The Deposition of Liposomes on Glass Microbeads Using Chemoselective Ligation

by

Khalid Shamiyah

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Chemistry
University of Toronto

© Copyright by Khalid Shamiyah (2017)
The Deposition of Liposomes on Glass Microbeads Using Chemoselective Ligation

Khalid Shamiyah

Master of Science
Department of Chemistry
The University of Toronto
2017

Abstract

Bicelles and liposomes have proven to have potential as membrane models, drug delivery reservoirs, and therapeutic vectors. Recently, our lab was able to deposit a lipid bilayer on 3μm polystyrene beads. This was done via hydrophobic anchoring of bicelles containing CHOLOA, a modified cholesterol moiety covalently bonded to a PEG₁₅₀ linker with an oxyamine functional group, reacting with aldehyde groups on the surface of PS beads. The reaction of both functional groups lead to the covalent binding of the polystyrene beads to the bicelles.

This thesis extends the efforts of this project by altering a couple of aspects of this system. Most importantly, CHOLOA’s ethylene glycol length was increased to assess the effect that had on the efficiency of binding to the beads. A CHOLOA derivative with ten ethylene glycol units was successfully synthesized, incorporated into liposomes, and bound to glass microbeads.
Acknowledgments

بسم الله الرحمن الرحيم

وَمَا تَوَفِّيقِي إِلَّا بِاللَّهِ وَعَلَيْهِ تَوَكَّلْتِ وَإِلَيْهِ أُنْبِئُ

“And my success is not but through Allah. Upon him I have relied, and to him I return.”

The Holly Quran
Surat Hud
11:8

To acknowledge everyone whom I have relied on for help and support throughout the past year of my graduate journey would require a chapter on its own. If I forgot to mention you in this section, I sincerely apologize, and I would like to assure you that I am grateful for your help and contribution.

I would first and foremost like to praise and thank Allah for His countless blessings throughout my graduate journey and for giving me the ability, strength, and power to successfully proceed.

Second, I would like to thank my supervisor, Dr. Peter Macdonald, for giving me the opportunity to do research under his supervision and for his endless support and guidance. Professor Macdonald’s office door was always open whenever I ran into dead ends or had any questions. He would always provide the right guidance, advice, and recommendation. He gave me the freedom and allowed for this project to be my own, but at the same time made sure to steer me into the right direction with his endless knowledge, intellect, and wisdom. It has been a great honor to work with you Sir!

I would also like to thank my colleagues Angel Lai and Advait Hasabnis for being great friends and providing me with unlimited support and mentorship. Through them I was able to
learn many of the knowledge I was able to grasp throughout the past year. I am also grateful to Kamalpreet Singh for sharing this experience with me and for being a good friend.

My research would not have been possible without the help and contributions of specific individuals. First, I would like to thank Dr. Peter Mitrakos for always maintaining the NMR spectrometers and the mass spectrometer. I would also like to thank the McMillen group for allowing me to use their fluorimeter.

I would like to extend my gratitude to all my friends and colleagues inside and outside the lab environment. A special and warm thank you goes to Jalal Al Noubani, who has been a brother to me throughout my entire journey. I appreciate your ability to tolerate all my nagging and stress and transforming it into positivity and motivation from halfway across the world.

Last but definitely not least, I would like to thank my wonderful family for always believing in me and cheering me on till the finish line. My beautiful sisters and best friends, I appreciate you putting up with my mood swings and giving me the emotional love and support I always needed. I hope to one day see you both accomplish amazing things in your own academic journeys and professions. Mama and Baba, no words or paper can ever reflect my gratitude and appreciation for all the sacrifices you have ever made for me. Mama, I would not have been able to get this far without your emotional support and your continuous Dua’a for me. Your love and dedication for knowledge and persistence to memorizing the Quran has been one of my biggest motivations and inspirations towards my goals in life. Baba, I appreciate your continuous hard work and dedication to provide the best standards of living for our family. You are my role model and the person I aspire to be. If it wasn’t for you, I would not be half the man I am today.
# Table of Contents

Abstract .......................................................................................................................... ii

Acknowledgments .......................................................................................................... iii

Table of Contents .......................................................................................................... v

List of Tables ................................................................................................................ x

List of Figures ................................................................................................................. x

List of Abbreviations ..................................................................................................... xii

Chapter 1: Introduction .................................................................................................. 1

1.1 Supported Phospholipid Bilayers (SPBs) ................................................................. 3

1.2 Lipids, Bicelles, and Liposomes ............................................................................... 4

1.2.1 Lipids .................................................................................................................. 4

1.2.2 Liposomes .......................................................................................................... 5

1.2.3 Bicelles ................................................................................................................ 8

Chapter 2: Materials and Methods .............................................................................. 9

2.1 Materials ................................................................................................................ 9

2.1.1 NMR Spectroscopy ........................................................................................... 9

2.1.2 Mass Spectrometry ............................................................................................ 9

2.1.3 Fluorescence ...................................................................................................... 9

2.2 Methods .................................................................................................................. 10

2.2.1 CHOLOA_{150} .................................................................................................. 10

2.2.1.1 Cholesterol-O-Tosylate (2) .......................................................................... 10

2.2.1.2 Cholesterol-O-PEG_{150} (3) ....................................................................... 10

2.2.1.3 Cholesterol-O-PEG_{150}-O-Tosylate (4) ..................................................... 11
## Chapter 3: Results and Discussion

### 3.1 Results

<table>
<thead>
<tr>
<th>Reaction Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1.4 Cholesterol-O-PEG\textsubscript{150} - O-N-hydroxyphthalimide (5)</td>
<td>11</td>
</tr>
<tr>
<td>2.2.1.5 Cholesterol-O-PEG\textsubscript{150} - Oxyamine (6)</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2 \textsc{cho}lo\textsubscript{400}</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2.1 Cholesterol-O-Tosylate (2)</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2.2 Cholesterol-O-PEG\textsubscript{400} (3)</td>
<td>12</td>
</tr>
<tr>
<td>2.2.2.3 Cholesterol-O-PEG\textsubscript{400} - O-N-hydroxyphthalimide (4)</td>
<td>13</td>
</tr>
<tr>
<td>2.2.2.4 Cholesterol-O-PEG\textsubscript{400} - Oxyamine (5)</td>
<td>14</td>
</tr>
<tr>
<td>2.3 \textsc{cho}lo\textsubscript{400}-\textit{glutaraldehyde Reaction}</td>
<td>14</td>
</tr>
<tr>
<td>2.4 Modifying Amine Functionalized Glass Beads with Glutaraldehyde</td>
<td>15</td>
</tr>
<tr>
<td>2.5 Preparation of Liposomes</td>
<td>16</td>
</tr>
<tr>
<td>2.5 Binding Assay</td>
<td>17</td>
</tr>
<tr>
<td>2.5.1 Calibration Curve for Binding Assay</td>
<td>18</td>
</tr>
<tr>
<td>2.5.2 Microplate Preparation for Fluorescence Readings</td>
<td>19</td>
</tr>
<tr>
<td>2.6 Fluorescence Measurements</td>
<td>20</td>
</tr>
</tbody>
</table>

### 3.1.2 \textsuperscript{1}H NMR (400 MHz) and Mass Spectra

<table>
<thead>
<tr>
<th>Reaction Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.2.1 \textsc{cho}lo\textsubscript{150}</td>
<td>24</td>
</tr>
<tr>
<td>3.1.2.1.1 Cholesterol (1)</td>
<td>24</td>
</tr>
<tr>
<td>3.1.2.1.2 Cholesterol-O-Tosylate (2)</td>
<td>25</td>
</tr>
<tr>
<td>3.1.2.1.3 Cholesterol-O-PEG\textsubscript{150} (3)</td>
<td>26</td>
</tr>
<tr>
<td>3.1.2.1.4 Cholesterol-O-PEG\textsubscript{150} - O-Tosylate (4)</td>
<td>28</td>
</tr>
<tr>
<td>3.1.2.1.5 Cholesterol-O-PEG\textsubscript{150} - O-N-Hydroxyphthalimide (5)</td>
<td>30</td>
</tr>
</tbody>
</table>
3.2 Discussion

3.2.1 Synthesis and Purification Complications

3.2.1.1 Avoid the Running of Columns

3.2.1.2 Cholesterol-O-PEG_{400}

3.2.1.2.1 Synthesis of Cholesterol-O-PEG_{400}

3.2.1.2.1.1 Modifying the Fold Excess

3.2.1.2.2 Purifying Cholesterol-O-PEG_{400}

3.2.1.2.2.1 Two-Phase Systems

3.2.1.2.2.1.1 Chloroform-Water Wash

3.2.1.2.2.1.2 Hexane-Water Wash

3.2.1.2.2.1.3 Diethyl Ether-Water Wash

3.2.1.2.2.1.4 Diethyl Ether- NaHCO_{3} Wash

3.2.1.2.2.2 Running a Silica Column

3.2.1.3 Reaction Routes to the N-Hydroxyphthalimide Activated Intermediate

3.2.1.3.1 Tosylation Instead of Mitsunobu for PEG_{150}

3.2.1.3.2 Using the Alternative Route for Cholesterol-O-PEG_{400}

3.2.1.3.3 Going Back to The Mitsunobu

3.2.1.3.3.1 Precipitation of TPPO Using Hexanes

3.2.1.3.3.2 Running a Silica Column to Get Rid of TPPO

3.2.1.3.3.3 Using Resin Bound TPP

3.2.1.3.3.4 Using Resin Bound TPP

3.2.1.6 Cholesterol-O-PEG_{150}-Oxyamine/CHOLOA_{150}
List of Tables

TABLE 2.1 QUANTITIES OF COMPONENTS DURING THE BINDING ASSAY OF LIPOSOMES AND GM-BEADS ......................................................... 17
TABLE 2.2 QUANTITIES OF COMPONENTS USED TO PREPARE SOLUTIONS FOR CALIBRATION CURVE FOR THE BINDING ASSAY ............ 18
TABLE 2.3 QUANTITIES OF DIFFERENT COMPONENTS USED FOR THE PREPARATION OF THE MICROPLATE FOR FLUORESCENCE READINGS.. 20
TABLE 2.4 FLUORIMETER SETTING USED FOR THE FLUORESCENCE MEASUREMENTS ................................................................. 20
List of Figures

FIGURE 1.1 CHEMICAL STRUCTURE OF CHOLOA ........................................................................................................ 1
FIGURE 1.2 THE FORMATION OF A LIPID BILAYER OVER GLASS MICROBEADS ........................................................................... 2
FIGURE 1.3 SUPPORTED PHOSPHOLIPID BILAYERS ........................................................................................................ 3
FIGURE 1.4 LIPOSOME ..................................................................................................................................................... 4
FIGURE 1.5 DIFFERENT TYPES OF LIPOSOMES ........................................................................................................ 6
FIGURE 1.6 LIPOSOMAL DRUG DELIVERY SYSTEM ................................................................................................... 7
FIGURE 2.1 FUNCTIONALIZATION OF GLASS MICROBEADS ....................................................................................... 16
FIGURE 3.1 SYNTHETIC SCHEME FOR CHOLOA$_{150}$ ........................................................................................................ 22
FIGURE 3.2 SYNTHETIC SCHEME FOR CHOLOA$_{400}$ ........................................................................................................ 23
FIGURE 3.3 $^1$H NMR (400 MHz) OF CHOLESTEROL ......................................................................................................... 24
FIGURE 3.4 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-TOSYLATE .................................................................................... 25
FIGURE 3.5 STRUCTURE OF CHOLESTEROL-O-TOSYLATE ......................................................................................... 25
FIGURE 3.6 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-PEG$_{150}$ .......................................................................................... 26
FIGURE 3.7 STRUCTURE OF CHOLESTEROL-O-PEG$_{150}$ ............................................................................................. 27
FIGURE 3.8 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-PEG$_{150}$-O-TOSYLATE ................................................................. 28
FIGURE 3.9 STRUCTURE OF CHOLESTEROL-O-PEG$_{150}$-O-TOSYLATE ........................................................................ 29
FIGURE 3.10 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-PEG$_{150}$-O-N-HYDROXYPTHALIMIDE ..................................................... 30
FIGURE 3.11 STRUCTURE OF CHOLESTEROL-O-PEG$_{150}$-O-N-HYDROXYPTHALIMIDE ....................................................... 30
FIGURE 3.12 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-PEG$_{150}$-OXYAMINE/CHOLOA$_{150}$ ....................................................... 32
FIGURE 3.13 STRUCTURE OF CHOLESTEROL-O-PEG$_{150}$-OXYAMINE/CHOLOA$_{150}$ ............................................................ 33
FIGURE 3.14 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-PEG$_{400}$ ......................................................................................... 34
FIGURE 3.15 STRUCTURE OF CHOLESTEROL-O-PEG$_{400}$ ............................................................................................. 35
FIGURE 3.16 LCMS OF FREE PEG$_{400}$ AND CHOLESTEROL-O-PEG$_{400}$ .................................................................................. 36
FIGURE 3.17 STRUCTURE OF CHOLESTEROL-O-PEG$_{400}$-N-HYDROXYPTHALIMIDE .............................................................. 36
FIGURE 3.18 $^1$H NMR (400 MHz) OF CHOLESTEROL-O-PEG$_{400}$-O-N-HYDROXYPTHALIMIDE .................................................. 37
FIGURE 3.19 LCMS OF CHOLESTEROL-O-PEG$_{400}$ AND CHOLESTEROL-O-PEG$_{400}$-N-HYDROXYPTHALIMIDE .......... 38
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOLOA&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Cholesterol-PEG&lt;sub&gt;n&lt;/sub&gt;-Oxyamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>OTs</td>
<td>Tosylate</td>
</tr>
<tr>
<td>TPP</td>
<td>Triphenylphosphine</td>
</tr>
<tr>
<td>TPPO</td>
<td>Triphenylphosphine oxide</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>NBD-PE</td>
<td>(1,2-dipalmitoyl-&lt;i&gt;sn&lt;/i&gt;-glycerol-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>PS beads</td>
<td>Polystyrene beads</td>
</tr>
<tr>
<td>GM-beads</td>
<td>Glass microbeads</td>
</tr>
<tr>
<td>SPB</td>
<td>Supported phospholipid bilayer</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micellar concentration</td>
</tr>
<tr>
<td>Rf</td>
<td>Retardation factor</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

Cell membranes are crucial systems in many living organisms as they house numerous biochemical reactions and processes of the cell. The main element of a cell membrane is the lipid bilayer, which acts as the main gateway for many ions, molecules, and other components controlling what comes in and out of the cell. Over the past couple of decades, studies regarding the deposition of lipid bilayers on solid surfaces have been intensely researched and developed to aid in the understanding of biological membranes. Such systems have proved many potential applications, such as model membrane mimetics and drug delivery systems\textsuperscript{1-3}.

A lipid bilayer is made of amphiphilic phospholipids that are composed of hydrophobic fatty acid chains (referred to as the tail) and hydrophilic phosphate groups (referred to as the head) that spontaneously form a two-layered sheet when in solution. When exposed to an aqueous environment, the amphiphilic phospholipids simultaneously assemble in a way to decrease the system’s overall potential energy. This is done by having the hydrophobic tails assemble towards each other, minimizing unfavourable exposure with the aqueous environment, while having the hydrophilic heads assemble outwards towards the aqueous environment, forming a two-layered sheet.

![Figure 1.1 Chemical structure of CHOLOA](image)

The chemical structure of CHOLOA is shown, where “n” stands for the number of ethylene glycol units.
This report will discuss the process of synthesizing an oxyamine functionalized cholesterol moiety, referred to as CHOLOA (figure 1.1) and its deposition, along with other phospholipids, on 1.01µm diameter GM-beads. This was done by hydrophobically incorporating CHOLOA in liposomes and allowing them to react with aldehyde functionalized GM-beads. CHOLOA’s oxyamine functional group allowed for chemoselective ligation between the liposomes and aldehyde functionalized GM-beads. This resulted in the formation of an oxime linkage and the two bodies were attached. Following attachment, the liposomes spontaneously fused into a continuous single lipid bilayer, fully encapsulating the GM-beads (figure1.2).

This chemoselective method was developed and established in our lab and was initially tested out with a CHOLOA derivative with three ethylene glycol units (CHOLOA$_{150}$). However, after running a series of different experiments, it was concluded that three was not the optimum number of ethylene glycol units to separate the cholesterol moiety from the oxyamine bond for several reasons. A longer ethylene glycol chain is hypothesized to increase the size and
flexibility of the supported lipid bilayer and decrease any steric interactions that could have taken place due to the close proximity of the liposomes and the beads. Also, if proven to be successful, this can increase the loading capacity of the system and allow the addition of larger amount of drugs and therapeutics. In attempt to discover the optimum number of ethylene glycol units, a CHOLOA derivative with ten ethylene glycol units was synthesized and tested.

1.1 Supported Phospholipid Bilayers (SPBs)

Being one of the most crucial structures assembled by nature, lipid membranes are very important systems to be studied. Lipid membranes provide a protective layer between the inside and outside of the cell, are a platform for many of the machinery for cellular transport, and control intercellular communication.

SPBs are composed of phospholipid bilayers deposited on a hard or soft and planar or spherical surface, such as glass or silica (figure 1.3). The hydrophilic heads of the phospholipids on one side of the bilayer face the solid surface. The hydrophilic heads and the solid surface are separated by a small hydration layer. The hydrophobic tails of the phospholipids face the other hydrophobic tails of the other side of the bilayer, whose hydrophilic heads interact with the aqueous environment. Due to their characteristics combining between the mechanical stability of solid surfaces and the fluidity of phospholipid bilayers, SPBs have been able to showcase a vast variety of applications. SPBs have been used as model systems that mimic lipid membranes to study

Figure 1.3 Supported phospholipid bilayers
A schematic of a planar and spherical supported lipid bilayers are shown.
the physical and chemical properties of the cell, and processes such as signaling, ligand-receptor interactions, and enzymatic reactions that can take place at the surface of the cell membrane 5-6.

Supported phospholipid bilayers (SPBs) have been subject to intense research for the past half a century. SPBs were first deposited on a solid surface in the 1980’s by McConnel et al7. Spinke et al. further improved this system in the 1990’s by using soft highly hydrated polymeric systems to increase the layer of hydration between the surface and the phospholipid bilayer, making it more natural 8. Many different efforts have been made using hard 9 and soft materials10.

1.2 Lipids, Bicelles, and Liposomes

1.2.1 Lipids

Lipids are a broad and diverse group of amphiphilic molecules that contain hydrophilic heads and hydrophobic tails. The hydrophobic tails are usually composed of fatty acid chains of different lengths, that can either be saturated (do no possess double bonds) or unsaturated (possess double bonds). The hydrophilic head could be made of various groups that are zwitterionic or charged. The different classes of lipids are classified based on their functional or structural characteristics.

During this project, phospholipids were the main type of lipids used to form liposomes due to their tendency to spontaneously aggregate in

Figure 1.4 Liposome
Schematic presentation of the structure of a liposome. Hydrophilic heads are exposed to the aqueous environment while the hydrophobic tails are buried towards each other to form a lipid bilayer.
aqueous solution at a specific concentration called the critical micellar concentration (CMC).

When placed in aqueous solution, the unfavorable interactions of the hydrophobic segments of the molecule with the solvent result in the self-assembly of the liposomes. The reason why they self-assemble can be accredited to the hydrophobic effect, which states that the segregation of water molecules from hydrophobic groups is more thermodynamically favorable. The way phospholipids achieve that is via the burial of the hydrophobic tails and exposure of the hydrophilic heads to the aqueous environment (figure 1.4). The shape that the aggregates come to form are determined based on different characteristics of the phospholipids used. Usually, short chain phospholipids (5-9) carbon units form micelles while larger chain phospholipids form bilayers.

1.2.2 Liposomes

A liposome consists of one or many spherical shaped lipid bilayer/s with an aqueous core. The main types of liposomes are multilamellar vesicle (MLV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV).

MLVs are usually larger than a 1μm in size, consist of more than one lipid bilayers, and are the most thermodynamically stable form. They are formed by simply hydrating a dry lipid film as they are the most thermodynamically stable form of liposomes and no external forces are required.

LUVs are approximately 80-800 nm in size and consist of only one lipid bilayer. They are formed by hydrating a dry lipid film to from MLVs, and then extruding them through polycarbonate membranes with the pore size desired.
SUVs are approximately 20-50 nm in size and like LUVs, also consist of only one lipid bilayer. They are formed by extruding MLVs through polycarbonate membranes with the pore size desired (<50 nm) \(^{11}\).

**Figure 1.5 Different types of liposomes**

Illustrations of the different types of liposomes. From left to right: Large multilamellar vesicle (MLV), large unilamellar vesicle (LUV), and small unilamellar vesicle (SUV).

Liposomes have been widely developed and used by researchers due to their versatility. The ability to control the composition, size, and stability of the lipids making up the liposomes have made using them as lipid membrane mimetics very popular. However, using liposomes as drug delivery vectors has been more intensely subject to research due to their many advantages. The customizability and the ease of surface modification of the liposomes plays a crucial role in their ability to become delivery vectors, as shown in figure 1.6. The addition of target ligands, antibodies, peptides, and carbohydrates can control the distribution of the drug based on the liposome’s properties, rather than on the properties of the drug itself. Also, liposomes can act as an additional layer of protection for the drug, protecting it from the body’s immune system, prolonging its half-life, and reducing its metabolism. For example, the PEG-ylation of the
surface of liposomes can reduce clearance rates in vivo and decrease the immune response of the body.\textsuperscript{12-14}

Due to the existence of both hydrophobic and hydrophilic regions in liposomes, drugs of either properties can be added to different parts of the liposome. Hydrophobic drugs can be incorporated inside the lipid bilayer of the liposomes along with the lipids’ hydrophobic tails. On the other hand, hydrophilic drugs can either be stored in the aqueous core or on the surface of the liposome.\textsuperscript{15}

**Figure 1.6 Liposomal drug delivery system**

The illustration of different structural possibilities of a liposomal drug delivery system. Liposomes can be surface functionalized with many different targeting ligands, such as antibodies, peptides, proteins, and carbohydrates. Therapeutics can be encapsulated into the aqueous core or incorporated into the lipid bilayer, depending on their polarities.
1.2.3 Bicelles

Bicelles are composed of long and short chain lipids that form disc shaped aggregates when suspended in aqueous solution. Depending on the ratio of long chain to short chain lipids (q), a bicelles can take on a variety of different shapes, such as micelles, discoidal bicelles, and lamellae. Bicelles are usually formed by first, hydrating a lipid film with buffer, and then applying a few cycles of heating and cooling. This is done to homogenize the sample.

Bicelles have been in the spotlight as biological membrane mimetics for a long period of time. Their ability to mimic biological membranes have allowed researchers to study the structure and dynamics of membrane proteins in their natural settings. Also, due to their ability to spontaneously align in a magnetic field, bicelles have also been subject to many NMR related studies.
Chapter 2: Materials and Methods

2.1 Materials

Cholesterol, p-toluenesulfonyl chloride, N-hydroxyphthalimide, resin bound TPP, diisopropyl azodicarboxylate (DIAD), hydrazine monohydrate, anhydrous pyridine, anhydrous 1-4 dioxane, anhydrous DCM and all other chemical reagents and solvents were obtained from Sigma-Aldrich (Oakville, ON). PEG<sub>400</sub> was purchased from Thermo Fisher Scientific (Mississauga, ON). The amino functionalized glass microbeads were purchased from Bangs Laboratories, Inc. The beads had a mean diameter of 1.01 µm and a density of 2 g/cm<sup>3</sup>. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and NBD-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) were purchased from Avanti Polar Lipids (Alabaster, AL).

2.1.1 NMR Spectroscopy

All <sup>31</sup>P, <sup>13</sup>C and <sup>1</sup>H spectra were collected on a Microbay Avance III 400 MHz Bruker system NMR spectrometer.

2.1.2 Mass Spectrometry

All mass spectra were collected on a Waters Micromass ZQ mass spectrometer.

2.1.3 Fluorescence

Fluorescence measurements were performed on a Tecan Infinite M1000Pro microplate fluorimeter with 200 µL wells.
2.2 Methods

2.2.1 CHOLOA$_{150}$

2.2.1.1 Cholesterol-O-Tosylate (2)

To a solution of cholesterol (5.002g, 1.29 mmol) in 40 mL of anhydrous pyridine (99.8%), p-toluenesulfonyl chloride (5.021g, 2.63 mmol) was added. The mixture was stirred under inert atmosphere and a nitrogen balloon overnight at room temperature. The solvent was then evaporated off under vacuum. The residue (white mass) was filtered off and washed with a 2:1 mixture of water:pyridine to precipitate out the product. The precipitate was filtered off and collected. Using minimum amount of chloroform, the white mass was taken up and added to a 150 mL of cold methanol to recrystallize as white crystals. The crystals were collected over a Buchner funnel to give Cholesterol-O-Tosylate (2) (4.10g, 0.0076 moles, 81.9%).

2.2.1.2 Cholesterol-O-PEG$_{150}$ (3)

8 mL of PEG$_{150}$ (8.8g, 56.5 mmol) were dried by heating to 70°C under vacuum overnight to remove any moisture. This was then added to a solution of (2) (1.5 g, 2.8 mmol) in 30 mL of anhydrous 1,4-dioxane (99.8%). The mixture was stirred under reflux overnight in an inert atmosphere and a nitrogen balloon at a 110°C. After the completion of the reaction, the 1,4-dioxane was evaporated off under vacuum. The resulting brown oil was taken up in hexanes and washed twice with 10% NaHCO$_3$. The organic layer was then washed with water (50 mL), and 10% brine. The hexanes layer was then evaporated off under vacuum to obtain a brownish/yellow paste. The paste was taken up with a 1:1 ethylacetate:hexanes mixture and was purified using a silica column eluted with 1:1 to 9:1 (ethylacetate : hexanes) gradient. The fractions collected were ran on TLC’s to check for (3) at an $R_f$ (retardation factor).
value of 0.3-0.4. Fractions that contained (3) were collected in a round bottom flask and the solvent was evaporated off under vacuum to give (3) as a brownish/yellowish paste (1.236g, 2.3 mmol, 58.8%).

2.2.1.3 Cholesterol-O-PEG$_{150}$-O-Tosylate (4)

(3) (0.988g, 1.85 mmol) was dissolved in 13 mL anhydrous DCM (99.8%) in a round bottom flask. To the flask, p-toluenesulfonyl chloride (0.704g, 3.7 mmol) and 2 mL of anhydrous pyridine (99.8%) were added. The reaction mixture was stirred at room temperature under inert atmosphere conditions and a nitrogen balloon overnight. The mixture was then partitioned between DCM and 1M HCl. The aqueous phase (top) was then re-washed with DCM and reunited with the organic phase from the previous wash. The organic phases were then grouped and washed with 10% NaHCO$_3$, 10% brine, dried over MgSO$_4$, filtered and concentrated. The filtrate was purified over a silica column eluted with a 3:1 petroleum ether:ethylacetate to give the pure product. The fractions collected were ran on TLC’s to check for (4) at an R$_f$ value of about 0.6 in a 1:9 (petroleum ether:ethylacetate). Fractions that contained a single spot at around 0.6 were collected and concentrated to give (4) as a brown oil (0.783g, 1.0 mmol, 73%).

2.2.1.4 Cholesterol-O-PEG$_{150}$-O-N-hydroxyphthalimide (5)

To a stirred solution of N-hydroxyphthalimide (0.169g, 1.04 mmoles) in 0.77 mL of anhydrous DMF, NaHCO$_3$ (0.087g, 0.00104 moles) was added. The mixture was heated to 80°C under reflux for half an hour until the mixture turned dark brown. A solution of (4) (0.36g, 0.52 mmol) dissolved in 0.257 mL of anhydrous DCM was then added to the heated mixture and kept at 80°C overnight under reflux and a nitrogen balloon. The crude mixture was then filtered and
the residue was concentrated under vacuum. The product was then taken up with DCM and was washed with NH₄Cl (10%), water, and brine (10%) and was then dried using MgSO₄, filtered, and concentrated under vacuum. This gave a cloudy white oil that was purified on a silica column with a 1:2 and 1:1 (ethylacetate:hexane) gradient yielding (5) as a colorless oil (0.28g, 0.37 mmol, 77%).

2.2.1.5 Cholesterol-O-PEG₁₅₀-Oxyamine (6)

(5) (0.12g, 0.17 mmol) was taken up in 2 mL of anhydrous DCM and hydrazine monohydrate (0.042g, 0.84 mmol) was then added to the reaction mixture along with a stir bar. The precipitation of the phthalhydrazide formed immediately. The reaction was allowed to react overnight. The crude mixture was then filtered and the residue was disposed. The filtrate was diluted with 5 mL of chloroform and was washed 5 times with water, and 3 times with brine (10%). The organic phase was then dried using MgSO₄. The solvent was evaporated under vacuum to yield (6) as a gum (0.075g, 0.000126 moles, 66%).

2.2.2 CHOLOA₄₀₀

2.2.2.1 Cholesterol-O-Tosylate (2)

The same procedure mentioned on page 10 was followed.

2.2.2.2 Cholesterol-O-PEG₄₀₀ (3)

20 mL of PEG₄₀₀ (22.6g, 56.5 mmol) were dried by heating to 70°C under vacuum overnight to remove any moisture. This was then added to a solution of (2) (1.5 g, 2.8 mmol) in 50 mL of anhydrous 1,4-dioxane (99.8%). The mixture was stirred under reflux overnight in an inert atmosphere and a nitrogen balloon at a 110°C. After the completion of the reaction, the 1,4-dioxane was evaporated off under vacuum. The resulting brown oil was then take up in
diethyl ether and transferred to a separatory funnel. Two partitions resulted from this step due to the different polarities of diethyl ether and the excess PEG₄₀₀. The PEG₄₀₀ partition (bottom) was removed and washed two more times with diethyl ether to make sure that none of the desired product is lost. The diethyl ether partitions were then grouped and washed with 10% NaHCO₃ three times and dried with MgSO₄. The diethyl ether was then evaporated off under vacuum to obtain a brownish/yellow oil. The oil was then taken up in DCM and was purified on a silica column eluted with a 100% DCM transitioning to 10% methanol in DCM gradient. The fractions collected were ran on TLC’s to check for (3) at an R_f value of 0.4-0.5. Fractions containing (3) were collected and concentrated to give (3) as a brown/yellow oil (1.24g, 1.63 mmol, 58.2%).

2.2.2.3 Cholesterol-O-PEG₄₀₀-O-N-hydroxyphthalimide (4)

(4) was prepared by first dissolving (3) (0.80g, 1.05 mmol), N-hydroxyphthalimide (0.34g, 2.10 mmol), and resin-bound TPP (1.6 mmol/g, 1.31g, 2.10 mmol) in 30 mL of anhydrous THF (98%) in a round bottom flask. The reaction mixture was left to dissolve at room temperature and a nitrogen balloon. Upon full dissolution, the reaction mixture was cooled to 0°C and a dilute solution of diisopropyl azidocarboxylate (0.5 mL in 4 mL of anhydrous THF, 2.10 mmol) was added in a dropwise fashion. The reaction was then allowed to react overnight. Using 20 mL of ethanol, the reaction was quenched upon completion and was left for half an hour. The polymer-bound TPPO was filtered off and the filtrate was collected and concentrated under vacuum. The product was applied to a silica column and eluted with a 1:1 (ethylacetate : hexanes), 100% DCM, and 10% methanol in DCM gradient to yield 0.76 g of (4) (79% yield) as a brown coloured oil.
2.2.2.4 Cholesterol-O-PEG<sub>400</sub>-Oxyamine (5)

(4) (0.76 g, 0.84 mmol) was taken up in 12 mL of anhydrous DCM and hydrazine monohydrate (0.042g, 0.84 mmol) was then added to reaction mixture under inert conditions. The precipitation of the phthalhydrazide formed immediately. The reaction was allowed to react overnight. Upon completion of the reaction, the crude mixture was then filtered and the residue was disposed. The filtrate was diluted with 5 mL of chloroform and was washed 5 times with water, and three times with brine (10%). The organic phase was then dried using MgSO<sub>4</sub> and filtered. The solvent was evaporated under vacuum to yield (5) as a paste (0.075g, 0.000126 moles, 66%).

2.3 CHOLO<sub>400</sub>-Glutaraldehyde Reaction

Glutaraldehyde (0.013 g, 0.13 mmol) and CHOLOA<sub>400</sub> (0.01 g, 0.013 mmol) were each separately dissolved in 1 mL of ethanol. The glutaraldehyde solution was added to a small vial along with a stir bar under inert conditions. The CHOLOA<sub>400</sub> solution was then added to the vial in a dropwise fashion over a period of half an hour to avoid the formation of the bis-compound. After it was all added, the solutions were allowed to react for an hour. Following the completion of the reaction, the ethanol was evaporated under vacuum. The reaction mixture was then taken up with 5 mL of hexanes and washed with water twice to remove the excess glutaraldehyde. The organic phase was collected and concentrated to yield the CHOLOA<sub>400</sub>-Glutaraldehyde conjugate.
2.4 Modifying Amine Functionalized Glass Beads with Glutaraldehyde

10 mg of amine functionalized GM-beads (1.01μm in diameter) were weighed into an eppendorf. Then, 1 mL of pH 7.4 HEPES buffer was added to the GM-bead and were vortexed and sonicated until the microbeads broke up and were dispersed throughout the solution. They were then centrifuged for 10 minutes at 10 kpm. The supernatant was discarded and this process was repeated two more times to make sure that the microbeads have been washed thoroughly. After discarding the supernatant from the third wash, 0.25 g of Na(CN)BH₃ (3.9 mmol), 0.25 g of glutaraldehyde (2.5 mmol), and 1 mL of MilliQ water were added to the microbeads in the eppendorf. The eppendorf was then vortexed and sonicated until the microbeads were completely dispersed throughout the solution. The eppendorf was then wrapped with aluminum foil due to the reactions photosensitivity and placed on a rocking platform overnight to ensure continuous mixing. Upon completion of the reaction, the reaction mixture was centrifuged for 10 minutes at 10 kpm. The supernatant was disposed and the reaction mixture was washed twice with HEPES buffer as mentioned earlier. Following the washing steps, the beads were suspended in 1 mL of HEPES buffer to yield a 10 mg/mL solution and were stored in the refrigerator at 4°C.
2.5 Preparation of Liposomes

Liposomes were prepared in an eppendorf by dissolving appropriate amounts of POPC, CHOLOA₄₀₀, and NBD-PE (99, 1, and 0.05 mol % respectively) in chloroform. Chloroform was then removed under a stream of nitrogen leaving behind a lipid film along the walls of the eppendorf. The eppendorf was left in a desiccator overnight to ensure all the remaining traces of solvent have been removed. The following day, 1 mL of pH 7.4 HEPES buffer was added to the lipid film. The eppendorf was vortexed and was left for an hour to hydrate the lipid film. The resulting hydrated lipid was subjected to five freeze-thaw cycles where they were frozen in liquid nitrogen and thawed in a 60°C water bath to mix the lipid components well. The hydrated lipids...
l lipid was then extruded using an extruder through a 0.1μm membrane filter, going back and forth 25 times to convert the generate LUVs. The LUV solution was then transferred into an eppendorf and stored in the refrigerator at 4°C until use.

2.5 Binding Assay

The binding assay was performed by adding appropriate amounts of liposome and microbeads stocks, according to the fold excess of liposomes that was being achieved. Then, different amount of HEPES buffer were added to different samples to maintain the final volume and keep it consistent with all the other samples. For example:

<table>
<thead>
<tr>
<th>Fold-Excess</th>
<th>Liposome Stock Concentration (mg/mL)</th>
<th>Liposome Stock Volume (μL)</th>
<th>HEPES Buffer Volume (μL)</th>
<th>Microbeads Stock Concentration (mg/mL)</th>
<th>Microbeads Stock Volume (μL)</th>
<th>Final Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.866</td>
<td>10</td>
<td>90</td>
<td></td>
<td></td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>20</td>
<td>80</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>30</td>
<td>70</td>
<td>10</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>40</td>
<td>60</td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Following the addition of the correct amounts of liposome stock, microbeads stock, and HEPES buffer, samples were then vortexed and sonicated to ensure that the microbeads were dispersed throughout the samples and left on a rocking plate for an hour to allow binding to occur. During this period, the samples were vortexed every 15 minutes. Samples were then centrifuged for 10 minutes at 10 kpm. The supernatants were carefully transferred to another eppendorf and labelled “Supernatant 1”. The volumes of all the “Supernatant 1” obtained from
all the different samples were then doubles using 0.4M sodium cholate, to result in a final concentration of 0.2M. 100µL of HEPES buffer was then added to the pellets from all the samples and were then sonicated, vortexed, and centrifuged. The supernatants were then collected and added to new eppendorfs and were labelled “Supernatant 2”. The volumes of all “Supernatant 2” obtained from all the different samples were then doubled using 0.4M sodium cholate, to result in a final concentration of 0.2M. To the pellets, 200µL of 0.2M sodium cholate were added and were each vortexed, sonicated, and centrifuged. The supernatants were then transferred to a new eppendorfs labelled “Bound”.

### 2.5.1 Calibration Curve for Binding Assay

Calibration curves were prepared by using different amount of liposome stock in different wells, but at the same time ensuring that the sodium cholate and the buffer concentration remained constant throughout. For example:

**Table 2.2 Quantities of components used to prepare solutions for calibration curve for the binding assay**

<table>
<thead>
<tr>
<th>Liposomes Stock Concentration (mg/mL)</th>
<th>Liposome Stock Volume(µL)</th>
<th>Sodium Cholate Concentration (M)</th>
<th>Sodium Cholate Volume(µL)</th>
<th>HEPES Buffer (µL)</th>
<th>Final Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.4</td>
<td>100</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>100</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>100</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td>100</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td>100</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td></td>
<td>100</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td></td>
<td>100</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>
2.5.2 Microplate Preparation for Fluorescence Readings

The microplate was prepared by pipetting the appropriate amounts of Supernatant 1, Supernatant 2, and Bound solutions along with HEPES buffer and sodium cholate. The solutions were first prepared in eppendorfs and were then transferred into the wells of the microplate. The final volume of each sample was kept consistent as well as the final concentration of sodium cholate. The following table is an example:
Table 2.3 Quantities of different components used for the preparation of the microplate for fluorescence readings

<table>
<thead>
<tr>
<th>Supernatant (uL)</th>
<th>HEPES Volume (μL)</th>
<th>Initial Sodium Cholate Concentration (M)</th>
<th>Sodium Cholate Volume (μL)</th>
<th>Final Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>90</td>
<td>0.2</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6 Fluorescence Measurements

Fluorescence measurements were performed on a Tecan Infinite M1000Pro microplate fluorimeter with 200μL wells. The following parameters were used for fluorescence measurements:

Table 2.4 Fluorimeter setting used for the fluorescence measurements

<table>
<thead>
<tr>
<th>Excitation Wavelength</th>
<th>470 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emission Wavelength</td>
<td>535 nm</td>
</tr>
<tr>
<td>Bandwidth Excitation</td>
<td>5 nm</td>
</tr>
<tr>
<td>Bandwidth Emission</td>
<td>2.5 nm</td>
</tr>
<tr>
<td>Number of Flashes</td>
<td>50</td>
</tr>
<tr>
<td>Integration Time</td>
<td>20 μs</td>
</tr>
<tr>
<td>Settle Time</td>
<td>50 μs</td>
</tr>
</tbody>
</table>
Chapter 3: Results and Discussion

3.1 Results

3.1.1 Reaction Schemes

Different reaction schemes were used to synthesize CHOLOA\textsubscript{150} and CHOLOA\textsubscript{400}. CHOLOA\textsubscript{400} required a different synthesis approach due to the incomplete conversion of Cholesterol-O-PEG\textsubscript{400} to Cholesterol-O-PEG\textsubscript{400}-O-tosylate and the complexity of the purification of the N-hydroxyphthalimide intermediate. To overcome these issues, the N-hydroxyphthalimide intermediate was successfully synthesized using the Mitsunobu reaction. Resin-bound TPP was used instead of unbound TPP to ease the purification of the desired product.
3.1.1.1 CHOLOA

Figure 3.1 Synthetic scheme for CHOLOA

Figure 3.2 Synthetic scheme for CHOLOA\textsubscript{400}

The synthetic scheme of CHOLOA\textsubscript{400} using the Mitsunobu reaction. All the different reactants and solvents used are included in the diagram. (1) Cholesterol (2) Cholesterol-O-Tosylate (3) Cholesterol-O-PEG\textsubscript{400} (4) Cholesterol-O-PEG\textsubscript{400}-O-N-Hydroxyphthalimide (6) Cholesterol-O-PEG\textsubscript{400}-Oxyamine/CHOLOA\textsubscript{400}. 
3.1.2 $^1$H NMR (400 MHz) and Mass Spectra

3.1.2.1 CHLOA$_{150}$

3.1.2.1.1 Cholesterol (1)

The $^1$H NMR spectrum of free cholesterol was obtained to ensure the purity of the starting material. The obtained peaks match the expected peaks in the literature, confirming 100% purity.

Figure 3.3 $^1$H NMR (400 MHz) of Cholesterol

$^1$H (400 MHz, CDCl3): δ 0.65 (3H, s); 0.85-1.57 (33H, m); 1.78-2.01 (6H, m); 2.23-2.29 (2H, m); 4.28-4.33 (1H, m); 5.29 (1H, d,J=4.5Hz)$^{17}$. 
3.1.2.1.2 Cholesterol-O-Tosylate (2)

All expected peaks were observed in the obtained spectrum (figure 3.4)\(^{18}\). Proton #1 in figure 3.5 resonates at about 3.53 ppm before the tosylate group was attached. Figure 3.4 shows a change in proton #1’s chemical shift to about 4.30 ppm. The change in chemical shift indicates that a reaction has taken place at this location and that the tosylate group was successfully attached.

**Figure 3.4** \(^1\text{H} \text{NMR (400 MHz)} \) of Cholesterol-O-Tosylate

\(^1\text{H} \text{(400 MHz, CDCl}_3\text{): } \delta \text{ 0.65 (3H, s); 0.85-1.57 (33H, m); 1.78-2.01 (6H, m); 2.23-2.29 (2H, m); 2.44 (3H, s); 4.28-4.33 (1H, m); 5.29 (1H,d,} J=4.5\text{Hz);7.32(2H,d,} J=8\text{Hz);7.79(2H,d,} J=8 \text{ Hz).}

**Figure 3.5** Structure of Cholesterol-O-Tosylate
Also, aromatic peaks at 7.32 and 7.79 ppm correspond to the 4 aromatic protons on the tosyl group (#3). The three protons on the methyl group on the tosyl (#2) are clearly resolved in figure 3.4 at around 2.44 ppm. There are only one set of tosyl peaks in the aromatic region, indicating that there is no excess p-tulenesulfonyl chloride is present and that the product is pure.

3.1.2.1.3 Cholesterol-O-PEG$_{150}$ (3)

\textit{Cholesterol-PEG(150)}

![NMR Spectrum](image)

**Figure 3.6** $^1$H NMR (400 MHz) of Cholesterol-O-PEG$_{150}$

$^1$H (400 MHz, CDCl$_3$); $\delta$ 0.85-1.60 (33H, m); 1.79-2.06 (6H, m); 2.18-2.29 (1H, m); 2.37-2.41 (1H, m); 3.16-3.23 (1H, m); 3.60-3.78 (12H, m); 5.37 (1H, d, J=4.5 Hz).

All expected peaks from the literature were observed in the spectrum obtained (figure 3.6)\textsuperscript{18}. Proton #2 in figure 3.7 resonates at around 3.2 ppm in the NMR spectrum, which
shows a change from the chemical shift it had in Cholesterol-O-Tosylate’s spectrum (4.33ppm). This indicates that a reaction has taken place at this location and that the PEG\textsubscript{150} subunit was successfully attached.

The terminal OH group (#1) shows a chemical shift at around 5.4 ppm, which is clearly resolved in the NMR spectrum. Also, the PEG\textsubscript{150} peaks (#3) are clearly resolved at around 3.78 ppm, which match the expected value in the literature. No aromatic peaks were observed indicating that the tosyl groups have been successfully substituted by PEG\textsubscript{150}, and that the product is pure.

Figure 3.7 Structure of Cholesterol-O-PEG\textsubscript{150}
3.1.2.1.4 Cholesterol-O-PEG150-O-Tosylate (4)

The synthesis of Cholesterol-O-PEG150-O-Tosylate (4) was confirmed using 1H NMR (400 MHz). After analyzing the spectrum, the peaks obtained matched the peaks expected. Proton # 1 in figure 3.9 resonates at about 3.11-3.20 ppm, which matches the literature value.
The first 8 PEG\textsubscript{150} protons (#2) show a chemical shift of about 3.5 ppm. The later 4 PEG\textsubscript{150} protons (#3) are shifted more downfield at 3.6 and 4.15 ppm. These results indicate the successful attachment of the tosylate subunit to the PEG\textsubscript{150} subunit attached to the Cholesterol, as the tosyl subunit deshields the later 4 PEG\textsubscript{150} protons (#3), therefore shifting them more downfield.

The aromatic peaks at 7.31-7.36 ppm and 7.7-7.81 ppm correspond to the 4 aromatic protons on the tosyl group (#4). The three methyl protons of the tosyl group (#5) are clearly resolved at around 2.47 ppm. One set of tosyl peaks are present indicating the absence of any extra tosyl.
3.1.2.1.5 Cholesterol-O-PEG$_{150}$-O-N-Hydroxyphthalimide (5)

![Chemical structure](image)

The synthesis of Cholesterol-O-PEG$_{150}$-O-N-Hydroxyphthalimide (5) was confirmed by using $^1$H NMR (400 MHz) and peaks obtained were compared with peaks given in the literature. The PEG$_{150}$ (#1) show 3 different chemical shifts from 3.6 to 4.4 ppm (shown in figure 3.10). However,

![NMR spectrum](image)

Figure 3.10 $^1$H NMR (400 MHz) of Cholesterol-O-PEG$_{150}$-O-N-Hydroxyphthalimide

$^1$H NMR (400MHz, CDCl$_3$): δ 0.67 (3H, s); 0.85 (3H, d, J=6.6 Hz); 0.86 (3H, d, J=6.6Hz); 0.90 (3H, d, J=6.5Hz); 0.99 (3H, s); 0.83-2.39 (28H, m); 2.44 (3H, s); 3.13-3.20 (1H, m); 3.55-3.70 (m, 10H), 3.83 (t, 2H), 4.45 (t, 2H), 7.72 (m, 2H), 7.81 (m, 2H).
the sum of the integrals of the 3 PEG₁₅₀ peaks is equal to 12.2, which is what is expected. This pattern is observed due to the fact that the protons closer to the hydroxyphthalimide ring are more shielded by the electron density and are therefore shifted more downfield than the earlier protons. The difference in chemical shifts indicate that the PEG₁₅₀ protons are within different environments due to the successful attachment of the hydroxyphthalimide subunit.

Proton # 2 in figure 3.11 shows a chemical shift of about 3.11-3.20 ppm, which matches the expected value. The protons of the hydroxyphthalimide ring (#3) correspond to the two peaks between 7.6-8.0 ppm. The integrals of each of these correspond to 4 aromatic protons.
3.1.2.1.6 Cholesterol-O-PEG$_{150}$-Oxyamine/CHOLOA$_{150}$ (6)

The synthesis of Cholesterol-O-PEG$_{150}$-Oxyamine/CHOLOA$_{150}$ (5) was confirmed by using $^1$H NMR (400 MHz) and peaks obtained were compared with peaks given in the literature.$^{18}$

The PEG$_{150}$ protons (#1) in figure 3.13 resonate at two different ppm, 3.6 and 4.2, in figure 3.12 due to the difference in shielding caused by the nitrogen on some of the protons.

**Figure 3.12 $^1$H NMR (400 MHz) of Cholesterol-O-PEG$_{150}$-Oxyamine/CHOLOA$_{150}$**

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 0.67 (3H, s); 0.85 (3H, d, J=6.6 Hz); 0.86 (3H, d, J=6.6Hz); 0.90 (3H, d, J=6.5Hz); 0.99 (3H, s); 0.83-2.39 (28H, m); 2.44 (3H, s); 3.13-3.20 (1H, m); 3.55-3.70 (m, 12H), 4.45 (t, 2H).
The protons on the nitrogen (#2) are expected to resonate upfield at around 1-2 ppm. Unfortunately, due to the presence of the cholesterol peaks in that range the protons on the nitrogen cannot be identified.

Also, figure 3.12 also shows the disappearance of the aromatic protons that correspond to the hydroxyphthalimide at around 7.6-8.0 ppm. This indicates that the substitution reaction has occurred successfully and that the product is pure.

3.1.2.2 CHOLOA₄₀₀

3.1.2.2.1 Cholesterol (1)

For the ¹H NMR spectrum of free cholesterol, refer to page 24.

3.1.2.2.2 Cholesterol-O-Tosylate (2)

The synthesis of Cholesterol-O-Tosylate was confirmed in the same way as discussed on page 25.
3.1.2.2.3 Cholesterol-O-PEG\textsubscript{400} (3)

![Cholesterol-PEG(400)](image)

Figure 3.14 \textsuperscript{1}H NMR (400 MHz) of Cholesterol-O-PEG\textsubscript{400}

\textsuperscript{1}H (400 MHz, CDCl\textsubscript{3}); \(\delta\) 0.85-1.60 (33H, m); 1.79-2.06 (6H, m); 2.18-2.29 (1H, m); 2.37-2.41 (1H, m); 3.16-3.23 (1H, m); 3.60-3.78 (40H, m); 5.37 (1H, d, J=4.5 Hz).

All expected peaks were observed in the spectrum obtained (figure 3.14). Proton #2 in figure 3.15 resonates at around 3.3 ppm in the NMR spectrum, which shows a change from the chemical shift it had in Cholesterol-O-Tosylate’s spectrum (4.33ppm). This indicates that a reaction has taken place at this location and that the PEG\textsubscript{400} subunit was successfully attached. The terminal OH group (#1) shows a chemical shift at around 5.4 ppm, which is clearly resolved in the NMR spectrum. Also, the PEG\textsubscript{400} peaks (#3) are clearly resolved at around 3.70 ppm,
which match the expected value in the literature. No aromatic peaks were observed indicating that the tosyl groups have been successfully substituted by PEG$_{400}$, and that the product is pure.

To further confirm the synthesis of Cholesterol-O-PEG$_{400}$ (3), mass spectrum was ran (figure 3.16). A mass spectrum of free PEG$_{400}$ is shown on the top of the figure. A Gaussian distribution is observed due to the fact that the PEG$_{400}$ used was polydispersed, meaning that it was an average of 10 ethylene glycol units, or an average of 400 in molecular weight. The difference between each peak and the peak prior or later to it is 44 units, which is the weight of a single ethylene glycol unit.

The mass spectrum on the bottom shows a clear shift in the Gaussian distribution towards the right by approximately 324 units. This happens to be approximately the weight of the cholesterol group that is being added onto PEG$_{400}$, therefore further confirming the conjugation between the two and the successful synthesis of Cholesterol-O-PEG$_{400}$.
3.1.2.2.4 Cholesterol-O-PEG<sub>400</sub>-O-N-Hydroxyphthalimide (4)

The synthesis Cholesterol-O-PEG<sub>400</sub>-O-N-Hydroxyphthalimide (4) was confirmed by using <sup>1</sup>H NMR (400 MHz) and mass spectra. Peaks obtained in matched peaks expected.

The PEG<sub>400</sub> protons (#1) shown in figure 3.17 show 3 different chemical shifts from 3.6 to 4.4 ppm in figure 3.18.
However, the sum of the integrals of the 3 peaks add up to the expected number of proton. The PEG$_{400}$ peaks do not show a single peak due to the shielding effects of the electron dense hydroxyphthalimide ring, causing some protons to resonate more downfield. The difference in chemical shifts indicate that the PEG$_{400}$ protons are within different environments due to the successful attachment of the N-Hydroxyphthalimide subunit.

Proton #2 in figure 3.17 shows a chemical shift of about 3.11-3.20 ppm, which matches the expected value. The protons of the hydroxyphthalimide ring (#3) correspond to the two peaks between 7.5-7.7 ppm.

**Figure 3.18** $^1$H NMR (400 MHz) of Cholesterol-O-PEG$_{400}$-O-N-Hydroxyphthalimide

$^1$H NMR (400MHz, CDCl3): δ 0.67 (3H, s); 0.85 (3H, d, J=6.6 Hz); 0.86 (3H, d, J=6.6Hz); 0.90 (3H, d, J=6.5Hz); 0.99 (3H, s); 0.83-2.39 (28H, m); 2.44 (3H, s); 3.13-3.20 (1H, m); 3.55-3.70 (m, 20H), 3.83 (t, 10H), 4.10 (t, 10H), 7.72 (m, 2H), 7.81 (m, 2H).
The synthesis of Cholesterol-O-PEG$_{400}$ was further confirmed by running mass spectra. Figure 3.19 above shows the shifting of Cholesterol-O-PEG$_{400}$’s peaks by approximately 145 units, which is the size of the N-Hydroxyphthalimide group.

**Figure 3.19 LCMS of Cholesterol-O-PEG$_{400}$ and Cholesterol-O-PEG$_{400}$-N-Hydroxyphthalimide**

The LCMS of Cholesterol-O-PEG$_{400}$ (top) and Cholesterol-O-PEG$_{400}$-O-N-Hydroxyphthalimide (bottom) are shown. The conjugation was confirmed by the clear shift observed by Cholesterol-O-PEG$_{400}$’s peaks by approximately 145 units, which is the size of the N-Hydroxyphthalimide unit being added.

The synthesis Cholesterol-O-PEG$_{400}$-O-N-Hydroxyphthalimide (4) was further confirmed by running mass spectra. Figure 3.19 above shows the shifting of Cholesterol-O-PEG$_{400}$ (3)’s peaks by 145 units to the right, confirming the addition of the N-Hydroxyphthalimide group.
3.1.2.2.5 Cholesterol-O-PEG<sub>400</sub>-Oxyamine/CHOLOA<sub>400</sub> (5)

The synthesis of Cholesterol-O-PEG<sub>400</sub>-Oxyamine/CHOLOA<sub>400</sub> (5) was confirmed by using <sup>1</sup>H NMR (400 MHz) and mass spectrum. The peaks obtained matched the peaks expected.

The PEG<sub>400</sub> protons (#1) in figure 3.21 resonate at two different ppm, 3.6 and 4.2, in figure 3.20 due to the difference in shielding caused by the oxyamine nitrogen on some of the protons.
The protons on the nitrogen (#2) are expected to resonate upfield at around 1-2 ppm, however, due to the presence of the cholesterol peaks in that range the protons on the nitrogen were not identified.

Also, figure 3.20 also shows the disappearance of the aromatic protons that correspond to the N-Hydroxyphthalimide group at around 7.6-8.0 ppm. This indicates that the substitution reaction has occurred successfully and that the product is pure.

**Figure 3.21 Structure of Cholesterol-O-PEG400-Oxyamine/CHOLOA400**

**Figure 3.22 LCMS of free Cholesterol-O-PEG400-N-Hydroxyphthalimide and Cholesterol-O-PEG400-Oxyamine/CHOLOA400**

The LCMS Cholesterol-O-PEG400-O-N-Hydroxyphthalimide (top) and Cholesterol-O-PEG400-Oxyamine/CHOLOA400 (bottom) are shown. The conjugation was confirmed by the clear backwards shift observed by Cholesterol-O-PEG400-O-N-Hydroxyphthalimide’s peaks by approximately 132 units, which is the size of the N-Hydroxyphthalimide unit being displaced.

CHOLOA400 (5)’s synthesis was also confirmed by running mass spectra. Figure 3.22 above shows the backward shift Cholesterol-O-PEG400-O-N-hydroxyphthalimide (4)’s peaks by
approximately 132 units, which exactly matches the weight of the N-Hydroxyphthalimide group that is being displaced during the hydrazine reaction.
3.2 Discussion

3.2.1 Synthesis and Purification Complications

3.2.1.1 Avoid the Running of Columns

The synthesis of CHOLOA is a five-step reaction scheme, of which three require purification via column chromatography. When attempting to purify using columns, it was noted that due to the similarity in polarity of the components that are being separated, too much time was being spent on developing optimum conditions and solvent systems for successful separations and that lead to the loss of yield and the waste of time. In interest to maximize efficiency, save time, and increase reaction yields, a new approach was taken. The plan of action was to purify the products of each step of the reaction scheme using a two-phase wash, catered to each reaction's specific circumstance, without running any columns. Eventually, a column would be ran at the very last step of the reaction scheme to ensure high purity of CHOLOA (figure 3.23). This way less time would be spent on the development of solvent systems for columns and the synthesis of CHOLOA would be achieved faster, therefore maximizing efficiency.

**Figure 3.23 Avoiding silica columns**
The figure clarifies the approach of not running silica columns throughout the synthetic route, until the very last step to separate CHOLOA from all the other impurities.
The ideas supporting this approach were that a two-phase system should alone be sufficient to purify the desired product, and if not, byproducts of each of the steps should not affect consecutive reactions, the incorporation of CHOLOA into liposomes, nor the chemoselective ability of CHOLOA to form oxime linkages with the aldehyde functionalized beads.

After attempting this approach, it was decided that it was not the correct way of action. This decision was made due to the complications faced when trying to synthesize later intermediates within the reaction scheme and the appearance of various unknown impurities. This made the completion of CHOLOA’s synthesis very difficult, if not impossible. Also, it was thought that if CHOLOA was synthesized successfully, having all other intermediates within the reaction mixture would make it even harder to purify over a silica column, due to their very small difference in structures and polarity. Therefore, the synthesis of CHOLOA was continued by purifying crude products fully, even if that meant by running a column, before moving onto the next reaction.

3.2.1.2 Cholesterol-O-PEG400

3.2.1.2.1 Synthesis of Cholesterol-O-PEG400

To synthesize Cholesterol-O-PEG400 a good leaving group was first required on Cholesterol’s terminal alcohol group. This was achieved by the tosylation of the alcohol group to yield the tosylated intermediate. Then, it was reacted it with PEG400, which in turn substituted the tosyl group via an SN₂ attack.
The molar ratio of Cholesterol-O-Tosylate to PEG\textsubscript{400} during the reaction was approximately 1:20. Such a large fold excess of PEG\textsubscript{400} while performing this reaction is required to avoid the bis-substitution of Cholesterol. The bis-product consists of a PEG\textsubscript{400} molecule with two cholesterol molecules attached to each of its terminal oxygens. This byproduct is not desired as it is not possible to proceed on with further reactions due to the burial of the functional groups required for later steps of the synthetic scheme.

### 3.2.1.2.1.1 Modifying the Fold Excess

When attempts were made to purify the crude product of the reaction, many problems were faced. The first problem faced was due to the large amount of excess PEG\textsubscript{400}, the crude product weighed approximately 25-30g. To purify this amount on a silica column, at least 500g of silica was required, which is equivalent to around six to seven columns, to avoid over saturating the column and obtaining a good separation. Obviously running seven different columns to purify one reaction was not reasonable and an alternative method was needed to be found.

One of the alternative methods attempted was the modification of the fold excess of PEG\textsubscript{400} used. After going through the literature, a couple of sources were found that were using...
as low as three fold-excess of PEG$_{400}$ and still avoiding the bis-product. The PEG-ylation reaction was repeated with only three-fold excess of PEG$_{400}$. Upon completion of the reaction, NMR spectra of the crude product showed that only 20% of the reactants were converted to products.

The experiment was then repeated with a ten-fold excess PEG$_{400}$ to increase the percentage of reactant conversion, thus increasing the yield of the reaction. This lead to a 65% conversion of reactants, which made the purification steps a lot easier, however, gave a moderate reaction yield.

After several trials, it was concluded that having a larger fold excess of PEG$_{400}$ did not only prevent the formation of the bis-product, but also insured a higher reactant to product conversion rate. Therefore, the approach of using less than twenty-fold excess was abandoned and other alternatives had to be considered.

### 3.2.1.2.2 Purifying Cholesterol-O-PEG$_{400}$

Assuming a 100% conversion, nineteen folds of PEG$_{400}$ is expected to be in the crude mixture. To purify the product of interest (Cholesterol-O-PEG$_{400}$) all the excess PEG$_{400}$ and other byproducts, such as the tosyl groups, have to somehow be removed. Usually, crude products are purified using a two-phase wash system, either on its own or followed by a silica column. Ideally, it is preferred that a two-phase washing system is established to fully purify the product of interest without having to further rely on running a silica column, as they can sometimes be very complex.
3.2.1.2.2.1 Two-Phase Systems

Purification via a two-phase system, or as often called extraction, is a separation method that is first relied on when trying to purify a crude mixture. It works by extracting a desired compound out of a mixture using a solvent it prefers. Extraction relies on the phenomenon of partitioning and the different solubilities of different components in the mixture in two different immiscible solvents. Being immiscible, water and most organic solvents, such as diethyl ether, form two distinct layers because the two don’t dissolve well in one another. If a solute was added to this system, the solute would distribute itself between the two immiscible solvents according to a partitioning coefficient for that specific system, $K$.

A partitioning coefficient ($K$) of a specific substance ($S$) in a two solvent system ($X$ and $Y$) is the ratio of the concentration of $S$ in solvent $X$ compared to the concentration in solvent $Y$.

$$K = \frac{\text{Concentration of } S \text{ in } X}{\text{Concentration of } S \text{ in } Y}$$

Equation (1)

For example, if $S$ had a partitioning coefficient equal to 1 for solvents $X$ and $Y$, then $S$ would be equally distributed between the two solvents.

To purify Cholesterol-O-PEG$_{400}$, two impurities have to be removed, the substituted tosyl group and the excess PEG$_{400}$. When approaching this issue and trying to figure out an appropriate two-phase system to use, it was clear at first that the PEG$_{400}$ would easily be removed by a simple water wash, since PEG is known to be very water soluble. A couple of two-phase systems were attempted till an optimum system was achieved.

3.2.1.2.2.1.1 Chloroform-Water Wash

When attempting to wash the crude product using chloroform and water, it was hypothesized that the desired product, Cholesterol-O-PEG$_{400}$, would remain in the organic phase (chloroform) due to its highly hydrophobic characteristics, while the excess PEG$_{400}$ would move
into the aqueous phase (water) due to its highly hydrophilic characteristics. After performing the wash, NMR spectra confirmed that the organic layer still contained excess PEG$_{400}$ and that the separation was not successful. Also, the spectra showed that the tosyl groups also preferred the organic phase, meaning that this specific system did not accomplish any purification.

### 3.2.1.2.1.2 Hexane-Water Wash

After attempting to wash with chloroform and water and learning from that experiment, the results stated that a less polar organic solvent must be used in order to make the excess PEG$_{400}$ prefer the aqueous phase. By checking the polarity indices of several organic solvents, hexanes seemed to be one of the more non-polar options with a polarity index of 0.1, compared to 4.1 of that of chloroform. It was also found that the partition coefficient of PEG$_{400}$ between hexanes and water is $1.5 \times 10^{-5}$, meaning that for 1.5 parts of PEG$_{400}$ in hexanes, there are a 100,000 part of PEG$_{400}$ in water.$^{21}$

Performing this wash proved that indeed, most of the excess PEG$_{400}$ did move to the aqueous phase. However, due to its very low polarity index, hexanes were also attracting more byproduct and unreacted material, such as tosyl groups and unreacted Cholesterol-O-Tosylate to the organic layer. This two-phase system did help get rid of the excess PEG$_{400}$, however raised other issues.

### 3.2.1.2.1.3 Diethyl Ether-Water Wash

The experience obtained from the first two washing systems performed was used to develop a third washing system that is more suitable for this purification. Results showed that chloroform was too polar, dissolving the majority of the excess PEG$_{400}$, and hexanes were too non-polar, dissolving other impurities. A middle ground was needed to be established, a solvent
that was not too polar nor too non-polar. With the polarity index of 2.8, diethyl ether seemed like the perfect balance.

Washing the crude product with diethyl ether and water showed promising results. The organic phase showed a lot less of tosyl groups and excess PEG\textsubscript{400} than that of the crude product. By performing sequential washes with the same system, it was noticed that impurity peaks would keep on decreasing.

3.2.1.2.1.4 Diethyl Ether- NaHCO\textsubscript{3} Wash

Even though the diethyl ether – water wash proved to be successful, an improvement was made to increase its efficiency. Instead of having to perform many sequential washes, an extra wash with 10\% NaHCO\textsubscript{3} was added. NaHCO\textsubscript{3} is an alkaline wash that is usually used to neutralize unwanted acid impurities, which in this case were the tosyl groups. By doing so, NMR spectra proved no sign of tosyl peaks and that the crude product was successfully purified.
3.2.1.2.2 Running a Silica Column

To reassure that the crude product has been successfully purified using the diethyl ether-NaHCO₃ wash, a simple TLC plate was ran to check if any unanticipated spots would appear. Unfortunately, an unknown impurity at an R_f value of 0.8-0.9 showed up (shown in figure 3.25). This value matched the R_f value of free cholesterol, which could have been unreacted from the initial tosylation reaction. This was not detected using NMR due to the fact that free cholesterol and conjugated cholesterol peaks resonate at approximately the same region (0-2.5ppm). This was an indicator that developing a silica column system at this stage was vital, as the two-phase washing system optimized was not sufficient enough to fully purify the reaction. Having all the excess PEG₄₀₀ removed using the wash, it was straight forward to develop a silica column system to remove an impurity with such difference in polarity from the desired product. A 100% DCM to 10% methanol in DCM gradient was used to first get rid of any free cholesterol and then elute the desired product second.

**Figure 3.25 TLC plate of Cholesterol-O-PEG₄₀₀**

A TLC experiment in 1:1 (hexanes:ethylacetate) was ran on Cholesterol-O-PEG₄₀₀ to check for its purity. The TLC plate shows a slight impurity at an R_f value of 0.9, which happens to be unreacted cholesterol.
3.2.1.3 Reaction Routes to the N-Hydroxyphthalimide Activated Intermediate

3.2.1.3.1 Tosylation Instead of Mitsunobu for PEG\textsubscript{150}

While attempting the synthesis of Cholesterol-PEG\textsubscript{150}, a cleaner and easier approach to get the N-Hydroxyphthalimide intermediate was developed. Initially, the N-Hydroxyphthalimide activated intermediate was obtained using the Mitsunobu reaction, a vastly used reaction use to re-functionalize alcohols. Even though it worked, it was very tedious and required many different washes and columns to purify the desired product. The new reaction scheme developed made a small detour to get the N-Hydroxyphthalimide activated intermediate via the tosylation of Cholesterol-PEG\textsubscript{150} followed by the substitution of the tosyl group by N-Hydroxyphthalimide (shown in figure 3.26).
Following the alternate reaction route was found easier and straightforward. Also, the synthesis of the \( N \)-Hydroxyphthalimide intermediate was achieved at a yield of 81\%, in contrast to 72\% yield with the Mitsunobu reaction. Thus, it was clear that the alternate route developed was the way to go due to the ease of synthesis and purification.

3.2.1.3.2 Using the Alternative Route for Cholesterol-O-PEG\textsubscript{400}

The alternative route developed was followed the same way when the ethylene glycol unit was extended in length from three to ten. However, when a mass spectrum was obtained of the crude product, higher abundance of reactant peaks was observed, indicating that the reaction was not going to completion. Reaction conditions, such as duration and fold excess of tosyl, were altered in attempt to push the reaction to full completion. Increasing the reaction duration up to 48 hours (4 times the instructed time) and the fold-excess 5 times (5 times the instructed

**Figure 3.26 Alternative synthetic routes to obtain the N-Hydroxyphthalimide activated intermediate**

The figure shows an alternative to the Mitsunobu reaction (red) to get to the \( N \)-Hydroxyphthalimide activated intermediate (blue).
amount) showed no change in the extent of the reaction. In interest to avoid the waste of time, alternative methods were considered to synthesize the N-Hydroxyphthalimide intermediate for Cholesterol-O-PEG$_{400}$.

### 3.2.1.3.3 Going Back to The Mitsunobu

Due to the lack of time, it was not possible to go back and change the chemoselective chemistry or the approach for synthesizing CHOLOA$_{400}$. Therefore, going back to the Mitsunobu reaction was the best option to complete the synthesis of the N-Hydroxyphthalimide activated intermediate as completing the synthesis was a bigger priority than having higher reaction yields.

The most challenging aspect of performing the Mitsunobu reaction was the purification of the desired product from the reaction’s byproducts. The major byproduct that was an issue to get rid of was TPPO generated from TPP. The phosphor in plays an important role in the Mitsunobu reaction. The phosphor in initiates the reaction by making a nucleophilic attack on one of diisopropyl azido carboxylate’s (DIAD) nitrogens to generate the phosphonium intermediate. The other nitrogen in the phosphonium intermediate then deprotonates Cholesterol-O-PEG$_{400}$’s terminal alcohol, which in turn binds to the phosphonium intermediate, making it a good leaving group. N-Hydroxyphthalimide, the nucleophile, then substitutes the phosphonium intermediate via an SN$_2$ attack to yield the final product. The formation of TPPO drives the reaction forward due to the formation of the strong phosphor-oxygen double bond, however does hinder the purification of the desired product $^{22-23}$.

First, two-phase system attempts, such as diethyl ether and water, along with other combinations were unsuccessful to get rid of the TPPO. Both TPPO and the N-
Hydroxyphthalimide activated intermediate are hydrophobically dominated, however, still poses hydrophilic groups (phosphor in TPPO, and PEG in the activated intermediate). Due to the lack of a difference in polarity, they both tend to dissolve in the organic phase.

3.2.1.3.3.1 Precipitation of TPPO Using Hexanes

The low solubility of TPPO in hexanes inspired the next approach. The crude mixture was dissolved in minimal amount of hexanes and cooled to 0 °C, in attempt to precipitate out the TPPO. A white precipitate crashed out of solution at a low temperature.

The precipitation process was monitored using two different methods, $^{31}$P-NMR and mass spectrometry. $^{31}$P-NMR samples of starting product, TPP, and the white precipitate, thought to be TPPO, were taken. Spectra show that TPP resonates at approximately -6 ppm while TPPO at 27 ppm, which agrees with the peaks expected (shown in figure 3.27).
However, mass spectra (figure 3.28) obtained were not as easy to analyze for the reason being that three unaccounted peaks kept appearing. What was interesting was the fact that the three peaks would get smaller with every sequential precipitation round and that the difference between them was 278 units, which is exactly the mass of a single TPPO molecule.

The pattern noticed lead to developing the hypothesis that the three peaks can be accounted for TPPO monomers, dimers, and trimers. We postulate that the different TPPO monomers are physically bonded to each other via pi-pi stacking between their phenyl rings (shown in figure 3.29), leading to the unaccounted mass spectrum peaks.

Even though with sequential rounds of precipitation, the amount of TPPO that would precipitate decreased, indicating that less and less TPPO was present, this was not the optimum way to get rid of TPPO. This was due to the fact that more and more product was being lost during each round of precipitation.
Running a Silica Column to Get Rid of TPPO

The next obvious thing to do was to find an appropriate solvent system to run a silica column that was able to get rid of the TPPO and purify the N-Hydroxypthalimide intermediate. Many different solvents and gradients, like DCM and methanol and ethyl acetate and hexanes, were first tested out on TLCs. Unfortunately, an appropriate system was not achieved as with all the different systems, the N-Hydroxyphthalimide intermediate would run very close to TPPO. As mentioned earlier, the polarity of both are very similar, therefore not finding an appropriate system was not a surprise.
3.2.1.3.3.3 Using Less Fold Excess

The original reaction conditions required a two-fold excess of TPP with respect to Cholesterol-O-PEG400. It was hypothesized that the reaction did not need that much TPP to go to full completion and that using less excess would be okay. This would also mean that by using slightly more than the required amount would make the purification process easier as only a small amount of TPPO would remain.

Figure 3.30 Monitoring the attempt of reacting Cholesterol-O-PEG400 with 1.2 fold excess of TPP using LCMS

The figure shows that the reaction did not go through while using 1.2 fold excess of TPP as the mass spectra did not change after the reaction.
The reaction was ran with 1.2 fold excess of TPP under original reaction conditions. After completion, mass spectrometry (figure 3.30) indicated that the reaction did not go full completion and disproved the hypothesis made earlier.

### 3.2.1.3.3.4 Using Resin Bound TPP

The approach of using resin-bound TPP has been made in order to simplify the purification of the product and increase the yield of the reaction. Resin-bound TPP is a TPP reagent supported on styrene and divinylbenzene copolymers. The reaction mechanism is identical to the reaction mechanism of the original reaction, however, upon completion the TPPO byproduct remains bound to the resin, allowing for easy purification by simple filtration (shown in figure 3.31).

Most of the reaction conditions remained unchanged from the original procedure, however, more solvent was required to compensate for the excess volume resulting from the resin. Initially, it was thought that the reaction would take longer to go to full completion due to the hindrance that may be caused by the bulky resin. After monitoring the reaction time using mass spectrometry, it was determined that the initial reaction time used was sufficient to reach full completion. The use of resin-bound TPP proved the ability to synthesize and purify the N-Hydroxyphthalimide intermediate very easily.
Figure 3.31 The use of resin-bound TPP for the Mitsunobu reaction

The figure clarifies the simplicity of carrying out the Mitsunobu reaction using resin-bound TPP due to the ability to filter its byproduct, TPPO, from the rest of the product mixture.
3.2.2 CHOLO\textsubscript{400}-Glutaraldehyde Chemistry

3.2.2.1 Glutaraldehyde Polydispersity

Prior to incorporating CHOLO\textsubscript{400} into liposomes, testing out the chemoselective chemistry of CHOLO\textsubscript{400} was mandatory. Instead of incorporating CHOLO\textsubscript{400} in liposomes and then testing whether or not it works, CHOLO\textsubscript{400} was first reacted with what is going to be on the surface of the GM-beads, glutaraldehyde. By doing so, it made it a lot easier to reach a conclusion about the chemoselective chemistry as less parameters are involved in this more controlled experiment.

To start off the experiment, a mass spectrum of free glutaraldehyde was obtained. The mass spectrum was obtained to check for the purity of the reactant and as a control experiment to compare to the spectra of the products. Strange enough, the mass spectrum showed a number of peaks separated by 100 units, which also happens to be exact mass of glutaraldehyde. This indicated that a chemical or a physical interaction between the glutaraldehyde molecules was taking place. This was something that was not acceptable as this would affect the formation of liposomes in later experiments. Glutaraldehyde is used to functionalize the amine GM-beads. If different species of glutaraldehyde exist, this can cause the exposure of different lengths of aldehyde groups on the microbeads, leading to the formation of non-uniform and uneven lipid bilayers.

The peaks from the mass spectrum could be due to two different things, either that the different glutaraldehyde molecules are forming permanent chemical bonds, such as covalent bonds, or that they are forming temporary physical bonds, such as hydrogen bonds.

To answer this question, a simple experiment was ran. Different concentrations of glutaraldehyde solutions were prepared and mass spectra of each were obtained. After obtaining the mass spectra (shown in figure 3.32), it was noticed that all the different peaks were present
in all different concentrations, however, they were at different intensities. In other words, the peak intensities were a function of the concentration of the glutaraldehyde solutions. This made the answer very clear, as if the glutaraldehyde molecules were forming permanent chemical bonds, the distribution and the intensity of the peaks would be consistent independent of the concentration of the solution. However, if it was a temporary physical interaction, then the concentration of the glutaraldehyde solution would affect the distribution of the peaks, which is what was observed.

Therefore, it was concluded that the glutaraldehyde molecules were forming physical interactions between each other, and more specifically, hydrogen bonds.

![Glutaraldehyde solutions LCMS](image)

**Figure 3.32 LCMS of Glutaraldehyde solutions of different concentrations**
The spectra show the LCMS of 1mg/mL, 10 mg/mL, and 100 mg/mL (top to bottom) glutaraldehyde solutions.
3.2.2.2 CHOLOA\textsubscript{400}-Glutaraldehyde Reaction

Proceeding after running glutaraldehyde’s control experiment, CHOLOA\textsubscript{400} and glutaraldehyde were reacted in a water/ethanol solution. Due to the symmetrical nature of glutaraldehyde, CHOLOA\textsubscript{400} was added in a dropwise to fashion to avoid the formation of the bis-product.

The duration of the reaction was monitored using mass spectrometry and the reaction seemed to go to full completion immediately (figure 3.33). At first, it was difficult to tell whether or not the reaction proceeded because the Gaussian distribution did not seem to shift to the right, indicating the conjugation between CHOLOA\textsubscript{400} and glutaraldehyde. However, after taking a closer look, the peak intensities at time 0 and 2 hours don’t seem to match. At time 0, the highest peak was 817, while at two hours, it became 861. As stated earlier, the change of peak intensities indicates a permanent chemical bond has formed.
The fact the values of the peaks have not changed can be attributed to the fact that the glutaraldehyde unit adding onto CHLOA\textsubscript{400} is approximately 84 units, which is approximately the same amount as two ethylene glycol units (44 each). The extra couple of units can be attributed to a loss/gain of proton/s. Therefore, the shift in the Gaussian distribution did occur, however it was camouflaged due to the very close resemblance in weight between glutaraldehyde and two ethylene glycol subunits.

To hit the point home, a \textsuperscript{13}C-NMR was ran on the reactants and the products (figure 3.34). The CHLOA\textsubscript{400}-Glutaraldehyde spectrum showed an appearance of a peak at 147ppm.
that does not exist in either of the reactants spectra. The value of 147ppm matches the predicted values of the oxime carbon formed, reassuring that conjugation did take place.

Figure 3.34 Monitoring the reaction between CHOLOA$_{400}$ and Glutaraldehyde using $^{13}$C NMR (400MHz)

The figure shows the $^{13}$C NMR of CHOLOA$_{400}$, glutaraldehyde, and CHOLOA$_{400}$-Glutaraldehyde (top to bottom).

3.2.3 Formation of Lipid Bilayers on Supported Systems

3.2.3.1 The Encapsulation of GM-beads by Liposomes

The liposomes used to encapsulate GM-beads were made of two different long chain lipids, phosphatidylcholine (POPC) and CHOLOA$_{400}$. The GM-beads were initially amine
functionalized, however, were reacted with glutaraldehyde to lead to the exposure of aldehyde on the surface of the microbeads, shown in figure 3.35.

The encapsulation process occurs via chemoselective ligation between the oxyamine functional group on CHOLOA400, in the liposomes, and the aldehyde functional groups on the surface of the GM-beads. The reaction of the two functional groups lead to the formation of oxime linkages between the GM-beads and the liposomes. Following the oxime bond formation of different liposomes with the different aldehyde groups on the surface of the GM-bead, the fusion of the liposomes occurred to form a continuous lipid bilayer, shown earlier in figure1.2.

3.2.4 Why Liposomes Not Bicelles?

It was found by other members in the lab that bicelles were unstable in dilute conditions. This was an issue as several dilution steps occur throughout the binding assay procedure. Liposomes were a better option because they not only do they spontaneously self-assemble at dilute conditions, they also trap some aqueous solution in their cores. Having an aqueous environment makes them better candidates as drug delivery vehicles for hydrophilic drugs.
3.2.5 Why GM-beads Not PS Beads?

Several experiments ran by other members in the lab have proven that the deposition a lipid bilayer on PS beads resulted in a non-continuous lipid bilayer. The reason behind this was hypothesized to be attributed to the fact that PS beads are made of long polymer chains that wind into the solid structures. The winding process may cause irregularities and uneven surfaces that could hinder the formation of a continuous lipid bilayer and exert pressure on the lipid bilayer due to uneven attachment levels via the oxime linkage. GM-beads are a better candidate for this job as they have a more even and smooth surface.

3.2.6 Binding Assay

After the confirmation of the synthesis of CHOLOA$_{400}$ and its ability to form an oxime bond with a glutaraldehyde molecule chemoselectively, it was time to put the system to the test. The binding assay procedure was followed as mentioned earlier in the procedures section. Upon completion of the experiment, 3 different supernatants were obtained; Supernatant 1, Supernatant 2, and Bound. “Supernatant 1 and 2” contain all the unbound liposomes that were left in solution and were stuck in-between the GM-beads, respectively. The two supernatants are expected to contain the fold excess used in the binding assay. Prior to collecting the “Bound” supernatant, detergent was used to strip off all the lipids that had bound on to the GM-beads, therefore all of the liposomes bound were collected in the “Bound” fraction.

Fluorescence measurements of all three samples were then taken to quantify the amount of liposome present in each of them.
3.2.6.1 Calibration Curve

To be able to quantify the amount of lipid from fluorescence measurements, a calibration curve is first prepared. Liposome stock, sodium cholate, and HEPES buffer were used to make the calibration curve samples. The concentration of HEPES and sodium cholate was kept consistent in all the samples, 10mM and 0.2, respectively. Duplicate samples were prepared to increase the reliability of the date obtained.

The calibration curves plotted the fluorescence (amu) versus the volume, mass, or the concentration of the liposome stock. The curve below is an example of a calibration curve obtained and shows a linear trend:

![Calibration Curve](image.png)

Figure 3.36 Calibration curve showing the relationship between concentration and fluorescence

3.2.6.1.1 Using the Calibration Curve to Find the Mass/Concentration of Lipid in Each Sample

To calculate the mass of lipids in a specific sample, the following equation can be used:

\[
Mass \ of \ Lipid \ (\mu g) = \frac{S_1 \times \text{amu}}{S_2 \times \mu L} \times C \ \frac{\mu g}{\mu L} \times V \ \mu L \quad \text{Equation (2)}
\]
where $S_1$ represents the slope of the sample calibration curve, which is made after the sample is prepared, $S_2$ represents the slope of the liposome stock calibration curve, $C$ represents the concentration of the liposome stock, and $V$ represents the volume of the sample. By canceling out the units, mass is indeed the value obtained from the equation.

### 3.2.6.2 Binding Assay Expected Results

Before running the binding assay, it is important to know what to expect the results to look like. The factor that is measured in this experiment is the fluorescence, which depends on the concentration, amount, or volume of lipids in a specific sample. The more the lipid, the more NBD-PE (fluorophore), hence the higher the fluorescence reading.

As an example, a prediction of the binding assay curves obtained for each of the samples of a 3-fold excess of lipids required to fully coat a known amount of GM-beads with a single lipid bilayer, will be analyzed. The theoretical amount of lipids needed to fully coat 1 mg of GM-beads with a lipid bilayer was calculated to be approximately $1.596 \times 10^{-8}$ moles (Calculation shown in Appendix A.1). Therefore, for this specific example, $4.8 \times 10^{-8} (1.596 \times 10^{-8} \times 3)$ moles of lipid were added to the binding assay. After running the experiment and collecting supernatants 1, 2, and bound solutions, a calibration curve for each was prepared while making sure that the final volume and the concentration of HEPES and sodium cholate stay constant throughout the samples.
The fluorescence readings for all the samples should look similar to the curves in figure 3.37 shown below. After calculating the amount of lipids in each of the samples using the equation above, they should all add up to the total amount of lipids initially added, which is $4.8 \times 10^{-8}$ moles. The amount of lipid bound to the GM-beads should approximately equal the theoretical amount, which is $1.596 \times 10^{-8}$ moles and the remaining amount of lipids, $3.204 \times 10^{-8}$ moles ($4.8 \times 10^{-8} - 1.596 \times 10^{-8}$), should be the sum of the amount of lipids found in supernatant 1 and 2.

![Figure 3.37 Expected fluorescence curve to be obtained from binding assay solutions](image)

### 3.2.6.3 Results Obtained

After running the binding assay experiment and taking fluorescence readings of each of the obtained supernatants, the curves below were obtained (figure 3.38). As shown, the results obtained did not follow the pattern of the expected results. The most obvious thing that can be noted by looking at the curves is the very low fluorescence recovery. Using the calibration curves, it can be noticed that fluorescence up to 70000 amu was obtained, where in the binding
assay curves, the maximum fluorescence obtained was approximately around 4000 amu, almost 17 times less.

**Figure 3.38 Obtained fluorescence curves from binding assay solutions**

### 3.2.6.4 What Could Have Went Wrong?

A couple of things could have led to the unexpected binding assay curves obtained. First and foremost, glutaraldehyde could have not attached to the GM-beads during the modification step of the amine functional groups displayed on the surface. If so, then the hydrophobically attached CHLOA\textsubscript{400} in the liposomes would have no means of attaching chemoselectively to the GM-beads and would therefore have no way of forming a lipid bilayer. The lack of techniques to confirm the successful conjugation between the two make it impossible to check whether or not conjugation was successful.

Secondly, by looking at the binding assay data obtained, the fluorescence is approximately 17 times less than the fluorescence obtained from the calibration curves. After looking at the experimental procedure carefully, we have come to the conclusion that it is most likely due to the low molar % of NBD-PE used (0.05%). Having such low amount of
fluorophore makes it very hard to detect, especially that after the different supernatant fractions and bound fractions are continuously diluted as a part of the binding assay procedure. As a result, unreliable fluorescence intensities are obtained. Due to the shortage of time, no further experiments with higher molar % of NBD-PE were done.
Chapter 4: Conclusion and Future Directions

This report demonstrates the successful synthesis of CHOLOA\textsubscript{400} and its incorporation into large unilamellar vesicles (LUVs) using hydrophobic interactions. Upon successful formation of liposomes, they were bound to GM-beads using chemoselective ligation between CHOLOA\textsubscript{400}’s oxyamine bond and the aldehyde functional groups on the GM-beads, to form a stable oxime bond.

The many difficulties of synthesizing CHOLOA\textsubscript{400} were overcome either by trial and error or the use of a different approach. An example of trial and error was demonstrated by the experimentation of different two-phase systems in attempt to purify Cholesterol-O-PEG\textsubscript{400} from the large amount of excess PEG\textsubscript{400}. On the other hand, the synthesis of the N-Hydroxyphthalimide intermediate, Cholesterol-O-PEG\textsubscript{400}-O-N-Hydroxyphthalimide, demonstrated the use of different approaches to problem solve and achieve the desired product at high purity. The use of resin-bound TPP was used to avoid the hassle of having to deal with the inefficient ways of purifying the desired product from the TPPO.

The incorporation of CHOLOA\textsubscript{400} in liposomes was easily achieved as past procedures developed in the lab were used. However, the encapsulation of the GM-beads was not so straightforward as it was not successfully quantified using fluorescence. We hypothesize that this was due to the low molar % of fluorophore (NBD-PE) used and the continuous dilution of samples during the binding assay procedure. This lead to a very low amount of NBD-PE in the final solutions and barely gave any fluorescence feedback. To address this issue in the future, we would use a molar % of NBD-PE that would be appropriate and concentrated enough by the end of the binding assay to get reliable fluorescence data.

Amine functionalized GM-beads were used as the solid surfaces during this thesis project. However, due to the chemoselective chemistry used, having aldehyde functional groups
on the solid surfaces was required. Therefore, they were chemically functionalized using
symmetrical glutaraldehyde molecules. The use of aldehyde functionalized GM-beads instead of
amine functionalized glass microbeads would allow the bypass of having to chemically
functionalize the amine functionalized GM-beads. Also, this would eliminate any possibilities of
the unsuccessful or uneven distribution of functionalization of the GM-beads, as it was not
possible to prove the success or the extent of functionalization due to the lack of experimental
techniques.

During the synthesis of CHOLOA_{400}, polydisperssed PEG_{400} was used. Polydisperssed
PEG means that the molecules weighed 400 mole/gram on average, meaning that slightly
smaller and larger PEG chains also existed. The reason why polydisperessed PEG_{400} was used
was due to the extremely expensive prices of monodispersed PEG_{400}. However, the issue with
using polydisperssed PEG_{400} is that when incorporating them into liposomes and binding them
onto solid surfaces, the different lengths of PEG chains may affect how uniform and continuous
the lipid bilayer turns out to be. After successfully achieving the fluorescence quantification of
the binding assay and determining the feasibility of this supported bilayer system, the
investment in monodispersed PEG_{400} should be made. This would increase the chances of
forming a more uniform and continuous lipid bilayer over the glass microbeads.


Chapter 5: References


(15) Çağdaş, M.; Sezer, A. D.; Bucak, S. In *Application of Nanotechnology in Drug Delivery*; Sezer, A. D., Ed.; InTech: Rijeka, 2014; p Ch. 01.


Appendix

A.1 Calculation of theoretical 1-Fold saturation of 1 mg of GM-beads

Average diameter of the GM-beads:

\[ d = 1.01 \mu m \]
\[ = 1.01 \times 10^{-4} cm \]

Volume of a sphere:

\[ V = \frac{4}{3}\pi r^3 \]

Volume of GM-beads:

\[ V = \frac{4}{3}\pi \left(\frac{1.01 \times 10^{-4}}{2}\right)^3 \]
\[ V = 5.394 \times 10^{-13} cm^3 \]

Mass of GM-beads:

\[ Mass = Density \times Volume \]

\[ Mass = 2 \, g/cm^3 \times 5.394 \times 10^{-13} cm^3 \]
\[ Mass = 1.079 \times 10^{-12} g \]
\[ = 1.079 \times 10^{-9} mg/bead \]

For 1 mg of GM-beads there are:

\[ \frac{1 mg}{1.079 \times 10^{-9} mg/bead} = 9.268 \times 10^8 beads \]

Surface area of a sphere:

\[ Surface \, area \, of \, sphere = 4\pi r^2 \]
\[ Surface \, area \, of \, bead = 4\pi \left(\frac{1.01 \times 10^{-4}}{2}\right)^2 \]
\[ = 3.20 \times 10^{-8} \frac{cm^2}{bead} \]

The surface area of 1 mg of beads is:

\[ 9.268 \times 10^8 \text{beads} \times 3.20 \times 10^{-8} \frac{cm^2}{bead} = 29.72 \text{ cm}^2 \]

Each lipid occupies approximately:

\[ 65\text{Å}^2 = 6.5 \times 10^{-15} \text{cm}^2 \]

Number of lipids needed:

\[ \frac{29.72 \text{ cm}^2}{6.5 \times 10^{-15} \text{cm}^2} = 4.57 \times 10^{15} \text{lipids} \]

Moles of lipids needed:

\[ \frac{4.57 \times 10^{15} \text{lipids}}{6.022 \times 10^{23} \text{mol}^{-1}} = 8.00 \times 10^{-9} \text{ moles of lipids} \]

Moles of lipids needed for a bilayer:

\[ 8.00 \times 10^{-9} \text{ moles of lipids} \times 2 = 1.50 \times 10^{-8} \text{ moles of lipids needed for a bilayer} \]

Therefore:

\[ 1.50 \times 10^{-8} \text{ moles of lipids needed encapsulate 1mg of beads with a bilayer.} \]