Modification of Lipid Microenvironments on Solid Support Structures for Use in Transmembrane Protein Assays

William J. Houlihan
CUNY City College

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Modification of Lipid Microenvironments on Solid Support Structures for Use in Transmembrane Protein Assays

By William J. Houlihan

Research Mentor: M. Lane Gilchrist
Department of Chemical Engineering
The City College of New York

A Dissertation Submitted to the Graduate Faculty in Engineering in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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This manuscript has been read and accepted for the Graduate Faculty in Engineering in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

M. Lane Gilchrist, Chair of Examining Committee  
Date

Ardie D. Walser, Associate Dean for Academic Affairs  
Date

Professor M. Lane Gilchrist, Mentor, Department of Chemical Engineering  
Professor Ilona Kretzschmar, Department of Chemical Engineering  
Professor Raymond Tu, Department of Chemical Engineering  
Professor Charles Maldarelli, Department of Biomedical Engineering  
Dr. Yueming Li, Memorial Sloan-Kettering Cancer Center

Examining Committee

THE CITY COLLEGE OF NEW YORK
Abstract

Gamma-Secretase (γ-secretase) is a transmembrane protease of increasing interest, which has been shown to have significant connections to both cancer and Alzheimer’s disease. γ-secretase cleaves both Notch-1, a transmembrane signaling protein, and Amyloid precursor protein (APP), a transmembrane protein whose cleavage may result in the formation of β-amyloid plaques in the brain. Notch-1 and APP are widely studied proteins that have substantial impacts on the development and proliferation of cancer and Alzheimer’s disease, respectively. Notch-1 partakes in the signaling of apoptosis in damaged and mutated cells, thus its cleavage by γ-secretase within the plasma membrane has ramifications on cell growth and proliferation.

However, the APP molecule is the key protein in the metabolic pathway that produces small amyloid fragments. These fragments, in undesirable conditions, have the propensity to aggregate and form, as stated above, amyloid plaques, depending on the fragment length. These plaques have been long believed to inhibit neuronal function if they are not degraded or removed from the intracellular space, specifically in the brain.

Due to these widespread mental and physical health impacts, isolation and modulation of the cleavage of such proteins in intact, controlled bilayers in a highly reproducible, and potentially high-throughput, process is a key goal in understanding these and a vast array of intramembrane proteases for the development of pharmaceutical therapies. The work presented looks to the development of one such platform, yielding crucial spatial and temporal information within these complex lipid microenvironments. Synthetic, biomimetic membranes were studied and manipulated to develop biologically relevant systems in which to resuspend isolated proteins. A formulation of sphingomyelin, 1,2-Dioleoyl-sn-phosphatidylcholine (DOPC), and cholesterol was chosen due to its attributes in resembling fundamental lipodomics within a
human brain cell. It is shown that this canonical formulation and subsequent formulations with added complex mixtures, yield a lipid system that retains visible phase separation to a quantifiable degree. These lipid formulations, when fused with solid silica support structures such as planar surfaces or silica microbeads, allows for the reconstitution of the three of proteins of interest.

These assay and high throughput platforms are essential to understanding key functions and potential modulations of these protein pathways, however this approach does not fully replicate the biological environment these proteins experience within an active cell. Two approaches are shown in this work to increase the biological relevancy of these platforms. Tethering of the solid support structures with a series of polyethylene glycol (PEG) polymers culminating in a functionalized capping moiety that can yield overall increases to protein mobility, and added functionality of the platform. Additionally, added dopants of more complex lipid components into the basic lipid membrane analogue shows the ability to increase complexity of the formulation and closes the gap between the synthetic membrane and the protein’s true biological lipid environment.

These platforms are highly robust and rugged in nature and lend themselves to be useful in future high-throughput screening and functional assay processes in pharmaceutical research. The coupling of both planar surface support structures and micro bead structures in tandem can be analyzed through confocal, super-resolution, and atomic force microscopy, leading to a fuller understanding of these complex spatial reaction-diffusion systems prevalent within human cells. The systems developed in this research, apart from being tested with the aforementioned proteins, are not protein-specific and thus could yield a viable platform on which to test any number of isolated transmembrane proteins in a highly reproducible manner.
Preface

The intent of this thesis is to develop and expand the field of potential viable protein assay and analysis platforms due to their large potential as drug and therapy targets in the pharmaceutical industry. The experiments chosen in this work were chosen to exemplify the utility and potential uses of in tandem studies performed on both planar supported bilayers and proteolipobead constructs, as well as the use of atomic force microscopy and confocal microscopy to yield a well-defined and characterized, and modular high-throughput solution for further protein analysis. A major theme of the work presented throughout this research is the flexibility of a modular approach, developing base systems that can be made more complex or more functional at the future researcher’s behest. Flexibility in these systems yields a wider impact potential on the viability and function as a protein analysis platform.

Chapter 1 begins by introducing the systems and platforms developed over the course of this study, and the biological relevancy and importance of the specific proteins analyzed in this work. The focus here being to elucidate the approach to developing biologically relevant assay and analysis platforms which can be used in high-throughput screening testing to better understand the intricacies and functions of these proteins. The methods of characterization and study used in this work are introduced to lay the foundation on which the claims in the results and conclusions are developed.

Chapter 2 provides the approach and modifications developed on the basis of a canonically used lipid membrane formulation for the study of proteins in supported lipid bilayers, both on planar supports and microbead systems. Sphingomyelin (SM), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), and Cholesterol are combined here to for a well-studied
brain cell membrane analogue. This analogue, while useful, still lacks in fully capturing the cellular lipid environment, and thus more complex mixtures and dopants were added to increase the system’s overall biological relevance. Chapter 3 is the core protein work of this research. This chapter gives the account of using these biologically analogous lipid formulation in order to develop and express γ-secretase and two of its known substrates, derived from Notch-1 and the Amyloid precursor protein. These three proteins are shown to be reconstituted in both the planar supported bilayer, as well as the lipobeads construct yielding a tandem approach to characterization, functional assays, and high-throughput analysis and testing.

Chapter 4 presents the work performed in developing functional polymers tethering supports structures on the surface of the solid support structures. By using a series of relatively simple ester-linkage chemistries, a solid surface can be fully coated with a modular polymer support structure to help provide further functionality in assays and analysis of these biomimetic systems as well develop a system that more closely resembles the mechanical properties of the biological environment which is trying to be mimicked. The process shown here is straightforward and modular in an attempt to create a basis for a system that can afford a host of functionalities rather than one static system.

Finally, the overall conclusion and claims made in this work are summarized in chapter 5 along with any future works possible to further ratify claims or answer questions raised by this research.
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These have been a long, arduous, exhilarating, fun, and eventful 6 years throughout this PhD program at City College. My life has been a combination of intricate plans and sometimes split second decisions, as it is for most, but for me Grad school was not overall my end goal as it is for so many. But I found myself here nonetheless, after deliberation with friends, family, coworkers, and advisors it was clear to me that further education and research was my calling. If you would have asked me 10 years ago if I was going to be attending the City College of New York, I would have looked at you funny since truthfully I never knew this magnificent institution existed. However, I was lucky enough to have an undergrad mentor and some other family connections (my Aunt’s doctor is a CUNY grad) to the CUNY system and specifically CCNY, and my life has been all the better for it.

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Subsequently, I would like to specifically thank Dr. Kretzschmar, early on in my career at City College she took over as the chair of the Chemical Engineering department, and yet still seemed to have the time to help everyone individually with any problems they may be facing. She has been a wonderful resource to help me focus my work, helped me plan and execute all of my goals and aspirations, and has held me accountable for all of my actions, helping me get back up after research doesn’t necessarily go as planned.

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# Table of Contents

Abstract .............................................................................................................................. iv

Preface ............................................................................................................................... vi

Acknowledgements ........................................................................................................... viii

List of Tables and Figures ................................................................................................ xiv

Chapter 1. Motivation and Background ............................................................................. 1
  1. I. Motivation .................................................................................................................. 1
  1. II. Plasma Membranes .................................................................................................. 2
  1. III. Transmembrane Proteins ....................................................................................... 5
  1. IV. Gamma Secretase and its Substrates ..................................................................... 7
  1. V. Microscopy .............................................................................................................. 9
  1. VI. Tables ..................................................................................................................... 14

Chapter 2. Sphingomyelin/1, 2-Dioleoyl-sn-glycero-3-phosphocholine/Cholesterol Formulation and variants ........................................................................................................... 16
  2. I. Introduction .............................................................................................................. 16
  2. II. Materials and Methods .......................................................................................... 16
  2. III. Results and Discussion .......................................................................................... 20
  2. IV. Conclusions .......................................................................................................... 39
  2. V. Tables ..................................................................................................................... 41

Chapter 3. Gamma Secretase and Substrates .................................................................... 42
  3. I. Introduction .............................................................................................................. 42
  3. II. Material and Methods ............................................................................................ 42
  3. III. Results and Discussion ......................................................................................... 44
  3. IV. Conclusions .......................................................................................................... 50
  3. V. Tables ..................................................................................................................... 52

Chapter 4. Tethering Modalities ....................................................................................... 53
  4. I. Introduction .............................................................................................................. 53
  4. II. Material and Methods ............................................................................................ 53
List of Tables and Figures

Table 1.1. List of Materials and Molecular structures............................................................................. 14
Table 2.1 Additional Materials....................................................................................................................... 41
Table 2.2 Lipid bilayer formulations............................................................................................................. 41
Table 3.1 Additional Materials....................................................................................................................... 52

Figure 1.1 γ-secretase structure and domain distribution................................................................................. 7
Figure 1.2 APP and Notch-1 substrate structures.............................................................................................. 8
Figure 2.1 FRET dye pair validation.................................................................................................................. 21
Figure 2.2 Spectral comparison of simplified analogue versus biological membrane............................. 23
Figure 2.3 Overlay of Perylene and DiI detection in SM:DOPC:cholesterol formulations............................ 27
Figure 2.4 CLSM general intensity over a domain ripened lipobead.............................................................. 28
Figure 2.5 CLSM general intensity over a representative bead.......................................................... 29
Figure 2.6 Airyscan general intensity over a representative bead............................................................ 29
Figure 2.7 Ternary Phase Diagram of the Sphingomyelin:DOPC:Cholesterol lipid formulation...................... 32
Figure 2.8 Comparative phase separation of short and long fusion times on the SM:DOPC:cholesterol lipid formulation.................................................................................................................. 32
Figure 2.9 Histogram of coverage analysis.................................................................................................... 33
Figure 2.10 Comparison of STED super resolution to CLSM imaging of DiI fluorophore.................... 34
Figure 2.11 Comparison of Airyscan super resolution imaging versus CLSM of DiI fluorophore............... 35
Figure 2.12 Varying BPLE dopant concentration into canonical SM:DOPC:cholesterol formulation........ 36
Figure 2.13 Variation of Saturated lipid dopant into canonical SM:DOPC:cholesterol lipid formulation........ 38
Figure 2.14 FRAP analysis performed on SM:DOPC:cholesterol formulation.............................................. 39
Figure 3.1 Colocalization of TMPs and Ld phase highlighted lipid membrane........................................... 46
Figure 3.2 STED and CLSM microscopy of HEK293 cells over expressing γ-secretase labeled with RFP (as pHuji)......................................................................................................................... 48
Figure 3.3 FRAP analysis of \textit{in vivo} \(\gamma\)-secretase within HEK293 cells……………………………………… 49
Figure 3.4 Extracted RFP-tagged \(\gamma\)-secretase reconstituted in PLBs……………………………………… 49
Figure 3.5 FRAP analysis on RFP and DiO in the HEK293 extract PLBs……………………………………... 50
Figure 4.1 Schematic of effective tethering linkages…………………………………………………………… 55
Figure 4.2 Validation of the Biotin capped tethering modality……………………………………………… 58
Figure 4.3 Overlay of indentation tests performed on glass microscope slides containing a 4-arm PEG\textsubscript{20000} star polymer tether connected to a SM:DOPC:cholesterol lipid bilayer………………... 60
Figure 4.4 Overlay of indentation tests performed on glass microscope slides containing a single PEG\textsubscript{2000}-DSPE polymer tether connected to a SM:DOPC:cholesterol lipid bilayer………………... 61
Figure 4.5 3D reconstruction of a tapping mode AFM scan of a glass microscope slide containing a 4-arm PEG\textsubscript{20000} tether connected to a SM:DOPC:cholesterol lipid bilayer…………………... 62
Figure 4.6 Tapping mode image of a PEG2000-DSPE tether supported phase separated lipid bilayer with inserted \(\gamma\)-secretase on a thermally oxidized silicon wafer……………………………………... 64
Chapter 1. Introduction

I. Motivation

High throughput screening has become a staple in the study of chemical therapeutics and pharmacology[1]–[3]. This is a process by which large quantities of chemical species are tested for their efficacy in order to eliminate species with low efficiency or some level of toxicity, as these characteristics make for a poor therapeutic or commercial drug. High throughput screening is an ideal technology for the drug and pharmaceutical industry, as it allows for high volume testing, for low effective cost, and it simultaneously eliminates the need to further study compounds with little to no efficacy and push them through costly FDA approval trials.[4], [5] Microbead and particle-based high throughput screening has gained a prominent foothold within this field, as to harness the power of flow cytometry[6].

Transmembrane proteins are currently a class of proteins with which high throughput screening techniques have only been used for approximately 10-15 years. These proteins are difficult to isolate and test while maintaining similar levels of functionality as found in cell studies. The main difficulty with this category of protein arises from its necessity to be suspended within a cell membrane, due to its amino acid structure creating a sizeable hydrophobic domain, which in cells sits within the confines of the plasma membrane. Cell studies are time and money intensive due to the necessary genetic modifications required to isolate and explore singular protein pathways, however this is the most common screening method for transmembrane proteins at the present.

It is thus desirable to develop a platform on which transmembrane proteins can be reconstituted, while maintaining cellular levels of functionality in a highly controlled and
reproducible manner. Under these constraints, a process has been developed to engineer synthetic plasma membranes, fuse them to solid support structures, and reconstitute a transmembrane protein known as γ-secretase into these membranes along with two of its substrates in order to test key metrics that associate with function in in vivo studies[7]. Namely these metrics include lipid microenvironment, diffusion, partitioning, and enzyme activity.

II. Plasma Membranes

The study of plasma membranes, or lipid bilayer, has been an ongoing pursuit for many generations [8]. A lipid bilayer is formed when molecules with a hydrophilic head group attached to a hydrophobic tail, known as a lipid, begin to self-assemble in aqueous solutions as a way to minimize their free energy. The lipids self-assemble into double sided sheets with the hydrophobic tails towards the center and the hydrophilic head groups jutting out into the water, called lamella.

Since the identification of the cell and its constituents, lipid bilayers have been known to play an important role in many biological functions with the main functional unit arising in the form of the cell membrane. The cell membrane is considered to be a fluid mosaic [9]. This means that while it can be considered a solid barrier for some large molecules, it is actually mobile and comprised of numerous mobile moieties including, proteins, lipids, and sugar[10], [11]. These mobile units give the membrane its structure and function creating highly complex intertwining networks which are the root of various cell processes. It is this quality of the cell membrane which makes it an interesting topic of study.

In recent year, the hypothesis of lipid raft formation has been a hotly debated topic between lipid membrane researchers[9], [12], [13]. The lipid raft theory states that small,
sometimes nano-scale gradients of lipid species occur throughout the confluent bilayer of the plasma membrane, and that these spatial gradients are an active player in cellular dynamics\textsuperscript{[14]–[16]}. Recent studied into cell lipid fractions using lysed cells dissolved in detergent show that two regimes exist within cell plasma membranes, a more rigid and viscous regime, known as the liquid ordered domain, and a less detergent resistant, more mobile, liquid disordered domain\textsuperscript{[17]}. This work was further confirmed through the use of NMR, as these studies are what gave rise to the distinction of the liquid ordered versus disordered phase as the order and disorder are a reference to lipid acyl chain order parameters of lipids within the phases\textsuperscript{[18]–[20]}. These cholesterol-rich microdomain are thought to be thicker and more viscous than the liquid disordered phase\textsuperscript{[21]–[23]}. Cellular lipodomics reveal a multitude of lipids present in the entire cellular lipid biome, reaching levels of 10,000+ individual species within a single cell\textsuperscript{[24]}. This large array of lipids found in the cellular biome makes the domain structure practically unresolvable under most common imaging methodologies.

Synthetic lipid bilayers have been developed in various forms as a stable yet dynamic system for understanding some high level cellular functionality like cell to cell interactions, integral membrane protein function, and microenvironment changes \textsuperscript{[25]–[28]}. A large push in the study of lipid bilayers is in the form of studying giant unilamellar vesicles (GUVs), giant plasma membrane vesicles (GPMVs), and proteoliposomes \textsuperscript{[29]–[34]}. These systems are widely used due to their ease of formation, and are helped by the fact that GUVs and GPMVs are easy to image due to their large size. However, they have some drawbacks when it comes to their versatility in experimentation as these systems are not very robust as a testing platform due to their susceptibility to puncture, cavitation, and inherently have no support structure. Due to the
lack of support, a new platform was developed to create a more robust system that resists
deformation while maintaining native levels of bilayer fluidity.

Supported lipid bilayers have been used as membrane mimics for a handful of years now
[35]–[37]. The most common form of supported lipid bilayer is the planar bilayer, which is
created through one of two procedures. The first method is through the use of a Langmuir-
Blodgett trough, when a hydrophilic material is coated with the lipids a single layer at a
time[38]–[40]. The Langmuir Blodgett approach allows for an added level of control over each
layer deposited onto the hydrophilic surface, and this control has the potential to form
asymmetric lipid bilayers. Asymmetric lipid bilayers, commonly found in biological systems,
are bilayers forced into a state of non-equilibrium in which each leaflet of the bilayer has a
varying composition of lipids[41]–[44]. Asymmetric bilayers are difficult to maintain in situ as
most supported lipid systems tend towards an equilibrium between both leaflets of the bilayer as
well as all molecular species within the bilayer.

The more common method is through lipid vesicle adsorption onto the hydrophilic
surface, in which a suspension containing unilamellar vesicles is exposed to the hydrophilic
surface for a desired time[45]–[48]. The vesicles deform and rupture on the hydrophobic surface
and reform into a confluent lipid bilayer. Planar bilayers are useful for the study of many
systems due to the rigidity of the material and the ability to deposit the bilayer directly onto a
microscope glass slide or coverslip. Secondly, the planar hydrophobic surface affords the added
ability to functionalize and augment parts or all of the solid surface, giving rise to added levels of
control and versatility in the lipid platform[46], [49].

There is a drawback to these planar supported lipid bilayers in the characteristic that these
platforms are two dimensional. In some cases a three dimensional approach is beneficial as it
allows for a more precise mimic of the biological system in the case of cellular interactions with these types of platforms. To this end, a three dimensional platform has been developed in the form of a spherical bead which is used as the solid support for the lipid bilayer. This lipobeads (LB) is a lipid bilayer suspended on the surface of a hydrophilic surface much in the same way as the planar bilayer is suspended in the planar bilayer system[50]–[53]. The LBs can be formed through normal adsorption of vesicles onto the surface, or through a Langmuir Blodgett approach as well. The beads, much like the hydrophilic surface in the planar system, can also be functionalized to yield a higher level of control and versatility to the 3D platform, including integrating wash steps to remove excess membrane proteins and lipids that could give artifacts. Furthermore, this format is amenable to flow cytometric analysis and screening.

III. Transmembrane proteins

Soluble and transmembrane proteins make up 2-4 million molecules per cubic micron of the mammalian cell, and a large subspecies of these proteins are integral, or transmembrane proteins (TMPs) [54], [55]. These proteins span the width of the plasma membranes within cells and perform many tasks ranging from transport, to cell signaling, as well as housekeeping functions. Many of the larger protein structures such as aquaporin, an integral membrane protein which allows for a large flux of water to travel through the membrane, have been studied intently over the years [56]. Though, this work only scratches the surface of the entire class of TMPs available for study as these proteins are difficult to study and understand due to their complex nature and steep requirement for a fluid bilayer to remain functionally viable. Most integral membrane proteins lose functionality when removed from the confines of a lipid bilayer, also aggregation of hydrophobic domains and denaturation occurs with these proteins when they are no longer stabilized by a confluent lipid bilayer or by detergents. It has also been shown that the
surrounding environment has effects on protein function, the environmental effects could be due to a global effect of the homeostasis of the biological system or could arise from small micro or nano environmental changes within the lipid bilayer, such as lipid bilayer thickness changes and raft formation or dissipation[57]–[59]. In systems comprised of multiple protein interactions and pathway triggers, orientation of the protein within the bilayer is another important factor which can confound the study of these proteins outside of the cell structure. As in cells, this expression and insertion into the cell membranes is facilitated through a series of chaperone proteins helping during transcription.

Current TMP studies rely heavily on recombinant cell and bacterial culture studies, in which eukaryotic or prokaryotic cells are genetically modified to over express these proteins or to suppress the expression of other proteins in order to isolate a single TMP system. These studies are long, difficult, and expensive while also plagued with a sense of uncertainty[60]–[63]. Cell and bacterial cultures are incredibly dynamic and complex systems which are still not yet fully understood in some cases [64]. Many metabolic and signaling pathways have not been completely mapped as of this time, while other pathways which are understood have multiple levels of redundancies built into them in order to maintain cell viability in the absence of a molecular pathway [65]. These built in redundancies and failsafe pathways have been developed over hundreds of thousands of years of evolution in some cases and are thus very hard to account for in these types of assays.

A desirable assay for integral membrane protein study must include the ability to isolate the interesting proteins and its substrates, proper environmental control of the lipid or homeostatic system, and proper orientation control of the protein in native cellular formats.
IV. Gamma Secretase and its Substrates

A subset of these integral membrane proteins is a class of cleavage enzymes known as intramembrane proteases, and one in particular is of paramount interest. γ-secretase is a cleavage enzyme found throughout mammalian biological systems, it is a protease which cleaves other TMPs at an active site located within the hydrophobic domain of the lipid bilayer shown in figure 1.1. There are two proteins of interest which this enzyme is known to cleave, one being the amyloid precursor protein (APP) and the second being the protein Notch-1 shown in figure 1.2. Both of these substrates are interesting because they are believed to hold a role in the development of Alzheimer’s disease and the proliferation of cancer cells, respectively [67], [68]. γ-secretase is a large integral membrane protein on the order of 170 kDa, with multiple transmembrane helixes and extracellular domains. γ-Secretase is an enzymatic complex composed of at least four proteins: presenilin 1 or presenilin 2 (PS1 or PS2), Nicastrin, Pen2 and Aph1, with presenilin representing the catalytic core. In 2015, a 3.4 Angstrom resolution cryo-EM structure of the enzyme was obtained, and further structures have been obtained showing the protein in an active configuration [69]–[72]. This discovery has given insight into the function and processes of this enzyme, but native configuration testing has not yet been completed.

Figure 1.1 γ-secretase structure and domain distribution. This is a representation of the full γ-secretase structure as seen in the PDB file 5a63. Panel A Shows the γ-secretase protein positioned within a confluent lipid bilayer. In panel B the γ-secretase molecule is colored to represent the different charge, hydrophilic, and hydrophobic domains. Panel C displays the isolated γ-secretase shown here exposing the sizeable hydrophobic domain displayed in white.
Figure 1.2 APP and Notch-1 substrate structures. Panel A here is a truncated structure of the APP known as C99 (PDB: 2LP1) as it is 99 amino acid residues long. The arrow here indicates the cholesterol binding domain found in the APP structure which is suggested to effect partitioning and diffusion of the protein. Panel B shows the Notch-1 signaling protein structure (PDB: 5KZO)

The APP substrate is cleaved by γ-secretase within the cell membrane, but the cleavage specificity and efficiency are unknown outside of the fact that this cleavage can create 2 distinct byproducts and the productions of these fragments can be correlated to cholesterol content [73]–[75]. One byproduct of this cleavage is a harmless protein fragment which can be further digested and recycled into the cell, while the second possible byproduct is the formation of an amylogenic protein fragment which cannot be further digested by the cell or other biological enzymes. This second amylogenic protein has the propensity to form amyloid aggregates which can grow into amyloid plaques[76]–[78]. Amyloid plaques that form in the brain can disrupt neuronal function and health, resulting in the possible development of neurodegenerative diseases such as Alzheimer’s, dementia, and potentially many others. These different byproducts are a result of a switch in cleavage position along the peptide strand, called cleavage specificity. The efficiency of γ-secretase is related to the enzymatic rate of the cleavage. As stated above, it has been shown that cholesterol plays a significant role in determining the cleavage specificity of
\( \gamma \)-secretase, though it is unknown whether this effect is due to a global increase of cellular cholesterol concentration or micro/nano environmental effects which arise from a higher concentration of cholesterol in the lipid bilayer.

Notch-1 is a cell signaling protein [68], [79], [80]. Notch proteins are well known to contribute to cell proliferation, differentiation, and apoptosis. Healthy cells express an activated form of Notch-1, it is believed that \( \gamma \)-secretase cleavage is the activation step of the Notch-1 protein. When \( \gamma \)-secretase is blocked, Notch-1 cleavage and activation also becomes blocked causing the cell to signal for apoptosis. When healthy cells are damaged or begin to function improperly, signals are released which block the enzymatic capabilities of \( \gamma \)-secretase, thus causing a cascade effect leading to the destruction and apoptosis of the unhealthy cell. It has been shown however that in some cancerous cells, these damage signals are suppressed and thus \( \gamma \)-secretase continues to activate Notch-1 as it would during healthy cell function. This leads to the proliferation of cancerous cells in the body and potential tumorigenesis [73], [81], [82].

Understanding the function and enzymatic rate of \( \gamma \)-secretase and its substrates in these two systems could hold the key to a fuller understanding of these diseases and potential therapies.

V. Microscopy

Light microscopy is a well-defined and widely used experimental tool [83]. A key feature of light microscopy is the idea of fluorescence microscopy. Fluorescent molecules are molecules that can absorb the energy of certain wavelengths of light, this absorption is used as energy to cause electronic transitions between energy levels within the molecular structure. This excited electron state only lasts a brief period of time however, and when the system returns to ground
state, the energy release by the system has been diminished slightly and is released as a red
shifted photon. This means a fluorescent molecule can absorb light at one wavelength and emit
light at a longer, lower energy wavelength. Light microscopy and fluorescence can be used in
conjunction in another imaging technique named confocal scanning light microscopy
(CSLM)[84], [85]. This imaging modality was developed as a method to visualize 3D structures
from a 2D imaging modality. CLSM uses a double pinhole set up in which the position of the
pinholes can be tuned in such a way that the photon detector only captures light from a specified
focal plane through the sample at a specific voxel. A series of images can be taken by adjusting
the pinholes to focus on discreet neighboring focal planes, then the images can be combined with
computer software to produce a 3D image of the sample[86].

Light and confocal microscopy have been used extensively as an imaging modality in
biological systems since light microscopy is one of the few methods which does not require
sample fixation, and can be performed on a live cellular sample. With the correct choice of
fluorophores, the entire internal structure can be viewed in real time, and with this method, real
time assays can be performed on active systems. The correct choice of fluorophores is an
important aspect of fluorescence microscopy ever since the development of the Forster resonance
energy transfer (FRET) technique of imaging [87], [88]. FRET is the process by which a single
fluorophore, is raised to the excited state but rather than release this energy as a photon, the
donor fluorophore pairs with an acceptor within a threshold distance and undergoes an energy
transfer due to a resonant coupling. The second fluorophore then relaxes to ground state,
releasing a photon of light[88]. This transfer of energy not only occurs between two
fluorophores whose emission and excitation overlap, but it also only occurs when these two
fluorophores are within a specific distance from one another. The intensity of the emitted light
from the second fluorophore can be used to calculate the distance between the donor and acceptor at the time of imaging. This technique has helped understand the spatial separation of molecules and biological moieties in countless studies [88]–[94].

Biological systems are difficult to study in other forms of microscopy such as electron microscopy as the bombardment of the sample with a high powered electron beam causes serious damage to live cells and in some cases cannot even be performed on live samples. Yet this imaging modality comes at a cost of resolution. Light microscopy uses light in the visible, and near visible, spectrum in order to create images, and thus the possible resolution of these images is handcuffed to the wavelength of visible light. The resolution of images taken with light microscopy is on the order of >200 nanometers, while electron microscopy can reach resolution limits of <2 nanometers. As stated above, cellular structures and membrane domains can be on the order of 1-2 nanometers, and the lipid bilayer itself is approximately 4 nanometers thick depending on lipid composition. Light microscopy is limited by Abbe’s diffraction limitation which is shown in equation 1, meaning that resolution is directly proportional to wavelength of light detected and the limits of the mechanical system (numerical aperture, refractive index, etc.).

\[ d = \frac{\lambda}{2 \times n \times \sin(\alpha)} \]  

[1]

In the past 5 years, new imaging modalities have emerged within light microscopy. In 2014, Stefan Hell, Eric Bertzig, and William Moerner won the Nobel Prize in chemistry for the development of a light based imaging technique which breaks the law of Abbe’s diffraction limitation, and their work in single molecule microscopy. This imaging technique is called STimulated Emission Depletion (STED)[95]–[97]. This new technology uses a 2 beam system in which the excitation light travels through the sample as normal, while a second wavelength of
light, tuned to emission spectra of the imaged fluorophore is modulated as a ring around the excitation beam. This depletion “donut” quenches all of the fluorescence caught within the ring, and thus only a small focal point emits any light to the detector. Through this method of imaging, the resolution limit of light microscopy is able to reach approximately 50 nm, a shift of about one order of magnitude greater resolution in live systems[96]. However, this method cannot be ubiquitously implemented as the fluorophores applicable to this modality are limited and specific.

Similarly in 2014 a second approach to this question of super resolution light microscopy was developed and implemented by Zeiss[98], [99]. This procedure was named Airyscan, and this modality to super resolution yields resolutions of 140 nm laterally and 400nm axially, about 2-3 times less specific than the STED, but the Airyscan method is not restrictive in usable fluorophores[100]. The Airyscan method consists of using an array of pinholes in lieu of a single pinhole for imaging. This array allows for the detection of particle “movement” as a function of the laser scanning aspect of confocal, where the object does not move but rather the moving laser light during the scan illuminates the fluorophore in a quantifiably different orientation during the course of the scan. Due to the location and layout of the pinhole array, this allows for the reflected light to only pass through certain pinholes depending on the orientation of the laser illumination which allows for a recombination of the pinholes images to produce a super resolution image.

The final approach to microscopy for this work is Atomic Force Microscopy (AFM)[101]–[103]. AFM is an experimental modality with a variety of potential uses. A small functional probe is positioned to reflect a laser light onto a specialized detector, modulations in the probe tip cause the laser reflection to move along the detector and this movement is
quantifiable as a deflection from a normalized position. Understanding and testing the material characteristics and shape of the AFM probe tip allows for a variety of calculations to be made about the sample being tested. Attractive, repulsive, indentation, and extraction forces can be obtained from samples through the use of force curves. AFM also allows for the development of an effective image of the sample being tested. AFM tapping or constant contact mode both produce a type of image over a small scanned area. By measuring the probe tip deflection over a small area, an effective 2D height analysis image can be developed. These images have a relative resolution limit below 1 nm. This resolution limit is effected by the shape and design of the probe tip, and the accuracy of the photodiode detector.

A combination of the aforementioned microscopy modalities can be used in tandem in order to develop nano and micron scale characterizations of samples and platforms described herein.
### VI. Tables

**Table 1.1. List of Materials and Molecular structures.**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Name</th>
<th>Atomic Structure</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td></td>
<td><img src="image" alt="Cholesterol Image" /></td>
<td>Sterol molecule used in all sample formulations</td>
</tr>
<tr>
<td>Brain Polar Lipid Extract (BPLE)</td>
<td></td>
<td><img src="image" alt="BPLE Image" /></td>
<td>Lipids extract used as majority component of biomimetic system</td>
</tr>
<tr>
<td>L-α-Phosphatidylcholine (PC)</td>
<td></td>
<td><img src="image" alt="PC Image" /></td>
<td>Purified lipid used in the biomimetic system</td>
</tr>
<tr>
<td>Sphingomyelin (SM)</td>
<td></td>
<td><img src="image" alt="SM Image" /></td>
<td>Liquid ordered mimic lipid used in minimalist formulation</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)</td>
<td></td>
<td><img src="image" alt="DOPC Image" /></td>
<td>Liquid disordered mimic lipid used in minimalist formulation</td>
</tr>
<tr>
<td>1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)</td>
<td></td>
<td><img src="image" alt="DSPC Image" /></td>
<td>Long chain saturated lipid molecule for use in cancer lipidome tests</td>
</tr>
<tr>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)</td>
<td></td>
<td><img src="image" alt="DPPC Image" /></td>
<td>Long chain saturated lipid molecule for use in cancer lipidome tests</td>
</tr>
<tr>
<td><strong>3,3’- Dihexadecyloxacarbocyanine Perchlorate (DiO)</strong></td>
<td><img src="image" alt="DiO Structure" /></td>
<td>Lipophilic fluorescent tracer. Excitation/Emission: 488/506</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>--------------------------</td>
<td>--------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>1,1’-Dihexadecyl-3,3’,3’,3’-Tetramethylindocarbocyanine Perchlorate (DiI)</strong></td>
<td><img src="image" alt="DiI Structure" /></td>
<td>Lipophilic fluorescent tracer. Excitation/Emission: 551/570</td>
<td></td>
</tr>
<tr>
<td><strong>Streptavidin-AlexaFluor 660 (SA660)</strong></td>
<td>N/A</td>
<td>Protein fluorescent tag. Excitation/Emission: 660/690</td>
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<td><strong>(3- Aminopropyl)trimethoxysilane (APS)</strong></td>
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<tr>
<td><strong>bis(sulfosuccinimidyl)suberate (BS3)</strong></td>
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<td>zero-length cross linker</td>
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<tr>
<td><strong>Polyethylene Glycol (PEG)</strong></td>
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<td>Polymer spacer</td>
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</tr>
<tr>
<td><strong>N-hydroxysuccinimide-PEG4-Biotin (NHS-PEG4-Biotin)</strong></td>
<td><img src="image" alt="NHS-PEG4-Biotin Structure" /></td>
<td>tethering moiety/streptavidin functionalization</td>
<td></td>
</tr>
<tr>
<td><strong>N-hydroxysuccinimide-PEG4-1,2 Distearoyl-sn-glycero-3-Phosphoethanolamine (NHS-PEG4-DSPE)</strong></td>
<td><img src="image" alt="NHS-PEG4-DSPE Structure" /></td>
<td>tethering moiety/lipid film anchor</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2: Sphingomyelin/ 1, 2-Dioleoyl-sn-glycero-3-phosphocholine/

Cholesterol Formulation and variants

I. Introduction

This chapter focuses on the development and optimization of the synthetic lipid analogue environment built for control and reproducibility. This work focuses on a widely used and studied SM/DOPC/Cholesterol system, which has been used as a mimic for the lipid environment of a brain cell. It is shown here that synthetic lipid membranes are producible across a wide variety of components and molecules. These lipid formulations can be used as representative analogues of all possible cell and organelle membranes to further increase the biological relevancy of these lipid microenvironments. It is shown here that reproducible membranes with visibly quantifiable characteristics can be formed through a straightforward process of dissolution, mixing and drying to form starting materials for supported lipid bilayers on particles or surfaces.

II. Materials and Methods

Purified Sphingomyelin (SM), DOPC, cholesterol, Brain polar lipid extract (BPLE), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and L-α-phosphocoline (LPC) were purchased from Avanti Polar lipids in powdered form. Fluorescent lipophilic tracers DiO, DiI, and Perylene were purchased from Molecular Probes, and added at 0.2%, 0.2%, and 0.4% (molar or weight equivalence as shown in Table 2.1) respectively in the lipid films in which they were used. Chloroform 99.9%, extra dry was purchased from Fischer-Scientific.
Lipid films were created by dissolving the purified lipids and lipophilic tracers in chloroform and then combining them to a total weight of 4 mg of total lipid and desired concentrations within 4 ml amber glass vials. These mixtures, shown below in table 2.1 were then dried in a vacuum chamber overnight (minimum of 8 hours) protected from light to help preserve the fluorescence of the lipophilic tracers. This process removes the chloroform from the mixture while leaving a deposition of the lipids in the glass vial, thus a film. After drying, any unused stock solutions or films to be stored were placed in a positive pressure chamber and flushed with inert gas, sealed tightly in parafilm, and placed in desiccated jars in the freezer at -20°C. Any films prepared for immediate use were removed from the vacuum chamber and rehydrated with 2 mL of PBS.

The films, upon rehydration, were mixed vigorously using hand mixing and a vortex mixer to ensure that all deposited lipid was suspended into the PBS aqueous phase. The rehydrated multilamellar vesicle (MLV) solution was then transferred from the amber glass vial into a 15 mL conical tube and placed into a 37°C water bath. Allowing approximately 5 minutes for temperature equilibration, the MLV solution was then sonicated for 15 minutes in a BioLogics Inc. 150 V/T Ultrasonic Homogenizer. This sonication produces a solution of small unilamellar vesicles (SUV) liposomes of relatively uniform size. After sonication, the liposomal solution is placed in an Emerson centrifuge and spun at 1500 rpm for 2 minutes to remove any debris accumulated throughout the previous steps. The supernatant is then transferred to a fresh 15 mL conical tube. 500µL of the liposomal solution is taken from the newly centrifuged tube and placed in a 1.5 ml microcentrifuge tube. This microcentrifuge tube is then replaced back into the 37°C water bath to once again equilibrate, approximately 5 minutes. These liposomes are then used further for varying liposomal fusion studies.
Separately to the formation of lipid films and SUV solutions, glass surfaces and 5 um silica beads were prepped for lipid fusion. Glass slides, coverslips, and silicon dioxide chips were cleaned overnight in Piranha solution, the supports were then washed thoroughly with DI water. Piranha solution was made as a mixture of Sulfuric acid and Nochromix, (Fischer Scientific) at a ratio of 1 g of Nochromix for every 100 mL of sulfuric acid. To ensure the glass surfaces were free of piranha solution and any excess sulfuric acid, the washing was performed by submerging the glass support in pure DI water in an ultrasonic cleaning bath (Branson B1510 Ultrasonic Cleaner) for 15 minutes, followed by a series of dunk washes in fresh DI water, and finally dried using compressed inert gas. The 5 um silica beads (Bangs) were weighed out and mixed to a total concentration of 1 mg of beads per 1 mL of PBS in a 15 mL conical tube. This mixture was then placed in a bath sonication unit (Branson) for a minimum of 15 minutes, to break apart any microbeads that may have stuck together upon shipping and dry storage. After sonication, an aliquot of the microbead solution was then placed into a 1.5 mL microcentrifuge tube and placed in the 37°C water bath until fusion.

After the SUV solution, the microbead, and glass surfaces have been prepped, then the liposomal solution is fused to the solid support structures. To undergo fusion of the SUVs to the microbeads, 200 µL of the heated microbead suspension is pipetted dropwise into the 500 µL of SUV liposome solution. After dropwise addition, the new mixture is lightly hand shaken, briefly mixed on the vortex mixer, and then placed in an end-over-end rotomixer located within a repurposed cell incubation chamber held at a constant 37°C. This fusion was completed at 3 separate timed intervals, 30 minutes, 18 hours, and 24 hours. After these times have been met, the fused lipobeads solutions were spun down in a microcentrifuge, the unfused liposomal
supernatant was removed and replaced with fresh PBS, and then the pellet was then resuspended, this process was repeated 3 times.

To fuse the liposomal solution onto the planar glass surfaces, the heated liposomal solution was pipetted onto the clean glass and then placed into the above mentioned incubation chamber for 30 minutes. The glass surfaces were then removed from the chamber and washed. There were three approaches to washing due to the intricacy and care needed to not shear off any of the fused lipid bilayer from the bare glass surface. The first approach was a series of immersion in a petri dish filled with fresh PBS, after the immersion, the glass surface was gently removed from the petri dish, the contents of the dish were discarded and the surface was immersed again, repeated for a total of 3 washes. The second approach was to pipette off the any excess liquid, leaving enough to just cover the extents of the fused area, then looping a small piece of parafilm around the opening of the pipette and adding fresh PBS back onto the surface. The parafilm was used to limit the shear force of the PBS leaving the pipette tip and dampen it over a broader area to minimize any bilayer disruption. This process was once again repeated thrice. The final technique was to remove any excess liposomal solution, similar to the second technique, however in the samples prepared with this technique the sample area to be imaged or tested was demarcated on the glass surface, so the fresh PBS was pipetted outside of this demarcated and the natural spreading and surface tension of the PBS was used to wet the surface. This process was performed 3 times, like all other samples, to insure them removal of any excess unfused SUVs from the final sample.

These samples were then imaged using both CLSM and AFM modalities. The confocal images were obtained by excitation of the DiO and DiI probes with a 488nm laser, this laser would excite the DiO molecule which would then undergo FRET with the DiI probe, which
would then emit light between 550-600 nm collected by the detector, any light emitted by the DiO probe was collected between 500-549 nm. CLSM imaging was also coupled with 2 super resolution techniques, the first being STimulated Emission Depletion (STED) from Leica and the second being Airyscan from Zeiss. STED microscopy was performed for the DiI and DiO samples using an excitation beam of 488 nm, and a depletion laser of 592 and 660 nm in varying tests. The Airyscan images were obtained using a 488 nm excitation beam and the same 2 detection windows as stated in the base CLSM studies.

III. Results and Discussion

Throughout these tests a main staple of the experimental set up was the use of the DiO, donor, and DiI, acceptor, FRET method described in Baird et.al[88]. This method is a very powerful way to isolate out and quantify the apparent phase partitioning within our lipid systems. DiO and DiI do not fluoresce in solution, so there was no worry of any background excitation, and so the only source of emitted light would be from a lipid bilayer, on top of this fact the FRET technique yielded a secondary level of clarification and further delineation of the two phases believed to be present in these samples. Figure 2.1 shows the comparison between samples containing no tracer, acceptor only (DiI), donor only (DiO), and with both dyes present. It is visible that under acceptor only conditions, the DiI signal is weakly visible throughout the lipobeads while illuminated at 488 nm. In the donor only condition, the DiO signal is visible throughout the entire lipobead and shows a small, negligible amount of bleed through into the higher wavelength detection channel. However it is seen when the samples contain both DiO donor and DiI acceptor the lipobead yields bright signal in both channels, and in this case the DiO signal is slightly weaker than in the donor only case due to the FRET phenomenon. These two lipophilic probes have been shown to have an innate preference of one lipid phase over
another, this was a point of contention once or twice throughout all of these studies, and this preference is a tendency to partition into the L_d phase of the bilayer.

![Figure 2.1 FRET dye pair validation.](image)

Figure 2.1 FRET dye pair validation. This chart shows the use of the FRET fluorophore labeling structure. All lipobeads were illuminated with a 488nm laser and the detected emission windows are shown on the left. All of the lipobeads images have their intensities normalized. In samples containing neither the DiI nor the DiO probe, no emitted light is seen in either window. In samples containing strictly the DiI fluorophore, the fluorophore is shown to emit a very low and weak signal barely above the overall noise level of the samples. The samples containing only the DiO lipid show a very bright emission in the expected DiO detection range with a small amount of bleed through into the DiI detection window. Finally, samples containing both the DiI and DiO lipid show bright signal in both detection windows, however the signal seen in the DiO detection window is slightly dimmer than in the samples containing only DiO, this is due to the FRET phenomenon in which a large number of DiO molecules will not emit excess energy as light but transfer it to the DiI fluorophore.

Initial tests were performed on two systems to verify the existence of visual phase separation. Previous work had shown a system containing BPLE, LPC, and Cholesterol would yield a confluent lipid membrane for use in lipobeads systems, and maintained activity of key TMPs, most importantly γ-secretase. However, one drawbacks of this lipid system was the inability to discern lipid phases and thus lacked important spatial and microenvironmental information which was a desired trait, of the γ-secretase system, to study. BPLE is a desirable component for use in a synthetic mimic of a brain cell microenvironment due to the fact that it is
a total extract of lipid material from lysed brain cells, which affords any brain cell TMPs a more biologically relevant microenvironment.

Figure 2.2 shows the comparison of lipobeads of the SM:DOPC:cholesterol formulations(2.2 A, B) to those composed of the BPLE:LPC:cholesterol (2.2 B, C) lipid formulation. The fluorescent spectral scans in panels B and D shows that both of the lipid formulations contain the DiO donor and DiI acceptor pair of fluorophores, with emission maxim of 505 nm and 555 nm respectively. Panel B shows the comparative intensities calculated from the two highlighted inset regions, a readout of the FRET spectral signature of the L_o and L_d phases. The intensities of the dyes are approximately 4-fold higher in the L_d phase, and the relative intensity of the donor to acceptor decreases in the less concentrated L_o phase, indicative of a change in FRET efficiency.
Figure 2.2 Spectral comparison of simplified analogue versus biological membrane. A) Synthetic SM:DOPC membranes shown here with visual phase separation were spectrally imaged, the insets show the equatorial z section lambda scan from isolated beads from the indicated ROIs to elucidate the intensity difference in emission intensities between the $L_o$ and $L_d$ phases. Panel B shows the comparative intensities calculated from the two highlighted inset regions a readout of the FRET spectral signature of the $L_o$ and $L_d$ phases. Panel C shows a BPLE:LPC lipid bilayer formulation here shows no visible signs of phase separation and shows high levels of FRET intensity. Panel D displays graph showing the spectrum obtained from the BPLE:LPC lipid formulation as well as a comparative spectrum from a SM:DOPC lipid formulation which contained only the acceptor (DiI) fluorophore of the FRET pair.
The BPLE containing lipobeads in figure 2.2 C have a distinct lack of any visual phase separation at this resolution. Panel D displays a graph showing the spectrum obtained from the BPLE:LPC lipid formulation as well as comparative spectrum from a SM:DOPC lipid control formulation which contained only the acceptor (DiI) fluorophore of the FRET pair. It is believed that phase separation within this system does in fact occur, but that it occurs on a nanoscale length scale as opposed to the micron scale seen in the SM/DOPC lipid membrane system. This nanoscale phase separation is well below the resolution limits of light microscopy, even when factoring newly developed super resolution techniques such as STED and Airyscan. It is also believed that due to the multitude of varying lipid species in the BPLE membrane, that these rafts not only are nano-scale but may also be highly transient as it has been shown in previous work that rafts tend occur along critical points and energetic tie lines within the ternary lipid phase diagrams. This lack of visual phase separation led to the adoption and prominent use of the SM:DOPC:cholesterol, or 2/2/1, lipid membrane formulation throughout most of the studies performed.

A contention over this partitioning arose when reading Feigenson et al.[27], [104] which described that in synthetic, biomimetic membranes both DiO and DiI partition into the L_d phase in SM:DOPC:cholesterol, which is the precise system in which the work here is completed. The Baird et al. paper describes that the DiO and DiI probes are found more commonly in the detergent resistant, or L_o, phases of cell membranes, since there work was performed in cell cultures expressing both the DiO and DiI probes. This discrepancy between the two formats and difference in synthetic versus biological was debated vigorously, but upon further study of our systems, the DiO and DiI emission showed many more characteristics of presenting in the L_d phase, similar to the work of Feigenson.
This preference is a key aspect as to why we chose a 2 probe FRET approach. As stated, this partitioning of the fluorescent probes is a preference, but it does not eliminate the presence of the fluorescent probe in the opposite phase, thus making it more difficult to clearly see the delineation between L\textsubscript{o} and L\textsubscript{d} phases in the bilayer. With the 2 probe approach, the FRET readout of the probes means that the donor and acceptor dyes are distributed in the bilayer together, as this FRET phenomenon only occurs when two probes are approximately between 3-6 nm apart and is thus proportional to the concentration and therefore the partitioning of the probes between L\textsubscript{o} and L\textsubscript{d} phases. This phenomenon limits the amount of signal seen in areas that are sparsely populated by both fluorophores, and occurs only when a sufficient concentration exist within the membrane. If both probes have a preference to the L\textsubscript{d} phase, and only under FRET when in close proximity, it is understandable the fluorescence we collect from the acceptor DiI probe would happen almost exclusively in the L\textsubscript{d} phase of the lipid bilayer. While it is possible to isolate the fluorescent signal of the disordered phase, it leaves the ordered phase to be imaged by the examination of the lower registers of the detected fluorescence, utilizing the 16 bit depth of the modern confocal microscopes (0-65536 levels for 16 bit versus 0-256 levels for 8 bit detection). This would mean that what is seen in the data collected, is that the areas devoid of fluorescence on the planar surface or microbead constructs is the L\textsubscript{o} phase (shown as false red color in Figure 2.2 A).

Upon further study of this fluorescent dye system a secondary approach was attempted to verify the existence of a confluent lipid bilayer, while maintaining the ability to discern visual phase separation. A third fluorescent probe was added into the lipid film formulation, Perylene. From Feigenson’s work with fluorophores in lipid systems and its use in the Dimova paper, which heavily influenced the work performed, it was deemed a viable candidate to help elucidate
the confluency of the lipid membranes within the lipobeads systems[27], [104]. However Figure 2.3 shows that upon inclusion of this tracer the bilayers lost their visible phase separation throughout multiple different lipid membrane formulations which had repeated yielded quantifiable phase separation. Also visible in this figure is the presence of unfused liposomes containing what appears to be exclusively Perylene signal. The Feigenson and Dimova work was predominantly performed in giant unilamellar vesicle (GUV) systems, which have vastly different formation methods than the lipobeads of planar supported bilayer systems in this work. This difference could be the reason of the discrepancy between their work and the work performed here, as the fusion process of our planar surfaces and microbead systems is the main aspect believed to be causing this difference, and this step does not occur in GUV tests.

The fusion step in the samples presented, can cause non-homogeneity to occur within our samples, a problem that will be addressed later in this work. This lack of homogeneity arises from the randomness of the fusion process and sonication process within these tests. During the probe sonication process to produce SUVs which are then used in the fusion step, these SUVs are created through a series of rapid expansion and compression causing the cavitation and reformation of larger MLVs into the SUVs used. This cavitation and reformation process is done through no external control, outside of forcing cavitation, and thus is a pseudo-self-assembly process which is dictated ultimately by energetics of the system. It makes sense in this case that if given the size discrepancy and saturation differences of both the DOPC and SM lipids that reformation would be favorable for vesicles comprised of the same lipids, yielding a potential range of liposomes from only DOPC to only SM and the gamut in between. Furthermore, we expect mechanical differences where the more Lα-like liposomes are more rigid and thus would fuse at lower rates during lipobead formation. This heterogeneity in liposomes for fusion can
lead to a slight disparity in fusion times required by different liposomes in order to fully coat the solid support structures. The addition of Perylene into these systems could exacerbate this heterogeneity leading to an overall increase in the predominance of a single phase, L_d, microbead fusion. This heterogeneity is likely the cause of the unfused liposome seen in figure 2.3. The addition of Perylene was eliminated from further studies.

**Figure 2.3 Overlay of Perylene and DiI detection in SM:DOPC:cholesterol formulations.** A) Perylene signal obtained by illuminating the sample with a 405 nm wavelength light and detecting at 450-480 nm, B) DiI signal obtained by illuminating with 488 nm wavelength laser and detecting at 550-600 nm range. C) Composite overlay of the two showing no apparent phase partition. However, in the Perylene and composite images small unfused liposomes can be seen attached to the lipobeads and free floating in the sample. These free liposomes do not show any visible DiI signal, suggesting that they are predominantly L_o phase liposomes.

The question of confluency is however addressed by the presence of low levels of the DiO lipid tracer throughout the microbead system. As stated before, the DiO and DiI lipophilic tracers do not fluoresce in solution, and in all of the solid supported systems, excess and unfused liposomes other material is washed out from the system over the course of three washes. While analyzing the microbeads, the ability of the lipophilic tracers to partition into both phases of the lipid bilayer became a benefit. By increasing the low end intensity of the DiO signal, it becomes apparent that this signal can be seen surrounding the entirety of the microsphere. This low signal is above the noise levels inherent in CLSM detectors, and thus signifies the confluency of the bilayer spanning the entire microbead. This can be checked across all samples, and thus can be
used to insure proper fusion of the SUV solution onto the solid support systems. Figures 2.4, 2.5, and 2.6 show examples of this analysis across 2 tested samples. Figure 2.4 shows a 2 phase system in which the phases have each coalesced into separate domains, the average intensity over the line shows 2 distinct regions in terms of intensity, which are both above background levels. The region with high intensity is the $L_d$ phase, while the low intensity region is the $L_o$ phase of the lipid bilayer. Figure 2.5 shows a CLSM image with a similar intensity chart as the previous figure, however due to the interdigitating of the phase separation regions and the lack of sub 200 micron resolution, it is difficult to distinctly make out the phase regions, however a similar trend to what is seen in the previous figure is observable. To further ratify this claim, figure 2.6 shows an Airyscan image of the same lipobeads observed in figure 2.5, with much higher resolution and the ability to distinctly observe the intensity difference between the two phase regions.

![Figure 2.4 CLSM general intensity over a domain ripened lipobead.](image)

As shown, a DiO fluorophore intensity was obtained for the bisecting line through a hemispherical projection of a domain ripened lipobead. The intensity can be seen to have roughly 2 intensity regimes which correspond to the $L_d$ phase shown as the higher intensity or top portion of the lipobead, and a less intense $L_o$ phase shown as the bottom portion of the lipobead.
Figure 2.5 CLSM general intensity over a representative bead. Shown here is the DiO fluorophore intensity obtained along the line bisecting the hemispherical projection of a single representative bead. The intensity levels fluctuate over the entirety of the bead and due to resolution limitations it is difficult to isolate an average intensity value for the $L_o$ or $L_d$ phase.

Figure 2.6 Airyscan general intensity over a representative bead. This figure shows the associated Airyscan image of the representative bead in figure 2.5. With the resolution increase provided by the Airyscan modality it is possible to view the relative intensity level differences between the $L_o$ and $L_d$ phases.

Proceeding the confirmation of the confluent lipid bilayer the next hurdle necessary to overcome was the aforementioned discrepancy with homogeneity. A key concept of the supported bilayer approach is the repeatability and normalization of test platforms for potential use in high-throughput systems. Lipobeads ranging from total $L_o$ coverage to total $L_d$ coverage have the ability to confound samples specifically tailored to test the effects of lipid
microenvironment on protein function. In order to test this homogeneity in the samples, 3D z-stacks were taken using CLSM, and processed in ImageJ software by making hemispherical projects of the top and bottom hemispheres of the microbeads. These projections were thresholded to isolate the bright Ld phase areas, and then overlaid with a mask to normalize for the loss of geometrical data, when the images was reduced from 3D to 2D in the hemispherical projections. The thresholded images was then ratioed with the mask to produce a coverage percentage, signifying the percentage of the hemisphere covered by the Ld phase. Initial tests were performed by allowing the SUV solution to fuse with the silica microbeads for a maximum of 30 minutes, but following analysis of these samples, the Ld coverage obtained from these samples averaged out to approximately 77% ± 23% showing a distinct preference for Ld phase coverage. The standard error shows that the overall variance in lipid phase distribution is very high, yielding a wide range of lipid microenvironments which is not ideal for a potential high-throughput modality with control of lipid microenvironment. One suggested approach on refining this variance was to employ the use of flow cytometry, which had been shown as a valid method to isolate desirable test sets from a varied population of lipobeads. However, since the average coverage was well above the desired and predicted 50/50 split, garnered from the fact that there are equal parts SM and DOPC in the lipid formulation, and the prediction from the phase diagram presented in Dimova et.als. work, it was suggested to extend fusion times before continuing to cytometry.

As mentioned above, the discrepancy of fusion and heterogeneity problem was the potential cause of this high Ld phase fusion. It was proposed that extending fusion time to long times could afford the solutions a better chance to reach an equilibrium and allow for more rigid, largely SM/cholesterol based liposomes, more time to undergo deformation and fusion with the
microbead systems. A fusion time of approximately 18 hours, overnight fusion, at 37°C was tested, and upon analysis of the microbeads, the \( L_d \) phase coverage reduced from the 77% above, to a value of 55% ± 19%, much closer to the desired ~55% from the phase diagram shown in figure 2.7, this difference can be seen on the lipobead constructs in figure 2.8. Figure 2.9 shows a histogram of the 2 samples, the short fusion time samples in blue, and the long fusion samples in red. In this figure it can be seen that overall median coverage has shifted down closer to the projected 55/45 \( L_d/L_o \) split, and an overall decreased spread in overall coverages. To further understand the effects a long fusion time had on the characteristics of the fused lipid bilayer, Fluorescence Recovery after Photobleaching (FRAP) analysis was performed on both samples in the \( L_d \) phase. Both of the fusion samples were observed to have a strikingly similar effective diffusivity of approximately 0.103 ± 0.04 \( \mu \text{m}^2/\text{second} \) for the short fusion lipobeads (n=7), and approximately 0.104 ± 0.03 \( \mu \text{m}^2/\text{second} \) for the long fusion lipobeads (n=15). The mobile fraction of the short fusion sample was calculated to be 94 ± 4%, while the mobile fraction of the long fusion was calculated to be 88±9%. Both of the values, diffusion and mobile fraction, are not found to be statistically significantly different. This shows, as expected, an extended fusion time does not negatively impact the diffusive characteristics of the membrane.
Figure 2.7 Ternary Phase Diagram of the Sphingomyelin:DOPC:Cholesterol lipid formulation. This is the expected regime in which the SM:DOPC:Chol liposomes will be formed, as well as the distribution of phases found on the microbead system. It is shown (blue star) that the 40:40:20% formulation of these molecules should yield an even distribution of both $L_o$ and $L_d$ phases on the microbeads.

Figure 2.8 Comparative phase separation of short and long fusion times on the SM:DOPC:cholesterol lipid formulation. This image shows the comparison of the lipobeads fusion step performed at 30 minute and 18 hour intervals. These are top and bottom hemispherical projections of the 3D images obtained from the LSM800 Zeiss microscope. The phase separation is visible in both examples, however the 18 hour fusion sample shows a more consistent phase separation from PLB to PLB.
Figure 2.9 Histogram of coverage analysis. A histogram showing the overall liquid disordered phase coverage values of the 18 hour fusion (red) and 30 minute fusion (blue). The blue histogram shows a population with an average value of 77% ± 23% total L_d phase coverage, while the blue histogram shows a population with an average L_d coverage of 55% ± 19%. Shown is the normalizing effect a prolonged fusion step had on the samples overall yielding a tighter distribution of lipobeads as well as one centered more closely to 50% coverage, the expected value from the ternary phase diagram.

Due to the high randomness and interdigitating of the visible phase separation in the SM:DOPC:cholesterol samples, they were a prime candidate to analyze and test out the aforementioned super resolution technologies available, STED and Airyscan. The Leica STED was the first available option to use for this study. However, as seen in figure 2.10, STED did not yield any increase in resolution in any of the x, y, or z imaged planes. Upon further research into the STED functionality, due to its use a depletion laser, the fluorescent dyes used in imaging must be optimized for emission depletion by the available lasers. The depletion lasers available
were a 592 nm, 660 nm, and 775 nm laser. All three were used to test both the DiO and DiI probes, but none of them showed any increased resolution, even after deconvolution.

Figure 2.10 Comparison of STED super resolution to CLSM imaging of DiI fluorophore. Shown here is a comparison between A) base confocal microscopy using a Leica SP8 microscope, and B) a STED confocal image from the same microscope set up. These images show no visible signs of any resolution increase from using the DiO/DiO fluorophore lipid tracers as the fluorophore of interest.

The Zeiss Airyscan was the made available shortly after the STED, and its use of a detector and pinhole array did not require specific fluorophores nor for them to be optimized. The Airyscan method is more of a mathematical reconstruction method, as opposed to the STED direct measurement. As seen in figure 2.11, the difference between Airyscan and base CLSM is a significant increase in resolution. In the highlighted area it can be seen that under base CLSM imaging, the dark area (L_{o} phase) is comprised of a combination of both L_{o} and L_{d} phases, just at the resolution limitation of CLSM this area is predominantly L_{o} and is visually represented as such. This increase in resolution using the Airyscan technology allows for the refinement of the coverage analysis to more adequately represent and understand the complexities of phase separation and in further studies, allow a better quantification of phase and spatial preferences of reconstituted TMPs.
Figure 2.1 Comparison of Airyscan super resolution imaging versus CLSM of DiI fluorophore. This is a comparison of the A) Airyscan imaging technique used on the Zeiss LSM800 to B) confocal image obtained under the same conditions. The zoomed region highlighting one bead shows further conclusive evidence that there is a significant resolution increase between the two modalities allowing for the further elucidation and clarification of lipid phase separation. In these images the green color is the emission from the DiI FRET acceptor lipophilic probe while the system is illuminated by a 488nm laser.

Throughout these SM:DOPC:cholesterol studies, one main drawback of the system was considered: the effective biological relevancy of such a basic ternary lipid formulation. It was necessary to understand what happens when shifting from the BPLE lipid formulation seen above, to the ternary SM:DOPC lipid formulation. This was tested by creating a series of SM:DOPC lipid formulations with increasing concentrations of BPLE doped into the formulation. The system was doped from 0-75% BPLE, specifically 0%, 25%, 50%, and 75% to determine the effects of increasing the concentration of a more biologically relevant lipid species would have on the visible phase separation, and later the diffusivity of the lipid bilayer. Figure 2.12 shows the steadily increased dopant concentrations, and it can be seen that when the lipid begins to shift into a BPLE dominant membrane, starting at 50% doping, the fused lipids become
unstable on their microspherical solid supports and begin to slough off the microbead and “melt” onto the coverslip. However, in the samples containing 25% and less BPLE, visible phase separation is still achieved to a quantifiable degree similarly to the pure SM:DOPC lipid formulations tested above, indicating that these BPLE systems will provide a new framework to study membrane protein partitioning in more natural and brain-like lipid microenvironments.

Figure 2.12 Varying BPLE dopant concentration into canonical SM:DOPC:cholesterol formulation. This figure shows the comparison of varying levels of BPLE lipid mixtures on the structural integrity and phase separation visibility on normal SM/DOPC/cholesterol levels. Panel A is the 25% BPLE dopant mixture, Panel B is the 50% dopant mixture, and Panel C shows the 75% dopant. (Displayd using Amira 5.43 using the Volren technique.) At each tested concentration, visible phase separation is present on the lipobeads constructs, however at concentrations of 50% and above the lipid formulation becomes unstable in its fusion to the silica microsphere surface and begins to effectively slough off of the surface of the lipobead and mobile lipids move onto the glass coverslip surface.

Upon doping the SM:DOPC system with lipids extracted from living brain cells, it was also suggested to look into other possible dopants to increase the diversity of the lipid environment on the solid supports, while also allowing for the lipid system to more accurately mimic a biologically relevant system, all while maintaining low levels of complexity and high repeatability. The second dopants chosen where two fully saturated straight chain lipids, DSPC and DPPC. Research has shown that when a cell develops cancer, the lipidomics of the membranes shift wildly from the native healthy cell configurations, and in specific cases such as
prostate cancer, the amount of saturated lipids, such as DPPC and DSPC, within membranes increased[105]. To mimic this effect in lipobead systems, we used the SM:DOPC:cholesterol platform to investigate these changes. Figure 2.13 shows three separate samples containing low levels of these DSPC and DPPC lipids doped into the membrane. Panels A-C compare the effects of increasing addition of the long saturated straight chain DPPC and DSPC lipid into the SM:DOPC:cholesterol base mixture. Panel A is a 0.5% doping of the saturated lipids, panel B results from doubling the concentration to 1% doping, and panel C results from a 5% doping of the saturated lipids. Substantial nanoscale phase separation is visible in both the 0.5% and 1% doping level images but is no longer visible in the 5% doped sample. However, at a level of 5% DPPC and DSPC it is shown that these lipobeads undergo a phenomenon known as domain ripening, in which the phases coalesce and separate as much as possible leaving 2 large single phase structures as opposed to continuously interdigitated nano- to microscale phase separation we have seen in all of the other lipobead samples of the SM:DOPC:cholesterol formulation. If this process occurs in cancer cells, it would provide a mechanism for strongly perturbing cell signaling that could drastically affect malignant cell function and membrane protein partitioning and dynamics.
Figure 2.13 Variation of Saturated lipid dopant into canonical SM:DOPC:cholesterol (2:2:1) lipid formulation. Panels A-C compare the effects of increasing additions of the long straight chain DPPC and DSPC lipid into the SM/DOPC/cholesterol base mixture. Panel A is 0.5% doping of the saturated lipids, panel B results from doubling the concentration to 1% doping, and panel C results from 5% doping of the saturated lipids. Substantial nanoscale phase separation is visible in both the 0.5% and 1% doping level images but it is no longer visible in the 5%, doped sample. However at a level of 5% DPPC and DSPC it is shown that these lipobeads undergo a phenomenon known as domain ripening, in which the phases coalesce and separate as much as possible leaving 2 large single phase structures as opposed to the continuously interdigitated nano- to microscale phase separation seen in all of the other samples of the 2:2:1 lipid formulation.

Throughout these experiments, FRAP was also performed on a large number of these platforms. These FRAP experiments provide two key parameters of lipid bilayer fluidity and diffusivity. These parameters being the mobile fraction and the effective diffusivity of the photobleached area. In these samples it is expressed as “effective diffusivity” due to the existence of phase separation within the system, and the evidence suggested in the Airyscan images, which is that these phases exist below the resolution limitation of the CLSM, and in further studies is shown to propagate even below the resolution limitations of super resolution into the nanoscale regime. Figure 2.14 shows a representative FRAP analysis following the method in Klonis et al.[106]. The results were obtained by photobleaching 1 µm² areas along the equatorial region of the lipobeads. Using this cross section of the lipobeads afforded the ability to analyze multiple instances of FRAP across a single lipobeads, with enough distance between the bleached areas to avoid any over bleaching or cross talk between the analyzed areas. The representative FRAP profile in figure 2.14 was developed from a sample fused for 18 hours.
The x-axis is time in seconds and y-axis is overall normalized recovery. FRAP was performed on both the visually discreet Ld and Lo phases. The Lo phase shown in here in orange, has low effective diffusivity at approximately 0.001 \( \mu m^2/s \), ~2 orders of magnitude different to the Ld phase shown here in black with an effective diffusivity of 0.106 \( \mu m^2/s \).

![Figure 2.14 FRAP analysis performed on SM:DOPC:cholesterol formulation. A representative FRAP profile developed from a sample fused for 18 hours, the x-axis is time in seconds, and the y-axis is overall normalized recovery. Frap was performed on both visually discreet Ld and Lo phases. The Lo phase shown here in orange, has low effective diffusivity at approximately 0.001 \( \mu m^2/s \), a 2 orders of magnitude different to the Ld phase shown here with an effective diffusivity of 0.106 \( \mu m^2/s \).]

**IV. Conclusions**

The work described here shows significant strides in developing a stable, reproducible, and modular platform, on which effective high-throughput processes can be performed, ranging from microwell plating assays to flow cytometry. The development of mobile and diffusive membranes is possible on an array of solid support structures, tested here was the spherical microbead surface, but the approaches used are rather ubiquitous in nature, and thus could be applied to various tailored supports including planar and tethered systems. With the rise of
micromanipulation, 3D printing, lithography, and nanofabrication, there is very little limit to the potential applications of these lipid systems. A major drive in this work however is to not only develop synthetic membrane systems, but to increase their biomimetic capabilities while maintaining key advantages of the synthetic systems.

The highly reproducible, canonical SM:DOPC:chol lipid formulation used throughout years of lipid studies is shown to be a viable basis on which to build this modular biomimetic system. It is a widely used baseline mimic in brain membrane studies, and is shown to be highly effective. However, this is still just a ternary system compared to the tens of thousands of lipid components found in living cells. By taking this formulation and augmenting it slightly with a host of varying dopants, it is possible to develop vastly different and compelling microenvironments more similar to those in living cells, but maintaining L_o/L_d phase separation. These microenvironments are shown to have substantial effects on TMP function, specificity, and efficiency. Through the development of more biologically relevant membranes, it is possible to close the gap between cell culture and low through-put experimental methods and highly reproducible, rugged, synthetic high- throughput modalities.
V. Tables

Table 2.1 Additional Materials.

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<th>Name</th>
<th>Atomic Structure</th>
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Table 2.2 Lipid formulations. This table contains the overall lipid formulations used throughout the studies within this work. Due to the fact that the BPLE lipid was a mixture of all lipids obtained from a lysed brain cell, it was necessary for the formulations to be weight based in their distribution as there was no equivalent molar weight.

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<th>Sphingomyelin</th>
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<th>DPPC</th>
<th>DSPC</th>
<th>Cholesterol</th>
<th>BPLE</th>
<th>L-alpha-PC</th>
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<td></td>
<td></td>
<td></td>
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<td>10.75</td>
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<td>75</td>
<td></td>
<td></td>
<td></td>
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<td>50</td>
<td></td>
<td>Wgt %</td>
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Chapter 3. Gamma Secretase and Substrates in Proteolipobeads

I. Introduction

The transmembrane proteins studied in this work comprised of γ-secretase and truncated versions of two of its known cleavage substrates, Amyloid Precursor Protein (as SB4) and Notch-1 (as NTM2). These proteins have a significant impact on disruptive and deadly diseases and are thus prime candidates for study in the pharmaceutical industry as potential drug and therapy targets. The work in this chapter was performed by using optimized SM/DOPC/cholesterol brain lipid analogues, to understand and elucidate key native protein characteristics such as diffusivity throughout the membrane, potential partitioning of the protein into one of the two lipid phases, and activity within these synthetic constructs.

II. Material and methods

γ-secretase, the Amyloid Precursor Protein (APP) based substrate SB4, and the Notch-1 based substrate NTM2 were secured from the Li lab at Memorial Sloan Kettering Cancer Center. Proteins were obtained as extractions from genetically modified cell lines, which causes over expression of the proteins of interest[107], [108]. All samples were provided as surfactant stabilized extractions. The surfactant used in these studies is CHAPSO. Table 3.1 shows the auxiliary molecules used in this section.

Lipobead solid support structures were developed using the aforementioned methods in the previous chapter. These fused and washed samples were then incubated with the surfactant solubilized TMP extractions in a 0.25% mixture of CHAPSO for 1 hour at 37°C in the repurposed incubator, while constantly mixing in an end-over-end rotomixer. γ-secretase was incubated at 0.07 ng/ml, SB4 at 60µM, and NTM2 at 100 nM, within their respective studies.
Following the incubation, the samples were then washed thrice, using the same procedure as above, and fresh PBS.

During the hour long incubation, a mixture of Steptavidin conjugated with Alexafluor 660 (StAv-AF660) was prepared. 25 µl of a 1 mg/ml stock StAv-AF660 solution was diluted into 10 ml of fresh PBS in a 15 ml conical tube. The StAv-AF660 solution was then subjected to a Bio-Bead SM-2 Adsorbent (Bio-Rad) clean up regimen in order to remove any unfolded or damaged proteins ad fluorophores which may non-specifically bind to the protein or lipid components of the system. After mixing and incubation with the Bio-Beads, the StAv-AF660 solution was passed through a 0.2 micron filter, to remove the Bio-Beads and further eliminate any unusable complexes. This final 2.5 µg/ml StAv-AF660 solution is further diluted to various concentrations throughout the experiments herein.

After the washing of the lipobeads systems following incubation with the associated protein solution, the proteolipobead systems containing APP and Notch-1 were then mixed and incubated with the StAv-AF660 fluorophore solution in order to label the proteins which have been reconstituted into the lipid bilayer. The SB4 and NTM2 substrates obtained from the Li lab have an intrinsic biotinylation site developed \textit{in vivo}, through genetic modification. This exposed biotin molecule is used to bind the conjugated StAv-AF660 and thus label the protein within lipid bilayer in order to track it through CLSM.

Membrane fragments of HEK293 cells containing γ-secretase with the subunit nicastrin-pHuji red fluorescent protein (RFP) variant as a C-terminal fusion protein cells were solubilized with 1% CHAPSO[109]. The solubilized membrane fragments were mixed with the DiO-doped 2:2:1 DOPC/Sphingomyelin/Cholesterol liposomes followed by dilution to give less than 0.01% final CHAPSO concentration. Centrifugation was used to remove any aggregates to give a clear
solution of DIO-doped γ-secretase proteolipomes incorporating the subunit nicastrin-pHuji red fluorescent protein (RFP). These proteoliposomes were fused with 5 μm nominal size silica microspheres (Bangs Laboratories) for 30 min at a ratio of greater than 10:1 lipid bilayer area to total microsphere surface area, followed by four wash steps to remove excess proteoliposomes or any γ-secretase containing membrane fragments or other debris.

III. Results and Discussion

Initial tests were performed with γ-secretase substrates SB4 and NTM2, prior to experimenting with the larger multi subunit protein γ-secretase. Figure 3.1 shows the overlaid images of the phase separated SM:DOPC:cholesterol lipid bilayer and the StAv-AF660 labeled proteins. Using this approach it is relatively straightforward to see the colocalization of the substrates and the lipid phases. In the samples analyzed within this work, the intensity ratio was evaluated with respect to the ordered phase. We first form Lα/Ld phase separated lipobeads and then conduct direct membrane protein insertion, followed by localization with StAv-AF660 (yellow). Then confocal fluorescence microscopy was conducted of substrates loaded into phase separated PLBs with Lα/Ld microdomain forming SM:DOPC:cholesterol (2:2:1) composition. Figure 3.1 shows the results of representative confocal laser scanning microscopy (CLSM) 3D reconstructions. Shown in panels 3.1 A-F are 3D hemispherical projections of a representative PLBs where the extent of the Ld phase is indicated by DiI (Acceptor) FRET (blue: Panels 3.1A and 3.1D). The biotinylated labelled substrates SB4 and NTM2 are localized with StAv-AF660(yellow), excited by a 633 nm laser line to eliminate crosstalk, displayed in panels 3.1B and 3.1E respectively. The third column, panels 3.1C and 3.1F, were obtained by merging the first two columns. We obtained the apparent partition coefficient of the protein (K_{p,app}) by closer examination of signal intensities of the Lα/Ld phase partitioning in a random sampling of PLBs of
each kind (n=10). Using this data, the SB4 substrate partition coefficient $K_{p,app}$ is 0.55±0.03, indicating strong preferential partitioning to the DOPC-rich $L_d$ phase (based on SB4:StreptavidinAlexaFluor660 phase partitioning). In contrast, the non-amyloidgenic Notch NTM2 substrate shown in the panel shows major loading into both the $L_d$ and $L_o$ phases, Notch NTM2 substrate partition coefficient $K_{p,app}$ is 1.03±0.08, indicating only slight preferential partitioning to the DOPC-rich $L_d$ phase (based on Notch-NTM2:StreptavidinAlexaFluor660 phase partitioning). For reference, a $K_{p,app}$ value of 1 indicates no preference for either phase. The differences between $L_o/L_d$ $K_{p,app}$ were statistically significant in each sample (p < 0.05). After analyzing this data, it shows that the NTM2 substrate has no distinct preference for partitioning into either phase of the lipid bilayer while the SB4 substrate shows a minor yet significant preference for the $L_d$ phase. This preference of the studies of an APP-based substrate is similar to that seen in previous GUV studies performed by Schlebach et al.[110]
Figure 3.1 Colocalization of TMPs and L\textsubscript{d} phase highlighted lipid membrane. A&D) Phase separated SM:DOPC lipid bilayers shown here fused to a microbead. B&E) SB4 and NTM2 substrates shown here are reconstituted into the lipid bilayer and then fluorescently labeled with a StAv-AF660 molecule to bind to any exposed/outward facing biotin site. C&F) a merge of the 2 channels to show colocalization of SB4 in the L\textsubscript{d} phase, and no apparent partitioning preference for the NTM2 molecule.

During the implementation of γ-secretase supported biomembrane systems, the Li lab developed a new modified version of the γ-secretase complex which is expressed containing a pH dependent red fluorescent protein (RFP). Specifically, the enzyme complex contains Nicastrin-pHuji red fluorescent protein (RFP) variant as a C-terminal fusion protein. These constructs were developed in a cell modified to show significant upregulation and over expression of γ-secretase. A benefit of RFP is that it is a highly optimized fluorophore for use in the STED super resolution modality. Figure 3.2 displays STED and CLSM microscopy of a representative HEK293 cell over expressing γ-secretase labeled with RFP (as pHuji). The 3D reconstruction displays a representative HEK293 cell that contains overexpressed γ-secretase visualized due to
incorporation of the subunit nicastrin-pHuji red fluorescent protein (RFP) variant as a C-terminal fusion protein. Optical sectioning of the 3D cell was used to isolate the plasma membrane localization of γ-secretase, shown in the insets. A comparison of the same z section in CLSM and STED shows the significant resolution increase obtained. The diffusivity of the γ-secretase protein in the plasma membrane was obtained through FRAP and determined to be 0.025 μm²/sec (as shown in figure 3.3) with a mobile fraction of ~79%. To our knowledge, these studies constitute the first diffusivity measurements of γ-secretase. The γ-secretase from these cells was then extracted from membrane fragments in 1% CHAPSO and proteoliposomes were formed. The 2:2:1 DOPC/Sphingomyelin/Cholesterol lipid formulation doped with DiO tracer was used to form proteoliposomes. PLBs were formed via proteoliposome fusion and the assemblies were characterized with CLSM and confocal-FRAP. Figure 3.4 displays a representative PLB with the supported lipid bilayer imaged via DiO (green) and the γ-secretase localized via RFP-pHuji (red). Highly homogenous supported bilayers were formed. In this case, no significant signs of phase separation were evidenced under these conditions, presumably due to the complex mixture of MPs and endogenous lipids that accompany the CHAPSO extraction and proteoliposome construction. Figure 3.5 displays a comparison of DiO and RFP FRAP performed on the supported bilayers formed on silica microspheres as PLBs. The red data points and fit (right) are from the γ-secretase FRAP and the green data points and fit are from the DiO lipid tracer. The γ-secretase signal yields an effective diffusivity of 0.017 um²/s with a mobile fraction of 80%, slightly lower than that obtained from the HEK293 cell. These studies constitute the first diffusivity measurements of γ-secretase in supported lipid bilayers. One aspect of this disparity between the live cell diffusivity and lipobead effective diffusivity is the unknown phase composition of the lipobead versus the live cell membrane, as shown in the previous chapter the
phase mobility is approximately two orders of magnitude different between the two predominant phases, Lo and Ld. Another aspect to consider is the effect of interactions between the protein and the solid support structure, this interaction is addressed later in this research. However, the lipid tracer DiO yields an effective diffusivity of 0.083 um²/s with a mobile fraction of ~80%, establishing that the extracted γ-secretase does in fact insert into the intact lipid bilayer of PLB constructs, which is well within the range of effective diffusivity from the previous chapter.

Figure 3.2 STED and CLSM microscopy of a HEK293 cell over expressing γ-secretase labeled with RFP (as pHuji). The 3D reconstruction displays a representative HEK293 cell that contains overexpressed γ-secretase visualized due to incorporation of the subunit nicastrin-pHuji red fluorescent protein (RFP) variant as a C-terminal fusion protein. Optical sectioning of the 3D cell to isolate the plasma membrane localization of γ-secretase is shown in the insets. A comparison of the same z section in CLSM and STED shows the significant resolution increase obtained.
Figure 3.3 FRAP analysis of in vivo γ-secretase within the HEK293 cells. This figure shows the relative FRAP of the pHuji RFP tagged γ-secretase complex examined within the plasma membrane of the HEK293 cell under observation. This analysis yields an effective diffusivity of ~0.025 μm²/s with a mobile fraction of 79%.

Figure 3.4 Extracted RFP-tagged γ-secretase reconstituted in PLBs. Reconstituted cell extracts from the HEK293 cell containing γ-secretase, obtained using the 1% CHAPSO cellular solubilization method typically used to purify the complex from mammalian cells to form proteoliposomes. The 2:2:1 DOPC/Sphingomyelin/Cholesterol lipid formulation doped with DiO tracer was used to form liposomes that were used to obtain PLBs via fusion, pictured above in 3D reconstructions from the CLSM. In this case, no significant signs of phase separation were evidenced under these conditions. Scale bar is 1μm.
Figure 3.5 Effective FRAP analysis on RFP and DiO in the HEK293 extract PLBs. A comparison of DiO and RFP FRAP performed on the cell extracts reconstituted on the silica microspheres. The red data points and fit is from the $\gamma$-secretase FRAP and the green data points and fit are from is the DiO lipid tracer. The $\gamma$-secretase signal yields an effective diffusivity of 0.017 $\mu$m$^2$/s with a mobile fraction of 80%, and the DiO yields an effective diffusivity of 0.083 $\mu$m$^2$/s with a mobile fraction of 80%.

IV. Conclusions

Shown here, protein direct insertion into the lipid bilayer is a viable and readily usable approach to studying isolated TMPs under controlled conditions. While these systems are not a direct measurement from a biological source, these platforms are comparable as they maintain mobility and activity, as seen in various assays performed by the Li lab. While it has been shown that overall diffusion is reduced in these supported bilayer systems, it is possible to account for the overall differences between cell and synthetic systems. This approach can also yield reliable and necessary values for future modelling work on these complex spatial reaction-diffusion protein systems.
The ubiquitous nature of the approach through all of the aforementioned studies leaves this as a highly modular and interchangeable system. This flexibility in design can allow for the study of any and all TMPs that can be successfully extracted from cells and reconstituted without significant loses. This approach to TMP study has the potential to revolutionize pharmaceutical discovery and trials by allowing for the direct testing of these proteins in controlled lipid environments with exact concentration measurements of both enzyme and substrate on a rugged platform. In this system, single protein pathways can be isolated and studied without the use of genetically modified and augmented cells, in an environment which maintains biological relevance while affording high levels of control and heterogeneity.

However, this process is not completely ubiquitous as a few minor changes to the lipid formulation, or protein studied can have a marked effect on the effectiveness of this exact approach. Each time a modification would be made, a new cycle of optimization would have to be attempted. In some cases this approach is a tedious downfall of the system, but overall these changes are not substantial or large changes, and overall the modifications needed to develop a new platform for a different TMP would include finding the proper fluorophore label and what concentration range works best for the given microenvironment. This optimization can be easily performed through the use of high throughput screening systems, for which these platforms are expressly being developed.

This work shows that reproducible, and mass produced, test platforms for TMPs can be created simply and reliably. These platforms are robust, and rugged enough to undergo varying forms of mechanical processing such as the washing, to eliminate unfused lipids and non-inserted or unfolded proteins, up to flow cytometry. The ability to produce and examine these samples in tandem on planar surface and microspheres allows for further characterization of
these structures and has advantages over other TMP testing platforms such as GUVs, solubilized protein mixtures, or cell culture studies. γ-secretase proteins and, SB4 and NTM2 substrates, are all shown to directly insert into the lipid bilayer and maintain key biological relevancy factors such as mobility and phase partitioning. These platforms also allow for the removal, via washing, of key non-biologically relevant substances, such as detergents, which have unknown direct effects on these proteins and their structures and interactions.

V. Tables

Table 3.1 Additional Materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Atomic Structure</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB4</td>
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<td>APP analogue, contains a biotinylation and FLAG antibody site</td>
</tr>
<tr>
<td></td>
<td>Cholesterol Binding site</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ:A40 cleavage site</td>
<td></td>
</tr>
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<td></td>
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</tr>
<tr>
<td>NTM2</td>
<td><img src="image" alt="NTM2 Molecule" /></td>
<td>Notch-1 analogue, contains a biotinylation site</td>
</tr>
<tr>
<td></td>
<td>Cleavage sites</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S3, S4</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 4. Tethering modalities

I. Introduction

In this chapter, the focus is on the use polymer moieties to produce complex yet elegant tethering systems. Tethering is a process by which a solid support structure is chemically augmented to both minimize surface interactions between the support and the system it is supporting, in this case a lipid bilayer, and provide additional functionality to the solid support. It is posited in this work, that functional tethers can be developed and implemented on solid silicon dioxide surface for use with a canonical brain cell-analogous lipid structure. The two functionalities approached in this work was a tethering modality which fundamentally provided the lipid bilayer with numerous anchor points to facilitate a pseudo-binding between the glass surface and the bilayer, and a modality which could preferentially sequester proteins which express a key binding domain towards the silicon dioxide surface as opposed to the expressing this domain outward away from the support. These two orientations of the protein are facilitated by the direct insertion method used in studies explained in an earlier chapter.

II. Materials and Methods

Glass slides, coverslips, and silicon dioxide chips were cleaned overnight in Piranha solution, the supports were then washed thoroughly with DI water as stated above. (3-Aminopropyl)trimethoxysilane (APTMS) was then mixed to varying concentrations with DI water, toluene, and ethanol. In this study the mixtures all contained 5% APTMS v/v within the three solvents. The glass and silicon dioxide surfaces were then immersed in these solutions in order to develop a deposited layer of the APTMS solution in a process known as silanization for a total of 30 minutes. One test was performed by placing the clean glass supports in a vacuum
chamber with pure APTMS in an open container, the chamber was then placed under a slight vacuum, just enough to completely seal the vessel, and let to sit for 30 minutes. This process subsequently leaves a chemically attached amine group on the surface of the solid support. The amine is then used to undergo a series of amine to N-hydroxysuccinimide (NHS) dehydration reactions in order to develop a step by step layering of tethering polymers[111].

Specifically used in these studies were varying chain lengths and shapes of poly(ethylene glycol) (PEG) polymers purchased from Nanocs, Inc. Also various NHS linkers and conjugates were used throughout the studies. After the silanization step, the supports were then treated with either NHS-PEG₄-Biotin, NHS-PEG₂₀₀₀₀-DSPE (DSPE: 1,2-disteroyl-sn-glycero-3-posphoethanolamine), both at 0.5 mg/ml in DI water, or a solution of a homo-bifunctional crosslinker bis(sulfosuccinimidyl)suberate (BS3), at 0.2 mg/ml in DI water. The BS3 supports were then further treated with a solution contained Amine terminated PEG chains of varying molecular weights and shapes, at a consistent concentration of 1 mg/ml in DI water. The most predominant linker used in these studies was the 4-arm star PEG polymer at a molecular weight of 20,000 Daltons (4armPEG₂₀₀₀₀-NH₂). This star polymer was then capped with one of the previously stated capping agents.

Biotin and DSPE were chosen to be the tether ends due to their desired functionality within the systems studied. Biotin is a molecule which binds strongly and quickly to the molecule Avidin, named due to this high avidity relationship. In these studies, Streptavidin was used as the binder to the adhered biotin molecules. Streptavidin was chosen due to its innate resistance to detergents, which are used primarily in future process described herein. Streptavidin conjugates are commercially available, this affords the ability to label and visually inspect the degree of tethering, and allows for additional use of the streptavidin-biotin affinity as
the streptavidin molecule contains 4 biotin binding domains per molecule. The DPSE capped tether is used for direct insertion into the lipid membrane. During tests in which small unilamellar vesicles (SUVs) are fused to the surface of these supports, the DPSE lipid can insert itself into the lipid bilayer facilitating and promoting vesicle fusion and anchor points to attach the bilayer tenuously to the solid support. Figure 4.1 shows the effective modular linking process and final product schematics.

**Figure 4.1 Schematic of effective tethering linkages.** This figure shows the effective modular tethering support structure, the far left image depicts the base tethering structure of silination of the glass surface leaving amine residues followed by a small linking molecule to which a large polymeric moiety is attached. The middle schematic depicts the capping of the previous tether with the lipid anchoring functional end piece, while the picture to the right depicts the Biotin expressing functional cap which is then further utilized through the introduction of the streptavidin molecule.

Using this system of NHS-Amine ester linkages, allows for the development of a modular tethering system, similar to building blocks. At each consecutive step molecules can be introduced, deleted, or augmented in order to develop a more apt tethering moiety for the
designed purpose. This linkage can also be carried out in water without the help of any ancillary chemicals to add complexity and potential harm to more delicate platforms, such as biological systems. Tests were performed using various different tethering moieties with this modular technique to develop various platforms. 4 arm PEG\textsubscript{20000}-amine was the predominant second tier linker, but other amine-terminated PEG polymers were used throughout this study ranging from straight chain polymers, to 8 arm star polymers, from 2kDa up to 20kDa.

III. Results and Discussion

Throughout these studies, the most important concept for the use of tethering was the development of a polymeric cushion on the surface of these solid supports. Untreated glass and silica beads can have varying degrees of roughness on the nano and micron scale, and this roughness has the potential of creating diffusion “wells” and barriers which prevent proteins and other molecules from properly moving throughout the lipid bilayer, and can even perpetuate the coalescence of domains artificially within the bilayer. As shown in previous work from this group, the use of polymer cushions can have a significant effect on the overall diffusivity of lipids and proteins within the supported lipid bilayer. Specifically in the experiments shown here, the biotin tag and the DSPE cap were used as added functionalization on top of the development of the polymer cushion.

The biotin capped samples were the first attempted tethers in this study. It was the original goal of this work to develop a system in which non-biologically relevant protein orientations could be sequestered and ultimately removed from the PLB and planar surface testing of these TMPs. Proteins inherently have a specific orientation within the cell, which is controlled \textit{in vivo} during protein transcription, with the extensive use of chaperone proteins and directional transcription, proteins maintain their specified orientation within the framework of a
cell. However, in these studies the proteins are reconstituted into synthetic membranes through the process of direct insertion. This method does not yield a constant or specific orientation to most of the proteins inserted into the lipid bilayer. This inevitably means, in any direct insertion of proteins into a supported bilayer, that the resulting biomimetic membrane contains a mixture of biologically relevant protein orientations as well as the opposite orientation.

The SB4 and NTM2 substrates used in later tests, developed by collaborators in Dr. Yueming Li’s lab at Memorial Sloan-Kettering Cancer Center, contain a built in biotinylation site which natively adds a biotin molecule onto the N-terminal side of the protein. The APP molecule also contains a FLAG antibody binding domain on the C-terminal side of the transmembrane helix. This directionality in design was the target of the biotin capped tether studies, due to the ability of the streptavidin molecule to bind multiple biotin molecules. Figure 4.2 shows the effective development of a tethered glass surface coated with a layer of streptavidin which had been conjugated with Alexafluor 660, a red fluorescent molecule. This planar surface was developed by using an APTMS->BS3->4armPEG20000-NH2->NHS-PEG4-Biotin modular structure, to which a biomimetic lipid bilayer was fused and lipid coated silica microspheres were then introduced. This proof of concept shows that it is possible to develop systems containing both planar supported bilayers and lipobeads in tandem as well as the ability to label one surface with the biotin capped tether while allowing for the secondary structure, in this case the microbead, to contain different functional tether, such as the DSPE capped tether. This platform could allow for the sequestration of the non-biologically relevant oriented protein by effectively capturing the N-terminal exposed biotin and eliminating the potential for lateral diffusion throughout the system. In this scenario, those proteins with biological orientation maintain the ability for lateral diffusion throughout the lipid bilayer and support structure.
Figure 4.2 Validation of the Biotin capped tethering modality. This figure is an Amira reconfigured 3D image taken from a sample in which the glass surface was treated and the biotin expressing tethering moiety was developed. A) The isolated red signal shows the coverage of the glass surface with a streptavidin molecule which had been conjugated with an Alexafluor 660 fluorophore. B) The greenish-blue section is a SM/DOPC/cholesterol lipid bilayer containing a lipophilic tracer (DiO) which was fused onto both the planar bilayer support as well as microbeads visible in this image.

The use of tandem planar supported bilayers and lipobead constructs have the potential to isolate the proper protein orientation, thus that the improper orientation will diffuse and then become pinned on the support containing the streptavidin molecules, and when allowed to reach an equilibrium should provide that one support structure contains predominantly relevant proteins while the secondary structures contains the oppositely oriented proteins. The lipobeads supports can then be removed from the solid support thus yielding either a planar support or lipobead system containing the proteins of interest, depending on the further application of the platforms.

The second approach to tethering came through the DSPE capped tethers in an attempt to create a more rugged and robust platform in order to further the possible use in high-throughput techniques. The addition of the DSPE cap onto the polymer cushion using NHS-PEG2000-DSPE would allow for the lipid bilayer to essentially contain an anchor point to the solid support structure. In early tests performed on this work, it was seen that the lipid bilayer, when fused directly to the solid support structures, was very tenuously adhered to the support structure. If
samples underwent varying forms of stress outside of delicate handling techniques, the result could be that the lipid bilayer could wholly or partially slough off of the surface of the support. With DSPE lipid inserting itself into the bilayer during the fusion process, and the DPSE molecule being chemically attached to the solid support structure, this tethering structure has the capacity to relieve stresses on the bilayer by transferring the stresses into the PEG cushion and further onto the solid support while maintaining overall mobility of the lipid bilayer.

A major difficulty of this approach is that there is no visual marker of this tethering moiety unlike the tethers using the Alexafluor conjugated to streptavidin, making this DSPE capped structure unidentifiable under CLSM. In order to inspect this tethering moiety AFM was used to gather information on the presence of the tethered structure. Two approaches were taken with the AFM, a puncture test using the probe tip to depress through the lipid bilayer and the polymer cushion in order to deflect against the solid support beneath, and a tapping imaging mode in order to view the intact membrane on the surface of the support. The puncture test seen in figures 4.3 and 4.4, yield distinctive patterns when a lipid bilayer and polymer cushion is present on the surface of the planar support structure, as described by Alessandrini et al[112]. There is a constant, linear force exerted on the tip as it passes through the PBS buffer. Then, close to the support there is a small upward tick in the deflection of the probe tip, followed by a small window of undulation, culminating in the linear increase of deflection indicating that the solid surface has been reached. The brief increase in deflection is the tip coming into contact with the lipid bilayer, and the undulation following is the tip effectively moving through the bilayer and the polymer tether. The distance of the initial deflection to the steep deflection caused by the solid support and the force necessary to break through the bilayer are the important characteristics of this test. The distance between the “infinite force” line and the initial increase
in deflection yields the relative height and thickness of the bilayer and polymer cushion together, understanding the lipid composition of the bilayer, and estimated thickness can be assumed and thus any extraneous distance between initial deflection and the solid support is the relative thickness of the polymer cushion. In the figures below, the effective tether and bilayer thicknesses are calculated to be 64.1 ±10.2 nm for the 4-arm PEG 20K sample and 5.6 ±1.6 nm for the PEG_{2000}-DSPE tether which is comparable to the results obtained by Hertrich et al [113]. In this PEG_{2000} case, the thickness is larger than the Flory radius (3.5 nm). The force obtained from this deflection is ultimately the force required to puncture the lipid bilayer fused onto the support. It is suggested with this information that a chemically deposited PEG tethering support structure was created on the surface of the silica microscope slides.

![Figure 4.3 Overlay of indentation tests performed on glass microscope slides containing a 4-arm PEG_{20000} star polymer tether connected to a SM:DOPC:cholesterol lipid bilayer.](image)

Figure 4.3 Overlay of indentation tests performed on glass microscope slides containing a 4-arm PEG_{20000} star polymer tether connected to a SM:DOPC:cholesterol lipid bilayer. Indentation tests were performed on a system similar to that shown in figure 4.1 as the lipid tether containing the large 4-arm PEG_{20000}-NH_{2} star molecule. Analysis shows that the separation distance from the average breakthrough height to the glass surface is approximately 64 nm.
Figure 4.4 Overlay of indentation tests performed on glass microscope slides containing a single PEG$_{2000}$-DSPE polymer tether connected to a SM:DOPC:cholesterol lipid bilayer. Indentation studied performed on a system containing only one polymer tether building block, the NHS-Peg$_{2000}$-DSPE only. These indentation studies show that the average distance between the membrane and the solid planar support is approximately 5.6 nm.

Using the tapping imaging mode of the AFM, it is possible to verify the presence of a confluent lipid bilayer. This imaging method converts the received deflection information from the AFM probe into a height map of a scanned area. This height map can then be viewed as an image of the surface being scanned by the probe tip, using relative height differences of the scanned area it is possible to discern systems of phase separation and individual protein molecules imbedded in the lipid bilayer. Figure 4.5 shows a 3D reconstruction of the tapping mode height readout on a glass microscope slide. It is visible here that the surface roughness of microscope slides has a negative impact on the smoothness of the lipid bilayer, even with a moderate level of tethering. Phase separation is impossible to determine under these conditions, while it is readily apparent on the Ultra-flat thermal oxide wafers. Coupled with the puncture test data, a confluent lipid bilayer can be shown with a given height away from the solid support structuring, giving ample evidence of a tethered lipid bilayer system. The tapping imaging mode
of the AFM is used in future studies found here to discern protein partitioning and phase separation on planar systems, in order to gain a two-fold approach to understanding the complex spatial and diffusive system of γ-secretase and its substrates.

Figure 4.5 3D reconstruction of a tapping mode AFM scan of a glass microscope slide containing a 4-arm PEG20000 tether connected to a SM:DOPC:cholesterol lipid bilayer. A 3D reconstruction of a sample on a silica microscope slide treated with a tethering construct similar to that seen in figure 4.1 the lipid tether containing the large 4-arm PEG spacer. Microscope slides show an incredibly large level of roughness on the AFM scale, and at this level simple tethering technique do little to help minimize the overall roughness of the support.

The ultimate goal of this project is to embed γ-secretase and it substrates into these systems. Figure 4.6 shows AFM data from the 37°C reconstitution of γ-secretase into preformed sphingomyelin:DOPC:cholesterol (2:2:1) supported bilayers with polymer cushion tethering using 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-PEG2000-succinimidyl ester (NHS-PEG2000-DSPE). Tapping mode image of a PEG2000-DSPE tether supported phase separated lipid bilayer with inserted γ-secretase on a thermally oxidized silicon wafer. Panel A is AFM tapping mode image obtained from imaging a tether supported SM/DOPC/cholesterol lipid bilayer, with inserted γ-secretase proteins. The liquid ordered and disordered phases of the lipid bilayer can be seen here in the two distinct light and dark brown regions. The single star (*) here denotes γ-
secretase in the Lo phase, while the double star (**) is an indications of a γ-secretase molecule suspended in the Ld phase. Panel B is a set of height profiles of features consistent with single γ-secretase complexes embedded in the tethered bilayer, including the ones indicated with * and ** in panel A. A Nicastrin molecular volume of 147 nm$^3$ was obtained using the polymer size versus chain length arguments made by Saslowsky et al and the feature size obtained here is 164±23 nm$^3$, in line with the expected protruding volume of the Nicastrin domain [114]. The incorporation levels are lower than desired, however, we will further tune the conditions in future experiments.
Figure 4.6 Tapping mode image of a PEG\textsubscript{2000}-DSPE tether supported phase separated lipid bilayer with inserted γ-secretase on a thermally oxidized silicon wafer. Panel A is AFM tapping mode image obtained from imaging a tether supported SM/DOPC/cholesterol lipid bilayer, with inserted γ-secretase proteins. The liquid ordered and disordered phases of the lipid bilayer can be seen here in the two distinct light and dark brown regions. The single star (*) here denotes γ-secretase in the L\textsubscript{o} phase, while the double star (**) is an indication of a γ-secretase molecule suspended in the L\textsubscript{d} phase. Panel B is a set of height profiles of features consistent with single γ-secretase complexes embedded in the tethered bilayer, including the ones indicated with * and ** in panel A. This analysis yields a feature size of 164±23 nm\textsuperscript{3} which is in line with the expected volume of a protruding Nicastrin domain of 147 nm\textsuperscript{3}. 
IV. Conclusions

Tethering is a viable, and in some cases necessary, approach to supporting biologically relevant synthetic systems. Tethers promote a more fluid and biologically equivalent system by helping to negate any artificial affects caused by the solid support, which are not present in cell systems. Effects ranging from roughness to electrostatic interactions to pinning of a protein containing non-negligible intra or extra cellular domains. Tethering systems also afford the ability to functionalize the solid support while not seriously affecting the fluidity or rigidity of the synthetic platform.

It is shown from this work that it is possible to develop tethered support systems using straightforward and simple NHS-Amine crosslinker chemistries, which yield highly modular and adaptive moieties. The ends of these moieties can be functionalized to promote various effects deemed desirable to the application of these systems. Shown in this study are the effective labelling of the tether moieties, and potential use for protein sequestration, and the process by which cushion thickness can be obtained in order to verify the presence of the tethering moiety.

This work lays the foundation for further exploration into the usefulness and potential functionalization for tethered surfaces. In future studies discussed within, AFM is performed on two distinct platforms, mica and the glass surfaces described above. These two platforms are vastly different in both charge and roughness which can lead to markedly different systemic results and these disparities can be effectively eliminated through the use of tethering. Mica is atomically flat, but contains a high charge density, while the glass surfaces have significantly higher roughness but with an overall lower charge density. The PEG cushion can dampen the effects of the surface charge interactions of the mica by creating spatial separation between the solid support and the lipid bilayer through which these electrostatic interactions cannot span.
The cushion can also minimize the effects of surface roughness on the glass supports by essentially filling in the “wells” and normalizing the surface to be a uniform roughness. Developing a complimentary tethered system using both supports could allow the qualification of using planar glass supports in AFM studies, since currently mica platforms are preferred in these studies due to their flatness. Use of planar glass supports allows for the further edification of an in tandem approach of using AFM on planar surfaces to characterize identically treated microbead systems, which offers increased capabilities in terms of high throughput techniques and other important biological assays.
Chapter 5. Conclusions and Future work

Throughout this process, highly specialized and biological lipid biomes were researched and emulated in order to produce a synthetic, homogeneous, controllable platform on which to perform TMP assays for potential use in high-throughput screening and study techniques important to the pharmaceutical industry. TMPs are one of the more difficult family of proteins to study and for the development drug modifications, due to the necessity of these proteins to remain within a confluent lipid bilayer so as to not denature and lose functionality. Current techniques available for the testing of TMPs on a large scale are not optimized and in some cases not possible to be utilized in high-throughput fashion. Cell culture studies are difficult to control, even with techniques such as genetic modification, and in most cases are intensely resource heavy to facilitate. Other intact biomembranes approaches such as GUV, proteoliposomes, or GPMVs, do not have the specificity or ruggedness to be used in a high-throughput format or in flow cytometry. The approaches shown in this work afford a relatively simple and effective option containing important factors from these other techniques.

Tethering systems are not a predominantly used technique in the field of TMP and lipid systems research, due to the fact that most test platforms do not utilize the solid support structures on which this research relies. These solid support structures, microbead and planar systems, while a readily viable platform for use in these protein studies as shown, can be augmented to more closely resemble and match key characteristics of the biological environment. In previous work it was shown that tethering large PEG moieties onto silica surfaces facilitated an environment with a higher overall diffusivity, the work performed herein shows that similar tethering moieties can be developed in a modular, building block like approach in order to help eliminate surface interactions of the lipid and protein with the solid
support, as well as introduce functional molecules onto the ends of these polymer cushion to increase overall assay and platform performance.

SM:DOPC:cholesterol lipid systems have been widely used as a brain cell analogue in various biological and protein studies throughout the past decade. This system yields a key advantage for studying systems such as γ-secretase and its substrates, where under specific conditions this lipid formulation forms two distinct semi-miscible phases, liquid ordered and disordered. This phenomenon is highly desirable as it has been shown that effective cholesterol levels in cells has an effect on the cleavage efficiency and specificity of proteins like γ-secretase. Using this lipid formulation, the work described shows that not only can a confluent lipid bilayer be deposited on various solid structures, but also that the effective phase separation can be visually studied and quantified. This aspect of the SM:DOPC:cholesterol lipid formulation is indispensable in the study of TMPs as each phase has substantial differences which can have marked effects on protein performance as a function of lipid microenvironment. Understanding key factors such as preferential partitioning of proteins, diffusion speed, and activity within given environments can readily be obtained with the systems developed. In addition to the SM:DOPC:cholesterol lipid formulation, it is shown that this canonical analogue can be modified with more complex lipid structures and cell extracts, yet still maintain this visible and quantifiable phase separation. This has led to the development of systems which are much closer to biological relevancy in terms of overall lipid environment and yield important spatial information about the lipid microenvironment and eventually proteins.

Direct protein insertion into a confluent lipid bilayer with the help of specialized detergents, namely CHAPSO, is a straightforward a reproducible process on both the planar supported bilayer and the lipobeads constructs. The process described in this work affirms the
ability to directly insert γ-secretase protein, SB4 and NTM2 substrates into a canonical SM:DOPC:cholesterol lipid bilayer. The insertion of these proteins into the lipobead and planar supported bilayer systems allows for the study of apparent phase partitioning of the proteins, protein localization, diffusivity, characterization of enzyme and substrate concentrations for elucidation of enzyme kinetics, and flow cytometry or other high through-put assays. This work was completed on γ-secretase and two of its substrates; however, this process is not restrictive to these three proteins. The procedure and treatment of the proteins in order to insert them into the lipid bilayer was not augmented between the three significantly different proteins. The fact that this procedure is unchanged between tests is a positive sign for the possibility of using these formulations and this detergent for a wide range of TMPs and other biological moieties which require a confluent bilayer in which to be studied in a relevant manner.

The experiments performed herein developed a malleable platform on which to perform this protein analysis on γ-secretase and its substrates. It was approached in a way to initially test if this process of lipid fusion of the SM:DOPC:cholesterol was applicable to silica microbeads and planar bilayers, followed by the introduction of proteins into the platform. Upon determining that this basic approach was valid, the optimization and bilayer changes then became a focus. For this reason, the more complex, doped lipid systems are in early stages of development and study to fully understand their usefulness and applicability to the tests described. This however is the first work performed to our knowledge of the development of proteolipobead systems with phase separation and lipid configurations with high levels of biological relevance, for the use in studying γ-secretase and two of its substrates. The basic system of SM:DOPC:cholesterol displays the ability to reconstitute mobile and active purified protein extracts on a platform, shown in previous studies, designed for use in a multitude of high-
throughput screening studies used heavily in the pharmaceutical industry. This simplified systemic approach is bolstered through the ability to be highly modular along every step of the process. Each step of this work is built for flexibility, from the building block aspect of the tethering support structures, to the canonical brain cell analogue, which still retains its beneficial qualities, doped with various more complex lipid systems used to model assorted biologically relevant lipidomes, and finally to the direct insertion of the \( \gamma \)-secretase and substrates into the lipid bilayer, a process which is not specific to the proteins studies and is an ubiquitous approach to TMP insertion into membranes. The tandem approach of studying these systems under both CLSM and AFM, while still in its infancy, is a highly effective tool in understanding and characterizing the lipid environment and understanding the details and dynamics of the system on the nanoscale.

Going forward, these systems do require further study and understanding before they are deployable test beds for an array of protein testing. The research here lays the groundwork required for the development of model and modular systems, though many more building blocks are necessary to develop a full-fledged analytical high-throughput testing modality.

Firstly, on the topic of tethering, it would be important to understand the effectiveness of tethering on solid supports outside of the silica glass based supports tested here. A litany of materials are currently used in biological studies, each could afford a new and unique approach to developing tethering moieties with high functionalization, but specifically for this work understanding and eliminated the differences between mica and silica surfaces for AFM use should minimize the discrepancies seen between the two modalities. In line with the further tethering studies, optimization of the tethering procedure for use on the microbead systems is required, all studies in this work were performed on planar silica glass surfaces and all
microbead studies did not yield highly reproducible lipobeads constructs. It would be pertinent to know the effect these tethering moieties have on these specific proteins in terms of the PLB constructs. The testing performed in this work primarily focused on the idea of sequestering the non-biologically relevant protein orientations in an attempt to eliminate them from any further experimental steps and was not continued through the entirety of the research reaching to the protein insertion steps. Similar tethering systems have been studied previously in this lab showing that this tethering did not heavily disrupt the protein insertion procedure, so this was considered a non-issue throughout this work.

Secondly, on the topic of confluent lipid bilayers and fusion techniques and fluorescent labelling. This work touches on the small window of potential modifications to the canonical SM:DOPC:cholesterol system, dopants such as BPLE, DSPC, and DPPC studied here are minor changes to the core system, however they still build platforms which are arguably very different from the lipid micro/nano environment these proteins experience within cell membranes. Continual study of relevant cellular lipidomes is necessary to build controllable, simplified, yet still relevant biological systems that maintain protein function and are highly relatable to the cell functionality. In this work, the DiO/DiI FRET method was used to understand phase separation, lipid mobility, and coverage of the solid supports. This method does have its disadvantages, which became apparent throughout this work particularly in that these molecules are a predominantly single phase label, these molecules are not STED applicable fluorophores, and they take up a large window of the available detector bandwidth. A complimentary fluorophore to label the L₀ phase would further validate the work here and help elucidate the two phase system. While Airyscan is an effective and highly functional super resolution method, having the ability to validate the phases on two separate systems is beneficial. The combination of the
FRET pairing required a detector imaging window from 500-600nm, this window is in the very center of the available spectral space. The proteins tested in this work were all labeled with the binding of a conjugated streptavidin to a biotin molecule, this process is straightforward, but as shown can contain variance. Other labeling systems can be studied, including but not limited to antibody labeling, genetic modification to include fluorescent proteins, and direct labelling prior to protein insertion.

Finally, on the topic of protein insertion and assays, the protein work completed in this research revolved around use in the canonical SM:DOPC:cholesterol lipid formulation, as this was the best studied and most optimized formulation throughout the entirety of this work. This lipid formulation while robust and effective, is still only a ternary or pseudo-ternary system compared to a biological system with upwards of 10,000 varying components. Testing of these proteins in the doped samples developed in this work could be useful in elucidating whether these more complex systems will be more effective in understanding the protein functionality in cell systems and verify a correlation between these developed synthetic systems and the cellular environments in which these proteins reside. Also suggested would be the testing of these proteins on more tethered surfaces, both in complexity and in structure. Furthermore, testing of this direct insertion method using the CHAPSO detergent on other more variable protein will determine the extent of use. \(\gamma\)-secretase is a multi-subunit with 19 helical passes through the lipid bilayer, while both APP and Notch-1 are comprised of a single helical pass through the bilayer with both intracellular and extracellular extremities. This range in proteins tested does suggest that a large variety of TMPs would follow the same pattern of facilitated insertion into the lipobead systems, however variations in size of the transmembrane portion, large or highly charged extra/intra cellular domains, and varying secondary/tertiary/quaternary structures of
TMPs may impose further difficulties on this procedure. In the case of studying a wider range of proteins, this aspect is closely tied with the further development and optimization of a wider variety of synthetic membrane analogues. Together the eventual goal of this work is to build a catalog of proteins and lipid membrane analogs which can be selected and prepared readily to closely mimic a highly reproducible biologically relevant environment through close control and synthetic means.
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