyl acetate/hexanes gave the clear product 2.31 (45%). Characterization matched prior literature. 91
$^1$H NMR (500 MHz, CDCl$_3$) δ 7.65 (d, $J = 7.4$ Hz, 4H), 7.42 (t, $J = 7.3$ Hz, 2H), 7.40 – 7.33 (m, 4H), 7.21 (d, $J = 8.6$ Hz, 2H), 6.85 (d, $J = 8.6$ Hz, 2H), 4.46 (s, 2H), 3.90 (m, 1H), 3.78 (s, 3H), 3.71 (d, $J = 5.4$ Hz, 2H), 3.61 – 3.46 (m, 2H), 2.50 (d, $J = 4.9$ Hz, 1H), 1.05 (s, 9H). $^{13}$C NMR (126 MHz, CDCl$_3$) δ 159.11, 135.41, 133.09, 130.00, 129.64, 129.21, 127.61, 113.67, 72.93, 70.67, 64.67, 55.13, 26.72, 19.14.

Trans-2-Phenyl-1,3-dioxan-5-ol. Compound 2.41.

![Chemical structure of the reaction](image)

Compound 2.40 (10.3 g, 97.11 mmol, 1.3 eq) and compound 2.39 (12.2 g, 133 mmol) were dissolved in 40 mL of dichloromethane and p-toluenesulfonic acid (2.53 g, 13.29 mmol) was added, along with some 4Å molecular sieves. The reaction was refluxed for 16 hours. The reaction crude was then filtered through Celite and extracted with 200 mL of a 1% potassium carbonate aqueous solution. The aqueous portion was extracted (2 x 100 mL). The combined organic layer was dried with magnesium sulfate and filtered. After concentrating, it was separated on normal phase column chromatography using a 25-50% ethyl acetate/hexane gradient. Benzaldehyde eluted at 10% and a mixture of condensed products eluted at 50%. The mixture of the cis and trans dioxane and dioxolane spots (four spots total) were collected. Recrystallization from 1:1 petroleum ether/benzene, heating to 50° C and cooling to -20° C, gave pure trans dioxane product 2.41 as a white solid after two rounds of recrystallization (12%). Characterization matched previous literature.\(^{17,92}\)

$^1$H NMR (300 MHz, CDCl$_3$) δ: 7.52 – 7.45 (m, 2H), 7.45 – 7.35 (m, 3H), 5.39 (s, 1H), 4.22 (dd, $J = 11.1, 5.0$ Hz, 2H), 3.94 – 3.78 (m, 1H), 3.52 (t, $J = 10.0$ Hz, 2H), 2.71 (d, $J = 4.8$ Hz, 0H). $^{13}$C NMR (75 MHz, ) δ: 137.34, 129.19, 128.42, 126.15, 100.98, 71.59, 61.10.

Octadecyl 4-methylbenzenesulfonate. Compound 2.42.
Stearyl alcohol (2.50, 3.0 g, 11.19 mmol) was dissolved in 100 mL of dichloromethane. Triethylamine (19.2 mL, 137.69 mmol) and \( \text{para-} \)toluenesulfonyl chloride (3.30 g, 17.3 mmol) were added. The next day, 200 mL of 1 M hydrochloric acid was added, and this crude was extracted with dichloromethane (2 x 100 mL). The combined organic layers were dried with magnesium sulfate, filtered, and concentrated via rotary evaporation. Column chromatography with 10–25% ethyl acetate/hexanes gave 3.8258 g of product 2.42 as a white solid (81%). Characterization matched a similar procedure.  

\[ ^1H \text{ NMR (300 MHz, CDCl}_3\) \delta 7.79 (d, \( J = 7.9 \) Hz, 2H), 7.34 (d, \( J = 7.6 \) Hz, 2H), 4.01 (t, \( J = 6.5 \) Hz, 2H), 2.45 (s, 3H), 1.68 – 1.54 (m, 2H), 1.23 (d, \( J = 11.9 \) Hz, 30H), 0.88 (s, 3H). \]  

\[ ^{13}C \text{ NMR (75 MHz, CDCl}_3\) \delta 212.76, 144.48, 129.65, 127.75, 70.59, 31.80, 29.57, 29.48, 29.38, 29.25, 28.80, 28.66, 25.18, 22.58, 21.53, 14.02. \]  

5-(Octadecyloxy)-2-phenyl-1,3-dioxane. Compound 2.43.

To compound 2.41 (100 mg, 0.555 mmol) was added 60 mL of toluene, compound 2.42 (589 mg, 1.39 mmol), and potassium hydroxide (150 mg, 1.67 mmol). The reaction was heated to 100 °C overnight. It was then heated to 125 °C for 9 more hours and then cooled to room temperature. The filtrate was poured through Celite and then concentrated. The organic layer was then diluted with 100 mL diethyl ether and was washed (2 x 50 mL) with saturated sodium chloride solution. Then the organic layer was dried with magnesium sulfate, filtered, and concentrated. Column chromatography with 10% ethyl acetate/hexanes gave 122 mg of product 2.43 as a white solid (51%).
$^1$H NMR (300 MHz, CDCl$_3$) δ: 7.63 – 7.44 (m, 2H), 7.43 – 7.27 (m, 3H), 5.54 (s, 1H), 4.33 (d, $J$ = 13.9 Hz, 2H), 4.04 (d, $J$ = 12.4 Hz, 2H), 3.54 (t, $J$ = 6.8 Hz, 2H), 3.34 – 3.18 (m, 1H), 1.77 – 1.55 (m, 2H), 1.43 – 1.17 (m, 30H), 0.89 (s, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ: 138.16, 128.81, 128.13, 126.19, 101.31, 70.58, 69.05, 68.99, 31.94, 29.80, 29.72, 29.70, 29.67, 29.64, 29.50, 29.38, 26.14, 22.71, 14.14.

2-(octadecyloxy)propane-1,3-diol. **Compound 2.52.**

To compound 2.51 (89.7 mg, 0.207 mmol) was added 2 drops of concentrated hydrochloric acid in 10 mL of methanol. After reaction overnight, the solvent was concentrated under vacuum and the residue was diluted with 10 mL water and extracted (3 x 10 mL) with diethyl ether. The combined organic layer was dried with magnesium sulfate, filtered and concentrated using a rotary evaporator. Column chromatography with 50–100% ethyl acetate/hexanes eluted the product, which was found by staining TLC plates with phosphomolybdic acid. After concentrating and drying the relevant fractions under vacuum, 60.8 mg of the white solid product 2.52 was collected (85%).

$^1$H NMR (300 MHz, CDCl$_3$) δ: 3.77 (dd, $J$ = 11.6, 4.5 Hz, 2H), 3.68 (dd, $J$ = 11.6, 4.9 Hz, 2H), 3.57 (t, $J$ = 6.7 Hz, 2H), 3.46 (p, $J$ = 4.7 Hz, 1H), 2.17 (s, 2H), 1.60 (p, $J$ = 6.8 Hz, 2H), 1.40 – 1.15 (m, 30H), 0.88 (t, $J$ = 6.4 Hz, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ: 78.62, 69.34, 61.26, 31.08, 29.19, 28.86, 28.84, 28.82, 28.78, 28.75, 28.63, 28.53, 25.26, 21.86, 13.30.

**2.8 Fluorescence-Based Irradiation Kinetics Assay of NB-PC**

Stock solutions were initiated by weighing out samples of DOPC, DOPE, cholesterol, DSPE-PEG$_{2000}$ amine, and/or NB-PC along with Nile red in vials. To the appropriate lipid components corresponding to each liposome sample was added 500 μL of ethanol-free chloroform, and after brief vortexing, proper
volumes of each lipid were pipetted into a clean vial per calculations on a 5 mM, 500 μL total lipid scale, to obtain the desired molar percentage of each component. Next, a solution of Nile red was added per calculations on a 250 μM, 500 μL scale.

As an example, for liposomes comprised of 50% NB-PC and 50% DOPE, 500 μL of ethanol-free chloroform were added to separate vials containing 4.9 mg NB-PC, 3.6 mg DOPE, and 4.2 mg Nile red, producing stock solutions of 11, 9.7, and 26 mM concentrations, respectively. After 30 seconds of vortexing, 109.4 μL of NB-PC, 129.2 μL of DOPE, and 4.74 μL of Nile red stock solutions were combined in a new vial. The chloroform was dried with a nitrogen stream, and the lipids were subsequently dried overnight under vacuum. The next day, the lipids were hydrated with 500 μL of MilliQ purified water, vortexed, and incubated on a rotary evaporator at 60 °C for 3 sets of 20 min, with vortexing after each set. Liposomes were frozen in a -40° C dry ice bath and then transferred to a 60° C water bath for 10 cycles and extruded through a 200 nm membrane for 21 passes using a LiposoFast extruder (Avestin, Inc.), placing the uniform-sized vesicles into a fresh vial. DLS scans were performed to confirm the formation of stable liposomes.

Next, 70 μL of this liposomal solution was diluted to 7 mL with ultrapure water. Two identical samples were made by placing 3 mL of this dilute solution into quartz cuvettes and were sealed with parafilm to minimize atmospheric exposure. After an initial fluorescence scan (λ_ex = 595 nm; λ_em = 612 nm), one sample was irradiated with 350 nm light while suspended and covered by Pyrex beakers between four 350 nm bulbs in a Rayonet Preparative Type RS photoreactor. The other sample was placed in a dark container. For each fluorescence scan (every 5 minutes), the sample was removed from the reactor or dark container and placed in the fluorimeter for scanning. Total amounts of time plotted for release experiments represent the amount of time the sample spent in the photoreactor. Experiments were run at least four times each, including runs with different batches of liposomes, and
averaged to obtain the results shown in Figures 2.1 to 2.5, with error bars included to depict standard error. Data were then curve fit using an exponential decay equation in SigmaPlot to obtain the values shown in Tables 2.1-2.5, according to Equation 2.1 and Equation 2.2.
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Spectra 2.16: gHSQC NMR. Compound 2.17. 1-Palmitoyl-2-((4-((4-hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoyl)-sn-glycero-3-phosphocholine (NB-PC) ............................................................... 79
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1H NMR (500 MHz, DMSO): δ 12.93 (s, 1H), 10.08 (d, J = 6.1 Hz, 1H), 7.93 (d, J = 8.3 Hz, 2H), 7.39 (d, J = 8.3 Hz, 2H), 4.46 (dd, J = 6.1 Hz, 2H).

Spectra 2.1: ¹H NMR. Compound 2.23. 4-((2,2,2-trifluoroacetamido)methyl)benzoic acid
Spectra 2.2: $^{13}$C NMR. **Compound 2.23. 4-((2,2,2-trifluoroacetamido)methyl)benzoic acid**
$^1$H NMR (500 MHz, DMSO-$d_6$): δ 10.12 (s, 1H), 8.47 (s, 1H), 8.25 (dd; 7.9, 1.9 Hz, 1H), 7.63 (dd, $J = 8.2$, 3.1 Hz, 1H), 4.77 (dt; 5.2 Hz, 2H).

Spectra 2.3: $^1$H NMR. Compound 2.24, 3-Nitro-4-((2,2,2-trifluoroacetamido)methyl)benzoic acid
\[^{13}\text{C} \text{NMR (126 MHz, dClO}_{2}F\text{dClO}_{2}F 166.97, 158.35, 149.56, 147.40, 138.00, 135.72, 133.09, 131.74, 127.06, 41.82.}\]

**Spectra 2.4: \(^{13}\text{C} \text{NMR. Compound 2.24. 3-Nitro-4-((2,2,2-trifluoroacetamido)methyl)benzoic acid**
$^1$H NMR (300 MHz, Chloroform-$d$) $\delta$ 8.73 (s, 1H), 8.29 (dd, $J$ = 7.8 Hz, 1H), 7.75 (d, $J$ = 8.0 Hz, 1H), 5.36 (s, 1H), 4.65 (dd, $J$ = 6.5 Hz, 2H), 1.44 (s, 8H).

Spectra 2.5: $^1$H NMR. **Compound 2.25.** 4-(((tert-Butoxycarbonyl)amino)methyl)-3-nitrobenzoic acid
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Spectra 2.7: \( ^1 \text{H NMR.} \) Compound 2.26. 4-((4-(Hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid
Compound 2.26. 4-((4-Hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid

$^{13}$C NMR (300 MHz, CDCl$_3$):
165.01, 156.07, 147.69, 137.61, 135.15, 132.17, 130.89, 123.68
1H NMR (500 MHz, Chloroform-d) δ 8.40 (s, 1H), 7.99 (s, 2H), 7.67 (s, 1H), 6.49 (s, 1H), 5.00 (dd, J = 402.9, 6.4 Hz, 1H), 3.45 (s, 2H), 1.72 – 1.57 (m, 2H), 1.44 (s, 9H), 1.36 – 1.14 (m, 28H), 0.88 (t, J = 7.0 Hz, 3H).

Spectra 2.9: 1H NMR. Compound 2.47. 4-((4-(Dodecylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid
Spectra 2.10: ¹H NMR. **Compound 2.27.** 4-((4-(Hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid
\[ \text{Spectra 2.11: } ^{13}\text{C NMR. Compound 2.27. 4-((4-Hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid} \]
Spectra 2.12: $^1$H NMR. Compound 2.17. 1-Palmitoyl-2-((4-(hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoyl)-sn-glycero-3-phosphocholine (NB-PC)
13C NMR (500 MHz, 60% CDCl₃/OD, δ): 174.45, 173.55, 172.81, 166.31, 148.43, 137.34, 135.37, 132.33, 130.35, 124.44, 71.53, 66.78, 64.23, 62.72, 59.48, 54.34, 41.16, 40.86, 34.34, 32.30, 31.93, 30.73, 30.68, 30.05, 30.02, 30.01, 28.99, 29.91, 29.87, 29.72, 29.66, 29.50, 27.11, 25.21, 22.94, 14.22.

Spectra 2.13: 13C NMR. **Compound 2.17.** 1-Palmitoyl-2-(4-((hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanylo)-sn-glycero-3-phosphocholine (NB-PC)
$^{31}\text{P} \text{ NMR (500 MHz, 60\% CDCl}_3\text{OD, }\delta)$:

-1.35, referenced to triphenyl phosphate at -17.70 ppm.

Spectra 2.14: $^{31}\text{P} \text{ NMR. Compound 2.17, 1-Palmitoyl-2-}$(4-(4-(hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanylo) $sn$-glycero-3-phosphocholine (NB-PC)
Spectra 2.15: gCOSY NMR. **Compound 2.17.** 1-Palmitoyl-2-((4-(hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoyl)-sn-glycero-3-phosphocholine (NB-PC)
Spectra 2.16: gHSQC NMR. **Compound 2.17.** 1-Palmitoyl-2-((4-(hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoyl)-sn-glycero-3-phosphocholine (NB-PC)
Spectra 2.17: gHMBC. Compound 2.17. 1-Palmitoyl-2-(4-((4-(hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoyl)-sn-glycero-3-phosphocholine (NB-PC)
$^1$H NMR (300 MHz, δ 7.32 – 7.13 (m, 2H), 6.84 (s, 8.6 Hz, 2H), 4.42 (s, 2H), 3.84 (s, 2H), 3.75 (s, 3H), 3.68 – 3.48 (m, 3H), 3.48 – 3.38 (m, 1H).

Spectra 2.18: $^1$H NMR. Compound 2.30. (R)-3-((4-methoxybenzyl)oxy)propane-1,2-diol


$\^13$C NMR (75 MHz, $\delta$ 159.25, 129.87, 129.43, 113.80, 73.05, 71.27, 70.87, 63.94, 55.20).

Spectra 2.19: $\^13$C NMR. **Compound 2.30.** (R)-3-((4-methoxybenzyl)oxy)propane-1,2-diol
\[ \text{Compound 2.31}: (S)-1-((\text{tert-Butyldiphenylsilyl})\text{oxy})-3-((4\text{-methoxybenzyl})\text{oxy})\text{propan-2-ol} \]
\[\text{Compound } 2.31, \ (S)-1-((\text{tert-Butyldiphenylsilyl})\text{oxy})-3-((4\text{-methoxybenzyl})\text{oxy})\text{propan-2-ol}\]
Spectra 2.22: $^1$H NMR. **Compound 2.41.** *trans*-2-Phenyl-1,3-dioxan-5-ol
Spectra 2.23: $^{13}$C NMR. **Compound 2.41.** trans-2-Phenyl-1,3-dioxan-5-ol


Spectra 2.24: $^1$H NMR. **Compound 2.42.** Octadecyl 4-methylbenzenesulfonate
$^{13}$C NMR (75 MHz) δ 212.76, 144.48, 129.65, 127.75, 70.59, 31.80, 29.57, 29.48, 29.38, 29.25, 28.80, 28.66, 25.18, 22.58, 21.53, 14.02.

Spectra 2.25: $^{13}$C NMR. Compound 2.42. Octadecyl 4-methylbenzenesulfonate
H NMR (300 MHz, CDCl₃) δ 6.3 – 7.44 (m, 2H), 7.43 – 7.27 (m, 3H), 5.54 (s, 1H), 4.53 (d, J = 13.9 Hz, 2H), 4.04 (d, J = 12.4 Hz, 2H), 3.54 (d, J = 6.8 Hz, 2H), 3.34 – 3.18 (m, 1H), 1.77 – 1.55 (m, 2H), 1.43 – 1.17 (m, 30H), 0.89 (s, 3H).

Spectra 2.26: Compound 2.43. 5-(Octadecyloxy)-2-phenyl-1,3-dioxane
Spectra 2.27: Compound 2.43. 5-(Octadeoxyloxy)-2-phenyl-1,3-dioxane
$^1$H NMR (300 MHz, Chloroform-d) $\delta$ 3.77 (dd, $J = 11.6, 4.5$ Hz, 2H), 3.68 (dd, $J = 11.6, 4.9$ Hz, 2H), 3.57 ($J = 6.7$ Hz, 2H), 3.46 ($J = 4.7$ Hz, 1H), 2.17 (s, 2H), 1.60 ($J = 6.8$ Hz, 2H), 1.40 – 1.15 (m, 30H), 0.88 ($J = 6.4$ Hz, 3H).

Spectra 2.28: $^1$H NMR. **Compound 2.44. 2-(Octadecyloxy)propane-1,3-diol**

Spectra 2.29: 1H NMR. **Compound 2.44.** 2-(Octadeoxyloxy)propane-1,3-diol
Spectra 2.30: Dynamic light scattering of NB-PC (Compound 2.17), kept in dark. Mean = 164.9 nm.
Spectra 2.31: Dynamic light scattering of NB-PC (Compound 2.17) irradiated at 350 nm for 90 minutes. Mean = 186.3 nm.
Chapter 3: Phosphatidylcholine-Analog Lipid with a Nitrophenethyl Moiety within the sn-2 Fatty Acid Chain for One and Two Photon Release

3.1 Introduction

The NB-PC lipid described in chapter 2 was designed with a one photon release in mind, as the α-NB protecting group releases caged substrates within the 300 to 400 nm range. The efficiency, however, of one-photon uncaging lies between 0.1 to 1%. The α-NB groups also do not have very efficient releases, with most derivatives of 0.1 to 0.3 GM at the far end of the visible region or the near end of the infrared region. The intensity of the laser must also be carefully controlled, because the intensity required for obtaining a good release of α-NB groups can be powerful enough to cause cell death above 7 mW. Damage occurs because the probability of excitation depends on the square of the intensity used, and photobleaching is probable at high power. For biological applications it is ideal to have the wavelength of release in the visible or near infrared range. This will minimize damage to proteins and nucleic acids with aromatic groups.

3.2 Two-Photon Excitation

An alternative to traditional absorbance irradiation is two-photon excitation (2PE), which has many advantages. It utilizes infrared light, so it penetrates further into tissue, up to 1 mm as reported by Svoda et al., or up to 8 mm in bovine tissue. It also causes less scattering of light. Furthermore, it can also be used on a femtoliter volume scale. TPA’s uncaging cross-section, which measures its usefulness for release, has units of Goeppert-Mayer (GM), and it is the product of the cross sectional area of the photon-absorbing portion of the molecule, \( \delta_u \), and the efficiency of substrates released per photon, \( \Phi_u \) (\( 1 \text{ GM} = 10^{-50} \text{ cm}^2/\text{photon} \)). Compounds need to have at least 0.01 GM to be considered for biological application, but at least 3 GM is desirable.

Two-photon excitation was first developed by Webb et al. One of the most important features of this technique for chemical and biochemical applications is that only more biologically inert infrared
light is used. The technique involves using excitation using two photons with twice the wavelength (half energy) compared to the energy of an ultraviolet photon. The two photons must impact the same chromophore on a small enough time scale to create an exited state. The absorbance is limited by the properties of the chromophore itself. Additionally, due to its nonlinear nature in which the probability of excitation depends on the square of the intensity of the laser beam, excitation can be localized to the focal volume when using dual laser beams for absorbance. This also significantly lowers the probability of photobleaching. The width of the focal volume will be directly proportional to the wavelength of irradiation and inversely related to the numerical aperture of the lens.

The method of improving $\phi_u$ is not completely understood yet, but $\delta_a$ seems to be generally improved by extension of the conjugated system or by adding strong donor and acceptor moieties on the opposite sides of the planar system. On the other hand, this is not always the case, as elongating the nitrobenzyl system with a styrene substituent did not have an appreciable effect on the cross action section.

### 3.3 Two Photon-Driven Release

A select few examples of two-photon release systems exist, but the field is still developing. One of these examples is the protection of the 7-hydroxycoumarin-3-carboxamide derivatives with the 1-(2-nitrophenyl)ethyl cage, which has been reported at 0.6 GM at 740 nm (Figure 3.1). More recently, the 3-(2-propyl)-4’-tris-ethoxy(methoxy)-4-nitrophenyl (PEMB, 3.1) protecting group was used with a fluorophore, having an uncaging cross-section of 3.7 GM at 740 nm. The producers of this cage, the Goueldner group, also later also synthesized a 3-2-(propyl-1-ol)-4’-methoxy-4-nitro biphenyl cage (EANBP, 3.2), exhibiting 11 GM at 800 nm.

In another published paper, Buhler et al. envisioning the potential for microelectronics and DNA analysis, synthesized a number of 2-(2-nitrophenyl)propoxy)carbonyl (NPPOC) derivatives (3.3) and
Figure 3.1: Different caging groups with extended π systems

Compounds 3.1 to 3.3 are different forms of nitrophenethyl (NBP) systems. Compound 3.4 is a styryl coumarin derivative with a high cross-section. Compound 3.5 eliminates the leaving group by cyclizing. Additionally are nitrobenzofuran (3.6) and coumarin derivatives (3.7 and 3.8).
used them to release thymidine under laboratory conditions. It was discovered that the substituents exhibited a broad effect on the photocleavage rate. Additionally, Gagey et al. used an o-hydroxycinnamic chromophore (3.5) in zebrafish, having 1 to 10 GM at 750 nm.  

Lusic et al. redeveloped a new synthesis from the 3-nitro-2-ethylbenzofuran (3.6), originally developed by Momotake et al., and found that this group releases its substrate at 0.6 GM at 710 nm. A couple of coumarin derivatives have also been used, including the brominated 7-hydroxycoumarin-4-ylmethyl (Bhc, 3.7) and the 8-bromo-7-hydroxyquinolone (BHQ, 3.8). These cages have two-photon cross section values at 0.72 GM and 0.59 GM at 740 nm. Another extension of the coumarin system utilized a styryl-conjugated derivative (3.4), which claims a two-photon cross section of 309 GM, by far the highest cross-section known.

3.4 Comparison of the o-Nitrobenzyl and Nitrophenethyl  
The nitrobenzyl and the nitrophenethyl (3.1 to 3.3 in Figure 3.1) both have similar mechanisms of photocleavage (Figure 3.2). After irradiation of the original molecule (3.9, 3.17), both compounds are placed into the excited state. This occurs at the weakest bond in the molecule, the N-O π bond (3.10, 3.18). The benzyl hydrogen is then abstracted via a six-membered ring transition state (3.11, 3.19). These structures are in resonance with the E-nitronic form (3.12, 3.20). For the o-NB system, proton transfer then produces the aci-nitro intermediate (3.13), then reprotonation occurs to the Z-nitronic form (3.14). With the o-NB system from the previous lipid NB-PC, discussed in chapter 2, the anionic species will cyclize into a five membered ring (3.15), simultaneously reestablishing aromaticity. Deprotonation and breakage of the ring forms the nitroso byproduct (3.16) and cleaves a bond at the benzylic position. The nitrobiphenethyl from the current lipid NBP-PC, however, does not have the leaving group at the benzylic position, but instead has an extra methylene. Instead of the five-membered ring, it undergoes elimination, reestablishing aromaticity and releasing the leaving group to 3.21.
For the 2-nitrobenzyl (NB) system, excitation of 3.9 leads to 3.10. Structure 3.11 is in resonance with $E$-nitronic acid 3.12. This then deprotonates to 3.13 and reprotonates to form $Z$-nitronic acid 3.14. Cyclization then occurs to 3.15, and nitroso product 3.16 forms. On the other hand, for the nitrobiphenethyl (NBP) system, the steps are parallel from the excitation of 3.17 to 3.18, abstraction to 3.19, and resonance to 3.20. Instead of proton transfer, aromaticity is reestablished and the leaving group, X, is expelled by forming a double bond.
3.5 Discussion of Synthesis of NBP-PC

Because the original lipid NB-PC was useful for photochemical release in the ultraviolet region, and not in the biologically friendly infrared region, we then sought to develop a photocleavable lipid for this purpose with a nitrophenethyl chromophore and a push-pull system containing nitro and methoxy groups at opposite ends. The synthesis (Figure 3.3) began with commercially available 4-ethylaniline (3.22) and followed the procedures of other similar papers.101, 108 Acidic nitration first protonates the aniline so that it becomes an electron-withdrawing group and then meta-direction reinforces the ethyl group to direct nitration to the position ortho to the ethyl position (3.23). Leaving the product in the protonated ammonium state gave the molecule more solubility for the following Sandmeyer reaction, performed in aqueous solution. After the iodide (3.24) was produced, hydroxymethylation with catalytic potassium tert-butoxide and paraformaldehyde gave the best yields for the production of 3.25. Other methods attempted were Triton B with paraformaldehyde or aqueous formaldehyde with potassium hydroxide.

Now that the triggerable nitrophenethyl handle was built, it was time to extend to system in order to enhance \( \delta_a \). Thus the Suzuki reaction was employed to conjugate the aryl halide (3.25) with an aromatic boronic acid (3.26) containing an electron-donating methoxy group, producing 3.27. To give the fatty acid chain some flexibility and further hydrophobicity, 4-aminobutyric acid was conjugated to the terminal hydroxy group of the nitrobiphenethyl system utilizing carbonyl diimidazole chemistry to form 3.28. The LPC itself was attached in the same manner as NB-PC, by using carbodiimide coupling under Steglich conditions to produce 3.29, or NBP-PC.

A different synthetic route was also attempted, starting the with commercially available 5-amino-2-nitrobenzoic acid. Trouble with recrystallization, however, made this synthetic route less facile. The finished product might still be interesting for synthesis, since the substitution of the final product would be different, with the nitro group on the opposite side of the biphenyl system instead of the
Compound 3.22 was nitrated to form the sulfuric salt 3.23. A sandmeyer reaction then produced 3.24. Hydroxymethylation from the acidic benzyl position led to 3.25. Coupling of the boronic acid 3.26 resulted in product 3.27. Coupling with carbonyldiimidazole, followed by 4-aminobutyric acid produced 3.28. Ester coupling then resulted in final compound 3.29 (NBP-PC).
carboxamide-attached linker.

Liposomes were then formed from the synthesized NBP-PC lipids. The kinetic studies were also carried out under the same conditions as NB-PC. Briefly, to a vial was aliquoted NBP-PC in chloroform, based on a 5 mM, 500 μL basis. Nile red was also added based on a 250 μM basis. After drying the solution, the lipids were suspended in water with vortexing to form liposomes. Incubation above the Tc, freeze-thaw cycles, and extrusion were carried out to produce uniform 200 nm unilamellar vesicles. Liposome solutions were then diluted and transferred to a cuvette. The sample was then irradiated at different intervals under UV irradiation, followed by reading the fluorescence of the sample. The same experiment was performed with DOPC instead as a control. Dynamic light scattering spectra were also taken of samples of 100% NBP-PC both kept in the dark and irradiated at 350 nm for 90 minutes. Minimal size change was seen in the distribution, which could just infer that the action of photodisruption was not fast enough to effect a change in liposome size.

The results of the photo-kinetics study showed faster and more extensive release than NB-PC. The release of Nile red from the 100% NBP-PC sample plateaued in about five minutes rather than 30 minutes for NB-PC (Figure 3.4, Table 3.1). The 100% DOPC control sample, on the other hand, did not appreciably release at all. What is also interesting is that the passive leakage of Nile red is additionally reduced in the dark for NBP-PC samples over DOPC samples. This may be explained by the π stacking of the NBP planar system with aromatic system of Nile red itself. The Tc will need to be measured to determine stability relative to natural lipid formulation, since it is possible that the biphenyl system stability does not have a Tc due to its resistance to passive leakage. Since this sample is a significant improvement over the NB-PC, it is a good candidate for two-photon irradiation studies, with a fast-pulsed infrared laser as the irradiation source. The flux must be significantly high for photocleavage to occur. Studies are currently underway to optimize the conditions for infrared release of this system.
Figure 3.4: Irradiation of 100% NBP-PC, NB-PC, and DOPC, along with corresponding dark controls. The NBP-PC exhibits more extensive and faster release with UV irradiation than NB-PC. Additionally, it also demonstrates a decreased leakage of Nile red in the dark.

Table 3.1: Irradiation and control for 100% NBP-PC, NB-PC, and DOPC.

Data was tabulated from Figure 3.4 according to Equation 2.1 and Equation 2.2

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<th>A</th>
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<th>k (s$^{-1}$)</th>
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3.6 Materials and Methods

Reagents and solvents were generally purchased from Acros, Aldrich, or Fisher Scientific and used as received. Palmitoyl-lysophosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Ablabster, AL) and 4-ethylaniline was purchased from Fisher Scientific. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Varian Mercury 300 MHz, Varian 500, or Varian 600 MHz spectrometer. Mass spectra were obtained with a JEOL AccuTof DART or a JEOL AccuTOF ESI spectrometer with high-resolution capabilities. Optical rotations were measured with a Perkin-Elmer 241 Polarimeter using the sodium D line. Ultrapure water was purified via a Millipore water system (≥ 18 MΩ·cm) triple water purification system. Detection of Nile red emission decreases attributed to release were performed using a Perkin Elmer LS55 fluorescence spectrometer. Samples were irradiated with a Rayonet Preparative Type RS photoreactor while suspended in a cuvette with Pyrex as a filter.

4-Ethyl-3-nitrobezeneammonium hydrogen sulfate. **Compound 3.23.**

![Chemical Reaction]

In a 50 mL round-bottom flask, commercially available 4-ethylaniline (**3.22**, 5.11 g, 42.2 mmol) was slowly dissolved in 22 mL of concentrated sulfuric acid and chilled to 0°C. Next, 6 mL of concentrated nitric acid was carefully added dropwise to the solution, minimizing smoke produced. After two hours the mixture was poured over crushed ice and the orange solid was filtered through a Büchner funnel lined with two pieces of filter paper. The filter cake was washed with small amounts of ice cold water, and then traces of water were removed by coevaporation with several portions of toluene (benzene was avoided due to toxicity issues). The resultant brown solid material (**3.23**, 10.1 g,
91%) was carried to the next step as the hydrogen sulfate salt, according to a similar literature precedent.\textsuperscript{108a}

\[^1\text{H} \text{ NMR (500 MHz, DMSO-}\text{d}_6\text{)} \delta: 8.10 \text{ (s, 3H), 7.56} \text{ (d, } J=2.2 \text{ Hz, 1H), 7.43} \text{ (d, } J=8.3 \text{ Hz, 1H), 7.29} \text{ (dd, } J=8.3, 2.3 \text{ Hz, 1H), 2.75} \text{ (q, } J=7.5 \text{ Hz, 2H), 2.17} \text{ (t, } J=7.5 \text{ Hz, 3H).} \]

\[^{13}\text{C} \text{ NMR (126 MHz, DMSO-}\text{d}_6\text{)} \delta: 149.07, 138.24, 132.26, 131.96, 124.24, 114.39, 24.73, 15.00.\]

1-Ethyl-4-iodo-2-nitrobenzene. \textbf{Compound 3.24.}

The ammonium salt from the previous reaction (3.23, 10.11 g, 38.3 mmol) was suspended in 170 mL of water and 20 mL of 50% hydrochloric acid (v/v), and the reaction was cooled to 0°C. After one hour, sodium nitrite (3.53 g, 5.12 mmol, in 16 mL of water) was added dropwise to the reaction, still at 0°C. After one more hour, 11.1 g of potassium iodide (11.1 g, 66.9 mmol) in a solution of 60 mL of saturated sodium acetate was added, upon which vigorous bubbling occurred due to evolution of nitrogen and orange smoke. The reaction was then allowed to stir overnight. It was then washed with 250 mL of 1M hydrochloric acid. The aqueous portion was extracted with ethyl acetate (3 x 150 mL). The organic layer was then washed with 100 mL of a 20% w/v solution of sodium thiosulfate to reduce the free iodide in solution. This was evident by the aqueous layer turning clear to yellow. The organic layer was then dried with magnesium sulfate and filtered. After concentration, column chromatography with 5–10% ethyl acetate/hexanes gave 4.40 g of pure product (3.24, 69%) as a red oil that solidified upon standing. NMR data for the product matched previous literature.\textsuperscript{101}

\[^1\text{H} \text{ NMR (300 MHz, CDCl}_3\text{)} \delta: 8.16 \text{ (s, 1H), 7.82} \text{ (d, } J=8.2 \text{ Hz, 1H), 7.12} \text{ (d, } J=8.2 \text{ Hz, 3H), 2.85} \text{ (q, } J=7.5 \text{ Hz, 7H), 1.26} \text{ (t, } J=7.5 \text{ Hz, 6H).} \]

\[^{13}\text{C} \text{ NMR (75 MHz, CDCl}_3\text{): } \delta^{^{13}\text{C} \text{ NMR (75 MHz, CDCl}_3\text{)} \delta 149.60,\]

105
141.80, 138.55, 132.98, 132.80, 89.88, 25.91, 14.77. HRMS-DART: [M+H]^+, calcd for C_{8}H_{8}NO_{2}I, 277.9673; found 277.96634.

2-(4-Iodo-2-nitrophenyl)propan-1-ol. **Compound 3.25.**

![Chemical structure](image)

Compound 3.24 (687 mg, 2.67 mmol) was dissolved in 10 mL of 4 Å sieve-dried dimethylsulfoxide. After 15 minutes, potassium tert-butoxide (42 mg, 0.374 mmol) was added in 1 mL of tert-butanol. The solution turned a dark purple upon addition. After 30 minutes, the solution was heated to 80°C, and after 30 minutes more, paraformaldehyde (81.0 mg, 2.67 mmol) was added, and the reaction was allowed to reflux overnight. One equivalent more of tert-butoxide and paraformaldehyde was added the next day when TLC confirms only starting material was present, and the reaction was allowed to reflux until the next morning, at which time no starting material was detected by TLC. The reaction was acidified with a saturated ammonium chloride solution until a pH of 3 or less was achieved. The crude material was then extracted with ethyl acetate (3 x 50 mL), and the combined organic layers were washed with 50 mL of saturated sodium chloride. After column chromatography with 25% ethyl acetate/hexanes, the product 3.25 was collected as red oil (562 mg, 68%). NMR data matched previous literature.\(^{101}\)

\[^1\text{H} \text{NMR (300 MHz, CDCl}_3 \delta: 8.05 \text{ (s, 1H)}, 7.87 \text{ (d, } J = 8.3 \text{ Hz, 1H)}, 7.24 \text{ (d, } J = 8.3 \text{ Hz, 1H)}, 3.85 - 3.66 \text{ (m, 2H)}, 3.46 \text{ (h, } J = 6.6 \text{ Hz, 1H)}, 1.82 \text{ (s, 1H)}, 1.30 \text{ (d, } J = 6.9 \text{ Hz, 3H)}. \[^{13}\text{C} \text{NMR (75 MHz, CDCl}_3 \delta: 150.98, 141.48, 137.81, 132.51, 129.95, 90.44, 67.43, 36.15, 17.27) \text{ HRMS-DART [M+H]^+, calcd for C}_{9}\text{H}_{10}\text{NO}_{3}I, 307.97781; found 307.97808.}

4-ethyl-4'-methoxy-3-nitro-1,1'-biphenyl-2-(4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propan-1-ol.
Compound 3.25 (521 mg, 1.92 mmol, 1 eq) was dissolved in 3 mL of 1,2-dimethoxyethane. Then 4-methoxyphenylboronic acid (442 mg, 2.00 mmol, 1.1 eq) and tetrakis(triphenylphosphorous) palladium (50 mg, 0.0433 mmol, 0.03 eq) were added and the solution was stirred for 10 minutes at room temperature. Next 1.46 g sodium carbonate and 7 mL water were added, and the reaction was stirred under reflux until the reaction was complete, as determined by TLC. It was then filtered through Celite with ethyl acetate and concentrated. Column chromatography with 10–25% ethyl acetate/hexanes gave 436 mg of product 3.26 as red oil (89%). NMR characterization matched literature precedent.  

$^1$H NMR (300 MHz, CDCl$_3$) δ: 7.91 (s, 1H), 7.74 (dd, $J = 8.2, 2.1$ Hz, 1H), 7.52 (d, $J = 8.8$ Hz, 3H), 6.99 (d, $J = 8.9$ Hz, 2H), 3.86 (s, 3H), 3.81 (dd, $J = 6.6, 3.8$ Hz, 2H), 3.53 (q, $J = 6.8$ Hz, 1H), 1.86 (s, 1H), 1.35 (d, $J = 6.9$ Hz, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 159.82, 140.98, 140.14, 135.94, 130.58, 128.57, 128.05, 121.89, 120.12, 114.46, 111.03, 104.97, 67.85, 55.38, 36.14, 17.55. HRMS-DART [M+H]$^+$, calcd for C$_{16}$H$_{17}$NO$_4$, 288.1230; found 288.1212.

2-(4’-Methoxy-3-nitro-[1,1’-biphenyl]-4-yl)propyl 1H-imidazole-1-carboxylate. Compound 3.30.
Alcohol 3.26 (259 mg, 0.900 mmol) was dissolved in 4.5 mL of toluene, and carbonyldiimidazole (175 mg, 1.08 mmol) and potassium carbonate (249 mg, 1.799 mmol) were added to the solution. After 2 hours, only partial conversion was evident by TLC. As a result, 100 mg more of carbonyldiimidazole was added, and the next day, reaction showed conversion to the imidazole carbamate via both TLC and AccuTOF-DART MS. The reaction was diluted between 50 mL ethyl acetate and 50 mL water, and ethyl acetate was used to extract the solution (3 x 50 mL). The combined organic solution was dried with sodium sulfate and the organic liquid decanted and concentrated and dried under vacuum. A crude NMR of the red residue 3.30 was taken to confirm product form, and then it was used for the next reaction. (286 mg, 83%) 

\[^{1}H\text{ NMR (300 MHz, CDCl}_3\text{): } \delta 8.07 (q, J = 1.1 \text{ Hz}, 1\text{H}), 7.97 (t, J = 2.0 \text{ Hz}, 1\text{H}), 7.83 – 7.73 (m, 1\text{H}), 7.70 \text{ (s, 1\text{H})}, 7.59 – 7.48 (m, 3\text{H}), 7.41 – 7.32 (m, 1\text{H}), 7.12 (s, 1\text{H}), 7.07 – 6.98 (m, 3\text{H}), 4.59 (dd, J = 6.7, 1.1 \text{ Hz}, 2\text{H}), 3.90 – 3.83 (m, 4\text{H}), 1.47 (d, J = 7.0 \text{ Hz}, 3\text{H}).\] 

\[^{13}C\text{ NMR (75 MHz, CDCl}_3\text{): } \delta 160.24, 150.48, 148.56, 141.17, 137.19, 133.92, 130.82, 130.50, 128.50, 128.23, 122.25, 117.21, 115.62, 114.72, 109.22, 103.91, 72.00, 55.54, 33.03, 17.79.\]

4-(((Benzyloxy)carbonyl)amino)butanoic acid. Compound 3.32.

\[
\begin{array}{c}
\text{H}_2\text{N} & \text{OH} \\
\text{C} & \text{O} \\
\text{CbzCl} & \text{CbzN} \\
aq \text{NaOH THF} \text{C} & \text{O} \\
49\% & \\
\end{array}
\]

 Compound 3.31 (7.50 g, 75.0 mmol) was dissolved in 18 mL tetrahydrofuran, and carboxybenzoyl chloride (CbzCl) was added (13.5 mL, 94.5 mmol). Afterwards, sodium hydroxide (6.04 g, 150 mmol) dissolved in 40 mL of water were cooled with an ice bath. Then 13.5 mL of carboxybenzoyl chloride was added dropwise in 18 mL of THF to the reaction, and the reaction was allowed to warm to room temperature overnight. The reaction was acidified with 1M hydrochloric acid, and the precipitate was collected on a fritted filter. The material was dried from water with toluene, and then
reconcentrated with methylene chloride. In all, 8.40 g of product 3.32 was collected as a white solid (49%).

\(^1\)H NMR (500 MHz, CDCl\(_3\)) δ: 7.36 – 7.27 (m, 5H), 5.13 (d, J = 6.8 Hz, 1H), 5.08 (s, 2H), 3.26 – 3.18 (m, 2H), 2.35 (t, J = 7.3 Hz, 2H), 1.87 – 1.78 (m, 2H).\(^13\)C NMR (126 MHz, CDCl\(_3\)) δ: 178.22, 156.73, 136.44, 128.48, 128.10, 128.06, 66.75, 40.25, 31.15, 24.88.

**tert-Butyl 4-(((benzyloxy)carbonyl)amino)butanoate. Compound 3.33.**

Carboxylic acid 3.32 (1.782 g, 7.51 mmol) was added to a flame-dried 10 mL flask and dissolved in 1.3 mL of toluene from a dry solvent system, added via syringe. Fresh, dry triethylamine from calcium hydride was added (1.05 mL, 7.51 mmol), followed by 2,3,5-trichloroacetyl chloride (2.35 mL, 15.0 mmol). After 40 minutes, dimethylaminopyridine (1.84 g, 15.0 mmol) in 2.5 mL of tert-butanol was added, and the solution turned yellow. The next day, the reaction was concentrated, and purification was carried out with column chromatography using 25% ethyl acetate/hexanes to produce 3.33 as a white solid (1.54 g, 70%). NMR characterization matched the literature procedure.

\(^1\)H NMR (300 MHz, CDCl\(_3\)): δ 7.39 – 7.24 (m, 5H), 5.08 (s, 3H), 3.21 (q, J = 6.7 Hz, 2H), 2.25 (t, J = 7.3 Hz, 2H), 1.77 (q, J = 7.1 Hz, 2H), 1.43 (d, J = 1.2 Hz, 9H). \(^13\)C NMR (126 MHz, CDCl\(_3\)): δ: 172.64, 169.34, 156.48, 136.65, 128.52, 128.13, 128.09, 80.51, 66.62, 40.53, 32.84, 28.98, 28.11, 27.28, 25.24. HRMS-DART [M+H]^+ calcd for C\(_{16}\)H\(_{23}\)NO\(_4\), 294.1687; found 294.1687.

**tert-Butyl 4-aminobutanoate. Compound 3.34.**
The fully protected 3.33 (825 mg, 2.81 mmol) was dissolved in 10 mL of methanol. 200 mg of palladium on carbon was added, and the atmosphere was evacuated and a hydrogen atmosphere in its place using a schlenk line and a hydrogen-filled balloon. After disappearance of starting material on TLC and concentrating under vacuum, 424 mg of product 3.34 was obtained of the free amine (95%), and the product matched the literature.101

1H NMR (300 MHz, CDCl3): δ 4.34 (s, 2H), 2.81 (t, J = 6.9 Hz, 2H), 2.30 (t, J = 7.4 Hz, 2H), 1.82 (p, J = 6.8 Hz, 2H), 1.44 (s, 9H). 13C NMR (126 MHz, CDCl3) δ 172.55, 80.31, 40.60, 32.75, 28.07, 27.05.

**tert-Butyl 4-(((2′-4′-methoxy-3-nitro-[1,1′-biphenyl]-4′-yl)propoxy)carbonyl)amino)butanoate.**

**Compound 3.35.**

Crude imidazole 3.30 (286 mg, 0.759 mmol), amine 3.34 (158 mg, 0.990 mmol), and dimethylaminopyridine (27 mg, 0.2244 mmol) were stirred in 1.5 mL of THF. The next day, the reaction was complete, as marked by disappearance of the starting material on TLC. After concentrating under vacuum, the crude was purified using column chromatography with 30–35% ethyl acetate/hexanes. This resulted in 228 mg of product 3.35 as a yellow solid (54% from 3.29)

1H NMR (500 MHz, CDCl3) δ: 7.89 (d, J = 1.6 Hz, 1H), 7.73 (dd, J = 8.3, 2.1 Hz, 1H), 7.58 – 7.44 (m, 3H), 7.05 – 6.94 (m, 2H), 4.85 (t, J = 6.0 Hz, 1H), 4.26 (dd, J = 10.5, 6.0 Hz, 1H), 3.85 (d, J = 0.7 Hz, 3H), 3.77 – 3.63 (m, 1H), 3.16 (q, J = 6.7 Hz, 2H), 2.23 (t, J = 7.2 Hz, 2H), 1.75 (p, J = 7.1 Hz, 2H), 1.42 (d, J = 0.8 Hz, 9H), 1.36 (d, J = 7.3 Hz, 2H). 13C NMR (126 MHz, CD3OD): δ 170.05, 157.46, 153.67, 148.59, 137.80,
132.79, 128.27, 127.93, 125.86, 125.58, 119.31, 112.04, 77.98, 66.22, 57.91, 50.95, 37.90, 25.56, 22.69, 18.10, 14.94. HRMS-DART [M+H]+, calcd for C_{25}H_{32}N_{2}O_{7}, 473.2282; found 473.2280.

4-(((2-(4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoic acid. **Compound 3.28.**

The tert-butyl ester **3.35** (139.4 mg, 0.2950 mmol) was dissolved in 3 mL of dichloromethane, and 0.3 mL of trifluoroacetic acid was added. After three hours, 7.8 mL more of trifluoroacetic acid was added. After 3 more hours, the reaction was marked as complete by TLC and concentrated. Column chromatography with 10% methanol in dichloromethane gave 122 mg of product (99%).

^1^H NMR (600 MHz, CD$_3$OD): δ 7.91 (s, 1H), 7.77 (d, $J = 8.2$ Hz, 1H), 7.54 (d, $J = 9.2$ Hz, 3H), 7.00 (d, $J = 8.8$ Hz, 2H), 4.26 (dd, $J = 10.6, 6.1$ Hz, 1H), 4.17 (dd, $J = 10.7, 7.7$ Hz, 1H), 3.85 (s, 3H), 3.73 – 3.62 (m, 1H), 3.15 (t, $J = 8.5$ Hz, 1H), 2.32 (t, $J = 7.4$ Hz, 2H), 1.78 (p, $J = 7.2$ Hz, 2H), 1.37 (d, $J = 7.1$ Hz, 3H). ^13^C NMR (75 MHz, CD$_3$OD): δ: 175.78, 159.87, 156.91, 150.80, 140.24, 135.14, 130.62, 130.40, 128.51, 127.92, 121.55, 114.42, 68.54, 55.15, 39.94, 33.17, 31.06, 24.86, 17.34. HRMS-DART [M+H]+, calcd for C$_{21}$H$_{24}$N$_{2}$O$_{7}$, 415.1511; found 415.1525.

4-(((2-(4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoic acid. **Compound 3.28.**
Compound 3.30 (318 mg, 1.11 mmol) was placed into a flame-dried flame with nitrogen atmosphere. To this was added dry potassium carbonate (125 mg, 0.906 mmol), and then 5.5 mL of toluene. Carboxyldiimidazole (175 mg, 1.08 mmol) was added in portions. An hour later, starting material was unconverted, so 70 mg potassium carbonate and 50 mg carboxyldiimidazole were added. After an hour 50 mg more carboxyldiimidazole was added. Finally, after one more hour, no more starting material was evident. The solvent was concentrated, and dichloromethane was added and subsequently concentrated. Next, 320 mg of potassium carbonate, 325 mg of 4-aminobutyric acid, and 3.2 mL of dry dimethylformamide were added. After 20 minutes, 46 mg of dimethylaminopyridine was also added. After 2.5 hours, conversion was slight and detectable by mass spectrometry (AccuTOF DART). The next day the intermediate was fully converted. The reaction was then acidified with 2 mL of 2M HCl and extracted with ethyl acetate (3 x 5 mL). Column chromatography with 100% ethyl acetate afforded the product 3.28 (48%, 2 steps) as a yellow solid.

\(^1\)H NMR (600 MHz, CD\(_3\)OD): δ 7.91 (s, 1H), 7.77 (d, \(J = 8.2\) Hz, 1H), 7.54 (d, \(J = 9.2\) Hz, 3H), 7.00 (d, \(J = 8.8\) Hz, 2H), 4.26 (dd, \(J = 10.6, 6.1\) Hz, 1H), 4.17 (dd, \(J = 10.7, 7.7\) Hz, 1H), 3.85 (s, 3H), 3.73 – 3.62 (m, 1H), 3.15 (t, \(J = 8.5\) Hz, 1H), 2.32 (t, \(J = 7.4\) Hz, 2H), 1.78 (p, \(J = 7.2\) Hz, 2H), 1.37 (d, \(J = 7.1\) Hz, 3H). \(^{13}\)C NMR (75 MHz, CD\(_3\)OD): δ: 175.78, 159.87, 156.91, 150.80, 140.24, 135.14, 130.62, 130.40, 128.51, 127.92, 121.55, 114.42, 68.54, 55.15, 39.94, 33.17, 31.06, 24.86, 17.34. HRMS-DART [M+H]\(^+\), calcd for C\(_{21}\)H\(_{24}\)N\(_2\)O\(_7\), 415.1511; found 415.1525.
1-Palmitoyl-2-(((2′-(4′-methoxy-3-nitro-1,1′-biphenyl)-4-yl)propoxy)carbonyl)amino)butanoyl)-sn-glycero-3-phosphocholine (NBP-PC). Compound 3.37.

Compound 3.36 (169 mg, 0.415 mmol) and LPC (46 mg, 0.13 mmol) were coevaporated from toluene several times and dried under vacuum. To this was added 6 mL chloroform freshly distilled from phosphorous pentoxide, dicyclohexylcarbodiimide (92 mg, 0.419 mmol) and dimethylaminopyridine (54 mg, 0.461 mmol) were added. After 2 days of stirring, the reaction finally showed no lyso-phosphatidylcholine (LPC) by TLC. Dowex 80Wx8 was added and stirred for 30 minutes, after which the reaction was cooled to 0° C and filtered through a Büchner funnel lined with filter paper and then concentrated under vacuum. Column chromatography on a two inch skinny column with 15% methanol in dichloromethane to remove excess fatty acid 3.36, and then 65:25:4 chloroform/methanol/water gave the product 3.37 as a yellow oil or crystals (48.5 mg, 58% yield).

$^1$H NMR (500 MHz, 40% CD$_3$OD -CDCl$_3$) δ: 7.88 (s, 1H), 7.72 (d, $J = 8.1$ Hz, 1H), 7.55 – 7.46 (m, 3H), 6.98 (d, $J = 8.3$ Hz, 2H), 5.75 (s, 1H), 5.18 (s, 1H), 4.38 – 4.32 (m, 1H), 4.26 – 4.19 (m, 1H), 4.15 – 4.08 (m, 2H), 3.99 – 3.89 (m, 2H), 3.84 (s, 3H), 3.79 – 3.72 (m, 2H), 3.68 – 3.61 (m, 2H), 3.31 (s, 10H), 3.04 – 2.97 (m, 1H), 2.39 – 2.28 (m, 2H), 2.24 (t, $J = 7.9$ Hz, 2H), 1.76 (s, 2H), 1.54 (s, 2H), 1.34 (d, $J = 7.0$ Hz, 3H), 1.28 – 1.20 (m, 24H), 1.14 (t, $J = 5.6$ Hz, 2H), 0.87 (q, $J = 7.3$ Hz, 3H). $^{13}$C (500 MHz, 40% CD$_3$OD -CHCl$_3$) δ: 173.60, 172.79, 159.92, 156.44, 150.80, 140.23, 135.23, 130.60, 130.48, 128.60, 128.39, 128.02, 127.59,

$^{31}$P NMR (121 MHz, 40% CD$_3$OD - CHCl$_3$): -0.44. Rotation: $[\alpha]^{24}_D = +1.5$ (c 2.1). HRMS AccuTOF-ESI: [M+Na+1]$^+$ calcd for $C_{65}H_{72}N_3O_{13}P$, 917.4740; found 917.4926.

### 3.7 Fluorescence-Based Irradiation Kinetics Assay of NBP-PC

Stock solutions were initiated by weighing out samples of DOPC, DOPE, cholesterol, DSPE-PEG$_{2000}$ amine, and/or NBP-PC along with Nile red in vials. To the appropriate lipid components corresponding to each liposome sample was added 500 μL of ethanol-free chloroform, and after brief vortexing, proper volumes of each lipid were pipetted into a clean vial per calculations on a 5 mM, 500 μL total lipid scale, to obtain the desired molar percentage of each component. Next, a solution of Nile red was added per calculations on a 250 μM, 500 μL scale.

As an example, for liposomes comprised of 100% NBP-PC, 500 μL of ethanol-free chloroform was added to separate vials containing 6.8 mg NBP-PC and 3.9 mg Nile red, producing stock solutions of 15 and 25 mM concentrations, respectively. After brief vortexing, 164 μL of NBP-PC and 5.10 μL of Nile red stock solutions were combined in a new vial. The chloroform was dried with a nitrogen stream, and the lipids were subsequently dried overnight under vacuum together in a 500 mL round bottom flask. The next day, the lipids were hydrated with 500 μL of MilliQ purified water, vortexed, and incubated on a rotary evaporatory at 60 °C for 3 sets of 20 min, with vortexing after each set. Liposomes were then freeze-dried between a -40° C dry ice bath and a 60° C water bath for 10 cycles and extruded through a 200 nm membrane for 21 passes using a LiposoFast extruder (Avestin, Inc.), placing the uniform-sized vesicles into a fresh vial. DLS scans were performed to confirm the formation of stable liposomes.

Next, 70 μL of this liposomal solution was diluted to 7 mL with ultrapure water. Two identical samples were made by placing 3 mL of this dilute solution into quartz cuvettes and were sealed with
parafilm to minimize atmospheric exposure. After an initial fluorescence scan ($\lambda_{ex} = 595 \text{ nm}; \lambda_{em} = 612 \text{ nm}$), one sample was irradiated with 350 nm light while suspended and covered by Pyrex beakers between four 350 nm bulbs in a Rayonet Preparative Type RS photoreactor. The other sample was placed in a dark container. For each fluorescence scan, the sample was removed from the reactor or dark container and placed in the fluorimeter for scanning. Total amounts of time plotted for release experiments represent the amount of time the sample spent in the photoreactor. The fluorescence was checked after the following total irradiation intervals: 0.5, 1, 1.5, 2, 3, 4, 5, 10, 15, 20, 25, 30, 60, and 90 minutes. Experiments were run at least four times each, including runs with different batches of liposomes, and averaged to obtain the results shown in Figure 3.4 and Table 3.1 with error bars included to depict standard error. Data were then curve fit using an exponential decay equation in SigmaPlot to obtain the values shown in Tables 2.1-2.5, according to Equation 2.1 and Equation 2.2.

3.8 Conclusion

The nitrobiphenethyl group is an extended system that can be used for better release under UV light over the nitrophenyl, due to the elongation of its pi system. As opposed to the NB-PC lipids, the NBP-PC lipid releases Nile red about six times fast, with a more extensive release. Since it works very well under UV light, it will be used in studies with two photon infrared light for more biocompatible release. Overall, this is a tunable liposome release system that is biocompatible because of the high degree of similarity of this lipid to natural PC.
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Specta 3.28: Dynamic light scattering of 100% NBP-PC (Compound 3.37) without irradiation. Mean = 154.0 nm.

Specta 3.29: Dynamic light scattering of 100% NBP-PC (Compound 3.37) irradiated at 350 nm. Mean = 145.2 nm.
Spectra 3.1: $^1$H NMR. **Compound 3.23.** 4-Ethyl-3-nitrobenzeneammonium hydrogen sulfate
Spectra 3.2: $^{13}$C NMR. **Compound 3.23.** 4-Ethyl-3-nitrobenzenearmonium hydrogen sulfate
\[ ^1H\ NMR\ (300\ MHz,\ Chloroform-\d)\ \delta\ 8.16\ (s,\ 1H),\ 7.82\ (d,\ J = 8.2\ Hz,\ 1H),\ 7.12\ (d,\ J = 8.2\ Hz,\ 3H),\ 2.85\ (q,\ J = 7.5\ Hz,\ 7H),\ 1.26\ (t,\ J = 7.5\ Hz,\ 6H).\]
$^{13}$C NMR (75 MHz, cdpal 149.60, 141.80, 138.55, 132.98, 132.80, 89.88, 25.91, 14.77.

Spectra 3.4: $^{13}$C NMR. **Compound 3.24.** 1-Ethyl-4-iodo-2-nitrobenzene
1H NMR (300 MHz, Chloroform-d) δ 8.05 (s, 1H), 7.87 (d, J = 8.3 Hz, 1H), 7.24 (d, J = 8.3 Hz, 1H), 3.85 – 3.66 (m, 2H), 3.46 (t, 6.6 Hz, 1H), 1.82 (s, 1H), 1.30 (d, 6.9 Hz, 3H).
Spectra 3.6: $^{13}$C NMR. **Compound 3.25.** 2-(4-iodo-2-nitrophenyl)propan-1-ol

$^{13}$C NMR (75 MHz, $d_6$-DMSO) 150.98, 141.48, 137.81, 132.51, 129.95, 90.44, 67.43, 36.15, 17.27.
\textbf{Spectra 3.7.} $^1$H NMR. \textbf{Compound 3.26.} 4-Ethyl-4′-methoxy-3-nitro-1,1′-biphenyl-2-(4′-methoxy-3-nitro-[1,1′-biphenyl]-4-yl)propan-1-ol
Spectra 3.8. $^{13}$C NMR. **Compound 3.26.** 4-Ethyl-4′-methoxy-3-nitro-1,1′-biphenyl-2-(4′-methoxy-3-nitro-[1,1′-biphenyl]-4-yl)propan-1-ol
\[ \text{Spectra 3. 9: } ^1\text{H NMR (crude). Compound 3.30. } 2-(4'-\text{Methoxy-3-nitro-[1,1'-biphenyl]-4-yl})\text{propyl 1H-imidazole-1-carboxylate} \]
$^{13}$C NMR (75 MHz, dglδ 160.24, 150.84, 148.56, 141.17, 137.19, 133.92, 130.82, 130.50, 128.50, 128.23, 122.25, 117.21, 115.62, 114.72, 109.22, 103.91, 72.00, 55.54, 33.03, 17.79.

$\text{Spectra 3.10: } ^{13}$C NMR (crude). \textbf{Compound 3.30.} 2-(4'-Methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propyl 1H-imidazole-1-carboxylate
Spectra 3.11: $^1$H NMR. **Compound 3.32.** 4-(((Benzyloxy)carbonyl)amino)butanoic acid
$\text{C NMR (126 MHz, } \text{cdCl}_3 \text{) 178.22, 156.73, 136.44, 128.48, 128.10, 128.06, 66.75, 40.25, 31.15, 24.88.}$

Spectra 3. 12: 1H NMR. Compound 3.32. 4-(((Benzyloxy)carbonyl)amino)butanoic acid.
Compound 3.33. tert-Butyl 4-(((benzyloxy)carbonyl)amino)butanoate

1H NMR (300 MHz, Chloroform-d) δ: 7.39 – 7.43 (m, 5H), 5.08 (s, 3H), 3.28 – 3.15 (m, 2H), 2.28 (t, 7.3 Hz, 2H), 1.77 (d, 7.1 Hz, 2H), 1.43 (d, 1.2 Hz, 9H).

Spectra 3.14. $^1$H NMR. Compound 3.33. tert-Butyl 4-(((benzyloxy)carbonyl)amino)butanoate
\[ 1^3\text{C NMR (300 MHz, CDCl}_3) \ 172.64, 169.34, 156.48, 136.65, 128.52, 128.13, 128.09, 80.51, 66.62, 40.53, 32.84, 28.98, 28.11, 27.28, 25.24. \]

Spectra 3.15: \( ^{13}\text{C NMR. Compound 3.33. tert-Butyl 4-((benzyloxy)carbonyl)amino)butanoate} \)
$^1$H NMR (300 MHz, Chloroform-$d$) δ 4.34 (s, 2H), 2.81 (d, $\delta$ 6.9 Hz, 2H), 2.30 (d, $\delta$ 7.4 Hz, 2H), 1.82 (p, $\delta$ 6.8 Hz, 2H), 1.44 (s, 9H).

Spectra 3.16: $^1$H NMR. Compound 3.34. tert-Butyl 4-aminobutanoate
$^{13}$C NMR (300 MHz, cδ6 172.55, 80.31, 40.60, 32.75, 28.07, 27.05.

Spectra 3.17: $^{13}$C NMR. Compound 3.34, tert-Butyl 4-aminobutanoate
H NMR (300 MHz, Chloroform) δ 7.89 (d, J = 1.6 Hz, 1H), 7.73 (d, J = 8.3, 2.1 Hz, 1H), 7.58 – 7.44 (m, 3H), 7.05 – 6.94 (m, 2H), 4.85 (6.0 Hz, 1H), 4.26 (d, J = 10.5, 6.0 Hz, 1H), 3.85 (d, J = 0.7 Hz, 3H), 3.77 – 3.63 (m, 1H), 3.18 (d, J = 9.7 Hz, 2H), 2.23 (d, J = 7.2 Hz, 2H), 1.75 (p, J = 7.1 Hz, 2H), 1.42 (d, J = 0.8 Hz, 9H), 1.36 (d, J = 7.3 Hz, 2H).

Spectra 3.18: 1H NMR. Compound 3.35. tert-Butyl 4-(((2'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoate
$^{13}$C NMR (75 MHz, qdd) at 172.46, 171.75, 159.89, 156.10, 151.00, 140.22, 135.23, 130.69, 130.35, 128.30, 128.00, 121.72, 114.47, 80.38, 68.62, 55.31, 40.29, 33.03, 32.68, 27.97.
Spectra 3. 20: $^1$H NMR. Compound 3.28. 4-(((2'-(4''-methoxy)-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoic acid
$^{13}$C NMR (151 MHz, 5dd) δ 175.78, 159.87, 156.91, 150.80, 140.24, 135.14, 130.62, 130.40, 128.51, 127.92, 121.55, 114.42, 68.54, 55.15, 39.94, 33.17, 31.06, 24.86, 17.34.

**Spectra 3.21: $^{13}$C NMR. Compound 3.28.** 4-(((2′-methoxy-3-nitro-[1,1′-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoic acid
Spectra 3.22: $^1$H NMR. Compound 3.37. 1-Palmitoyl-2-((2-(4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoyl-sn-glycero-3-phosphocholine (NBP-PC)
Spectra 3.23. $^{13}$C NMR. Compound 3.37. 1-Palmitoyl-2-(((2-(4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoyl)-sn-glycero-3-phosphocholine (NBP-PC)
$^3$P NMR (121 MHz, cd$_3$SO $d$-0.44).

Spectra 3.24: $^3$P NMR. **Compound 3.37.** 1-Palmitoyl-2-{$^-$}{(2-(4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl}amino)butanoyl)-sn-glycero-3-phosphocholine (NBP-PC)
Spectra 3.25: gCOSY NMR. **Compound 3.37.** 1-Palmitoyl-2-(((2-4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoyl)-sn-glycero-3-phosphocholine (NBP-PC)
Spectra 3.26: gHSQC NMR. **Compound 3.37.** 1-Palmitoyl-2-((2-((4'-methoxy-3-nitro-[1,1'-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoyl)-sn-glycero-3-phosphocholine (NBP-PC)
Specta 3.27. gHMBC NMR. **Compound 3.37.** 1-Palmitoyl-2-(((2-(4’-methoxy-3-nitro-[1,1’-biphenyl]-4-yl)propoxy)carbonyl)amino)butanoyl)-sn-glycero-3-phosphocholine (NBP-PC)
Specta 3.28: Dynamic light scattering of 100% NBP-PC (Compound 3.37) without irradiation. Mean = 154.0 nm.
Specta 3. 29: Dynamic light scattering of 100% NBP-PC (Compound 3.37) irradiated at 350 nm. Mean = 145.2 nm.
Chapter 4: Caging of Diacylglycerol Signaling with a 2-Nitrobenzyl Group

4.1 DAG and Its Role with PKC

Natural sn-1,2-diacylglycerol (DAG) is an important signaling lipid, consisting of a glycerol moiety with two esterified tails and an overall S configuration at the sn-2 methine carbon. DAG is produced \textit{in vivo} by one of three ways. Most notably, it is produced by phospholipase C (PLC) hydrolysis of phosphatidylcholine (PC), producing phosphorylcholine as a byproduct. Secondly, it can be produced from the PLC hydrolysis of phosphoinositol 4,5-bisphosphate (PIP$_2$) to inositol 1,4,5-trisphosphate (IP$_3$). Thirdly, it can be produced by phospholipase D (PLD) hydrolysis of PC to phosphatidic acid (PA), followed by hydrolysis to DAG by phosphohydrolase.$^{109}$

DAG is known for its role with the isoenzymes of protein kinase C. It binds either to a C1 domain of novel PKCs with or without calcium or to the C2 domain of classical PKC isoenzymes with calcium and phosphatidylserine (PS)$^{8,110}$ PKC has been found to participate in many roles, including cellular events such as apoptosis, survival, proliferation, as well as the migration of organelles within the membrane. PKC activity was originally discovered in response to an observation that phorbol esters, especially tetradecanoyl phorbol acetate (TPA), acted as a tumor promoter for infected mice. It was then found that DAG activates PKC in the presence of phosphatidylserine (PS). Also, it was found that DAG could compete out phorbol esters in PKC binding, although over time the binding of DAG diminishes.$^8$ Another role for DAG includes the activation of acidic sphingomyelinase (SMase) to ceramide and the possible reversal of ceramide-induced apoptosis.$^{109}$ Furthermore, DAG also binds other proteins, such as DGK$_\gamma$, Munc13, and RasGRP.$^{111}$

The photochemical protection of DAG may be useful for in situ quantification by biological studies to characterize PKC activation and binding. The Walker group used a nitrobenzyl-protected DAG to study the response of cardiac muscle to DAG, finding that it strengthened the contraction strength.$^{112}$
They also later used a coumarin-caged DAG for similar studies. The Schultz group also used nitrobenzyl and coumarin versions of DAG with different acyl chains of different degrees of saturation, lengths, and regiochemistry. Their studies with the C1 domain of a green fluorescent probe showed that there is a preference for certain acyl compositions over others, and the binding is not solely mediated by headgroup binding.

4.2 Discussion of NB-DAG Synthesis and Future Studies

The DAG we designed and synthesized was a nitrobenzyl version. The synthesis was completed in three steps, starting with 2-nitrobenzyl bromide (Figure 4.1). The most limiting step is the first one, which consists of making an ether bond from electron-deficient nitrobenzyl bromide (4.1) and (S)-glycerol acetonide (4.2) to form 4.3. The best yield resulted from silver(I) oxide coupling, though similar results could be obtained using phase transfer ammonium tetrabutylammonium salts. The biggest issue with this reaction is that the typical reagent for ether bonds, sodium hydride, produces decomposition products or polymerization reactions. Yields can be slightly improved by using a nitrobenzyl derivative with a better leaving group. After ether formation, acetal deprotection gave the free diol (4.4), and finally Steglich coupling with stearic acid gave the nitrobenzyl-protected DAG (4.5, NB-DAG) compound.

NMR spectroscopy has been used to visualize the spectra over time in response to irradiation (Figure 4.3). There is a general decrease of the original aromatic and benzylic peaks, and an increase of new peaks. Over time, more than one sn-3 peak is evident. This could be due to the 1,2 and 1,3-isomers of DAG. Preliminary results demonstrate an exponential increase over approximately 30 minutes. Future studies will focus on microplate characterization of PKC binding (Figure 4.2). Specifically, liposomes containing DAG will be incorporated into liposomes and transferred to microplates with a buffer containing phosphatidylserine (PS). PKC binding can be detected after irradiation in the microplate wells.
Ether formation between bromide 4.1 and alcohol 4.2 produces 4.3 with silver(I) oxide. Catalytic acid produces 4.4. Fatty acid coupling forms the final product 4.5.

Figure 4.2: Photochemical activation of PKC after UV Irradiation.
Figure 4.3: Irradiation of NB-DAG (Compound 4.5) over time (bottom to top) shows conversion of compound 4.5 to others compounds, including DAG isomers.
via a chemiluminescence assay according to a previous procedure\textsuperscript{116} or by live cells with GFP-tagged PKCs.\textsuperscript{114}

4.3 Materials and Methods

Reagents and solvents were generally purchased from Acros, Aldrich or Fisher Scientific and used as received. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230-400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using a Varian Mercury 300 MHz or Varian VNMRS 500 MHz spectrometer.

**(S)-2,2-dimethyl-4-(((2-nitrobenzyl)oxy)methyl)-1,3-dioxolane. Compound 4.3.**

![Chemical reaction](image)

In a 100 mL flask, commercially available (S)-glycerol acetonide (4.1, 0.10 mL, 0.810 mmol) was dissolved in 10 mL of dichloromethane. To this was added crushed sodium hydroxide (486 mg, 12.2 mmol) in 50 mL of water to make a 10% w/v solution, followed by 2-nitrobenzyl bromide (385 mg, 2.02 mmol, 2.5 eq) and Tetrabutylammonium hydrogen sulfate (385 mg, 1.133 mmol, 1.4 eq). After three days, the reaction was diluted with dichloromethane and extracted (3 x 10 mL) with the same organic solvent. The organic layer was then washed with 10 mL of saturated sodium chloride and dried with magnesium chloride and filtered. After concentration, the column was run with 15 to 25% ethyl acetate in hexanes. A yellow solid mixture of the bromide and acetonide was collected. The product was 71% of the collected material and was carried along, with the bromide, to the next reaction (17%). [NOTE: 2-nitrobenzyl alcohol was also produced, in a 2:1 ratio of the alcohol.]
\(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\): 8.06 (d, \(J = 8.7\) Hz, 1H), 7.80 (d, \(J = 7.8\) Hz, 1H), 7.65 (t, \(J = 8.1\) Hz, 1H), 7.44 (t, \(J = 8.2, 6.7\) Hz, 1H), 4.95 (s, 2H), 4.36 (p, \(J = 6.0\) Hz, 1H), 4.10 (dd, \(J = 8.3, 6.5\) Hz, 1H), 3.80 (dd, \(J = 8.3, 6.8\) Hz, 1H), 3.67 (dd, \(J = 9.9, 5.6\) Hz, 1H), 3.62 (dd, \(J = 9.9, 5.1\) Hz, 3H), 1.44 (s, 3H), 1.38 (s, 3H).

\(^{13}\)C (75 MHz, CDCl\(_3\)) \(\delta\): 134.76, 133.58, 128.68, 128.00, 124.62, 109.52, 74.60, 72.09, 70.00, 66.59, 26.71, 23.36. HRMS-DART: [M]\(^{+}\) calcd for C\(_{13}\)H\(_{17}\)NO\(_4\), 268.11795; found 268.11612.

(R)-3-((2-nitrobenzyl)oxy)propane-1,2-diol. Compound 4.4.

Acetonide 4.3 (29.7 mg, 0.111 mmol) was dissolved in 1 mL of methanol and p-toluenesulfonic acid (4.00 mg mg, 0.0245 mmol) was subsequently added. The reaction was stirred until completion as determined by TLC, and then it was quenched with sodium bicarbonate (4.1 mg, 0.0490 mol). After concentration, the crude was extracted (3 x 10 mL) with dichloromethane from 10 mL of water. The organic layer was then washed with saturated sodium chloride and dried with magnesium chloride, then filtered and concentrated via rotary evaporation. Column chromatography with 75%-100% ethyl acetate/hexanes, produced 25.2 mg of the yellow oil 4.4 (47%).

\(^{1}\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\): 8.05 (d, \(J = 8.2\) Hz, 1H), 7.72 (d, \(J = 7.7\) Hz, 1H), 7.65 (t, \(J = 7.5\) Hz, 1H), 7.53 – 7.40 (m, 1H), 4.93 (s, 2H), 3.97 (s, 1H), 3.76 (d, \(J = 11.8\) Hz, 1H), 3.73 – 3.61 (m, 3H), 2.83 (s, 1H), 2.39 (s, 1H). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\): 134.18, 133.68, 128.78, 128.35, 124.78, 111.09, 72.50, 70.65, 70.14, 63.82.

(S)-3-((2-nitrobenzyl)oxy)propane-1,2-diyldistearate. Compound 4.5.
Dimethylaminopyridine (DMAP, 418 mg, 3.423 mmol) and stearic acid (4.4, 974 mg, 3.42 mmol) were mixed together in chloroform. Then dicyclocarbodiimide (DCC, 0.31 mL, 2.04 mmol) was added along with the diol 4.11, and the reaction was refluxed at 50° C for four hours. Next the reaction was chilled in ice bath and filtered to remove urea, rinsing with cold dichloromethane, and the reaction was concentrated under rotary evaporation. Column chromatography with 10-25% ethyl acetate/hexane eluted product 4.5 (307 mg, 59%). $R_f = 0.31$ (10% ethyl acetate/hexanes).

$^1$H NMR (300 MHz, C$_6$D$_6$) $\delta$ 7.67 (dd, $J = 8.2$, 1.2 Hz, 1H), 7.53 (d, $J = 7.9$ Hz, 1H), 6.99 (td, $J = 7.8$, 1.1 Hz, 1H), 6.70 (t, $J = 7.8$ Hz, 1H), 5.44 (dt, $J = 9.1$, 5.1 Hz, 1H), 4.61 (d, $J = 5.7$ Hz, 2H), 4.45 (dd, $J = 11.9$, 3.6 Hz, 1H), 4.21 (dd, $J = 11.9$, 6.6 Hz, 1H), 3.43 – 3.25 (m, 2H), 2.23 (dt, $J = 18.0$, 7.4 Hz, 4H), 1.71 – 1.53 (m, 4H), 1.43 – 1.14 (m, 56H), 0.98 – 0.86 (m, 6H). $^{13}$C NMR (300 MHz, C$_6$D$_6$) $\delta$ 173.38, 172.95, 147.06, 134.61, 133.64, 128.42, 128.00, 124.65, 24.65, 69.67, 62.48, 31.91, 29.69, 29.67, 29.65, 29.47, 29.35, 29.28, 29.27, 22.67, 14.08.
4.4 List of Spectra (Chapter 4)
Spectra 4.1: $^1$H NMR. Compound 4.3. (S)-2,2-Dimethyl-4-(((2-nitrobenzyl)oxy)methyl)-1,3-dioxolane. 154
Spectra 4.2: $^{13}$C NMR. Compound 4.3. (S)-2,2-Dimethyl-4-(((2-nitrobenzyl)oxy)methyl)-1,3-dioxolane. 155
Spectra 4.3: $^1$H NMR. Compound 4.4. (R)-3-(((2-Nitrobenzyl)oxy)propane-1,2-diol. .......................... 156
Spectra 4.4: $^{13}$C NMR. Compound 4.4. (R)-3-(((2-Nitrobenzyl)oxy)propane-1,2-diol. .......................... 157
Spectra 4.5: $^1$H NMR. Compound 4.5. (S)-3-(((2-Nitrobenzyl)oxy)propane-1,2-diyl distearate. .............. 158
Spectra 4.6: $^{13}$C NMR. Compound 4.5 (S)-3-(((2-Nitrobenzyl)oxy)propane-1,2-diyl distearate. .............. 159
1H NMR (500 MHz, Chloroform-d) δ 8.06 (d, J = 8.7 Hz, 1H), 7.80 (d, J = 7.8 Hz, 1H), 7.65 (t, J = 8.1 Hz, 1H), 7.44 (d, J = 8.2, 6.7 Hz, 1H), 4.95 (s, 2H), 4.36 (t, J = 6.0 Hz, 1H), 4.10 (dd, J = 8.3, 6.5 Hz, 1H), 3.80 (dd, J = 8.3, 6.8 Hz, 1H), 3.67 (dd, J = 9.9, 5.6 Hz, 1H), 3.62 (dd, J = 9.9, 5.1 Hz, 3H), 1.44 (s, 3H), 1.38 (s, 3H).

Spectra 4.1: 1H NMR. Compound 4.3. (S)-2,2-Dimethyl-4-(((2-nitrobenzyl)oxy)methyl)-1,3-dioxolane
\[^{13}C\] NMR (126 MHz, cdCl\textsubscript{3}) 134.80, 133.62, 128.71, 128.03, 124.66, 109.56, 74.63, 72.13, 70.04, 66.64, 64.85, 26.75, 25.40.

Spectra 4.2: \[^{13}C\] NMR. **Compound 4.3.** (S)-2,2-Dimethyl-4-(((2-nitrobenzyl)oxy)methyl)-1,3-dioxolane
1H NMR (300 MHz, Chloroform-d4): 8.05 (d, J = 8.2 Hz, 1H), 7.72 (d, J = 7.7 Hz, 1H), 7.65 (d, J = 7.5 Hz, 1H), 7.53 – 7.40 (m, 1H), 4.93 (s, 2H), 3.97 (s, 1H), 3.66 (q, 8 Hz, 1H), 3.73 – 3.61 (m, 3H), 2.83 (s, 1H), 2.39 (s, 1H).

Spectra 4.3. $^1$H NMR. **Compound 4.4. (R)-3-((2-Nitrobenzyl)oxy)propane-1,2-diol**
\[1^1\text{C} \text{NMR} \ (75 \text{ MHz}, \text{cdCl}_6) \ 134.18, 133.68, 128.78, 128.35, 124.78, 111.09, 72.50, 70.65, 70.14, 63.82.\]

\[\text{Residual glycerol}\]

Spectra 4.4: $^{13}\text{C} \text{NMR. Compound 4.4. (R)-3-}((2\text{-Nitrobenzyl})\text{oxy})\text{propane-1,2-diol}$
$^1$H NMR (300 MHz, Benzen) δ 7.69 (d, J = 8.3 Hz, 1H), 7.53 (d, J = 5.9 Hz, 1H), 6.99 (d, J = 7.8 Hz, 1H), 6.70 (d, J = 7.8 Hz, 1H), 5.31 - 5.36 (m, 1H), 4.68 - 4.53 (m, 2H), 4.45 (dd, 3H), 3.69 (d, J = 5.1, 3.7 Hz, 2H), 2.23 (d, J = 18.0, 7.4 Hz, 4H), 1.62 (d, J = 7.2 Hz, 4H), 1.39 - 1.19 (m, 6H), 0.97 - 0.87 (m, 6H).

Spectra 4.5: $^1$H NMR. Compound 4.5. (S)-3-((2-Nitrobenzyl)oxy)propane-1,2-diyl distearate
Spectra 4.6: $^1$H NMR. **Compound 4.5** (S)-3-[(2-Nitrobenzyl)oxy]propane-1,2-diyl distearate
Chapter 5: Azide-Alkyne Linkers and Light-Interacting Compounds

5.1 Cyclooctyne Lipid Conjugate for Copper-Free Presentation of Functional Handle

The synthesis of linkers for surface decoration of liposomes is an important concept for drug delivery. Additionally, when a linker is attached to the exterior of the liposome, many factors affect reactivity of a tag or handle attached by a linker, including composition of the linkers, type of chemistry used to attach the tag or reactive handle to the linker, and length of the linker. If a linker is too short, this may bury the reactive handle in the membrane. Conversely, a longer linker does not always correspond to better reactivity. For example, Stefanick et al. found that tethering peptides on a PEG_{2000} attachment to liposomes was much less effective than a PEG_{350} strand for cellular uptake. The moiety used as the reactive handle is also important, because for in vivo studies, handles should be used that are bio-orthogonal to normal biological processes.

The azide-alkyne cycloaddition is a reaction that is classified as click chemistry, which refers to reactions with reactive units that quickly and efficiently react on different scales of reaction. The azide-alkyne reaction was developed by Huisgen et al. in 1967, but the introduction of the copper-catalyzed azide-alkyne reaction gave this reaction much more utility, especially for biological systems. It has been one of the forefront reactions of click chemistry decades afterwards. This copper-mediated azide-alkyne reaction was an improvement to the heat-mediated azide-alkyne cycloaddition, which led to a mixture of products with different regiochemistry. Other types of click chemistry include inverse electron demand reactions such as the trans-cyclooctene-tetrazine reaction, the norborene-tetrazine reaction, and the cyclobutene-tetrazine reaction. The copper-free cycloaddition was developed by the Bertozzi group, and it has been used widely in aqueous systems. The ring strain of cyclooctynes or cyclononyynes provides enough strain to make the reaction proceed without requiring the copper catalyst, which is toxic to living cells.
Various lipids with a PEG linker and a cyclic alkyne have been used. Feldborg et al. evaluated different reactive handles in conjunction with the tumor-targeting peptide.\textsuperscript{122} One important finding was that reaction of the surface of the liposome had a much bigger effect than reactivity in solution. Presentation of an alkyne on the surface of the liposomes was about 1.5 times as effective as an azide on the surface. Zhang et al.\textsuperscript{42} also used a system that involved a PEGylated lipid with a reactive cyclooctyne handle. They used copper-free cycloaddition to attach thrombomodulin onto to surface of liposomes, leading to better protein C activation than with thrombomodulin alone.\textsuperscript{123} Jølck et al. utilized an assay with PEGylated lipid conjugates containing terminal or cyclic alkynes that were efficiently evaluated with a pro-fluorogenic azido-coumarin species to validate conjugation of an RGD peptide.\textsuperscript{124}

The dibenzylcyclooctyne (DBCO) will be utilized as a copper-free lipid tether to azide-functionalized moieties. In the current work, a PE-PEG\textsubscript{2000}-amine (5.3) conjugate was reacted with a (DBCO) species, either donated from the Popik laboratory, or commercially purchased from Sigma Aldrich (Figure 5.1). This reaction was either attempted by HATU and triethylamine or by first activating the carboxylic acid (5.1) as the N-hydroxysuccinamide ester (5.2), then reacting of the activated ester with the amine conjugate. Reaction was verified by TLC and staining with ninhydrin or a solution of a coumarin azide compound\textsuperscript{125} at a 5 mM solution in dimethylsulfoxide. MALDI-MS also verified the presence of product (5.4) via a shift of the mass-to-charge ratio. Purification was performed by size exclusion chromatography and reverse phase chromatography. Normal phase chromatography was found to be problematic because it absorbed most of the longer distributions of 5.4. The presence of starting material was not detected by TLC, but the presence of reagents has not been ruled out, as the NMR is complex and the presence of small ions cannot be verified in the presence of fragmentation of the product via mass spectrometry techniques. Preliminary results have demonstrated that a pro-fluorescence assay is also a good method of testing for successful cycloaddition with a coumarin-azide,\textsuperscript{125} species, though further work will be performed to validate this result. The coumarin-
Acid 5.1 is activated to ester 5.2. This is reacted with amine 5.3 to produce cyclic alkyne lipid tether 5.4.
cycloalkyne mixture will be monitored over time as a dramatic increase in fluorescence. Further testing
will also be performed to characterize binding affinity using a microplate assay similar to the one used
by Bostic et al.,\(^\text{126}\) in which a biotin-alkyne linker was tethered on streptavidin-coated microplate wells
and liposomes containing PEGylated cyclooctyne lipids were reacted at different concentrations.

5.2 Materials and Methods (DBCO-PEG-PE)
Dibenzycyclooctyne-N-hydroxysuccinamide ester (DBCO-PEG-PE)

\[
\begin{align*}
\text{Compound 5.1} & \quad (3.1 \text{ mg, 9.3 \(\mu\text{mol}\)),} & \quad \text{1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC,} \\
& & \quad \text{5.0 mg, 0.026 mmol) were weighed into a 1.5 mL eppendorf tube and dissolved in 0.75 mL of dry} \\
& & \quad \text{dichloromethane. After 10 minutes, N-hydroxysuccinamide (8.9 mg, 0.077 mmol) was added. More} \\
& & \quad \text{1-ethyl-3-(3-dimethylaminopropyl)carbodiimide or N-hydroxysuccinamide were added as needed until the} \\
& & \quad \text{starting material was consumed. This was determined by TLC monitoring of the reaction with ethyl} \\
& & \quad \text{acetate as the solvent. An } R_f \text{ value of 0.81 in ethyl acetate corresponded to the crude product, an } R_f \text{ of} \\
& & \quad 0.61 \text{ corresponded to the acid, and HOSu stayed at the baseline. After completion, the crude was diluted} \\
& & \quad \text{with 5 mL dichloromethane, and then it was washed with water (2 x 5 mL), 2M hydrochloric acid (2 x 5} \\
& & \quad \text{mL), and again with water (2 x 5 mL). This crude was concentrated and dried under vacuum for one} \\
& & \quad \text{hour. The crude weight of 5.2 was 8.8 mg, and it was directly used for the next reaction.}
\end{align*}
\]

Dibenzycyclooctyne-polyethylene glycol\(_{2000}\)-phosphatidylethanolamine (DBCO-PEG-PE)
Compound 5.3 (12.8 mg, 4.6 μmol) was weighed into an eppendorf tube. Compound 5.2 was transferred with 0.6 mL dichloromethane in portions to the amine. Triethylamine (5 μL), freshly distilled from calcium hydride, was added. After disappearance of the amine via visualization on TLC, the dimethylformamide was distilled from the reaction. The crude was then separated via Sephadex G-50 size exclusion chromatography with 50% methanol-water. The fractions that were more highly visible under 365 nm light when stained with azido coumarin were collected and co-evaporated with acetonitrile to remove water. Then the crude was passed through a C8 reverse phase column to remove the leached Sephadex. The fractions containing product were collected, co-evaporated with acetonitrile and dried under vacuum.

$^1$H NMR (600 MHz, CDCl$_3$): the NMR is more difficult to analyze, but the important areas are the aromatic region between 6.5-8.0 ppm, the PEG$_{2000}$ peak at 3.7 ppm, and the methyl peak at 0.9 ppm. MALDI-TOF MS illustrates a shift from the amine 5.3 to the product 5.4.

5.3 Azide and Alkyne Lipids for Terminal Conjugation

Another alkyne-azide lipid project in the preliminary stages is the production of PC analogues presenting an azide or alkyne on the terminal end, similar to phospholipids synthesized by O’Neil et al. or Lampkins et al. Initial attempts to produce a fatty acid with a terminal azide (5.6) were successful in moderate yields, via 5.5. However, producing an alkynoic acid (5.9) has proved more difficult due to the presence of the carboxylic acid. This can be related to the pK$_a$ trend, as hydrazoic acid has a pK$_a$ of about 4.7, a simple carboxylic acid is also about 4.7, and a terminal alkyne is approximately 26. Since basicity parallels nucleophilicity in many cases, this can also be used to predict reactivity in this situation. Since hydrazoic acid, the conjugate acid of sodium azide, has a similar pK$_a$ with carboxylic acids, the reaction can be favored by an excess of sodium azide. On the other hand, deprotonation of the carboxylic acid is favored by the acetylide anion by more than a factor of five, so nucleophilic addition competes heavily with acid-base chemistry. In fact, the alkyne is readily made with a long chain alkyl bromide, without the
presence of the carboxylic acid. Therefore to remedy this situation, iodo acids have also been synthesized (Figure 5.2), in order to give a compound with a better leaving group. Then conjugation to commercially available LPC will be more favorable.

5.4 Methods and Materials (Alkyne and Azide Lipids)

6-Iodohexanoic acid.

![Chemical Structure](image1)

5.5

Commercially available 5.5 (390 mg, 2 mmol, 1 eq) was dissolved in 10 mL of acetone and sodium iodide (2.4 g, 16 mmol) was added. After reaction completion, as judged by AccuTOF-DART MS, the reaction was concentrated to about 2 mL, diluted with water, extracted with 3 mL hexanes twice, 3 mL diethyl ether twice, and 3 mL ethyl acetate twice. Organic portions from all three solvents exhibited product, so they were combined and washed twice with a total of 25 mL sodium thiosulfate, which turned the organic layer from yellow to clear in color. The organic layer was dried with sodium sulfate and dried under vacuum, giving 5.8 as a white solid (n = 5, 460 mg, 90%).

\[ ^1H \text{ NMR (500 MHz, CDCl}_3 \text{) } \delta 10.85 (s, 1H), 3.19 (t, J = 7.0 \text{ Hz, 2H}), 2.38 (t, J = 7.4 \text{ Hz, 2H}), 1.85 (p, J = 7.1 \text{ Hz, 2H}), 1.67 (p, J = 7.5 \text{ Hz, 2H}), 1.52 - 1.40 (m, 2H). \]

\[ ^13C \text{ NMR (126 MHz, CDCl}_3 \text{) } \delta 33.83, 33.05, 30.92, 29.86, 23.55, 6.41. \]

11-Iodoundecanoic acid

![Chemical Structure](image2)

5.5

11-Iodoundecanoic acid

5.8 (n=10)
ω-Bromocarboxylic acid 5.5 can be derivatized with an azide to produce 5.6 for further work to make PC analogue 5.7. Otherwise 5.5 can be activated to the better leaving iodide group 5.8, then lithium acetylide will give the alkynoic acid 5.9, and final coupling will give 5.10.

Figure 5.2: Synthesis of terminal azide PC or terminal alkyne PC.
11-Iodoundecanoic acid

![Chemical Structure]

Commercially available 5.5 (390 mg, 2 mmol, 1 eq) was dissolved in 10 mL of acetone and sodium iodide (2.4 g, 16 mmol) was added. After reaction completion, as judged by AccuTOF-DART MS, the reaction was concentrated to about 2 mL, diluted with water, extracted with 3 mL hexanes twice, 3 mL diethyl ether twice, and 3 mL ethyl acetate twice. Organic portions from all three solvents exhibited product, so they were combined and washed twice with a total of 25 mL sodium thiosulfate, which turned the organic layer from yellow to clear in color. The organic layer was dried with sodium sulfate and dried under vacuum, giving 5.8 as a white solid (n = 10, 533 mg, 85%).

^1H NMR (500 MHz, CDCl₃) δ 11.56 (s, 1H), 3.17 (t, J = 7.0 Hz, 2H), 2.33 (t, J = 7.5 Hz, 2H), 1.80 (p, J = 7.1 Hz, 2H), 1.61 (p, J = 7.4 Hz, 2H), 1.41 – 1.22 (m, 12H). ^13C NMR (126 MHz, CDCl₃) δ: 180.54, 34.10, 33.51, 30.45, 29.29, 29.26, 29.14, 28.98, 28.47, 24.60, 7.23.

10-Azodecanoic acid

![Chemical Structure]

Commercially available 5.5 (1 g, 251 mmol, 1 eq) was dissolved in 25 mL dimethylsulfoxide, along with sodium azide (1.33 g, 19.8 mmol, 5 eq). After completion, the reaction was diluted with water, which caused an exothermic reaction. After the reaction cooled, the aqueous layer was acidified to pH 1 and extracted with ethyl acetate twice. The organic layer was washed with saturated sodium chloride and dried with sodium sulfate. After decanting and drying under vacuum, the weight was found
to be more than the theoretical yield. Chromatography with 50% ethyl acetate/hexanes gave the product 5.6 (512.0 mg, 60% yield).

$^1$H NMR (300 MHz, CDCl$_3$) δ 10.84 (s, 1H), 3.25 (t, $J = 6.9$ Hz, 2H), 2.34 (t, $J = 7.5$ Hz, 2H), 1.73 – 1.51 (m, 4H), 1.30 (s, 10H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ 180.35, 51.43, 34.06, 29.21, 29.09, 29.04, 28.96, 28.79, 26.65, 24.60.

5.5 Biotin-Azide Linker

Biotin-azide linker 5.18, which was also been previously synthesized in the Stubbs lab to efficiently link biotin to a thalidomide derivative,\textsuperscript{129} has also been synthesized by a different route and is currently in use by our laboratory (Figure 5.3). Commercially available diamine 5.11 with ethylene glycol units was protected on one terminus with a tert-butoxycarbonyl (Boc) group to yield 5.13. Amino conversion to azide was carried out with an azide transfer reagent, either in-situ with trifluoromethanesulfonyl azide (Tf-N$_3$)\textsuperscript{130} or a shelf-stable imidazole azide (Im-N$_3$)\textsuperscript{131}. The Tf-N$_3$ produced the best results, although the hazards are higher, as azides are known to be potentially explosive. Once the azide 5.15 was produced, the Boc group was removed to 5.17, and biotin was attached via a pentafluorophenyl ester 5.16 to 5.18. Several novel stains were used in this synthesis, including ones for biotin\textsuperscript{132} or azides.\textsuperscript{133}

5.6 Methods and Materials (Biotin-Azide)

Biotin pentafluorophenyl ester

Biotin (5.12, 1.00 g, 4.093 mmol) was added to 20 mL of DMF and heated to 80°C in a 50 mL round bottom flask. After dissolution, the reaction was cooled to room temperature (23°C), and triethylamine (1 mL) was added. Pentafluorophenyl trifluoroacetate (1 mL) was then added. The
solution turned orange and then pink. After one hour, the DMF was distilled and the resulting solids were washed with diethyl ether to give crude 5.16 (781 mg, 46%). Because of the high polarity of this compound, it was directly used for the next reaction.

\[ ^1H \text{NMR (500 MHz, DMSO-}d_6 \text{): } \delta: 6.26 \text{ (dd, } J = 36.9, 8.4 \text{ Hz, } 2H), 4.18 \text{ (t, } J = 6.2 \text{ Hz, } 1H), 4.09 - 3.93 \text{ (m, } 1H), 2.97 \text{ (dd, } J = 9.1, 4.8 \text{ Hz, } 1H), 2.81 - 2.58 \text{ (m, } 2H), 2.51 - 2.40 \text{ (m, } 1H), 2.06 \text{ (t, } J = 7.3 \text{ Hz, } 1H), 1.55 \text{ (d, } J = 6.3 \text{ Hz, } 2H), 1.47 - 1.25 \text{ (m, } 2H), 1.25 - 1.12 \text{ (m, } 2H). \]

\[ ^13C \text{NMR (126 MHz, DMSO-}d_6 \text{): } \delta 174.62, 169.66, 162.93, 160.66, 162.93, 61.20, 59.35, 55.53, 55.40, 33.61, 32.44, 28.25, 28.16, 28.04, 27.83, 24.65, 24.42, 8.74. \]

**tert-Butyl (3-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate. Compound 5.13.**

\[ \text{H}_2\text{N} \overset{\text{Boc}_2\text{O, dioxane}}{\longrightarrow} \overset{\text{Et}_3\text{N, 60\%}}{\longrightarrow} \overset{\text{NH}_2}{\longrightarrow} \]

Diamine 5.11 (9.54 mL, 43.53 mmol), triethylamine (400 µL, 55.38 mmol), and 25 mL of methanol were combined together. Di-tert-butyl dicarbonate (946 mg, 4.34 mmol, 1 eq) was then added in 15 mL of methanol dropwise over 40 minutes. The reaction was stirred overnight and then concentrated with rotary evaporation. Column chromatography with 10:40:50 triethylamine/ethyl acetate/methanol, then a second column with 89:10:1 CHCl₃/MeOH/NH₄OH produced product 5.13 as a slightly yellowish oil, as detected by ninhydrin (1.19 g, 85%).

\[ ^1H \text{NMR (500 MHz,CDCl}_3\text{): } \delta 3.69 \text{ (t, } J = 5.5 \text{ Hz, } 2H), 3.64 \text{ (s, } 6H), 3.62 - 3.59 \text{ (m, } 2H), 3.55 \text{ (t, } J = 6.0 \text{ Hz, } 2H), 3.20 \text{ (dd, } J = 7.8, 4.4 \text{ Hz, } 2H), 2.08 - 2.00 \text{ (m, } 2H), 1.77 \text{ (p, } J = 6.4 \text{ Hz, } 2H), 1.43 \text{ (s, } 9H). \]

\[ ^13C \text{NMR (500 MHz, CDCl}_3\text{): } \delta 156.06, 78.66, 70.53, 70.49, 70.14, 70.10, 69.51, 69.39, 39.56, 33.24, 29.51. \]


**Tert-butyl (3-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamate**

\[ \text{Boc} \overset{\text{H}_2\text{O-MeOH-toluene, 73\%}}{\longrightarrow} \overset{\text{Et}_3\text{N}_2}{\longrightarrow} \overset{\text{NH}_3}{\longrightarrow} \]

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Activated of biotin (5.12) with trifluoropentafluorophenyl ester gave biotin pentafluorophenyl ester (5.16). Separately, diamine 5.11 was selectively protected on one end to produce 5.13. An azide transfer reagent then gave 5.15, and deprotection of the Boc group gave 5.17. Reaction of this compound with the activated 5.16 then produced biotin-TEG-azide 5.18.
Amine 5.13 (319 mg, 0.9946 mmol), sodium bicarbonate (280 mg, 0.119 mmol), and copper(II) sulfate (7.0 mg, 0.0280 mmol) were added to 1 mL of water and 7 mL of methanol. To this 1.7 mL of trifluoromethanesulfonyl azide, prepared from a literature procedure,\textsuperscript{130} was added. After 6 hours, AccuTOF-DART MS revealed the disappearance of the amine. Concentration via rotary evaporation and column chromatography with 10-25% ethyl acetate/hexanes gave product 5.15 as a clear oil, as judged by a TLC stain for azides.\textsuperscript{133} (253 mg, 73%).

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\): 5.11 (s, 1H), 3.70 – 3.49 (m, 12H), 3.40 (t, \(J = 6.7\) Hz, 2H), 3.22 \(q, J = 6.3\) Hz, 2H), 1.86 \(p, J = 6.4\) Hz, 2H), 1.76 \(p, J = 6.2\) Hz, 2H), 1.44 \(s, 9H\). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\): 70.56, 70.52, 70.31, 70.15, 69.43, 67.82, 48.43, 39.48, 32.78, 29.10. HRMS-DART [M-H]\textsuperscript{+} calcd for C\textsubscript{15}H\textsubscript{30}N\textsubscript{4}O\textsubscript{5}, 347.2289; found 347.2286.

3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propan-1-amine

Azide 5.17 (234 mg, 1.05 mmol) was dissolved in 2.5 mL of ethanol, and 0.5 mL of concentrated hydrochloric acid was slowly added, along with 2.5 mL of diethyl ether. After disappearance of the starting material was confirmed by TLC, the reaction was concentrated under rotary evaporation. Then to neutralize the hydrochloric acid, 2.3 mL of sodium bicarbonate was added to the residue, upon which bubbling occurred. The organic material was extracted with dichloromethane (2 x 20 mL), dried with sodium sulfate, and decanted. The resulting solution was dried under rotary evaporation to give the product 5.17 (91 mg, 55%).

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \(\delta\): 5.11 (s, 12H), 3.70 – 3.49 (m, 2H), 3.40 (t, \(J = 6.7\) Hz, 2H), 3.22 \(q, J = 6.3\) Hz, 2H), 1.86 \(p, J = 6.4\) Hz, 2H), 1.76 \(p, J = 6.2\) Hz, 2H). \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\): 70.56, 70.52, 70.31, 70.15, 69.43, 67.82, 48.43, 39.48, 32.78, 29.10. HRMS-DART [M-H]\textsuperscript{+} calcd for C\textsubscript{10}H\textsubscript{12}N\textsubscript{4}O\textsubscript{3}, 247.1765; found 247.1781.
N-(3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)-5-(biotin)pentanamide

![Chemical Structure](image)

Compound 5.18 (60.5 mg, 0.1535 mmol) was dissolved in 1 mL dry dimethylformamide. The azide 5.17 (41.6 mg, 0.1689 mmol, 1.1 eq) was separately added to 1 mL dry dimethylformamide, along with 22 μL of triethylamine. The solution of 5.18 was chilled in a dry ice-acetone bath, and the azide solution was added to it. After disappearance of product was detected in the AccuTOF DART-MS, a spatula-tip full of dimethylaminopyridine was added to the solution. The next day the dimethylformamide was removed by vacuum distillation, and column chromatography with 25-50% methanol/ethyl acetate was used. Visualization of the product on TLC was aided by a cinnamaldehyde stain to detect biotin substrates. The azide stains mentioned earlier also gave visual confirmation of products. The relevant fractions were collected and dried under vacuum to give product 5.18 as an orange solid (21.1 mg, 29%).

$^1$H NMR (500 MHz, CD$_3$OD) δ 4.50 (dd, $J = 7.8$, 5.2 Hz, 1H), 4.31 (dd, $J = 7.9$, 4.4 Hz, 1H), 3.64 (dh, $J = 4.8$, 1.4 Hz, 4H), 3.61 – 3.58 (m, 4H), 3.56 (t, $J = 6.1$ Hz, 2H), 3.53 (t, $J = 6.2$ Hz, 2H), 3.40 (t, $J = 6.7$ Hz, 2H), 3.26 (t, $J = 6.8$ Hz, 2H), 3.21 (ddd, $J = 8.9$, 5.7, 4.4 Hz, 1H), 2.93 (dd, $J = 12.7$, 5.0 Hz, 1H), 2.71 (d, $J = 12.7$ Hz, 1H), 2.20 (t, $J = 7.4$ Hz, 2H), 1.83 (p, $J = 6.4$ Hz, 2H), 1.76 (p, $J = 6.5$ Hz, 3H), 1.72 – 1.55 (m, 3H), 1.44 (p, $J = 7.8$ Hz, 2H). $^{13}$C NMR (126 MHz, CD$_3$OD) δ 175.96, 166.07, 71.53, 71.52, 71.27, 71.23, 69.94, 68.92, 63.37, 61.61, 56.99, 49.48, 41.04, 37.82, 36.85, 30.40, 30.15, 29.79, 29.50, 26.87.

5.7 Azobenzenes for Chiral Polarimetry

Azobenzenes play a big role in the scientific community, and different types and some applications have been reviewed by Hamon et al. The property that makes them unique is that under ultraviolet light, they isomerize from the $E$ form to the higher energy $Z$ form. Even more unique is that
some azobenzenes can be converted back to the $E$ form by irradiation with visible light. One family of compounds that exhibits these characteristics include the $o$-fluoroazobenzenes and their derivatives. These fluorinated compounds (5.23) are being used in a collaboration with Dr. Bob Compton to test the effect of chiral plane-polarized light (Figure 5.4), along with an methylated azobenzene (5.23), a nitrated azobenzene (5.29), and a combination of the previous two (5.27). The goal of these compound is to find a combination that forms a chiral enantiomers under UV light, due to steric or electronics of the molecule causing the rings to remain in a staggered position (Figure 5.4). The first three of these compounds were synthesized in one step by oxidative coupling from the corresponding aniline. The last compound was synthesized in several steps from 2,5-dimethylaniline. The yields of all oxidative coupling reactions, however, were moderate to low yields under all conditions attempted. Either the reagents did not convert the starting materials well, or they converted them to a side product, such as phenazines.

5.8 Materials and Methods (Azobenzenes)

$2,2',6,6'$-Tetrafluoroazobenzene.

![Chemical structure](image)

2,6-Difluoroaniline (5.30, 662 uL, 1 g, 7.745 mmol) was dissolved in 40 mL of dichloromethane. Then using a mortar and pester, equal weight iron(II) sulphate heptahydrate and potassium permanganate (8 g total) were ground together and added into the flask. The reaction was heated to 40°C. The next day the reaction was filtered through Celite and washed with dichloromethane. Column chromatography with 50% dichloromethane in hexanes, then dichloromethane gave product 5.31 (113 mg, 11%).
Electronic or steric effects of the substituents of the azobenzene compounds could produce a non-meso form of the higher energy Z form of the azobenzenes, and with chiral light can produce enantiomers.
Figure 5.5: Synthesis of azobenzenes for the production of chiral enantiomers under UV light
$^{1}$H NMR (300 MHz, CDCl$_3$) $\delta$: 7.45-7.28 (m, 2H), 7.13-6.98 (m, 4H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$: 157.28 (d, $J = 4.1$ Hz), 153.82 (d, $J = 4.2$ Hz), 131.48 (t, $J = 10.5$ Hz), 112.60 (dd, $J = 20.7$, 3.4 Hz). 19F (282 MHz, CDCl$_3$) $\delta$: -124.94, referenced to 2,2,2-trifluoroethanol as an external standard. (There is a minor peak due to a partial existence of the $E$ isomer of the compound.) HRMS-DART: [$M^-$], calcd for C$_{12}$H$_6$F$_4$N$_2$, 254.0467, found 254.0467.

2,2',6,6'-Tetramethylandazobenzene.

2,6-Dimethylaniline (2 g, 2.03 mL, 16.5 mmol) was added to 125 mL of dichloromethane. With a mortar and pestle, copper sulfate pentahydrate (11.8 g, 0.0472 mmol) and potassium permanganate (11.1 g, 5.8 mmol) were ground together and then added to the solution. The next day, the reaction was filtered through Celite, rinsed with dichloromethane, and concentrated under rotary evaporation. Column chromatography with 25% ethyl acetate/hexanes produced product 5.23 (524 mg, 27%).

$^{1}$H NMR (300 MHz, CDCl$_3$) $\delta$: 7.18 – 7.12 (m, 6H), 2.42 (s, 12H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$: 151.31, 131.16, 129.28, 128.30, 19.70. AccuTOF DART MS: [$M^-$], calcd for C$_{16}$H$_{18}$N$_2$, 238.14700; found 238.1466.

4,4'-Dinitroazobenzene.

4-Nitroaniline (217 mg, 1 eq), copper(I) bromide (43 mg, 0.10 eq), and pyridine (72 uL, 0.30 mmol) were added to 12 mL of toluene. The reaction was refluxed at 60°C under air, then a balloon with oxygen was used when TLC showed little conversion. After 18 hours of reflux at 75°C with little
conversion, 43 mg more of copper(I) bromide and also 153 mg of L-ascorbic acid were added. After 1 hour, more conversion was evident. Then 110 mg more of L-ascorbic acid was added and the reaction was refluxed overnight. The reaction was then concentrated, column chromatography with 50 to 75% dichloromethane/hexanes gave product (116 mg, 54%).

\[ ^1H \text{ NMR (300 MHz, CDCl}_3 \delta: 8.46-8.41 (m, 2H), 8.14-8.08 (m, 2H). } ^{13}C \text{ (126 MHz, CDCl}_3 \delta: 155.12, 149.50, 124.50, 124.00. HRMS-DART [M], calcd for C}_{12}H_8N_4O_2, 272.0546; found 272.0540. \]

**2,2',6,6'-Tetramethyl-4,4'-Dinitroazobenzene.**

![Chemical structure](image)

2,6-dimethyl-4-nitroaniline (417.9 mg, 2.515 mmol) was dissolved in dichloromethane. A ground mixture of equal weight potassium permanganate and copper(II) sulfate heptahydrate (4g total) was added to the solution. The next day, the reaction was filter through Celite and concentrated. Column chromatography with a 10-25% dichloromethane/hexanes gradient gave pure product 5.27. (32.8 mg, 29%).

\[ ^1H \text{ NMR (500 MHz, CDCl}_3 \delta: 8.06 (s, 4H), 2.47 (s, 12H). } ^{13}C \text{ NMR (126 MHz, CDCl}_3 \delta: 154.84, 146.91, 132.58, 124.45, 19.60. AccuTOF DART: [M], calcd for C}_{16}H_{16}N_4O_4, 328.1172; found 328.1111. \]

**N-(2,6-dimethylphenyl)-4-methylbenzenesulfonamide. Compound 5.24.**

![Chemical structure](image)

2,6-Dimethylamine (2.03 mL, 2.0 g, 16.50 mmol, 1 eq) was added to pyridine (8 mL, 6 eq). Then p-toluene sulfonyl chloride (4.68 g, 24.55 mmol, 1.5 eq) was added to the solution. The reaction was refluxed at 80° C for slightly over one hour and cooled to room temperature. The reaction was then precipitated into ice, and the solids were collected on a fritted filter, rinsing them with ice cold water.
The solids were partitioned between water and ethyl acetate, and the aqueous layer was extracted with ethyl acetate (2 x 50 mL). The organic layer was then washed with 50 mL saturated sodium chloride, dried with sodium sulfate, and decanted. After drying using rotary evaporation, the resulting white solids were found to be product 5.24 as a white solid (3.97 g, 88%).

\[^1\text{H} \text{NMR} (300 \text{ MHz, CDCl}_3) \delta 7.60 (d, J = 8.4 \text{ Hz, 2H}), 7.23 (d, J = 8.7 \text{ Hz, 2H}), 7.13 – 6.94 (m, 3H), 6.42 (s, 1H), 2.41 (s, 3H), 2.04 (s, 6H).\]^13\text{C} \text{NMR} (75 \text{ MHz, CDCl}_3) \delta 143.62, 137.79, 132.61, 129.63, 128.76, 127.74, 127.17, 104.99, 21.58, 18.74. \text{HRMS-DART \ [M+H]^+}, \text{ calcld for C}_{15}\text{H}_8\text{N}_4\text{O}_4, 276.1053, \text{ found 276.1136.}

**N-(2,6-Dimethyl-4-nitrophenyl)-4-methylbenzenesulfonamide.**

![Reaction Scheme](image)

To 25 mL of a 60% aqueous acetic acid solution was added N-(2,6-dimethylphenyl)benzenesulphonamide (2.0 g, 7.263 mmol, 1 eq). To this was added sodium nitrite slowly (100 mg, 1.453 mmol, 0.2 eq). Concentrated nitric acid (1.0 mL, 2.2 eq) was then added dropwise. The reaction was refluxed at 80° C for 30 minutes, after which it was washed with ethyl acetate (2 x 50 mL). The organic layer was then washed with 100 mL of 10% w/v sodium hydroxide and then dried with sodium sulfate and decanted. Column chromatography with dichloromethane, then a second column with 25% dichloromethane-hexanes gave product 5.25 as a white solid (951 mg, 41%).

\[^1\text{H} \text{NMR} (300 \text{ MHz, CDCl}_3) \delta: 7.89 (s, 2H), 7.61 (d, J = 8.3 \text{ Hz, 2H}), 7.29 (d, J = 8.7 \text{ Hz, 2H}), 2.45 (s, 3H), 2.16 (s, 6H).\]^13\text{C} \text{NMR} (75 \text{ MHz, CDCl}_3) \delta 144.49, 139.25, 138.70, 137.20, 129.94, 127.06, 123.53, 105.00, 21.63, 19.13.

2,6-dimethyl-4-nitroaniline. **Compound 5.25.**
of concentrated sulfuric acid in two separate batches and refluxed for 75 minutes. The combined reaction was filtered and the solids collected. The solids were then basified with saturated sodium bicarbonate and extracted with ethyl acetate (2 x 50 mL). The organic layer was dried with magnesium sulfate and filtered. After concentration and drying under rotary evaporation, the resulting material was found to be product 5.25 as a yellow solid (418 mg, 87%).

5.9 \(N\text{-Nosylated Alanine Derivatives}

\(N\text{-Nosyl} \) alanine derivatives were also synthesized for studies of light with chiral molecules, in order to test if light has specific interactions with one enantiomer versus another. These compounds were synthesized in a collaborative work with Dr. Compton to characterize the photoelectric spectrum and collision-induced dissociation of these compounds and two others.\(^{137}\) \(N\)-nosyl derivatives of alanine have been synthesized previously. Gioia et al.\(^{138}\) utilized them as an efficient protecting group of the \(N\)-terminus in the synthesis of a peptides, using mercaptoacetic acid and sodium ethoxide to cleave it when necessary. Both were synthesized in one step from D- or L-alanine. Rotations were measured afterwards to ensure they were enantiomers. (D = -76.9, L = + 80.6)

5.10 Methods and Materials (\(N\)-Nosylated Alanine Derivatives)

\(\{(4\text{-nitrophenyl})\text{sulfonyl}\}\)-L-alanine. \textit{Compound 5.37}.

\(L\text{-Lysine (5.36, 0.21 g, 2.36 mmol)}\) was dissolved in 5 mL of 1M sodium hydroxide. After 10 minutes, \(p\text{-nosyl chloride (0.82 g)}\) was added. After 5 hours, insoluble solids were seen in the aqueous solution. The aqueous solution was washed with diethyl ether (3 x 20 mL). The aqueous layer was then acidified with 2 M hydrochloric acid to pH 1, as tested by litmus paper. The aqueous layer was then washed with ethyl acetate (3 x 20 mL). The organic layer was then dried with magnesium sulfate and
filtered. Column chromatography with 5% methanol–dichloromethane gave the product **5.37** as a yellow solid (265 mg, 53%).

$^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.60 – 8.51 (m, 1H), 8.37 (d, $J = 8.8$ Hz, 1H), 8.02 (d, $J = 8.7$ Hz, 1H), 3.91 – 3.81 (m, 1H), 1.18 (d, $J = 7.2$ Hz, 2H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) δ 172.98, 149.99, 128.10, 124.43, 51.37, 18.50. $[\alpha]^\text{D}_{20} = -80.6^\circ$

**((4-nitrophenyl)sulfonyl)-D-alanine.**

*D*-Lysine (**5.38**, 0.21 g, 2.36 mmol) was dissolved in 5 mL of 1M sodium hydroxide. After 10 minutes, p-nosyl chloride (0.82 g) was added. After 5 hours, insoluble solids were seen in the aqueous solution. The aqueous solution was washed with diethyl ether (3 x 20 mL). The aqueous layer was then acidified with 2 M hydrochloric acid to pH 1, as tested by litmus paper. The aqueous layer was then washed with ethyl acetate (3 x 20 mL). The organic layer was then dried with magnesium sulfate and filtered. Column chromatography with 5% methanol/dichloromethane gave the product (317 mg, 64%).

$^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.56 (s, 1H), 8.39 ($J=8.9$ Hz, 1H), 8.03 ($J=8.3$ Hz, 1H), 3.87 (q, $J=7.1$ Hz, 1H), 1.19 ($J=7.2$ Hz, 2H). $^{13}$C NMR (500 MHz, DMSO-$d_6$) δ 172.97, 149.50, 146.98, 128.10, 124.43, 51.37, 18.50. HRMS-DART: [M-H]$^-$, calcd for $C_9H_{10}N_2O_6S$, 273.0187; found 273.0577. $[\alpha]^\text{D}_{20} = -76.9^\circ$
Figure 5.6: \(N\)-Nosylation of \(D/L\) alanine
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Spectra 5.1: $^1$H NMR. **Compound 5.4.** Dibenzocyclooctyne-polyethylene glycol2000-phosphatidylethanolamine (DBCO-PEG-PE)
Spectra 5.2: MALDI-MS of Compound 5.3 (H₂N-PEG₂₀₀₀-PE)
Spectra 5.3: MALDI-MS of Compound 5.4 (DBCO-PEG\textsubscript{2000}-PE)
5.4 + PEG
3400
(reference)

Spectra 5.4: MALDI-MS of Compound 5.4 (DBCO-PEG_{2000}-PE) + PEG 3400 reference
$^1$H NMR (500 MHz, Chloroform-$d$): $\delta$ 7.68 (d, $J = 8.6$ Hz, 1H), 7.44 – 7.21 (m, 7H), 5.19 (t, 1H), 3.65 (d, $J = 13.8$ Hz, 1H), 2.26 – 2.15 (m, 1H), 2.10 (broad s, 2H), 1.95 – 1.85 (m, 1H), 1.52 – 1.29 (m, 4H).

Spectra 5.5: $^1$H NMR. Compound 5.1. DBCO-acid
$^{13}$C NMR (126 MHz, CDCl₃) 178.42, 173.14, 151.73, 147.96, 132.31, 128.91, 128.33, 128.29, 128.06, 127.71, 127.11, 125.48, 123.00, 122.61, 115.14, 107.70, 55.33, 34.36, 33.49, 24.55, 23.94.

Spectra 5.6: $^{13}$C NMR. **Compound 5.1.** DBCO-acid
1H NMR (500 MHz, Chloroform-d) δ 10.85 (s, 1H), 3.19 (q, J = 7.0 Hz, 2H), 2.38 (q, J = 7.4 Hz, 2H), 1.85 (p, J = 7.1 Hz, 2H), 1.67 (q, J = 7.5 Hz, 2H), 1.52 – 1.40 (m, 2H).

Spectra 5.7: 1H NMR. Compound 5.8 (n=5). 6-Iodohexanoic acid
$^{13}$C NMR (500 MHz, Chloroform-d$_6$): 10.85 (s, 1H), 3.19 (d, $J=7.0$ Hz, 2H), 2.38 (d, $J=7.4$ Hz, 2H), 1.85 (p, $J=7.1$ Hz, 2H), 1.67 (q, $J=7.5$ Hz, 2H), 1.52 – 1.40 (m, 2H).

Spectra 5.8: $^{13}$C NMR. **Compound 5.8 (n=5).** 6-Iodohexanoic acid
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2.33 ($^3$J = 7.5 Hz, 2H), 1.80 ($^3$J = 7.1 Hz, 2H), 1.61 ($^3$J = 7.4 Hz, 2H), 1.41 –
1.22 (m, 12H).

Spectra 5.12: $^1$H NMR. **Compound 5.8 (n=10).** 11-Iodoundecanoic acid
$^{13}$C NMR (126 MHz, CDCl$_3$) 180.54, 34.10, 33.51, 30.45, 29.29, 29.26, 29.14, 28.98, 28.47, 24.60, 7.23.

Spectra 5.13: $^{13}$C NMR. **Compound 5.8 (n=10).** 11-iodoundecanoic acid
\( ^1H \text{NMR (300 MHz, Chloroform-}d) 10.84 \text{ (s, 1H), 2.34 \delta} = 7.5 \text{ Hz, 2H), 1.73 - 1.51 \text{ (m, 3H), 1.30 \text{ (s, 9H).}} \)

**Spectra 5.14: \(^1H\text{ NMR. Compound 5.6. 10-Azidodecanoic acid}**
$^{13}$C NMR (75 MHz, CDCl$_3$) 180.35, 151.43, 34.06, 29.21, 29.09, 29.04, 28.96, 28.79, 26.65, 24.60.
Spectra 5. 16: $^1$H NMR (crude). Compound 5.16. Biotin pentafluorophenyl ester
Spectra 5. 17: $^{13}$C NMR (crude). **Compound 5.16.** Biotin pentafluorophenyl ester
Compound 5.13.

**tert-Butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate**

1H NMR (500 MHz, Chloroform-d): 3.69 (t, J = 5.5 Hz, 2H), 3.64 (s, 6H), 3.62 – 3.59 (m, 2H), 3.55 (t, J = 6.0 Hz, 2H), 3.20 (dd, J = 7.8, 4.4 Hz, 2H), 2.08 – 2.00 (m, 2H), 1.70 (s, 4H, 2H), 1.43 (s, 9H).

Spectra 5.18: 1H NMR. **Compound 5.13. tert-Butyl (3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate**

201
\[ \text{\(^{13}\text{C NMR (75 MHz, cdCl}_3\): 156.0, 78.66, 70.53, 70.49, 70.14, 70.10, 69.51, 69.39, 39.56, 38.38, 33.24, 29.51, 28.42.} \]

**Spectra 5.19: \(^{13}\text{C NMR. Compound 5.13. tert-Butyl (3-2-(3-aminopropoxy)ethoxy)ethoxy)propyl)carbamate**
\[ ^{1}H \text{ NMR (300 MHz, Chloroform-} \delta \text{)} \delta 5.11 (s, 1H), 3.70 - 3.49 (m, 12H), 3.4\# (t, 6.7 Hz, 2H), 3.22 (q, J = 6.3 Hz, 2H), 1.86 (dq, J = 6.4 Hz, 2H), 1.76 (dq, J = 6.2 Hz, 2H), 1.44 (s, 9H). \]
Spectra 5.21: $^{13}$C NMR. **Compound 5.15.** Tert-butyl (3-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)carbamate
$^{1}H$ NMR (300 MHz, Chlorofom-d$_6$, $\delta$ 3.70 - 3.50 (m, 12H), 3.40 (d, 6.7 Hz, 2H), 2.83 (d, 6.5 Hz, 2H), 2.28 (s, 2H), 1.85 (p, 11.6, 5.7, 5.0 Hz, 2H), 1.75 (p, 6.4 Hz, 2H).

\[ \text{H}_2\text{N} - \text{O} - \text{O} - \text{N}_3 \]

Spectra 5.22: $^{1}H$ NMR. **Compound 5.17.** 3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propan-1-amine.
Spectra 5.23: $^{13}$C. **Compound 5.17.** 3-[(2-(3-azidopropoxy)ethoxy)ethoxy]propan-1-amine
1H NMR (500 MHz, Methanol-d8) δ 4.50 (dd, J = 7.5, 5.2 Hz, 1H), 4.31 (dd, J = 7.9, 4.4 Hz, 1H), 3.64 (d, J = 4.8, 1.4 Hz, 4H), 3.51 – 3.58 (m, 4H), 3.39 (t, 6.1 Hz, 2H), 3.53 (t, 6.2 Hz, 2H), 3.40 (t, 6.7 Hz, 2H), 3.26 (t, 6.8 Hz, 2H), 3.21 (dd, J = 8.9, 5.7, 4.4 Hz, 1H), 2.84 (dd, J = 12.7, 5.0 Hz, 1H), 2.71 (d, 12.7 Hz, 1H), 2.27 (d, 7.4 Hz, 2H), 1.83 (d, 6.4 Hz, 2H), 1.76 (m, 2.5 Hz, 3H), 1.72 – 1.55 (m, 3H), 1.44 (t, 7.8 Hz, 2H).

Spectra 5.24: 1H NMR. Compound 5.18. N-[(3-[2-[(3-azidopropoxy)ethoxy]ethoxy]propyl]-5-(biotin)pentanamide
$^{13}$C NMR (126 MHz, $\delta$) $\delta$ 175.96, 166.07, 71.53, 71.52, 71.27, 71.23, 69.94, 68.92, 63.37, 61.61, 56.99, 49.48, 41.04, 37.82, 36.85, 30.40, 30.15, 29.79, 29.50, 26.87.

**Spectra 5.25: $^{13}$C NMR. Compound 5.18. N-(3-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)-5-(biotin)pentanamide**
Spectra 5.26: $^1$H NMR. Compound 5.31. 2,2',6,6'-Tetrafluorozobenzene

$^1$H NMR (300 MHz, Chloroform-$d$) δ 7.45 – 7.28 (m, 3H), 7.13 – 6.98 (m, 4H).
\(^{13}\)C NMR (75 MHz, Chloroform-\(d_8\)): 157.28 (d, \(J = 4.1\) Hz), 153.82 (d, \(J = 4.3\) Hz), 131.48 (d, \(J = 10.5\) Hz), 112.60 (dd, \(J = 20.7, 3.4\) Hz).

Spectra 5.27: \(^{13}\)C NMR. **Compound 5.31.** 2,2',6,6'-Tetrafluorobenzene
$^{19}$F NMR (282 MHz, DMSO) $\delta$ -123.20, -124.92, -124.94, -124.95, -124.97

Spectral data for Compound 5.31: 2,2',6,6'-Tetrafluoroazobenzene
Spectra 5.29: $^1$H NMR. Compound 5.23. 2,2',6,6'-Tetramethylazobenzene
$^1$H NMR (75 MHz, cdcl$_3$) 151.31, 131.16, 129.28, 128.30, 19.70.

$^{13}$C NMR. **Compound 5.23.** 2,2',6,6'-Tetramethylazobenzene

Spectra 5.30: $^{13}$C NMR. **Compound 5.23.** 2,2',6,6'-Tetramethylazobenzene
1H NMR (500 MHz, Chloroform-$d$, $\delta$ 8.46 – 8.40 (m, 1H), 8.15 – 8.08 (m, 1H).

Spectra 5.31. $^1$H NMR. **Compound 5.29.** 4,4'-Dinitroazobenzene
$^{13}$C NMR (126 MHz, CDCl$_3$) δ 155.12, 149.50, 124.88, 124.00.

Spectra 5.32: $^{13}$C NMR. **Compound 5.29.** 4,4'-Dinitroazobenzene
$^1$H NMR (300 MHz, Chloroform-$d$) δ 7.60 (d, $J = 8.4$ Hz, 2H), 7.23 (d, $J = 8.7$ Hz, 2H), 7.13 – 6.94 (m, 3H), 6.42 (s, 1H), 2.41 (s, 3H), 2.04 (s, 6H).
Spectra 5.34: $^{13}$C NMR. **Compound 5.25.** $N$-(2,6-dimethylphenyl)-4-methylbenzenesulfonamide
Compound 5.25.

N-(2,6-dimethyl-4-nitrophenyl)-4-methylbenzenesulfonamide

$^1$H NMR (300 MHz, Chlorofom-d$_6$): 7.89 (s, 2H), 7.61 (d, $J = 8.3$ Hz, 2H), 7.29 (d, $J = 8.7$ Hz, 2H), 2.45 (s, 3H), 2.16 (s, 6H).
Spectra 5.36: $^{13}$C NMR. **Compound 5.25.** $N$-(2,6-dimethyl-4-nitrophenyl)-4-methylbenzenesulfonamide
\textbf{Spectra 5.37: }$^1\text{H NMR. Compound 5.26. 2,6-dimethyl-4-nitroaniline}$

$^1\text{H NMR (300 MHz, Chloroform-}$d$) \delta$ 7.89 (s, 1H), 4.29 (s, 1H), 2.22 (s, 1H).
$^{13}$C NMR (75 MHz, cdcl$_3$) 160.91, 149.28, 124.54, 120.47, 17.46.

Spectra 5.38: $^{13}$C NMR. **Compound 5.26.** 2,6-dimethyl-4-nitroaniline
$^1$H NMR (500 MHz, Chloroform-$d$) 8 8.06 (s, 4H), 2.47 (s, 12H).

Spectra 5.39: $^1$H NMR. **Compound 5.27.** 2,2',6,6'-Tetramethyl-4,4'-dinitroazobenzene
$^{13}$C NMR (126 MHz, CDCl$_3$) 154.84, 146.91, 132.58, 124.45, 19.60.

**Compound 5.27.** 2,2',6,6'-Tetramethyl-4,4'-dinitroazobenzene

Spectra 5.40: $^{13}$C NMR. **Compound 5.27.** 2,2',6,6'-Tetramethyl-4,4'-dinitroazobenzene
\( ^1 \text{H NMR} \) (500 MHz, DMSO-\( d_6 \)) \( \delta 8.60 - 8.51 \text{ (m, 1H)}, 8.37 \text{ dd, } J = 8.8 \text{ Hz, 1H)}, 8.02 \text{ (dd, } J = 8.7 \text{ Hz, 1H)}, 3.91 - 3.81 \text{ (m, 1H), 1.18 (d, } J = 7.2 \text{ Hz, 2H).} \\

![](image)

Spectra 5.41: \(^1\text{H NMR. Compound 5.37, [(4-nitrophenyl)sulfonyl]-L-alanine}\)
$^{13}$C NMR (500 MHz, cdcl$_3$ 172.98, 149.51, 146.99, 128.10, 124.43, 51.37, 18.50.

Spectra 5.42: $^{13}$C NMR. Compound 5.37. ($4$-nitrophenyl)sulfonyl-$L$-alanine
\( ^1H\) NMR (500 MHz, DMSO-d\(_6\)) \( \delta \) 8.56 (s, 1H), 8.39 (d, \( J = 8.9 \text{ Hz}, 2\)H), 8.03 (d, \( J = 8.8 \text{ Hz}, 2\)H), 3.87 (q, \( J = 7.1 \text{ Hz}, 1\)H), 1.19 (d, \( J = 7.2 \text{ Hz}, 3\)H).

Spectra 5.43: \(^1H\) NMR. Compound 5.39. (4-nitrophenyl)sulfonyl)-D-alanine
\[ \text{\(^{13}\)C NMR (126 MHz, CDCl}_3 \text{ 172.97, 149.50,} \\
146.98, 128.10, 124.43, 51.37, 18.50, 4.06, 3.13}. \]

Spectra 5.44: \(^{13}\)C NMR. **Compound 5.39.** ((4-nitrophenyl)sulfonyl)-D-alanine


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Vita

Andrew Michael Bayer was born in Silvis, Illinois. After finishing his high school education in Waterloo, Iowa, he started his career first as a chemical engineer at the University of Iowa, and then later changed his major to chemistry upon a closer inspection of his own personal interests and goals. While attending Iowa, he performed undergraduate research of Dr. Johna Leddy with a zinc-air battery model system and with Dr. David F. Wiemer, synthesizing a portion of the natural compound Schweinfurthin A. Additionally, he spent a summer abroad in the Slovak Republic with under Dr. Ivan Ciznar at the Slovak Medical University. In December 2008, he earned his B.S. in Chemistry, with a minor in Spanish. He joined the University of Tennessee in Knoxville in 2009, lured by the biomedical emphasis in the organic chemistry department. He then joined the research group of Dr. Michael D. Best, researching and synthesizing photoreactive systems. During this time he published a first author paper entitled, “Triggered Liposomal Release through a Synthetic Phosphatidylcholine Analogue Bearing a Photocleavable Moiety Embedded within the sn-2 Acyl Chain,” as well as was involved in various poster presentations and talks, including SURMACS, the annual Board of Regents presentation at the University of Tennessee, and the Institute of BioMedical Engineering Symposium in Knoxville, Tennessee.