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| Title        | Studies on Tetrahedral Diagram Analysis on Interfacial Properties of the Quaternary Lipid System Related to the Brain Cell Membrane |
| Author(s)    | Ajaikumar, Arun                                                                                                                     |
| Citation     | 大阪大学, 2026, 博士論文                                                                                                                    |
| Version Type | VoR                                                                                                                                 |
| URL          | <a href="https://doi.org/10.18910/105518">https://doi.org/10.18910/105518</a>                                                       |
| rights       |                                                                                                                                     |
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**Studies on Tetrahedral Diagram Analysis on Interfacial  
Properties of the Quaternary Lipid System Related to the  
Brain Cell Membrane**

**ARUN AJAIKUMAR**

**MARCH 2026**

**Studies on Tetrahedral Diagram Analysis on Interfacial  
Properties of the Quaternary Lipid System Related to the  
Brain Cell Membrane**

**A dissertation submitted to**

**THE GRADUATE SCHOOL OF ENGINEERING SCIENCE**

**THE UNIVERSITY OF OSAKA**

**in partial fulfillment of the requirements for the degree of**

**DOCTOR OF PHILOSOPHY IN ENGINEERING**

**By**

**ARUN AJAIKUMAR**

**MARCH 2026**

## Preface

This dissertation work was conducted under the supervision of Professor Dr. Hiroshi Umakoshi in the Division of Chemical Engineering, Graduate School of Engineering Science, at The University of Osaka from 2021 to 2026.

This thesis has the objective to characterize the impact that lipid bilayer composition has on the various properties of the membrane such as membrane polarity, fluidity and phase state. Lipids present or related to those in the brain membrane were prioritized for potential future research and applications to the biomedical field. First, bilayer composition complexity was incrementally increased to analyze any relevant impact on membrane properties. Next, this work aimed to further expand methodology in the characterization of more complex membrane compositions via tetrahedron diagram. Finally, Membrane composition was examined to determine any link to brain pathology.

Ultimately, the author hopes that this work will serve the scientific and pharmaceutical community as fundamental work for further characterization of the brain lipid membrane and assist with building the groundwork for brain pathology therapeutics that focus on the membrane. Insight obtained in this work is expected to contribute to the further development of analytical methods for the study of complex lipid membranes.

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## **Abstract**

Various membrane properties are impacted by the specific composition of the lipid bilayer, and these properties are worth analyzing for their connection and implication in different pathologies. Particularly, the brain is an integral part of the central nervous system and thereby changes to any aspect of its anatomy, including lipid membrane properties, has the possibility of causing systemwide consequences. Liposomes are commonly used as a biomimetic environment that allows research to focus exclusively on the lipid membrane with minimal other molecular interference, thus liposomes made from brain lipids or their analogs were the primary platform of analysis in this work.

In chapter 1, a general introduction was provided about the relevance of lipid bilayer properties and its significant connection to the brain. An overview of the brain lipid membrane and relevant pathologies was discussed. Analytical techniques used in this thesis were introduced and their benefits were further discussed. Assessment on the limitations in current tools for interpretation and analysis of complex membrane properties was discussed. Finally, the overarching philosophy of this work was briefly introduced.

In chapter 2, emphasis was placed on how inter-lipid interactions with different ratios of lipids in the bilayer may impact the overall properties and functionality of a simple unary or binary membrane system. Brain sphingomyelin (bSM) and cholesterol (Chol) of different ratios were made into liposomes, and their polarity and fluidity were analyzed. Fluorescence spectroscopy and deconvolution analysis were both used for characterization and revealed a high possibility of heterogeneity in liposomes with 30% or more Chol content. Interactions that possibly act as precursors to lipid raft phenomena were briefly discussed.

In chapter 3, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) lipids were introduced as brain lipid analogs for further adding complex variation to liposome compositions. Liposomes of ternary lipid compositions of varying ratios were constructed and a ternary phase diagram for characterizing fluorescence spectroscopy results was briefly introduced. Both the significance of the properties of these ternary membrane systems, particularly PE systems, and the necessity of expanding the concept of ternary phase diagrams was discussed and a 2D “flattened tetrahedron phase diagram” was introduced as a tool for comparing multiple ternary phase diagrams with correlated lipid species. This flattened tetrahedron phase diagram was shown to show smooth transition in displaying the membrane polarity and relevant boundaries across the component ternary phase diagrams and was finally folded to propose the next step in data interpretation of increasingly complex membranes.

In chapter 4, the concept of the 2D “flattened tetrahedron phase diagram” was expanded into a more complex 3D tetrahedron phase diagram to analyze more complex quaternary lipid membrane compositions. Liposomes consisting of 4 lipid species were constructed and further analyzed with previously used fluorescence techniques. Phase states were discussed in the context of quaternary membrane compositions and depicted via the tetrahedron phase diagram. Quaternary membranes revealed more robustness in phase with temperature change. Insight was obtained into the interior of the tetrahedron diagram at specific temperatures. Ultimately, with the tetrahedron diagram, there is a future goal of a tetrahedron mapping analysis of the brain lipid membrane as well as other important membranes in anatomy.

In chapter 5, we first examined how the composition of the lipid bilayer of brain lipids or their analogs impacted the aggregation of amyloid- $\beta$  (A $\beta$ ) peptide on the liposome membrane. Temporal kinetic aspects of A $\beta$  aggregation were analyzed and correlated to different membrane

compositions. It was found that certain fluid membranes generally acted to enhance the speed of fibrillation kinetics of A $\beta$ . Next, to conversely analyze how the A $\beta$  impacted membrane properties, the membrane polarity at different stages of A $\beta$  aggregation was both quantified and qualified with Laurdan fluorescence spectroscopy and deconvolution analysis. It was found that during the lag phase ( $t_{lag}$ ) of A $\beta$  aggregation, there was slight hydration behavior of the membrane, followed by dehydration behavior as the aggregation progressed to its completion. This dehydration behavior revealed that surface level aggregation also impacts the solvent environment inside the membrane.

In chapter 6, we draw general conclusions from this work and how it is relevant to future research in both the microscopic/mesoscopic realm of lipid membrane research and the macroscopic realm of the biomedical field. Significance in the necessity for further development of the tetrahedron diagram for complex membrane composition analysis and tetrahedron mapping of complex systems was discussed. Suggestions for future works were also proposed to further examine properties of increasingly complex lipid membrane compositions.

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# **Chapter 1**

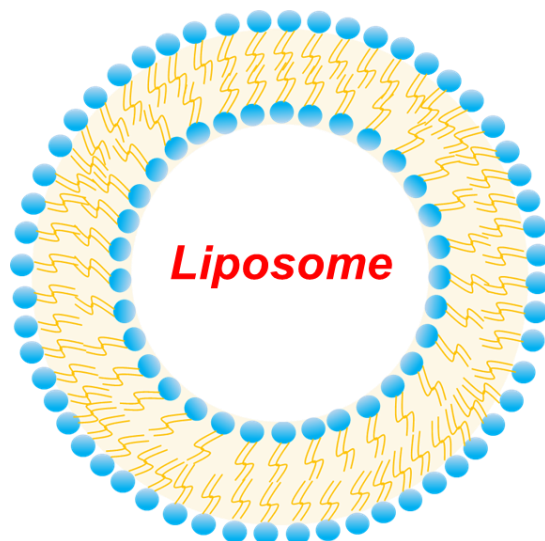
## **General Introduction**

Lipids are a major component of the dry mass of the brain and serve an integral function in the brain's various complex metabolic functions. Naturally, due to the necessity of healthy lipid metabolism for proper brain function, changes to brain lipid properties have the potential to have widespread downstream impact on various other anatomical and physiological processes. Due to the significant role of lipids in the brain, the study of brain lipid properties serves as a fundamental window into the realms of neurological homeostasis, pathology, and ultimately medicine. This chapter thus has the aim to serve as an introduction to our platform for analyzing the lipid membrane, the role of different brain relevant lipids, lipid characterization techniques, and lipid relevance in brain pathology.

### **1.1. Basics of brain anatomy and physiology**

One of the most important organs in anatomy and physiology is the brain due to its downstream impact on virtually all other anatomical systems and processes. Specifically, the brain belongs to the central nervous system (CNS), which also includes the spinal cord. Some of the most important functions that the brain is responsible for include but are not limited to learning, memory, sensory processing, and movement. These functions are generally carried out using neuronal signaling which involve signals going out of the brain or coming into the brain. Neurons, like other cells, have a plasma membrane composed of lipids and are thereby impacted by the various properties of these lipid membranes. Any variations to these lipid compositions have the possibility to have large-scale and widespread downstream effects, and thus the properties of these brain lipid compositions are an important area of neuronal research.

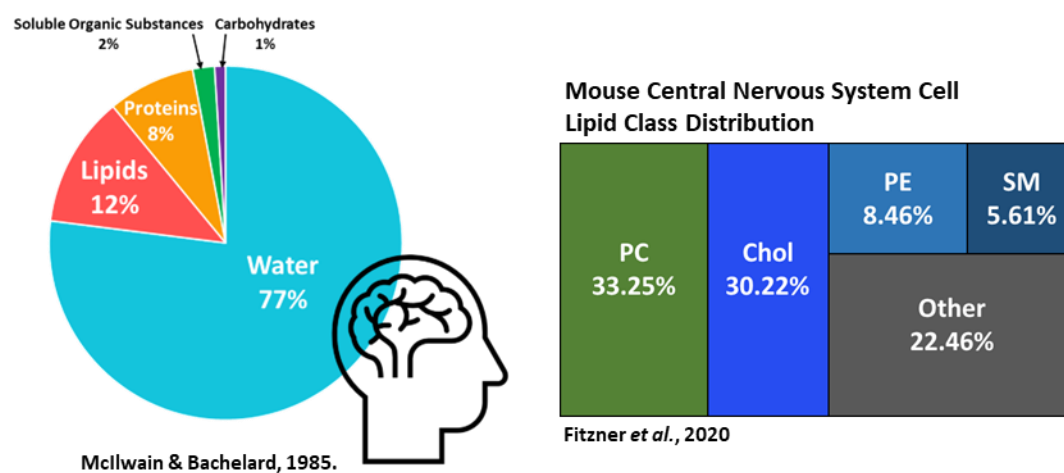
## 1.2. Liposomes as a biomimetic platform



**Fig. 1-1** 2D depiction of the primary platform of analysis in this study, the liposome. Liposomes are spherical lipid membrane structures used as a biomimetic environment.

Since the brain and neurons are involved in multiple biological processes and therefore possess extensively complex structures, the isolated study of lipid properties in the brain would be relatively difficult if using biologically sourced neuronal membranes. A popular method of examining the membrane in the absence of other biological processes or complexity would be the use of small unilamellar vesicles (liposomes) (**Fig. 1-1**). Liposomes are relatively easy to assemble with selectively chosen lipids, ranging from simple to more complex compositions, and are thereby an ideal platform to act as a biomimetic environment for the neuronal membrane for specifically characterizing lipid components. Liposomes are also compatible with various analytical techniques, such as fluorescence spectroscopy, that thereby connects them to well established methodology for the analysis of lipid properties. Due to this, liposomes were selected as the primary platform for analyzing lipid properties in this study.

### 1.3. Brain lipidomics



**Fig. 1-2** Human brain molecular composition (left) adapted from (McIlwain & Bachelard, 1985) and the typical composition in a mouse brain (right) adapted from (Fitzner *et al.*, 2020). Figures referenced from *lipotype.com*.

As previously mentioned, the dry mass of the brain is composed primarily of lipids (McIlwain & Bachelard, 1985). Brain lipidomic studies have revealed that among those brain lipids, the most dominant species include phosphatidylcholine (PC), cholesterol (Chol), phosphatidylethanolamine (PE), and brain sphingomyelin (bSM) (**Fig. 1-2**) (Fitzner *et al.*, 2020; McIlwain & Bachelard, 1985). Since these are the most dominant lipid species of the brain, they were selected as the primary components for membranes in this study. Each of these lipids have unique characteristics that are expected to play a role in the properties and overall efficiency of function of the brain lipid membrane.

bSM is a sphingolipid, meaning that unlike glycerophospholipid species it possesses a sphingosine backbone, giving it an amide and hydroxyl group that allow for strong hydrogen bond propensity with other lipids. Comparative to other lipids, this strong hydrogen bonding capability allows for bSM to have more stable interactions with Chol and thereby provide more stability to the membrane. Additionally, the long acyl chains of bSM consequentially gives the lipid high order

and a high phase transition temperature ( $T_m$ ) (Cevc, 1991). Due to these characteristics, bSM plays a key role in the formation and maintenance of ordered domains in the membrane, particularly when interacting with Chol cell membrane, some of the most dominant are sphin(Zidar et al., 2009).

PC is a glycerophospholipid, and unlike bSM, possesses a glycerol backbone and does not possess any hydrogen bond donors. Since this study relies on the use of liposomes as biomimetic environments rather than biologically sourced membranes, 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was utilized in this study as a synthetic analog of PC due to its widely researched characterization and behaviors across multiple previous research projects (Bui et al., 2016; Nyholm et al., 2011; Suga & Umakoshi, 2013). Due to its unsaturated nature, DOPC does not have large constriction of its acyl chain region and thereby has a relatively low  $T_m$  as a consequence. Additionally, since DOPC does not have the hydrogen bonding potential of bSM, the stability it provides to the membrane is relatively much weaker. With both of these characteristics in consideration, DOPC consequentially is responsible for the fluid nature of the lipid membrane across most temperature ranges and is a good predictor of fluidity as a result.

PE is another glycerophospholipid, however, compared to PC it is generally considered to be more of a “helper lipid,” a lipid that is not the primary structural component of a membrane but rather a secondary supportive component, with a closer functional role in the membrane to Chol. Structurally, PE tends to possess a smaller ethanolamine headgroup and therefore has a difficult time forming a flat membrane as its sole structural component and is more involved with curvature stability of certain membranes. However, since brain lipidomic studies have emphasized PE to be another major component among the lipids of the brain, its effects on other brain relevant lipids and their analogs is worth examining for impact on membrane properties. For its widespread research use, availability, and for internal experimental consistency with using the synthetic DOPC,

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was used as an analog for the examination of PE in this series of studies. DOPE, similar to DOPC, is an unsaturated lipid and consequently has a low  $T_m$ , meaning that for the temperature ranges used in this study it will remain in a relatively fluid phase.

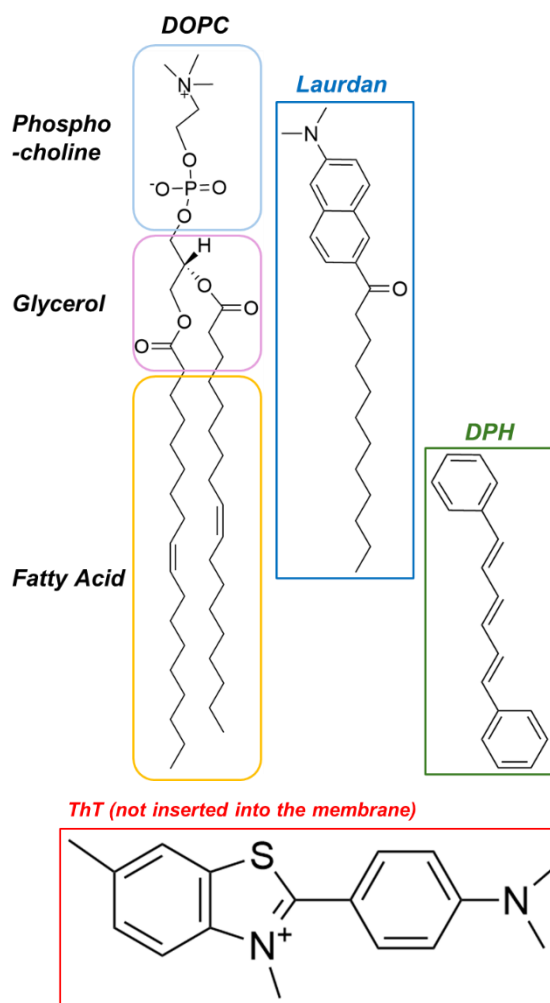
Chol will act as the 4<sup>th</sup> and final lipid in this study due to both its significant and well-documented interactions with bSM and its well-known role as a structural component of the lipid membrane (Marsh, 2009; Samsonov et al., 2001). Chol, although still in the classification of lipids, does not possess a traditional lipid structure of a phospholipid head with acyl chains, but rather has a sterol structure. In the lipid membrane, Chol has been observed to have a dual nature of providing disorder to ordered membranes and order to disordered membranes (Nyholm et al., 2011; Schachter et al., 2022). Similar to PE, Chol is primarily in the “helper” lipid category as its structure prevents it from forming a stable membrane structure if it is the primary component and will thereby be used as another secondary lipid, alongside PE, in the proceeding study.

#### **1.4. Brain Pathology**

There are believed to be many different brain pathologies that are associated with interactions with the lipid membrane. A frequently observed element of pathology in multiple brain pathologies such as Alzheimer’s disease (AD), Huntington’s disease (HD), multiple sclerosis (MS), among others is the amyloid- $\beta$  ( $A\beta$ ) peptide (Chen et al., 2017a; Frischer et al., 2009; Hardy & Higgins, 1992; Vonsattel & DiFiglia, 1998).  $A\beta$  is a peptide that is cleaved from the amyloid precursor protein (APP) and has well-known aggregation behavior on the surface of the membrane that results in increasingly severe damage, notably in the brain (Chen et al., 2017a; Esch et al., 1990; Hardy & Higgins, 1992; Koo et al., 1999; Matsuzaki, 2014; Sisodia, 1992). Aggregation in the brain has been observed extensively in patients with many of the aforementioned pathologies

and is thereby a key area of investigation in the medical field. Since membrane composition in the brain is complex, individual impact of certain lipids such as bSM, PC, PE, and Chol is an area that requires further characterization. Thus, this study has the aim to understand how alterations to the membrane composition may impact the aggregation kinetics and intensity of A $\beta$  and conversely, understand how increasing A $\beta$  may impact the properties of an otherwise healthy lipid membrane.

### 1.5. Fluorescence spectroscopy and fluorescent probes



**Fig. 1-3** Typical insertion regions of Laurdan and DPH. Laurdan inserts at the membrane interface region while DPH inserts deeper into the acyl chain region. ThT binds to  $\beta$ -sheets rather than inserting into the membrane.

Fluorescence spectroscopy is a well-established and non-intrusive technique that is commonly used for lipid membrane analytical studies for its ease of execution and ability to

investigate and characterize multiple membrane properties. Fluorescence spectroscopy generally relies on the employment of different fluorescence probes that interact with the selected molecular component being analyzed and reveal properties such as aggregation, interfacial membrane polarity, and membrane fluidity, among others via insertion into the membrane (**Fig. 1-3**). This study has utilized primarily 3 fluorescent probes to accomplish its investigation of brain lipid membrane properties: Thioflavin T (ThT), 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan), and 1,6-diphenyl-1,3,5-hexatriene (DPH) summarized in **Table 1-1**. Conceptually, fluorescence spectroscopy functions by excitation of the fluorescent probe at a certain wavelength, which is followed by the emission of the excited probe across a certain range of wavelengths that can be used to mathematically quantify and convey specific properties of the membrane.

| Fluorescent probe | Detection            | Excitation wavelengths (nm) | Emission wavelengths (nm) |
|-------------------|----------------------|-----------------------------|---------------------------|
| ThT               | $\beta$ -sheets      | 444                         | 460 – 600                 |
| Laurdan           | Interfacial polarity | 340                         | 400 – 600                 |
| DPH               | Inner fluidity       | 360                         | 430                       |

**Table 1-1** Description of the fluorescent probes used in this study (detection, excitation wavelengths, and emission wavelengths).

Laurdan inserts directly into the membrane interface and after its excitation and based on the efficiency of the solvent environment undergoing dipolar relaxation, can convey the polarity of the membrane based on the stability of the excited state of Laurdan causing emission at certain wavelengths. Generally, the emission at lower wavelengths is called a “blue shift” and corresponds to a more ordered membrane while at higher wavelengths it is called a “red shift” and corresponds to a disordered membrane. Membrane polarity is then quantified with the emission intensity of Laurdan at 440 nm (gel phase) and 490 nm (liquid-crystalline phase) to obtain the generalized polarization ( $GP_{340}$ ) (Parasassi et al., 1990, 1991, 1998). Additionally, Laurdan spectra are able to

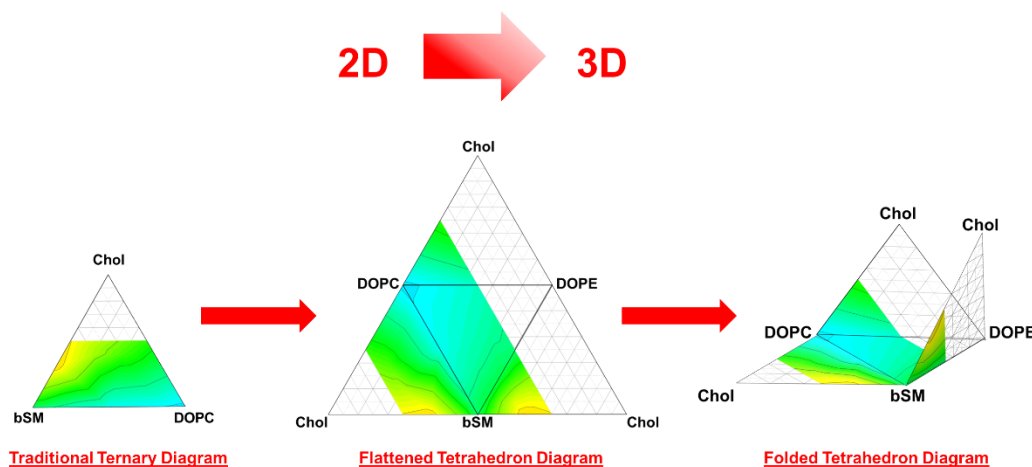
be further deconvoluted into 4 component peaks [Peak 1: non-polar ( $\epsilon_r < 5$ ), Peak 2: polar aprotic ( $5 < \epsilon_r < 20$ ), Peak 3: polar protic ( $10 < \epsilon_r < 20$ ), and Peak 4: polar protic ( $\epsilon_r > 20$ )] where  $\epsilon_r$  is the dielectric constant (Watanabe et al., 2019). DPH inserts into a deeper region of the membrane than Laurdan and is thereby less sensitive to specific interfacial changes. DPH relies on its rotational mobility in the acyl chain region of the membrane and is thereby able to convey the fluidity of the membrane with its anisotropy (He, 2023). Laurdan and DPH data are typically used in a complimentary fashion on figures such as a cartesian diagram to establish the order of a specific membrane [quadrant I (heterogeneous), quadrant II (ordered), and quadrant IV (disordered)], therefore these 2 probes are the backbone of this study (Suga & Umakoshi, 2013). ThT, on the other hand, is able to uniquely quantify the intensity of aggregation of A $\beta$  by interaction with the increasing presence of  $\beta$ -sheets that coincide with the progression of aggregation and is thus only used in this study, albeit importantly, in the final chapter to quantify A $\beta$  intensity rather than a quantification of membrane characteristics (LeVine, 1995).

## 1.6. Phase state characterization

One of the most important aspects of lipids and the membrane is the ability to vary phase state across a temperature range. There exist several commonly occurring phase states including solid ordered ( $s_o$ ), liquid ordered ( $l_o$ ), heterogeneous, and liquid disordered ( $l_d$ ) which generally correlate to an increase in temperature. Additionally, phase state coexistence, such as  $l_o + l_d$  is known to occur in more complex membrane compositions and is a prerequisite for important lipid functional behavior such as lipid rafts. Due to the extensive nature of phase states, methodology to display these phase states comparatively across lipid species is an important point of lipid analytical research.

One of the most commonly used methods for examination of lipid phase states is the ternary phase diagram (de Almeida et al., 2003a; Feigenson, 2009; Nyholm et al., 2011; Thewalt & Bloom, 1992). Traditional ternary phase diagrams can display up to 3 different lipids at their vertices, with edges representing the ratios of binary compositions, and the interior representing the more complex ternary ratios of lipids. Using techniques such as Laurdan and DPH fluorescence, phase boundaries can be established within this ternary diagram and depict at what ratios of lipids a phase variation may occur. Additionally, separate phase diagrams can be constructed for different temperatures, comparatively determining how temperature may alter the phase variation of certain lipid ratios. However, one of the main restrictions in the ternary phase diagram is the fact that it's limited to the depiction of a ternary lipid membrane composition, whereas biological membranes are increasingly more complex.

To expand the concept of a ternary phase diagram, our study examines first the use of a “flattened tetrahedron phase diagram” to incorporate all 4 of the lipids we decided to focus on in this work (bSM, DOPC, DOPE, and Chol). This flattened tetrahedron diagram is essentially a combination of 4 unique ternary diagrams connected at their overlapping binary edges. By aligning



**Fig. 1-4** Depiction of the progression of diagrams focused on in this study from 2D ternary diagram, to 2D flattened tetrahedron diagram, to 3D folded tetrahedron diagram.

these 4 ternary diagrams together, phase state trends can be examined more readily across multiple ternary diagrams and consequently more lipid compositions may be compared. Although this does not directly address the limitation of the 3-lipid membrane composition yet, it is a pivotal intermediary step in the introduction of the 4<sup>th</sup> lipid component to the membrane.

To finally solve the ternary phase diagram limitation, we attempt to support the concept of a tetrahedron diagram previously proposed in the field but that has yet to be widely incorporated into a practical means of lipid characterization techniques (Konyakhina et al., 2013). By folding the binary edges of the previously discussed 2D flattened tetrahedron phase diagram, a more complex 3D tetrahedron phase diagram may be established (**Fig. 1-4**). This tetrahedron diagram is composed of 4 separate ternary diagram faces with an interior space that is finally able to conceptually expand membrane composition analysis into the 3D realm by incorporation of a 4<sup>th</sup> lipid component.

### **1.7. Tetrahedron diagram mapping analysis**

Ultimately, this study aims to establish a tetrahedron diagram mapping analysis strategy for different anatomical organ systems. This concept involves utilizing any of the multitudes of analytical techniques (fluorescence spectroscopy, UV, NMR, etc.) with lipid membranes consisting of different levels of complexity, from unary to quaternary. By first selecting the complexity of the composition, then selecting the appropriate diagram for each bioenvironment, this study has the goal of providing a strategy to eventually have a comprehensive characterization of the lipids in the major organ systems of anatomy. Finally, the concepts set up in this study provide the essentials necessary to expand beyond the quaternary composition, with the aim to assist in the biomedical realm through membrane property classification. With proper classification of the lipid bioenvironment of the major organ systems, the idea of lipid membrane therapeutics is conceived

as a method to target pathologies by alteration or maintenance of certain properties associated with the homeostatic functions of the lipid membrane.

### **1.8. Overview of this study**

This study has the aim to both characterize the properties of lipid membranes composed of brain relevant lipids and to widely establish methodology that allows future researchers to more readily examine more complex 4-lipid composition lipid membranes. Characterization of brain-relevant lipid membrane compositions will allow for common phase states of these lipids membranes at normal homeostatic conditions to be established. Additionally, temperature variation will also provide insight into the sensitivity and variability of these phases that may coincide with the symptoms of different brain pathologies. Specific interactions of A $\beta$  and the lipid membrane was done to both quantify and qualify the effects of the membrane on the symptoms of brain pathologies such as AD, and vice versa. Next, the supporting study for the further development and establishment of more complex 4-lipid mixtures was done via expanding the concept of a ternary diagram, into a flat tetrahedron diagram, and finally into a 3D tetrahedron diagram. Ultimately this study has the goal to characterize the behaviors of brain-relevant lipids and provide insight into the analysis of increasingly complex membrane compositions.

In chapter 2, we first examine the simple binary composition of bSM and Chol. Using Laurdan and DPH fluorescence, we examine the changes to the properties of polarity and fluidity over a temperature range with variable compositions. This basic binary compositional analysis revealed that with temperature increase, both the polarity and fluidity increased proportionally. Additionally, this chapter showed that Chol concentrations of 30% and 50% in the bSM membrane were more robustly in an ordered phase with temperature increase. Additionally, deconvolution analysis also revealed that these 30% and 50% Chol membranes had a more heterogeneous spread

of characteristics at the membrane interface compared to membranes with less Chol. These results were important in hypothesizing about the  $l_o$  phase.

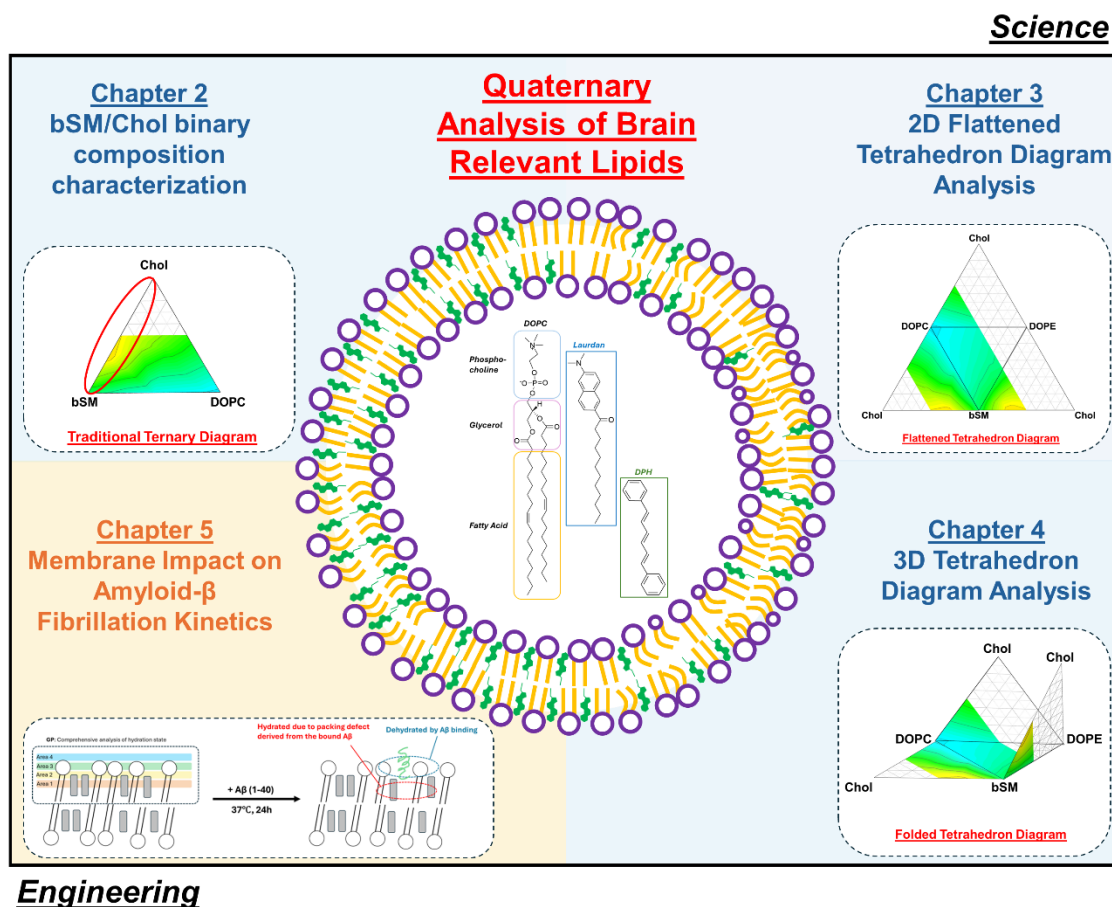
In chapter 3 an effort is made to advance to first examine the properties of ternary lipid compositions. Since there are 4 primary lipid species utilized in this study (bSM, DOPC, DOPE, and Chol), 4 separate ternary diagrams were able to be constructed at 20 °C, 40 °C, and 60 °C. These diagrams were compared for each of the combinations of the 4 lipids in their ternary pairs to determine how temperature change affected the  $GP_{340}$  of each system. Each  $GP_{340}$  value was plotted on a heat contour of the ternary diagrams to display the changes in value as they correspond to temperature and ratio variation. Emphasis was placed on DOPE and it was revealed that it acted in a similar way to Chol, inducing disorder at lower temperatures and order at higher temperatures. Lastly, a flattened tetrahedron diagram was constructed at the biologically relevant 35 °C and revealed phase trends across the 4 separate ternary diagrams, revealing its utility for comparative purposes in lipid research.

In chapter 4 the concept of the 2D flattened tetrahedron diagram was finally expanded into the concept of the 3D tetrahedron diagram. Liposomes of quaternary composition were made with microfluidic techniques and analyzed with Laurdan and DPH fluorescence. When compared to binary mixtures via cartesian diagram ( $GP_{340}$  vs  $1/P$ ), these quaternary membrane compositions seemed to be more robust in remaining in the heterogeneous phase at higher temperatures. Deconvolution analysis for the majority of these compositions, however, were dominated by the polar protic peak 4 as temperature increased, indicating that the membrane interface's solvent environment was primarily in a hydrated state. Peaks 1 and 2, however, were not completely diminished as the temperature was raised for the majority of the compositions, suggesting that there was still dehydration within the overall solvent environment of the quaternary membrane

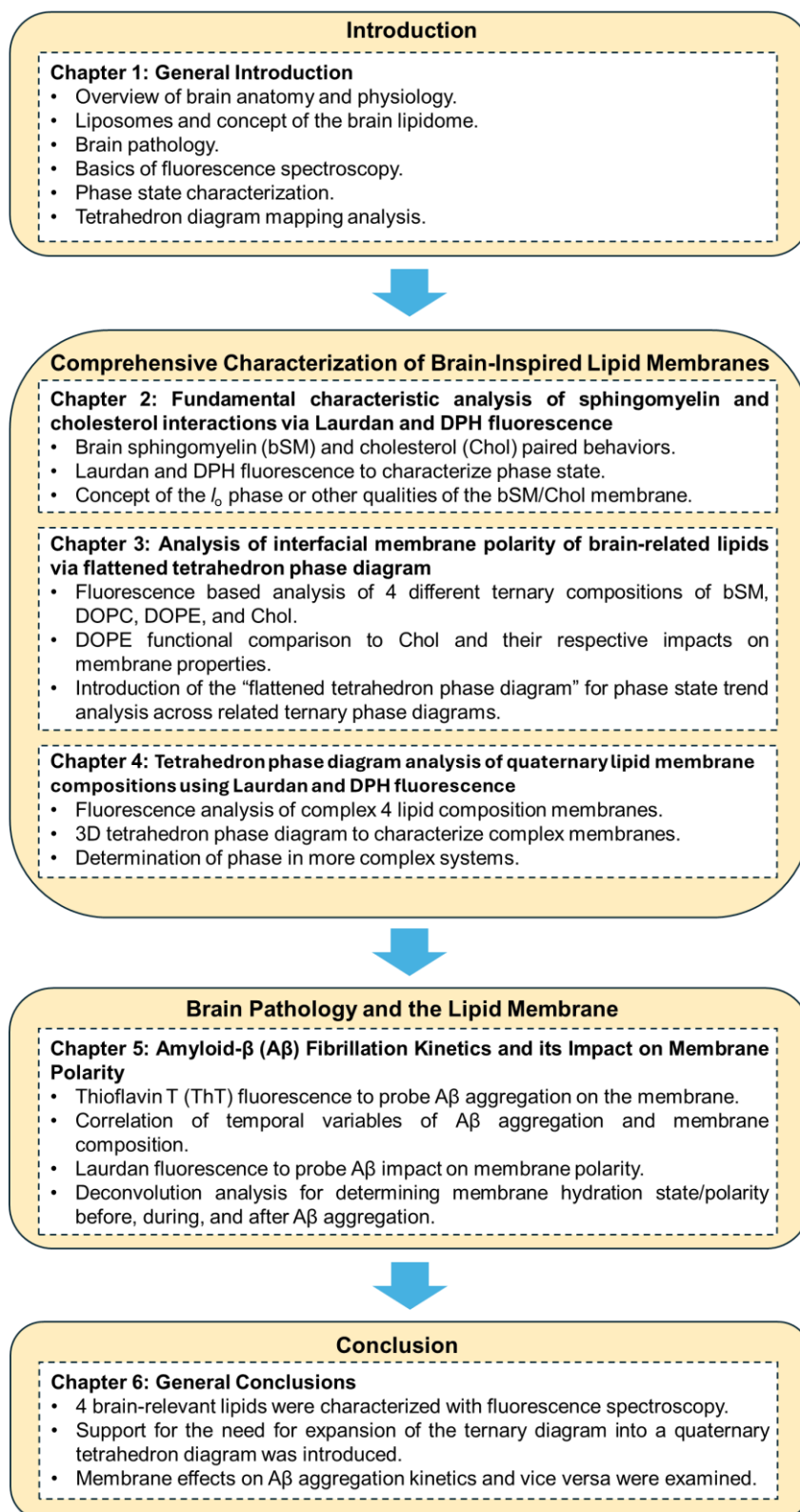
systems. Comparing the cartesian results with deconvolution results suggest that the acyl chain region detected by DPH fluorescence may not be the primary contributor in maintaining the heterogeneous nature of these membrane compositions as the  $1/P$  value seems to be largely increasing, however the points are not shifting downward (via decreased  $GP_{340}$ ) into quadrant IV.

In chapter 5, to transition the study into a more biologically relevant realm, we introduced binary membrane compositions of bSM/Chol and DOPC/Chol to A $\beta$ . It was noted by comparing a control (no membrane) with the membrane compositions that simply including a membrane increased the majority of temporal kinetic parameters for A $\beta$  fibrillation processes. It was found that the DOPC/Chol membrane may have more impact on the fibrillation kinetics of A $\beta$ , however, the kinetic process itself may have more impact on the polarity of SM/Chol.

In chapter 6 we provide general conclusions to this work regarding the significance of the selection of lipids in this study and the characteristics that their results revealed across the several studies performed. Significance of this study to the biomedical field was described with an emphasis on the neurological side of the field. Finally, several proposals for future work were also provided to expand on the concepts that were examined in this study. An overview of the concepts that will be discussed is provided in **Fig. 1-5 & 1-6**.



**Fig. 1-5** Concept map of the present study



**Fig. 1-6** Framework of the present study.

## **Chapter 2**

### **Fundamental characteristic analysis of sphingomyelin and cholesterol interactions via Laurdan and DPH fluorescence**

#### **1. Introduction**

Among the many different lipid species that are present in the brain's cell membrane, some of the most dominant are sphingomyelin (SM) and cholesterol (Chol) (Fitzner et al., 2020; O'Brien & Sampson, 1965; Svennerholm et al., 1994). Sphingomyelin, unlike glycerophospholipids, possesses a sphingosine backbone and thus can have more extensive and stabilizing hydrogen-bond interactions with Chol (Slotte, 2016). Chol, although not structurally the same as other membrane lipids, is another important component of a cell's membrane due to its role in fluidity and its ability to provide both order and disorder to membranes with its increased inclusion (Martin et al., 2010). Specifically, in the SM membrane Chol has been discovered to both disrupt the gel-phase and provide acyl-chain ordering to the liquid-crystalline phase (Guo et al., 2002). Due to the significance and abundance of both species of lipid in a cell membrane, and notably in the brain, there has been extensive research that has been done that has the aim to identify specific interactions between SM and Chol.

Lipid rafts have been described as a cluster of SM and Chol that form "raft-like" structures that are generally insoluble in detergents and are observed to be involved in the attachment of different proteins, membrane trafficking purposes, or membrane signaling purposes (Simons & Ikonen, 1997). Although raft domains do not form in binary mixtures of lipids, these binary

mixtures may have behaviors that are still worth analyzing for their potential similarities to behaviors in lipid raft membranes due to the presence of SM and Chol. Previous research has emphasized that there is a minimum concentration of Chol (above 30%) that is involved in the formation of a liquid-ordered ( $l_o$ ) phase, an important intermediate step in raft formation, and that the stability of this phase is due in part to the H-bond interactions of SM/Chol membranes (Lopez et al., 2018; Slotte, 2016; Zidar et al., 2009). However, determining the interactions of lower concentrations of Chol is also an important area of study for categorizing phase behavior in a lipid membrane and should further be explored.

Since temperature is able to influence the order of lipids in the membrane, and this  $l_o$  phase has been noted as a key precursor in the formation of lipid rafts, temperature alteration is yet another area that should be further analyzed to characterize the threshold of  $l_o$  phase state formation with different ratios of SM/Chol and varying temperature. Past research has examined phase behavior with techniques such as fluorescence spectroscopy, differential scanning calorimetry (DSC), and nuclear magnetic resonance (NMR) (Bartels et al., 2008; Lopez et al., 2018; Nyholm et al., 2011; Stott et al., 2008). Although many of these studies have elucidated the interactions of SM, Chol, and the  $l_o$  phase, there are still techniques such as cartesian analysis of phase and deconvolution analysis of Laurdan that have yet to receive widespread use as supplementary methodology. Thus, phase state analysis across a temperature range using fluorescent probes with both cartesian phase analysis and deconvolution analysis are the primary characterization techniques that are used in this work to further characterize why certain compositions of SM and Chol have  $l_o$  behaviors similar to those observed in lipid rafts membranes and at which temperatures these behaviors may be identified.

Liposomes are vesicle-like lipid structures that can function as a biomimetic environment for various membrane studies without the involvement of other biological factors that may be involved in a living cell's membrane. Liposomes are also advantageous in that they are relatively simple to assemble and alter their composition with different ratios of lipids. Since this research has the primary objective to observe the interactions between SM and Chol across a large temperature range (20 – 70 °C), liposomes are the ideal platform for fundamental study without the involvement of other biologically relevant factors in a cell's membrane.

Fluorescence spectroscopy, especially in recent years, has become one of the most popular and well-established methods for characterizing different membrane properties in lipids. Fluorescence probes such as 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan) and 1,6-diphenyl-1,3,5-hexatriene (DPH) can be inserted into the membrane relatively easily via time-based incubation and are thereby a popular method to assay different properties of the membrane. Laurdan is a probe that localizes at the membrane interface and is thereby able to convey the hydration states of that membrane via its emission being sensitive to the surrounding solvent environment (Parasassi et al., 1990, 1991, 1998). DPH is a probe that localizes deeper into the membrane and based on its anisotropy via polarization, is able to convey the fluidity of a membrane. These two probes are often used in conjunction to establish the phase state of a particular membrane and are thereby an important means of determining the potential for lipid rafts or other phenomena at different measured temperatures and ratios of lipids. This research therefore has the aim to use these probes for the fluorescence spectroscopy measurement of different ratios of brain SM and Chol to analyze changes to phase state with varying temperature and thereafter determine which ratios and temperatures are the most likely to have similar behaviors as more complex membranes capable of raft formation or other unique interactions.

## **2. Experimental Section**

### **2.1. Materials**

Sphingomyelin (porcine brain) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) and 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan) fluorescent probe was purchased from Sigma-Aldrich (St. Louis, MO, USA). Any other relevant chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

### **2.2. Liposome Preparation**

Liposome solutions were made for each ratio of lipid membrane according to the following protocols: stock solution of porcine brain SM and Chol was prepared by adding the lipid in its powder form to chloroform. An arbitrary amount of stock solution was transferred to a round bottom flask according to each ratio of SM/Chol (10:0, 9:1, 7:3, or 5:5) and rotary evaporated under vacuum conditions until a clear lipid film was visible. This film was placed in a vacuum environment for a minimum of 2 hours after which the film was rehydrated with water. This solution then underwent 5 cycles of freeze (-80 °C) and thaw (60 °C) to encourage the homogeneous formation of lipid vesicles. This solution was finally extruded using an extruding device (Liposofast, Avestin Inc., Ottawa, ON, CA) using a 100 nm pore filter to create the small unilamellar vesicles (SUVs). This process was repeated for each ratio of brain SM and Chol that were analyzed.

### **2.3. Laurdan and DPH sample preparation**

Laurdan in ethanol (100  $\mu$ M) was added in a 1:100 ratio and DPH in ethanol (100  $\mu$ M) a 1:250 ratio of probe:lipid. Each sample was then incubated for a minimum of 30 minutes to allow time for probe insertion into the membrane. Samples were shielded from light during and after the

incubation processes as much as possible to prevent any negative influence on their fluorescence. Temperature was maintained at around 25 °C to 37 °C during the incubation process. Samples were measured using a spectrophotometer (FP-8500; JASCO, Tokyo, Japan). Each sample underwent measurements from 20 °C to 70 °C in increments of 5 °C with minimal exposure to light.

#### 2.4. Evaluation of Membrane Polarity via Generalized Polarization ( $GP_{340}$ )

Polarity values were measured at the excitation and emission wavelengths of Laurdan (Ex = 340 nm, Em = 400 – 600 nm) and extracted from the spectrophotometer based on the intensity values of each Laurdan sample at  $I_{440}$  and  $I_{490}$ , the fluorescence intensities at 440 nm (gel phase) and 490 nm (liquid crystalline phase), respectively. These values were then used to estimate the membrane polarity using the following equation (Eqn. 1):

$$GP_{340} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (2-1)$$

This value was then plotted against the temperature to determine the influence of temperature change on the membrane polarity of each different composition of liposome.

#### 2.5. Peak Deconvolution of Laurdan Fluorescence Spectra

Additionally, each of the obtained Laurdan fluorescence spectra were deconvoluted to obtain a minimum of at least 4 component curves according to previous protocols (Watanabe et al., 2019). Each component curve represented the solvent environment detected by Laurdan [Peak 1: non-polar ( $\epsilon_r < 5$ ), Peak 2: polar aprotic ( $5 < \epsilon_r < 20$ ), Peak 3: polar protic ( $10 < \epsilon_r < 20$ ), and Peak 4: polar protic ( $\epsilon_r > 20$ )] where  $\epsilon_r$  is the dielectric constant. Deconvolutions were performed with OriginPro's peak deconvolution application. Peaks were deconvoluted with the following

parameters: second derivative, Gaussian, quadratic Savitzky-Golay smooth derivative, with upper bounds for the FWHM set at 60. Area fit %, the theoretical area from negative to positive infinity of wavelength, was obtained for each deconvoluted peak, averaged, and plotted.

## **2.6. Evaluation of membrane fluidity via inverse polarization $1/P$**

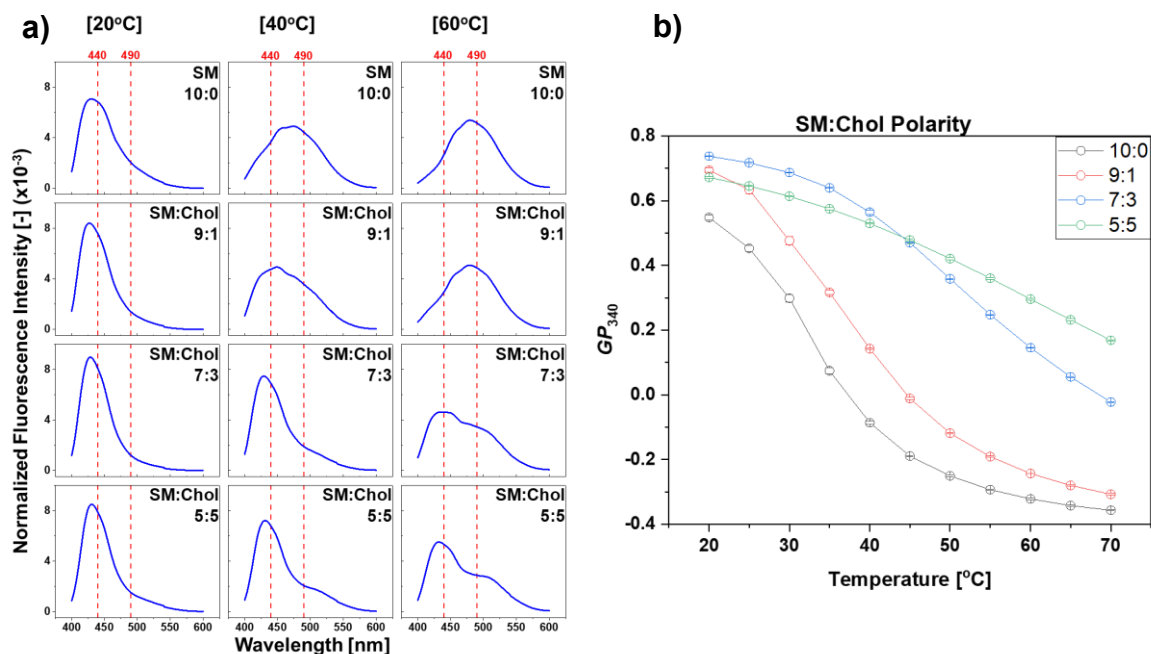
Membrane fluidity was evaluated with the DPH probe according to previously established protocols (Suga & Umakoshi, 2013). Fluorescence anisotropy values were measured according to the excitation and emission wavelength of DPH ( $E_x = 360$  nm,  $E_m = 430$  nm). Intensity perpendicular ( $I_{\perp}$ ) and parallel ( $I_{\parallel}$ ) were used alongside the correction factor ( $G$ ) to obtain the polarization ( $P$ ) (**Eqn. 2-3**).  $1/P$  was used rather than  $P$  to show a more proportional relationship between the fluidity and temperature.

$$P = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + GI_{\perp}) \quad (2-2)$$

$$G = i_{\perp} / i_{\parallel} \quad (2-3)$$

### 3. Results & Discussion

#### 3.1. Laurdan Analysis

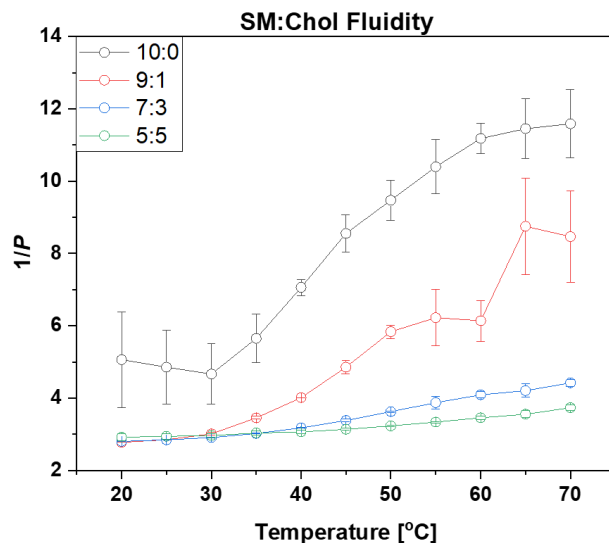


**Fig. 2-1** Polarity trend of varying compositions of SM/Chol liposomes. **(a)** Area normalized Laurdan spectra (1 iteration displayed out of 3 measured) depicting red shift wavelength variation of the  $GP_{340}$  region (dashed lines at 440 nm and 490 nm) that coincide with temperature changes. **(b)**  $GP_{340}$  graph of each of the different SM/Chol liposome combinations plotted against temperature change.

Porcine brain SM was incorporated with Chol in several ratios of SM:Chol (10:0, 9:1, 7:3, and 5:5). Each liposome was analyzed with Laurdan via the methodology described in the experimental section to obtain the  $GP_{340}$  (Eqn. 2-1). Normalized spectra for each ratio (1 sample spectrum displayed out of 3 measured) are shown to have their peak shift to a higher wavelength (“red shifted” emission) as the temperature is increased for every analyzed composition (Fig. 2-1a). When  $GP_{340}$  was plotted against temperature, there were noticeable differences based on increased inclusion of Chol (Fig. 2-1b). It was initially expected that across all temperatures, increased inclusion of Chol would result in an incrementally larger  $GP_{340}$  (lower polarity), however, at temperatures below 45 °C SM/Chol (5:5) had a lower  $GP_{340}$  value than SM/Chol (7:3). After

the 45 °C measurement, the results shifted back to the expectation of more Chol being proportional to a larger  $GP_{340}$  value.

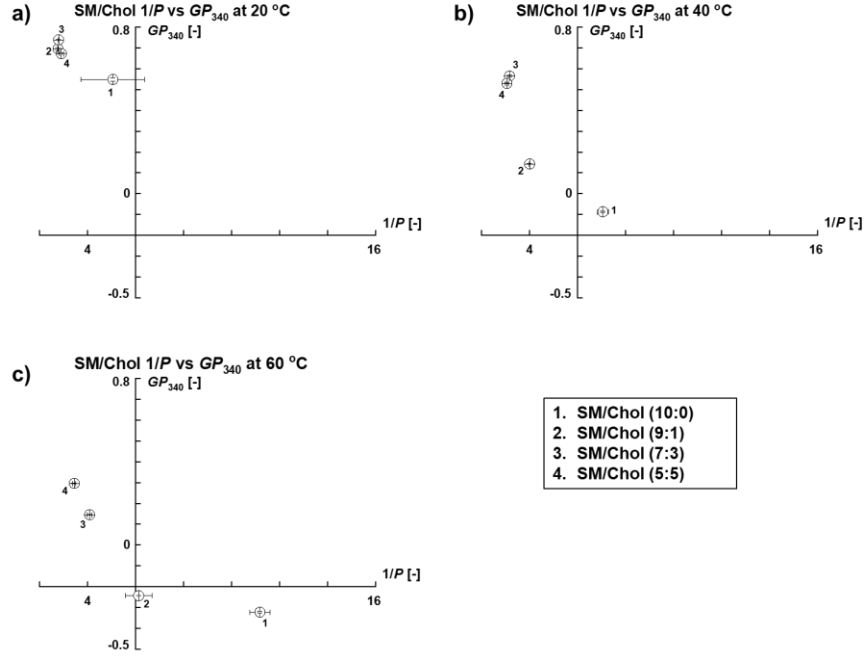
### 3.2. DPH analysis of SM/Chol liposomes



**Fig. 2-2** Fluidity trend of SM/Chol liposomes of varying ratios (10:0, 9:1, 7:3, 5:5). Temperature was plotted against the inverse polarization ( $1/P$ ) to display a linear relationship.

After obtaining the polarization value ( $P$ ) from DPH fluorescence via **Eqn. 2-2**, the inverse of  $P$  was taken to display a proportional relationship between the fluidity and temperature. Fluidity ( $1/P$ ) was plotted against temperature to reveal any variation (**Fig. 2-2**).  $1/P$  was only noticeably distinct for the liposome with no Chol at the lower temperatures. At 30-35 °C, the 9:1 liposome also began to diverge in its similarity. Finally, at 40-45 °C, the 7:3 and 5:5 compositions began to diverge, with the proceeding measurements having a proportional relationship between  $1/P$  and Chol inclusion, with more Chol causing a lower fluidity (lower  $1/P$ ).

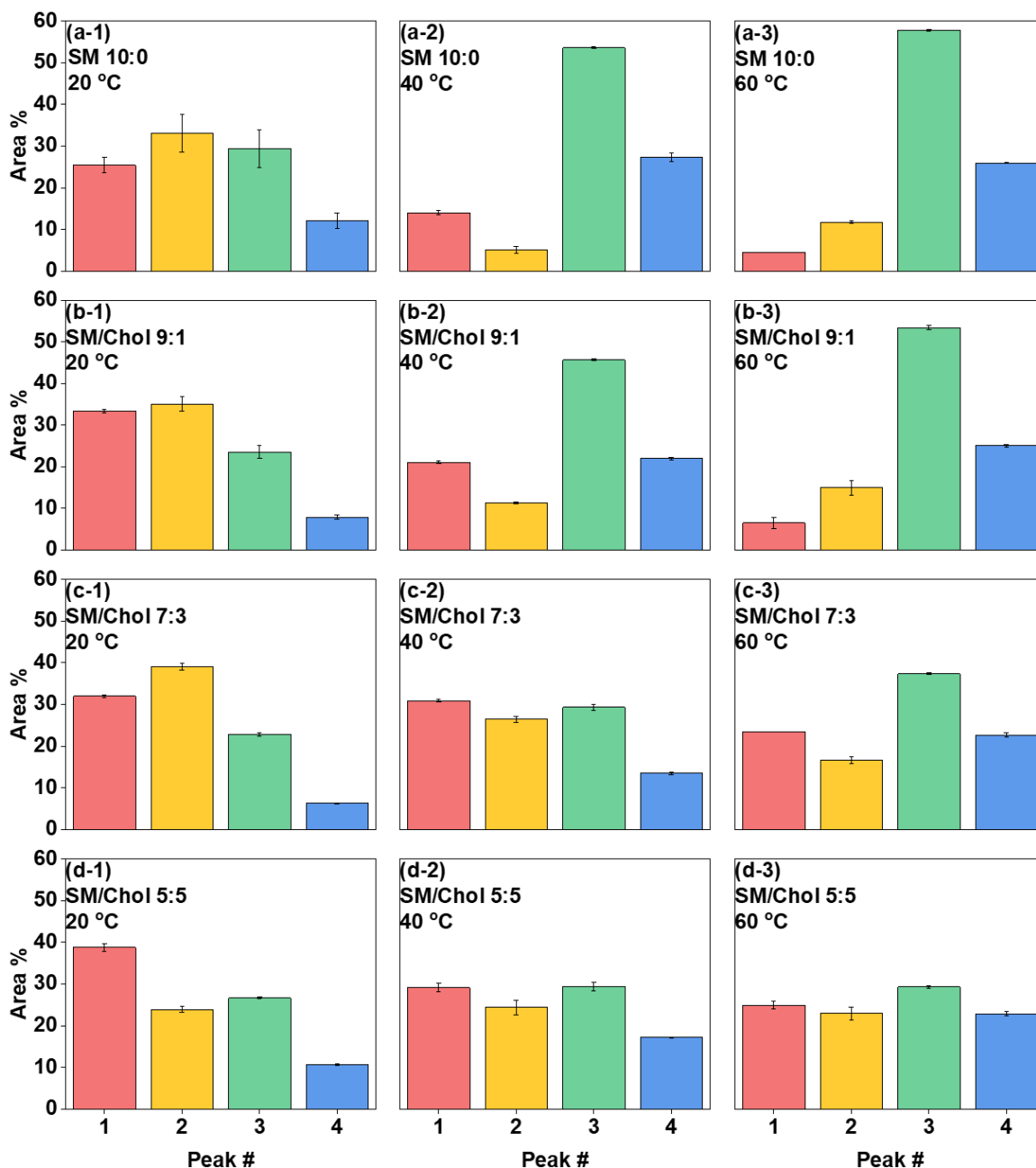
### 3.3. Cartesian diagrams of SM/Chol compositions at 20 °C, 40 °C, and 60 °C



**Fig. 2-3**  $GP_{340}$  vs  $1/P$  cartesian diagrams of SM/Chol (10:0, 9:1, 7:3, and 5:5) at **a)** 20 °C, **b)** 40 °C, and **c)** 60 °C.

Cartesian diagrams were constructed for each SM/Chol ratio (10:0, 9:1, 7:3, and 5:5) according to previous protocol using their threshold points of phase transition in DPPC liposomes for determining axes crossing points (**Fig. 2-3**) (Suga & Umakoshi, 2013). Polarity ( $GP_{340}$ ) was plotted on the y-axis against fluidity ( $1/P$ ) plotted on the x-axis. Each quadrant represents a specific phase state of the membrane according to the previously established protocols mentioned: quadrant I (heterogeneous phase), quadrant II (ordered phase), and quadrant IV (disordered phase). As the temperature increases from 20 °C to 60 °C, there is a clear transition from the ordered phase to disordered phase for SM/Chol (10:0) and SM/Chol (9:1) while SM/Chol (7:3) and SM/Chol (5:5) remain in the ordered phase.

### 3.4. Deconvolution Analysis



**Fig. 2-4** Deconvolutions of SM/Chol Laurdan spectra of each composition used to calculate the  $GP_{340}$  values at (1) 20 °C, (2) 40 °C, and (3) 60 °C: **a)** SM/Chol (10:0), **b)** SM/Chol (9:1), **c)** SM/Chol (7:3), **d)** SM/Chol (5:5). Peak 1 (red) represents the nonpolar region ( $\epsilon_r < 5$ ), peak 2 (orange) represents the polar aprotic region ( $5 < \epsilon_r < 20$ ), peak 3 (green) represents the polar protic region ( $\epsilon_r < 20$ ), and peak 4 (blue) represents the polar protic region ( $\epsilon_r > 20$ ).

To further reveal the solvent environment that contributed to the overall Laurdan fluorescence spectra, deconvolution analysis was performed on each iteration of the 3 iterations used to obtain the  $GP_{340}$  values. Each membrane composition of SM/Chol (10:0, 9:1, 7:3, and 5:5) was deconvoluted at 20 °C, 40 °C, and 60 °C according to previous protocols to determine the hydration state of the membrane at varying temperatures and compositions (**Fig. 2-4**) (Watanabe et al., 2019). Peak 1 (red) corresponds to a nonpolar environment ( $\epsilon_r < 5$ ), peak 2 (orange) corresponds to a polar aprotic environment ( $5 < \epsilon_r < 20$ ), peak 3 (green) corresponds to a polar protic environment ( $10 < \epsilon_r < 20$ ), and peak 4 (blue) corresponds to a polar protic environment ( $\epsilon_r > 20$ ). For SM/Chol (10:0) there was an initial similar dominance of peaks 1-3 and increasing the temperature resulted in a shift to the dominance of the polar protic peaks 3-4. For SM/Chol (9:1) there was once again an initial dominance of peaks 1-3, with an increasing temperature resulting in the increased dominance of the polar protic peaks 3-4 once again. For SM/Chol (7:3) there was also a dominance of peaks 1-3, however, increasing the temperature with this composition somewhat retained a larger presence of peaks 1-2 even at higher temperatures whilst peaks 3-4 increased with temperature once again. Lastly, for SM/Chol (5:5) peaks 1-3 were initially the most dominant, with peak 1 for the first time being the most dominant, after which temperature increase reduced peak 1 but maintained a similar level of dominance across all 4 peaks by 60 °C. Generally, across all compositions, by the 60 °C mark, peak 3 was the most dominant, emphasizing the change in membrane characteristics from an ordered to disordered environment proportionally with temperature increase.

### 3.5. General Discussion

For most liposomes increased inclusion of Chol generally resulted in the predictable results of Chol increasing the order of a given membrane (**Fig. 2-1, 2-2, 2-3**). It is generally expected that higher ratios of Chol would result in more ordered characteristics (lower polarity and fluidity), however, this does not seem to be the case in certain ratios of SM/Chol. In the given measurement range of 20 – 70 °C, porcine brain SM has its transition temperature between 35 – 40 °C and consequentially this transition temperature region range is where there appears to be some deviations from expected results, most apparent in the  $GP_{340}$  values of SM/Chol (7:3) and (5:5). Past studies using alternative saturated-lipid/Chol systems have also noted similar overall trends coinciding with temperature and the lowering of  $GP_{340}$  (Soto-Arriaza et al., 2013). Other studies with saturated lipids has indicated, however, that a transition temperature may not be responsible for interactions resulting in an ordered state at high temperatures (Majumder et al., 2024). Overall, these past studies with saturated lipids and Chol comparatively show similar overall trends, however, the strong H-bond interactions between SM and Chol cause SM/Chol membranes to remain a unique point of interest relative to other systems even with similar results. Similarities are also interesting since, compared to synthetic lipids like DPPC, the biologically derived porcine brain SM has a distribution of chain lengths and any similarities to synthetic/pure lipids like DPPC is worth noting.

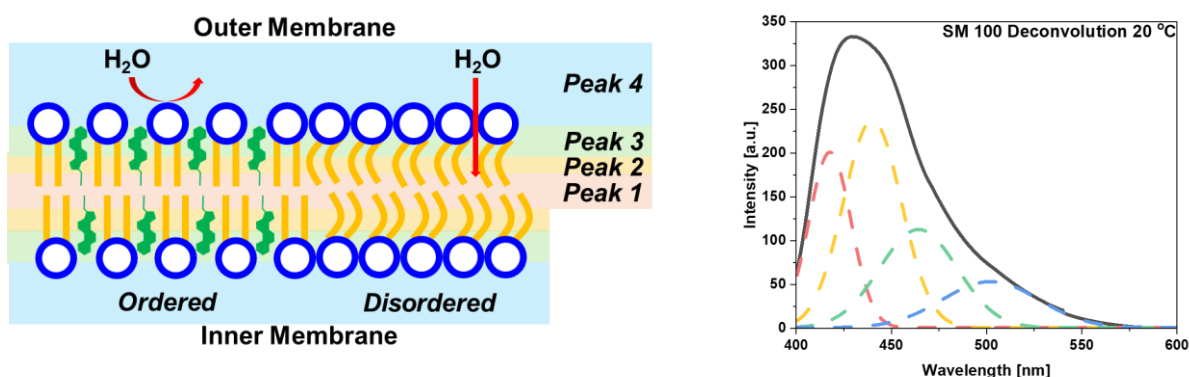
Area normalized Laurdan spectra showed a very clear and distinct red shift from lower wavelengths to higher wavelengths as the temperature was increased, indicating that the membrane is transitioning from the gel phase ( $I_{440}$ ) to the liquid crystalline phase ( $I_{490}$ ). Since previous research has indicated that the  $l_o$  phase state is an apparent precursor to lipid raft phenomena, it can be assumed that membranes and temperatures that are not clearly in the liquid-disordered ( $l_d$ )

phase range of values are the most likely contenders for raft formation in more complex membranes (Zidar et al., 2009). Generalized polarization ( $GP_{340}$ ) values were close at the starting temperature for every liposome that included Chol, however, brain SM with no Chol was the most distinctly different (lower) for  $GP_{340}$  (**Fig. 2-1b**). Most notable would be the result of the 5:5 composition having a lower  $GP_{340}$  value than the 7:3 composition at temperatures below 45 °C. In our binary system, SM and Chol are not expected to form raft domains. Thus, when contributing about 50% compositionally, Chol disruption of the ordered (pre-phase transition) SM membrane may result in a uniquely lower  $GP_{340}$  value (higher polarity). This result also suggests that at values between 30% and 50% Chol there may be a Chol concentration at which raft domains may be more likely to form in more complex membranes (membrane lipids  $\geq 3$ ) (Lopez et al., 2018; Zidar et al., 2009). This idea is connected to previous studies that indicate that above 30% Chol there is a formation of an  $l_o$  phase, a precursor to rafts, however our results suggest that this ordering may primarily occur at concentrations closer to 30% due to the relatively higher  $GP_{340}$  of SM/Chol 7:3 which suggests more order (at temperatures below the  $T_m$  of SM) (Lopez et al., 2018; Zidar et al., 2009). Previous studies have also suggested that the potential existence of rafts may cause fluctuations in the generalized polarization value, however, our binary mixture (no rafts) results suggesting that this may be due specifically to SM and Chol interactions (Sanchez et al., 2012). As previously mentioned, since the results become more predictable post SM phase transition temperature range, this interaction may be unique to the gel phase of SM, which consequently may link these SM and Chol interactions to the phase transition temperature of SM. However, we cannot completely rule out the possibility that the 50% Chol membrane is the unique composition, with the extra Chol interfering with packing at lower temperatures while maintaining membrane order at higher temperatures (Schachter et al., 2022).

DPH analysis via inverse polarization ( $1/P$ ) yielded similar results across the 20 – 70 °C temperature range in that the most distinctly different liposome composition was brain SM with no Chol. Similar to the  $GP_{340}$  variation, the  $1/P$  values for all membranes with Chol inclusion started similar, with the 9:1 membrane deviating first at around 30 °C to 35 °C followed by the 7:3 and 5:5 at around the 40 °C to 45 °C region of measurement. Unlike the relatively clear distinction that was observed with  $GP_{340}$ , the  $1/P$  values were more similar leading up to the transition temperature range of diversion, aside from the SM/Chol 10:0 liposome. Previous reports have indicated that compared to the large impact on Laurdan generalized polarization, Chol content had much less impact on DPH measurements (Stott et al., 2008). This may be due to the general insertion region of Laurdan vs the insertion region of DPH. Generally, Laurdan will insert into the interfacial region of the membrane while DPH will insert into the deeper acyl chain region of the membrane. Since our DPH ( $1/P$ ) data does not reveal any distinct abnormalities with temperature prior to the general phase transition temperature region of SM while Laurdan ( $GP_{340}$ ) does reveal the unique variation between the SM/Chol (7:3) and (5:5), we may assume that the primary effect or interactions of Chol on the membrane may be in the interfacial region prior to the phase transition of SM.

In the cartesian graphs that plotted  $GP_{340}$  vs  $1/P$ , both SM/Chol (7:3) and SM/Chol (5:5) were the compositions that remained in the ordered region when temperature was increased. Since Chol is responsible for ordering of the membrane, it is reasonable to conclude that the larger quantity of Chol resulted in the robustness of rigidity of the membrane at higher temperatures. Notably, however, there was a noticeable shift in the  $GP_{340}$  but not  $1/P$ , which suggests that the lipid compositions with 30% and 50% Chol may be undergoing phase transition while simultaneously staying in a relatively ordered state. Previous reports suggest that this may be the

$l_o$  phase and indicate that Chol inclusion in a membrane may induce different types of effects at low vs high temperatures (Schachter et al., 2022). Since the  $l_o$  phase is assumed to be a lipid raft precursor, we can speculate that from the variations in these binary compositions that lack lipid rafts, these SM/Chol interactions between 30% and 50% Chol may play a key role in establishing the environmental conditions necessary for raft formation to occur in more complex membrane systems.



**Fig. 2-5** Schematic depiction of the lipid membrane with incorporated Chol. Each peak corresponds to the representative solvent environment of a region in the membrane. Peak 1 would have a similar environment to the ordered acyl chain region, while peaks 2-4 are representative environments of the interfacial region from the bottom of the interfacial region to the head groups, respectively.

Lastly, deconvolution of the obtained Laurdan spectra into the 4 component peaks was a crucial step in strengthening the concept of the  $l_o$  phase (**Fig. 2-5**). When comparing component peaks of the Laurdan spectra with deconvolution analysis, it is noted that both SM/Chol (7:3) and (5:5) are also the most obvious compositions that maintain relatively similar area % across all 4 peaks as temperature is increased, whereas the other compositions shift dominance to the polar protic peaks primarily. This result suggests that there is a heterogeneous quality of the membrane's solvent environment as it suggests that there is both an ordered environment indicated with peak 1 (likely ordered acyl chains) and different magnitudes of a disordered environment represented

by peaks 2-4 (likely the interfacial region) which coincides and supports the idea of a  $l_o$  phase for these specific membrane compositions.

#### 4. Summary

Sphingomyelin and Chol are often implicated in the formation of lipid raft domains that play roles in the movement of proteins, membrane signaling, and membrane trafficking in complex membrane compositions. In this study's basic compositions inclusion of larger quantities of Chol were observed to cause unique polarity phenomena prior to the transition temperature of brain SM.  $GP_{340}$  from Laurdan and  $1/P$  from DPH plotted against each other on cartesian graphs indicate that both 30% and 50% Chol inclusion cause the SM membrane to remain in an ordered phase state. Deconvolution analysis supports the notion that for SM/Chol (7:3) and (5:5), there is heterogeneity present as the temperature is increased which is hypothesized to be the  $l_o$  phase. Overall, this work provides insights into the fluorescent characteristics that are commonly associated with the established precursor to lipid rafts, the  $l_o$  phase.

## **Chapter 3**

### **Analysis of interfacial membrane polarity of brain-related lipids via flattened tetrahedron phase diagram**

#### **1. Introduction**

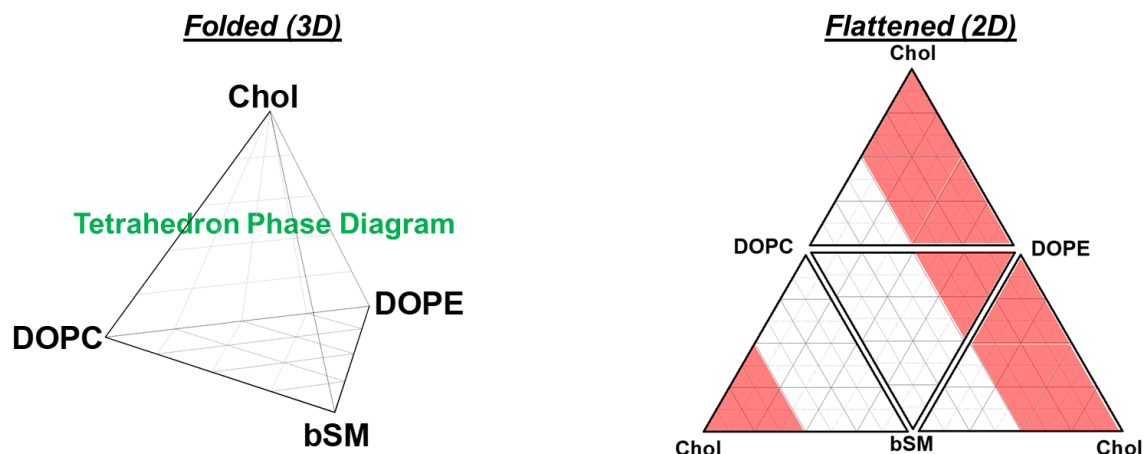
Plasma membranes are an important structure of cells that serve as an integral part of cellular homeostatic mechanisms such as transport and communication in addition to playing a vital role in acting as the cell's barrier (Ammendolia et al., 2021). One of the most widely accepted descriptions of the membrane's structure is the fluid mosaic model, which describes the membrane as solutions of oriented globular proteins and lipids (Singer & Nicolson, 1972). Although most membranes follow this basic model, there exist many unique characteristics of a membrane that can alter the overall qualities of its associated cell and allow the cell to be specialized. Neuronal membranes in particular are notable for their ability to propagate electrical signals with their high electrical resistance and capacitance and are thereby key factors in the central nervous system (CNS) (Lehninger, 1968). Damage to the functional ability of the neuron and neurodegeneration has been implicated in diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and multiple sclerosis (MS) among others (Dawson & Dawson, 2003; Frischer et al., 2009; Sciacca et al., 2018; Vonsattel & DiFiglia, 1998). Lipid homeostasis has been previously implicated in neurodegeneration and consequently suggests that the lipid membrane may thereby play a role in these brain diseases (Yadav & Tiwari, 2014). Due to the interwoven relationship between lipids, the membrane, and neurodegeneration, characterization of the

neuronal membrane is an important area of research that requires further clarification as a means to provide potential biomedical therapeutics to brain pathologies.

In brain lipidomic studies, about half of the dry mass of the brain is lipids with it also possessing the highest level of cholesterol (Chol) in the entire body, designating these molecules as a major contributor to brain function (Jin et al., 2019; Yoon et al., 2023). Among the variety of different brain lipids, the most dominant lipids of the brain include sphingolipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and Chol (Fitzner et al., 2020). A common method of isolating and analyzing the lipids of the membrane in the absence of other membrane structures is the use of small unilamellar vesicles (SUVs), also known as liposomes. Liposomes are commonly used as a biomimetic environment to study the biological membrane due to the simplicity of their structure alongside the ease of altering properties such as interfacial polarity and fluidity. Different lipid compositions of liposomes can be constructed that allow the researcher to study variations in characteristics such as polarity, fluidity, phase state, and hydrophobicity among others. By constructing liposomes with different compositions of brain membrane mimicking lipids, analysis may be performed on relevant brain membrane properties in isolation of proteins and other macromolecules that would be present in a living neuron. This study utilized porcine brain sphingomyelin (bSM), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and Chol as designated lipids to mimic commonly occurring brain lipid membranes. Although both DOPC and DOPE are synthetic lipids, DOPC's wide previous use, established behaviors across past research, and basic fundamental properties has made it a primary candidate for this study due to the ability to readily compare results in this study to those in previous studies, with DOPE being used thereafter for consistency with the selection of DOPC. Compared to biological lipids such as bSM, however, it must be noted

that the synthetic lipids used in this study do not have a distribution of variations within a lipid species in such things as chain length, which in biological systems would generally make a phase change occur over a range of temperatures rather than a fixed point. Thus, the proceeding experiments are not wholly representative of a biological system but rather an estimation of the properties of some of these systems.

Interfacial membrane polarity is a key property in understanding the overall structural characteristics of a specific lipid membrane since it can convey whether the membrane is in an ordered or disordered state. In this study, membrane polarity is examined and hereafter defined by the hydration state of the lipid membrane via the use of the 6-lauroyl-2-dimethylamino naphthalene (Laurdan) fluorescent probe. Laurdan is commonly used to probe the hydration state of the membrane by its fluorescence emission, which is sensitive to its surrounding solvent environment and quantified by generalized polarization ( $GP_{340}$ ) (Parasassi et al., 1990, 1998; Watanabe et al., 2019). Laurdan possesses an amphipathic structure and is thereby able to integrate itself into a lipid membrane at the interface of the hydrophilic head group and the hydrophobic tail. Through excitation, the Laurdan fluorophore moiety exhibits a large dipole moment that is relaxed by the surrounding solvent environment and is represented by the fluorescence emission (Parasassi et al., 1998; Vequi-Suplicy et al., 2014; Watanabe et al., 2019).



**Fig 3-1.** Folded 3D tetrahedron (left) and the flattened 2D tetrahedron (right). This work focuses on the flattened 2D variant. Highlighted red regions depict the compositions that are difficult to maintain a stable membrane due to the overabundance of small-head lipids (Chol and DOPE).

Ternary phase diagrams have commonly been utilized to display the changes in membrane characteristics such as fluidity and polarity. Conventional ternary phase diagrams, however, are limited to displaying a maximum of only three components per diagram, making it difficult to track trends across related ternary diagrams. Tracking these trends is an important preliminary step before increasing complexity of the membrane to biological relevance as these trends will first establish the basic relationships between multiple lipid components by thoroughly displaying how certain properties may vary across compositions of related ternary mixtures. Although previous publications have already proposed and emphasized the next step, the usefulness in developing a 3D tetrahedron in the analysis of lipid phases of quaternary lipid mixtures or in some surfactant related research studies, it is yet to be widely adapted or its concept explored (Ackerman & Feigenson, 2015; Hu et al., 2024; Konyakhina et al., 2013; Salager et al., 2005). Compared to previous publications that dealt with directly establishing a 3D tetrahedral representation, however, this study as previously noted has the aim to act as preliminary work for such a tetrahedral diagram by first confirming that the relationship between the 4 ternary diagram faces of the tetrahedron can be readily compared and remain consistent prior to work on the interior. Additionally, this work

focuses on interpretation of the interfacial membrane polarity by detection of the hydration state by the Laurdan fluorescent probe for brain lipid analogs, an alternative approach that emphasizes the analysis and characterization of the polarity of specific organ-relevant lipids for potential use in therapeutics. Thus, this work aims to ultimately build into the concept of a 3D tetrahedron by first proposing an unfolded or henceforth “flattened tetrahedron phase diagram” as a precursor to a tetrahedron to more thoroughly reveal membrane polarity trends across 4 separate but related membrane compositions (**Fig. 3-1**). Folding of the flattened two-dimensional tetrahedron phase diagram will hereafter enable more reliable and consistent analysis of ternary diagrams prior to the progression into complex four component membrane systems. This study’s logic has the potential to be applied in future works that equal or exceed 4 components into other geometric shaped phase diagrams via modern techniques such as machine learning and is thus an important area of preliminary and fundamental work in the eventual analysis of complex membrane systems.

## **2. Experimental Section**

### **2.1. Materials**

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) lipids were purchased from NOF Corporation (Tokyo, Japan). Porcine brain sphingomyelin (bSM) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) and 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan) fluorescent probe was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,6-diphenyl-1,3,5-hexatriene (DPH) and any other relevant chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

## 2.2. Liposome Preparation

Liposome solutions were made for each relevant lipid membrane according to the following protocol: stock solution of bSM, DOPC, DOPE, and Chol were dissolved with chloroform. An arbitrary amount of stock solution, according to the desired lipid ratio, was transferred to a round bottom flask and rotary evaporated under vacuum conditions until a clear lipid film was visible. This film was placed in a vacuum environment for a minimum of 2 hours after which the film was rehydrated with DI water. This solution then underwent 5 cycles of freeze (-80 °C) and thaw (60 °C) for the homogenous formation of the lipid vesicles. This solution was finally extruded via extrusion apparatus (Liposofast, Avestin Inc., Ottawa, ON, CA) using a 100 nm pore filter to create the small unilamellar vesicles (SUVs).

## 2.3. Laurdan and DPH Sample Preparation

Laurdan and DPH liposome samples were prepared in replicates of 3 for each unique lipid composition, generally with one stock liposome solution used to make the 3 replicates.  $GP_{340}$  and  $1/P$  values were the average of these 3 Laurdan and DPH replicates, respectively. Error (STDEVA) was also calculated based on the 3 replicates and plotted on relevant figures accordingly (omitted on contour figures). Laurdan or DPH dissolved in ethanol (100  $\mu$ M) was added in a 1:100 ratio of Laurdan:lipid or 1:250 ratio of DPH:lipid according to previous protocols, with DI water used for relevant dilution to achieve the desired ratio (Suga & Umakoshi, 2013; Watanabe et al., 2019). Each sample was then incubated for a minimum of 30 minutes in the 25 °C – 37 °C range for probe insertion into the membrane. Samples were shielded from light during and after the incubation processes as much as possible to prevent any possible influence on the lipid's structure or fluorescence of the probes. Samples were measured using a spectrophotometer (FP-8350; JASCO,

Tokyo, Japan). Each sample underwent multiple measurements from 20 °C to 70 °C in increments of 5 °C also with minimal exposure to light.

#### 2.4. Evaluation of Membrane Polarity via Generalized Polarization ( $GP_{340}$ )

Laurdan samples were measured by exciting the probe at 340 nm wavelength and measuring the emission at wavelengths 400-600 nm. Polarity values were extracted from the spectrophotometer based on the intensity values of each Laurdan sample at  $I_{440}$  and  $I_{490}$ , the fluorescence intensities at 440 nm (hydrophobic region) and 490 nm (hydrophilic region), respectively. Each sample composition was measured over a temperature range of 20 °C to 70 °C with an incremental adjustment of 5°C. These values were then used to estimate the membrane generalized polarization ( $GP_{340}$ ) at each temperature using the following equation (**Eqn. 3-1**):

$$GP_{340} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (3-1)$$

This value was then plotted vs the temperature to display the influence of temperature change on the generalized polarization of each different composition of liposome (**Fig. 3-8**).

#### 2.5. Evaluation of Membrane Fluidity via Inverse Polarization ( $1/P$ )

Membrane fluidity was evaluated with DPH according to previously established protocols. DPH samples were excited with vertically polarized light at 360 nm and emission intensities both perpendicular ( $I_{\perp}$ ) and parallel ( $I_{\parallel}$ ) to the excited light were recorded at emission wavelength 430 nm. Polarization ( $P$ ) was calculated according to **Eqn. 3-2 and Eqn. 3-3** where ( $G$ ) is the correction factor, and ( $i_{\perp}$ ) and ( $i_{\parallel}$ ) are the emission intensity perpendicular and parallel to the horizontally polarized light, respectively. To obtain a more linear relationship between temperature

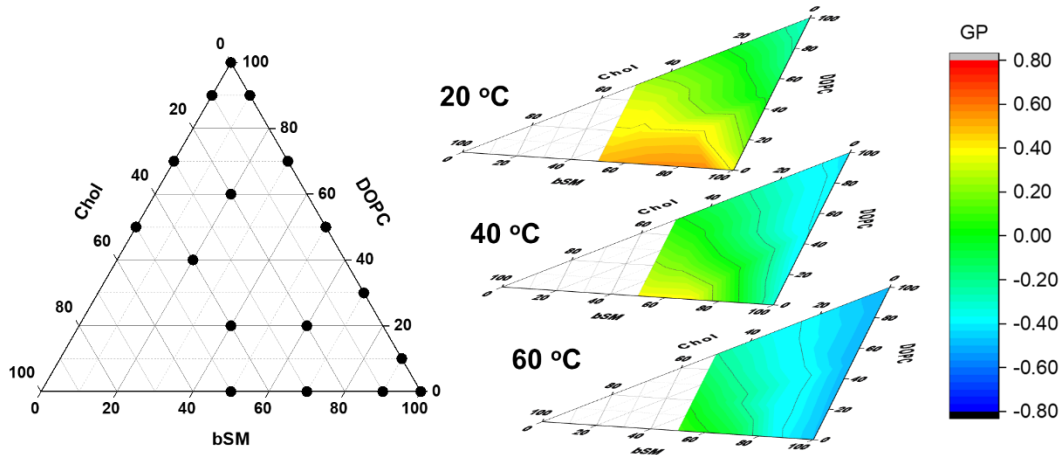
and polarization the inverse of polarization ( $1/P$ ) was plotted for any relevant figures.  $1/P$  was plotted vs temperature to then examine the influence of temperature on the fluidity (**Fig. 3-9**).

$$P = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + GI_{\perp}) \quad (3-2)$$

$$G = i_{\perp} / i_{\parallel} \quad (3-3)$$

### 3. Results & Discussion

#### 3.1. bSM/DOPC/Chol

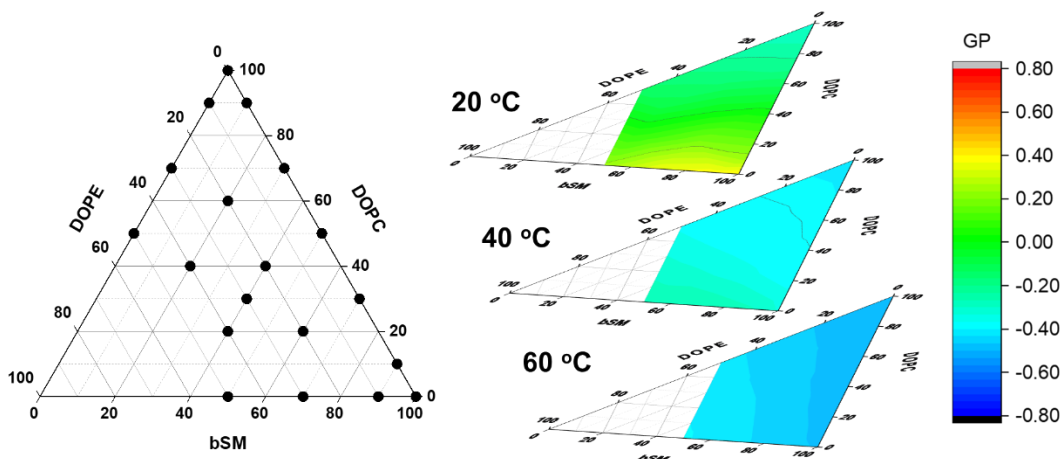


**Fig. 3-2 (a)** Ternary diagram with points representing the measured ratios for each composition of bSM/DOPC/Chol. **(b)** Ternary heat contour representing changes to the  $GP_{340}$  values of bSM/DOPC/Chol mixtures at 20/40/60 °C.

$GP_{340}$  variation of bSM/DOPC/Chol at 20 °C, 40 °C, and 60 °C are depicted using a heat contour to visualize changes to polarity that coincide with temperature increase (**Fig. 3-2b**). Among the three lipids that were used in this first ternary mixture, DOPC is the most fluid/disordered at the start of the measured temperature range (20 °C) due to its low transition temperature (-17 °C) compared to that of bSM (35 – 40 °C). Within the set of compositions, the more rigid nature of both bSM and Chol result in the highest  $GP_{340}$  values with their increased ratios in the membrane while increasing DOPC ratios resulted in lower  $GP_{340}$  values. As the

temperature range further exceeded the transition temperatures, the  $GP_{340}$  values of every analyzed composition expectedly decreased and was correlated to low membrane polarity.

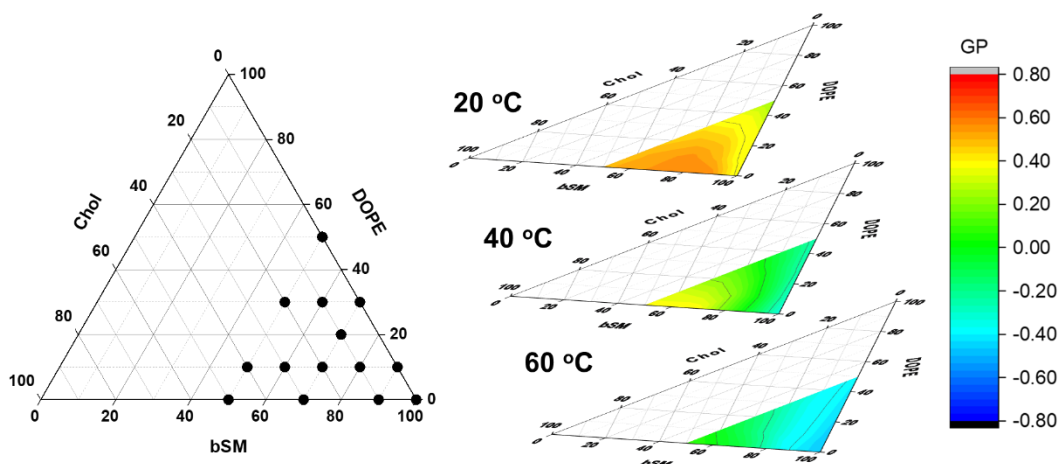
### 3.2. bSM/DOPC/DOPE



**Fig. 3-3 (a)** Ternary diagram with points representing the measured ratios for each composition of bSM/DOPC/DOPE. **(b)** Ternary heat contour representing changes to the  $GP_{340}$  values of bSM/DOPC/DOPE mixtures at 20/40/60 °C.

$GP_{340}$  variation of bSM/DOPC/DOPE at 20 °C, 40 °C, and 60 °C are depicted using a heat contour to visualize changes to polarity that coincide with temperature increase (**Fig. 3-3b**). Ternary mixtures of bSM/DOPC/DOPE possessed two fluid/disordered lipids (DOPC and DOPE) at the starting temperature of the measurement range. DOPE has a low transition temperature (-16 °C) and thereby, like DOPC, is a contributor in lowering the  $GP_{340}$  value with its increased ratio in the membrane compositions. Increasing the bSM ratio once again results in the highest  $GP_{340}$  values among the analyzed compositions. Once the transition temperature threshold of bSM is exceeded, interestingly, certain compositions with abundance of DOPE have a slightly higher  $GP_{340}$  which indicates slight ordering by DOPE at higher temperatures, most readily observed at 60 °C.

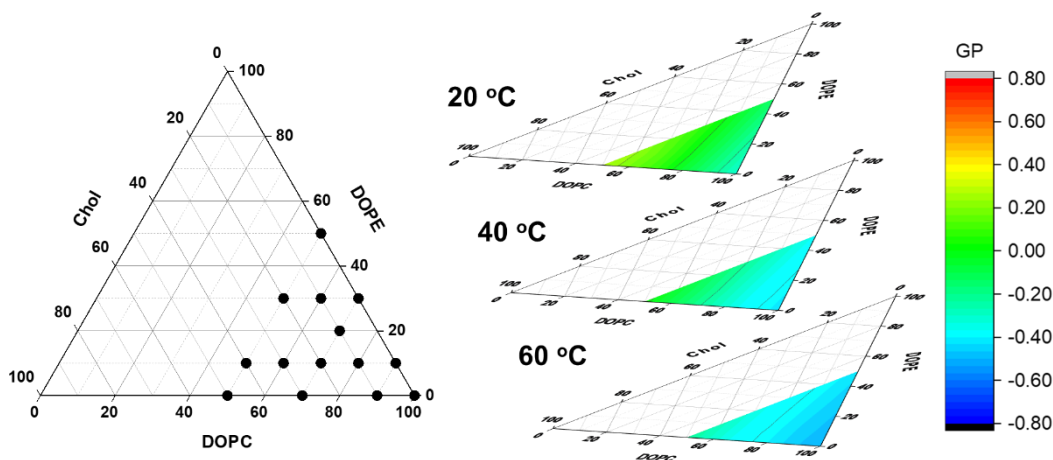
### 3.3. bSM/DOPE/Chol



**Fig. 3-4 (a)** Ternary diagram with points representing the measured ratios for each composition of bSM/DOPE/Chol. **(b)** Ternary heat contour representing changes to the  $GP_{340}$  values of bSM/DOPE/Chol mixtures at 20/40/60 °C.

$GP_{340}$  variation of bSM/DOPE/Chol at 20 °C, 40 °C, and 60 °C are depicted using a heat contour to visualize changes to polarity that coincide with temperature increase (**Fig. 3-4b**). Ternary mixtures of bSM/DOPE/Chol were unique in that both DOPE and Chol are considered “small-head” lipids and thereby cannot be the primary component (> 50%) of a membrane due to the restriction of their head groups being unable to interact due to their size, thus being unable to form a stable membrane without the inclusion of a “large-head” lipid such as bSM or DOPC. These ternary mixtures once again implicated bSM and Chol as the primary components that increase the  $GP_{340}$  value. DOPE, conversely, was the lipid component that was the most fluid/disordered due to its low transition temperature. Similar to the bSM/DOPC/Chol mixture, this bSM/DOPE/Chol mixture revealed increased bSM and Chol inclusion to be the most relevant component in the highest  $GP_{340}$  value across the temperature range while increased DOPE caused the lower  $GP_{340}$  values, aside from the higher temperature range such as 60 °C where once again there was a rigid-like effect by DOPE compared to pure bSM membranes.

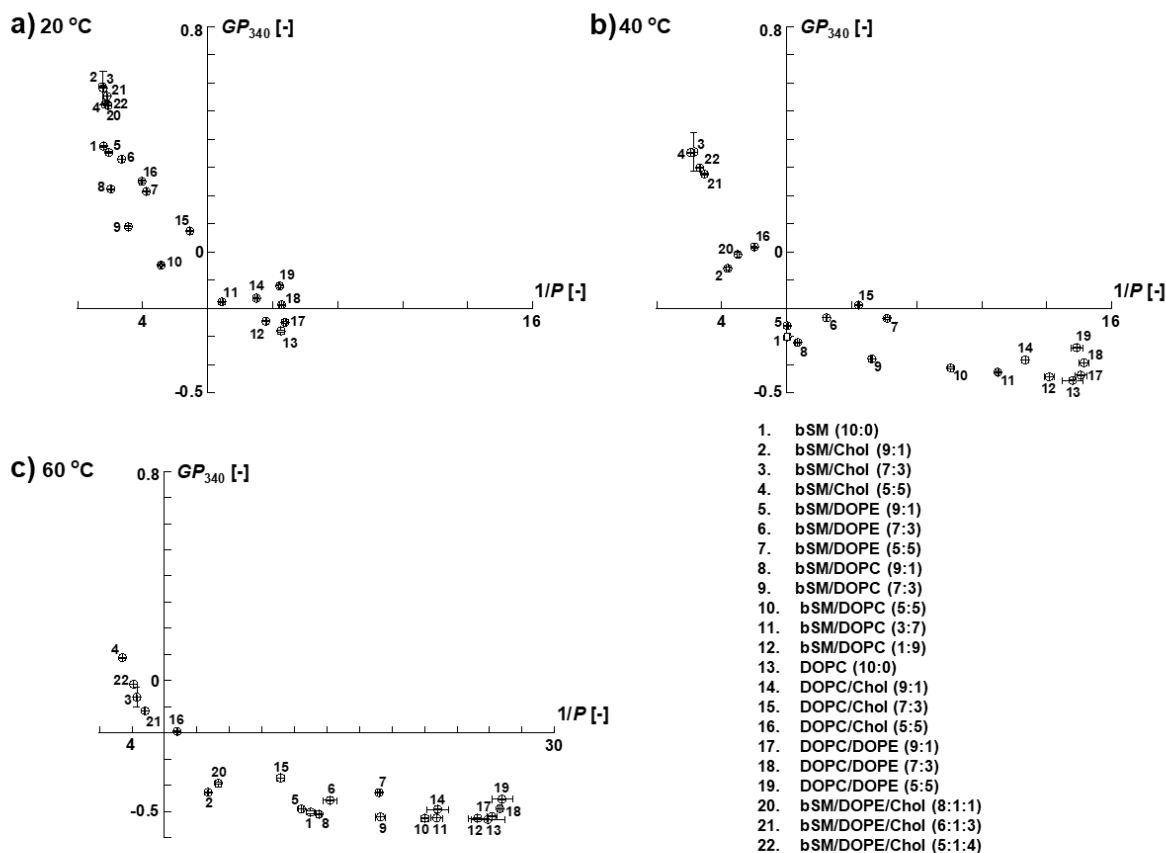
### 3.4. DOPC/DOPE/Chol



**Fig. 3-5 (a)** Ternary diagram with points representing the measured ratios for each composition of DOPC/DOPE/Chol. **(b)** Ternary heat contour representing changes to the  $GP_{340}$  values of DOPC/DOPE/Chol mixtures at 20/40/60 °C.

$GP_{340}$  variation of DOPC/DOPE/Chol at 20 °C, 40 °C, and 60 °C are depicted using a heat contour to visualize changes to polarity that coincide with temperature increase (**Fig. 3-5b**). Unlike the ternary mixtures with bSM, since DOPC and DOPE are both in a fluid/disordered state in the temperature range of measurements, the resulting  $GP_{340}$  values were all relatively low. In this set of data, Chol was the main contributor in the increasing  $GP_{340}$  values. Although Chol still acted to increase the  $GP_{340}$ , resulting values still remained primarily fluid/disordered due to DOPC being the only “large-head” lipid in the membrane compositions. Although much more slight than the bSM membranes, DOPE did also increase the  $GP_{340}$  compared to pure DOPC.

### 3.5. Cartesian coordinate analysis of $GP_{340}$ vs. $1/P$ on the flattened tetrahedron phase diagram borders

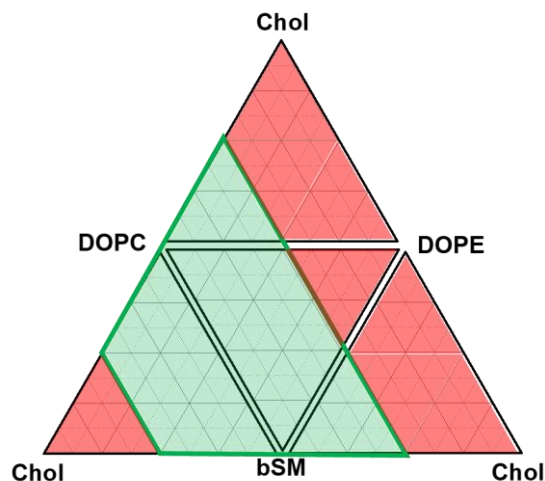


**Fig. 3-6** Cartesian diagrams of  $GP_{340}$  vs  $1/P$  of binary membrane compositions and several ternary compositions at **(a)** 20 °C **(b)** 40 °C and **(c)** 60 °C.

Membrane phase at the borders of each ternary diagram given by the  $GP_{340}$  value of binary liposome samples were further examined with DPH; the fluidity given by the  $1/P$  value of binary compositions was plotted against the  $GP_{340}$  in a cartesian coordinate plane with each quadrant representing a specific phase state boundary as determined in previous research (Suga & Umakoshi, 2013). Specific figures for both  $GP_{340}$  vs temperature and  $1/P$  vs temperature are shown later in **Figure 3-10**. Each quadrant of the cartesian coordinate system pertains to the order of a membrane: quadrant I (heterogeneous), quadrant II (ordered), and quadrant IV (disordered). Expectedly, increasing the temperature during measurement caused both the membrane polarity and fluidity to

increase (**Fig. 3-6**). Prior to the transition temperature of bSM, binary compositions with a dominance of bSM and/or Chol aggregated in quadrant II, confirming that bSM and Chol are the main contributions to the ordered phase. Measurements at 40 °C and 60 °C support this concept in that the few compositions remaining in quadrant II contain a large amount of Chol in a primarily bSM focused membrane. This information moreover aligns with most of the behavior observed at the border of the ternary phase contour diagrams. Notably, however, membranes of both bSM and DOPC that contain DOPE increase their fluidity much more readily than membranes with Chol. Whereas DOPC/DOPE  $1/P$  values are more clustered with temperature increase, bSM/DOPE show that increased DOPE clearly increase the  $1/P$  values, which seems to directly contradict the heat contour diagrams. However, since DPH inserts into a deeper region of the membrane than Laurdan, it can be assumed that both observations are true, with DOPE primarily retaining order in the interfacial region of the membrane while the deeper regions of the membrane still become fluid with temperature increases.

### 3.6. General Discussion

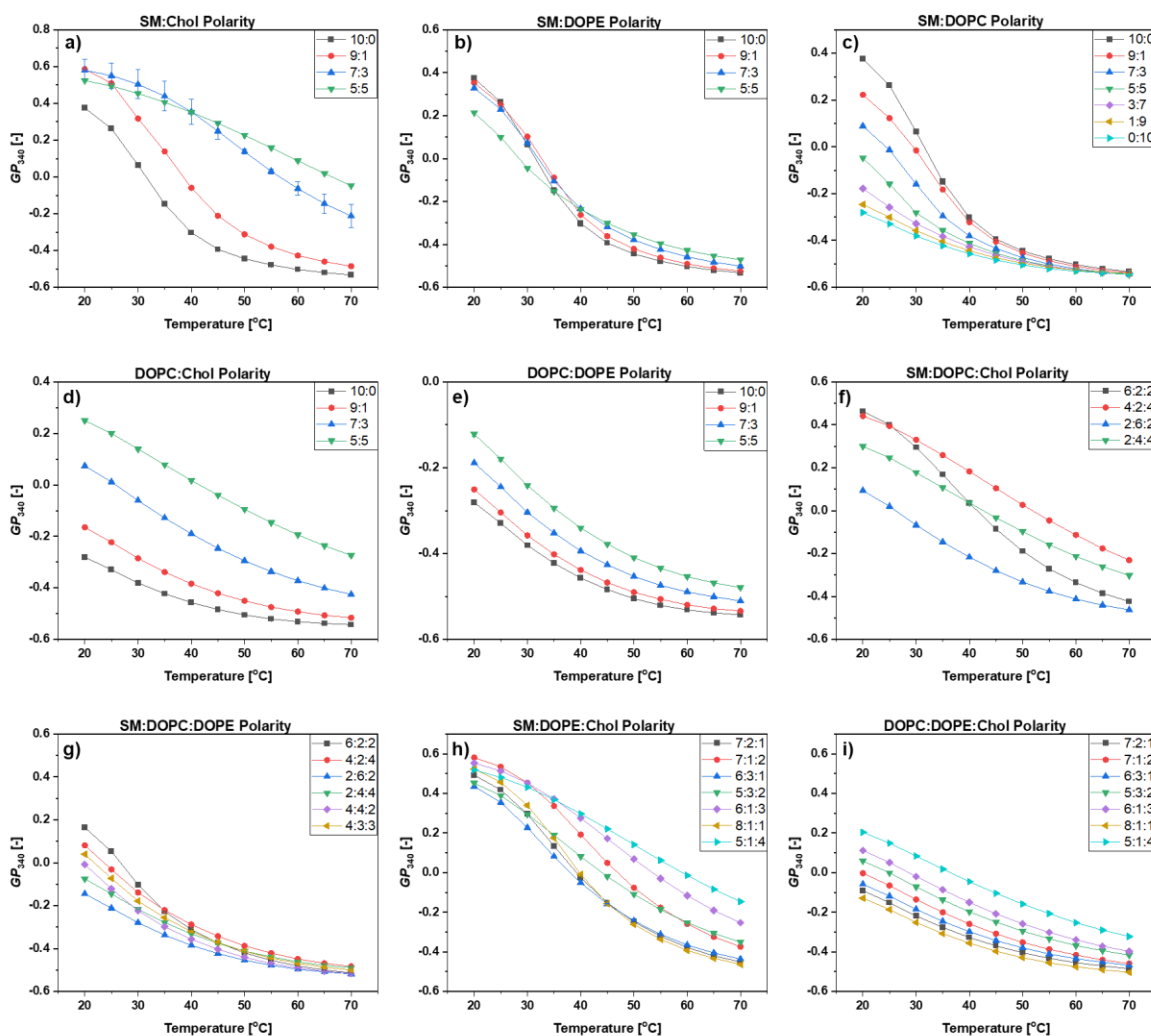


**Fig. 3-7** Flattened tetrahedron diagram representing the measured membrane region (green) and the region with over 50% DOPE or Chol (red) where the membrane is unable to be formed due to structural constraints.

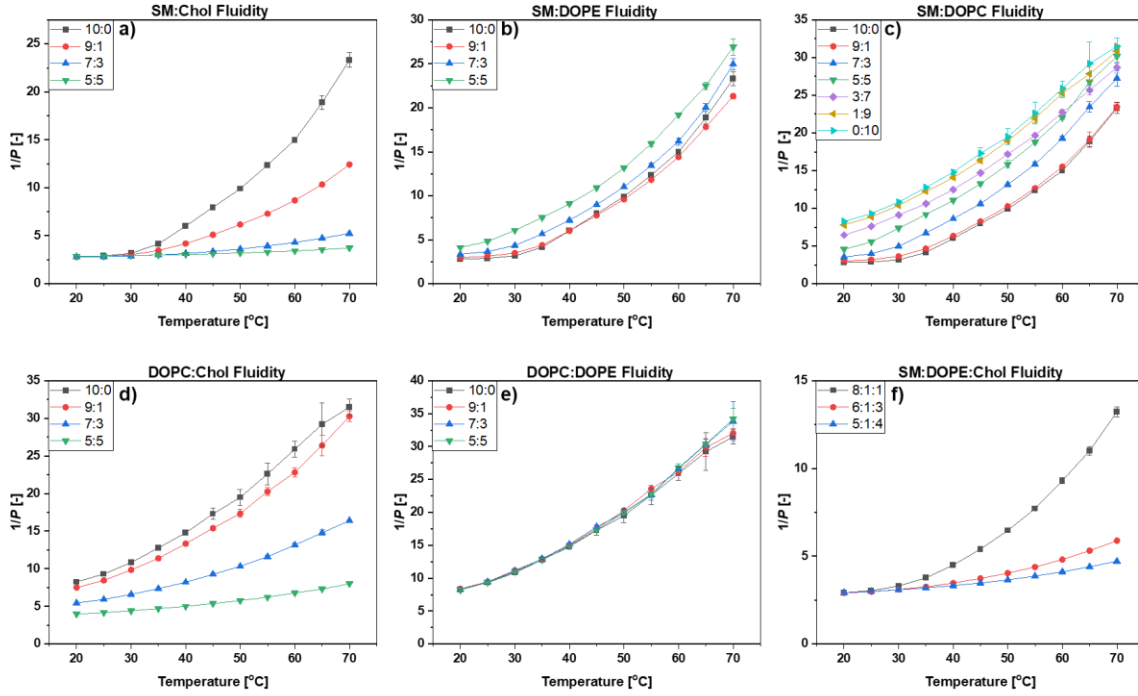
There have been numerous previous publications that have employed the use of phase diagrams to represent the trends observed in 3 lipid component membrane systems, however the inability to display more than three components per diagram therein is their fundamental limitation (de Almeida et al., 2003b; Feigenson, 2009; Marsh, 2009; Nyholm et al., 2011; Veatch & Keller, 2005). Although these past publications have revealed important trends in phase variation across their respective ternary phase diagrams, it may be both difficult and tedious to track phase trends across multiple independent phase diagrams within the same publication as well as with other publications. Before proceeding to methodology to analyze membrane systems exceeding 3 components (3D models), we must first ensure that the current methodology is utilized to its maximal potential. Herein we propose the use of a “flattened tetrahedron phase diagram” composed of 4 separate ternary phase diagrams that possess mutual overlapping components that allow the diagrams to be joined together at their borders as important and relevant methodology in analysis of lipid phase behavioral trends (**Fig. 3-7**). This flattened tetrahedron phase diagram is a pivotal preliminary step before establishing a quaternary analysis system because of both its ability

to display trends of different lipid compositional phase variations across the borders of separate phase diagrams while also allowing for the inclusion of a 4<sup>th</sup> lipid component in the 2D ternary space. Hereafter we will discuss the trends observed in the 4 faces of the tetrahedron and how adjoining these faces into the flattened tetrahedron is beneficial for future scientific analytical works.

### 3.7. Temperature Variation on Polarity ( $GP_{340}$ ) and Fluidity ( $1/P$ )



**Fig. 3-8 (a)-(i)**  $GP_{340}$  vs temperature for analyzed compositions in this work.

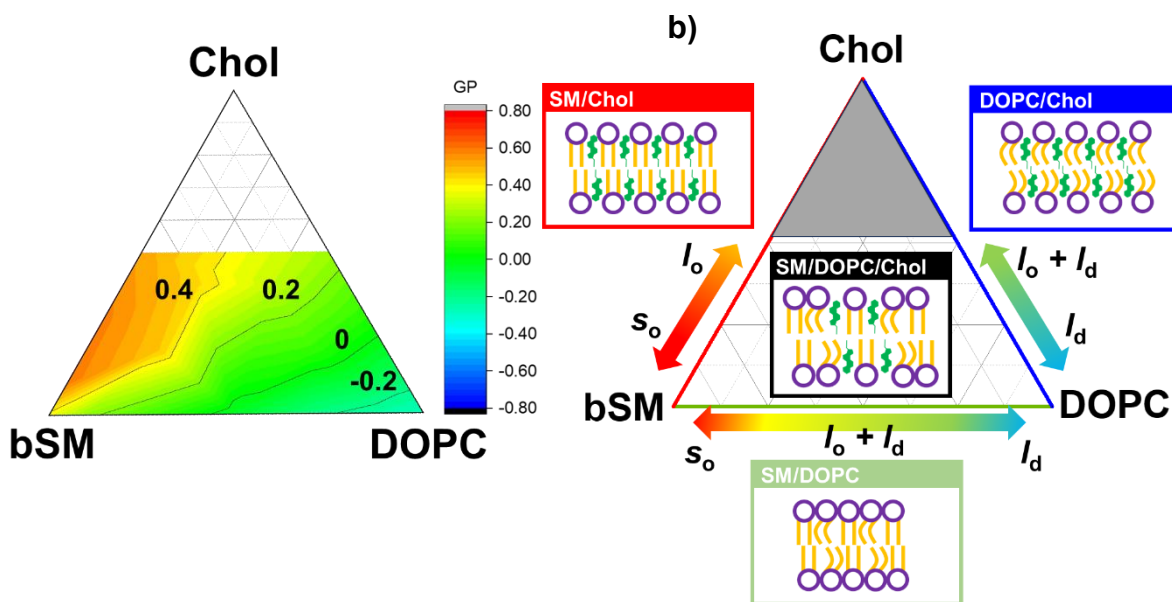


**Fig. 3-9 (a)-(f)**  $1/P$  vs temperature for several of the analyzed compositions in this work.

Lipids generally have different transition temperatures ( $T_m$ ) at which they transition from a gel phase to a liquid crystalline phase. This work measured the polarity of bSM, DOPC, DOPE, and Chol in the range of 20 °C to 70 °C. At the given measured temperature range, both DOPC ( $T_m = -17$  °C) and DOPE ( $T_m = -16$  °C) are already in a disordered phase while bSM ( $T_m = 35 - 40$  °C) starts at the ordered phase and transitioned during the measurement range. Chol does not have a set  $T_m$  within the measured range and is thereby used solely as a component meant for mediating order in the membrane. Due to the aforementioned  $T_m$  information, most membranes with dominance of DOPC/DOPE, due to their pre-existence in a disordered phase at the start of measurement, also become disordered readily. Past research has suggested that at lower temperatures, Chol induces disorder in the bSM membrane while inducing order at higher temperatures (Schachter et al., 2022). Interestingly, DOPE had a similar effect in inducing disorder to bSM at lower temperatures while slightly ordering bSM at higher temperatures, specifically

displayed with  $GP_{340}$  vs temperature (**Fig. 3-8**). When observing  $1/P$ , however, increased DOPE in the bSM membrane specifically seemed to contradict the initial observation by increasing fluidity, however, this may be attributed to the deeper location of DPH compared to Laurdan in the membrane, suggesting DOPE primarily maintains interfacial membrane order (**Fig. 3-8 & 3-9**). Overall, however, temperature increases maintained their expected effect of disordering the membrane by increasing both the polarity and fluidity. Membranes dominant in bSM and/or Chol overall seemed to be the most robust in remaining in the ordered phase.

### 3.8. Face 1: bSM/DOPC/Chol

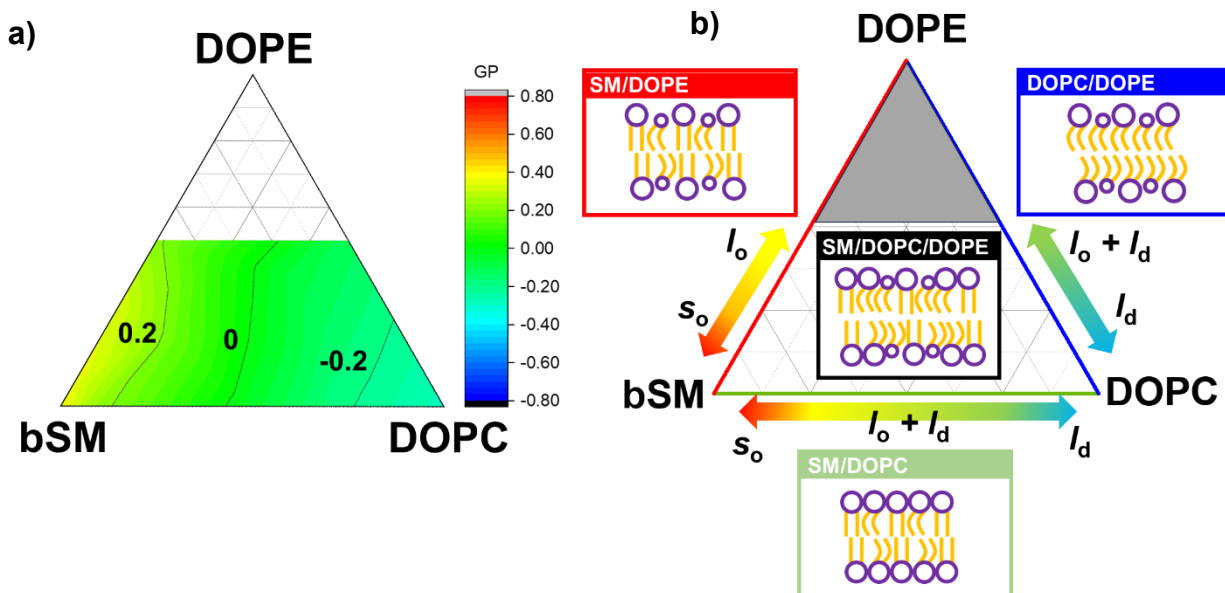


**Fig. 3-10 a)** Contour phase diagram at 20 °C, contour lines given in increments of 0.2. **b)** Hypothetical depiction of the liposome membranes for each given composition at the start of measurement (20 °C): Isolated bSM is assumed solid-ordered ( $s_o$ ), increased integration of Chol into bSM maintained a strong ordered phase, however this could also be the  $l_o$  phase. Increased integration of Chol into DOPC sustained a heterogeneous  $l_o$  phase and  $l_d$  phase.

Membranes with varying ratios of bSM, DOPC, and Chol were analyzed for the contrast in phase qualities of their component lipids, with Chol acting as a modification component for the former two stable membranes. Phases are abbreviated as follows:  $s_o$  (solid-ordered),  $l_o$  (liquid-ordered), and  $l_d$  (liquid-disordered). Classification and nomenclature of phase states

hereafter is adapted primarily on the calculated  $GP_{340}$  values [ $GP_{340} > 0$ , ordered ( $s_o$  or  $l_o$ );  $-0.2 < GP_{340} < 0$ , heterogeneous ( $l_o + l_d$ );  $GP_{340} < -0.2$ , disordered ( $l_d$ )] according to previously established protocol that utilizes a cartesian coordinate system similar to **Figure 3-6** (Suga & Umakoshi, 2013). These adapted bounds for the  $GP_{340}$  only serve as rough approximations for this study's points that are lacking  $1/P$  values. Previous publications using separate techniques such as Förster resonance energy transfer (FRET) have confirmed heterogeneous phase coexistence in bSM/DOPC/Chol membranes at temperature below 35 °C (Petruzielo et al., 2013). Other publications, although using different variants of SM (i.e. PSM/egg SM) instead of bSM, also show that there is a heterogeneous phase occurring below the transition temperature of SM (Nyholm et al., 2011; Veatch & Keller, 2005). At 20 °C (starting temperature), the majority of DOPC dominant face 1 compositions are roughly approximated via  $GP_{340}$  to be in the ( $l_d$ ) phase or heterogeneous phase ( $l_o + l_d$ ), agreeing with the aforementioned previous publications (**Fig. 3-10a**). Compositions containing higher ratios of bSM and Chol were distinctly in an ordered phase, which is assumed to be  $s_o$  or  $l_o$ . Although there are slight variations, most of the calculated  $GP_{340}$  values are consistent with previous works that indicate this ternary mixture as heavily reliant on DOPC for disordering and specific ratios of Chol for ordering or disordering of the membrane.

### 3.9. Face 2: bSM/DOPC/DOPE

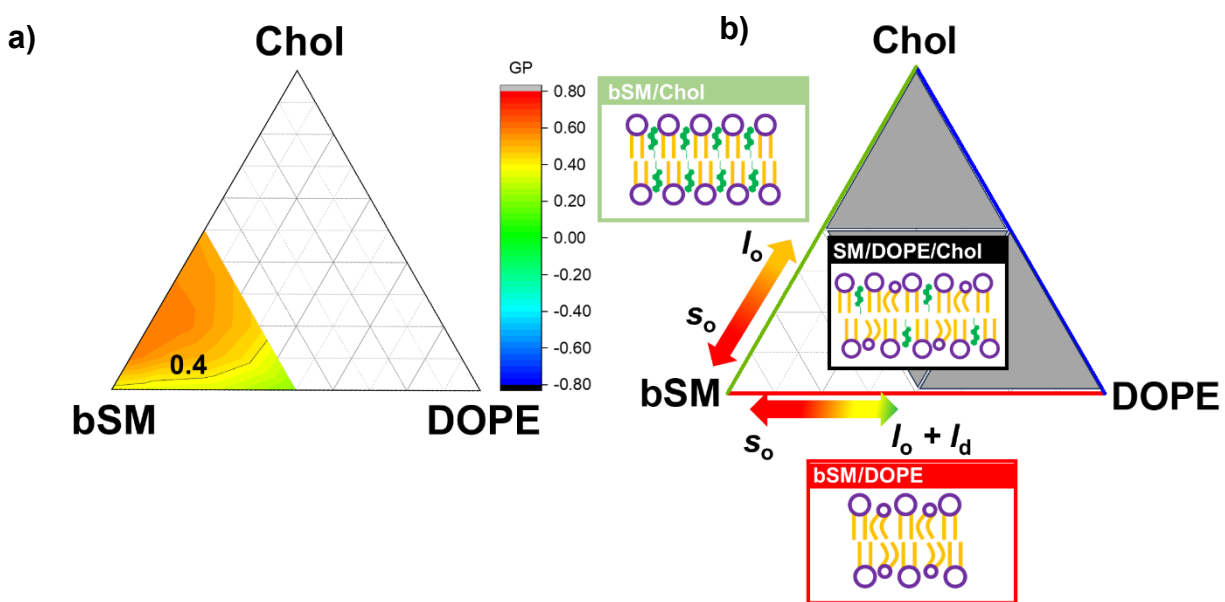


**Fig. 3-11 a)** Contour phase diagram at 20 °C, contour lines given in increments of 0.2. **b)** Hypothetical depiction of the liposome membranes for each given composition at the start of measurement (20 °C). Isolated bSM is solid-ordered ( $s_o$ ). Integration of DOPE into bSM introduced a liquid-ordered ( $l_o$ ) phase alongside the existing solid-ordered ( $s_o$ ) phase while integration into DOPC introduced an  $l_o$  phase into the existing  $l_d$  phase.

Most previous research chooses to emphasize Chol as the primary structural component of lipid membranes due to both its abundance in lipidomic studies as well as its unique interactions with bSM resulting in interesting phenomena such as lipid rafts. However, a less studied lipid class of the membrane that is the most abundant following PC and Chol is the PE lipid class (Fitzner et al., 2020). Past work has emphasized the importance of PE lipids in such things as gene therapy as well as various cellular homeostatic functions (Mochizuki et al., 2013; Vance & Tasseva, 2013). This work ultimately has the aim to establish a 4 lipid analysis of common brain lipid classes, thus DOPE was selected as the 4<sup>th</sup> major component. DOPE surprisingly provides a similar role to Chol as a “helper” lipid. Increased addition of DOPE to DOPC membranes made the membranes heterogeneous rather than changing the entire membrane dynamic at 20 °C (**Fig. 3-11a**). For bSM, increased addition of DOPE induced slight disorder at

20 °C but not enough to significantly alter membrane phase. Since both DOPC and DOPE have very similar transition temperatures (-17 °C and -16 °C, respectively), it is interesting to note that certain ratios of DOPE provides some order to bSM at higher temperatures and order to the DOPC membrane across all temperatures, showing that its function may be analogous to Chol, specifically at the membrane interfacial region. Since the ternary composition has 2/3 of the components (DOPC and DOPE) already existing well past their  $T_m$ , most ternary compositions favored disorder or heterogeneity with bSM acting to provide order to these domains.

### 3.10. Face 3: bSM/DOPE/Chol

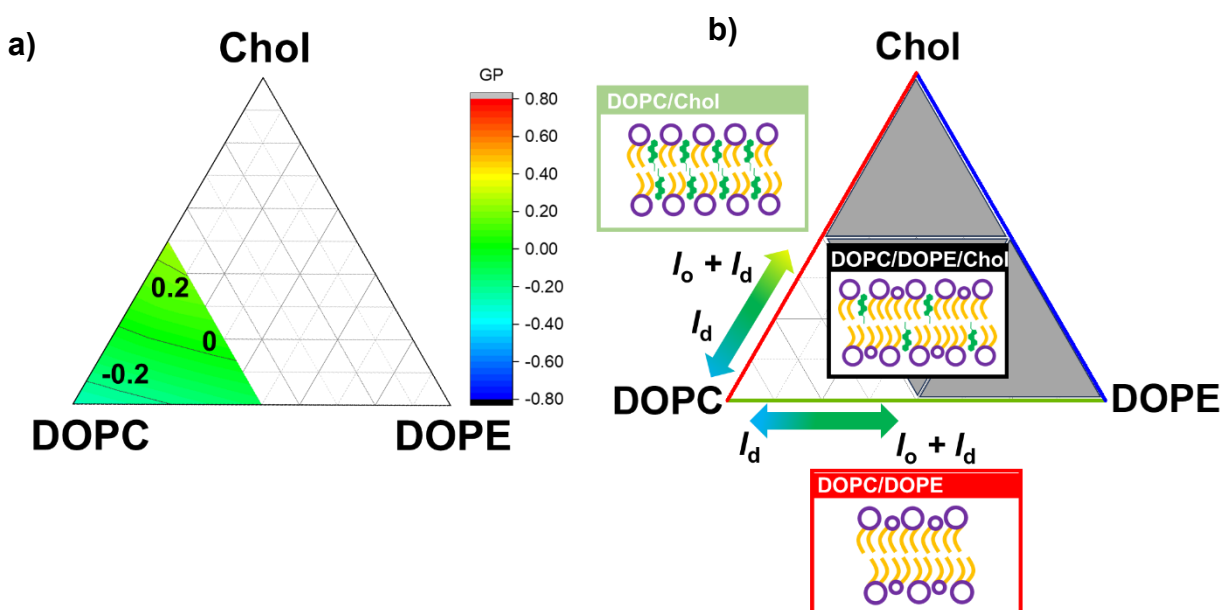


**Fig. 3-12 a)** Contour phase diagram at 20 °C, contour lines given in increments of 0.2. **b)** Hypothetical depiction of the liposome membranes for each given composition at the start of measurement (20 °C). Isolated bSM is solid-ordered ( $s_o$ ). Integration of Chol or DOPE into bSM introduced a liquid-ordered ( $l_o$ ) phase alongside the existing solid-ordered ( $s_o$ ) phase.

Compositions consisting of bSM, DOPE, and Chol were contained in a smaller region of the ternary phase diagram due to the nature of Chol and DOPE dominant membrane's being unable to maintain a stable structure, thereby the sum of DOPE and Chol could not exceed 50% of the membrane composition. Binary compositions of the axes of this phase diagram were discussed in

the face 1 and 2 discussions and thereby the focus of face 3 will be on the behavioral characteristics of the ternary compositions. Ternary compositions of bSM/DOPE/Chol always have at least 50% inclusion of bSM and are thereby revealed to remain within the ordered domain at 20 °C (**Fig. 3-12a**). However, increased integration of Chol and DOPE had an increased possibility of an  $l_o$  domain in the membrane as temperature was raised.

### 3.11. Face 4: DOPC/DOPE/Chol



**Fig. 3-13 a)** Contour phase diagram at 20 °C, contour lines given in increments of 0.2. **b)** Hypothetical depiction of the liposome membranes for each given composition at the start of measurement (20 °C). Isolated DOPC is liquid-disordered ( $l_d$ ). Integration of DOPE into DOPC introduced a liquid-ordered ( $l_o$ ) phase alongside the existing liquid-disordered ( $l_d$ ) phase while integration of Chol into DOPC introduced an  $l_o$  phase into the existing  $l_d$  phase.

Ternary compositions of DOPC/DOPE/Chol at the start of measurement (20 °C) seemed to be in the heterogeneous ( $l_o + l_d$ ) or ( $l_d$ ) phase when the dominant lipids are DOPC and DOPE (**Fig. 3-13a**). Both Chol and DOPE, in this instance, served to provide some order to the fluid membranes, which suggests that the membrane transitions from primarily  $l_d$  to heterogeneous. Although this was expected of the Chol, which generally is used to provide order to membranes, the fluid DOPE

surprisingly brought more order to the DOPC membrane. This might possibly be due to the small head group of DOPE slightly interfering with mobility of the fluid head groups of DOPC and ultimately acting to provide order by restricting the mobility of the membrane. Main points discussed for all the faces are summarized in **Table 3-1**.

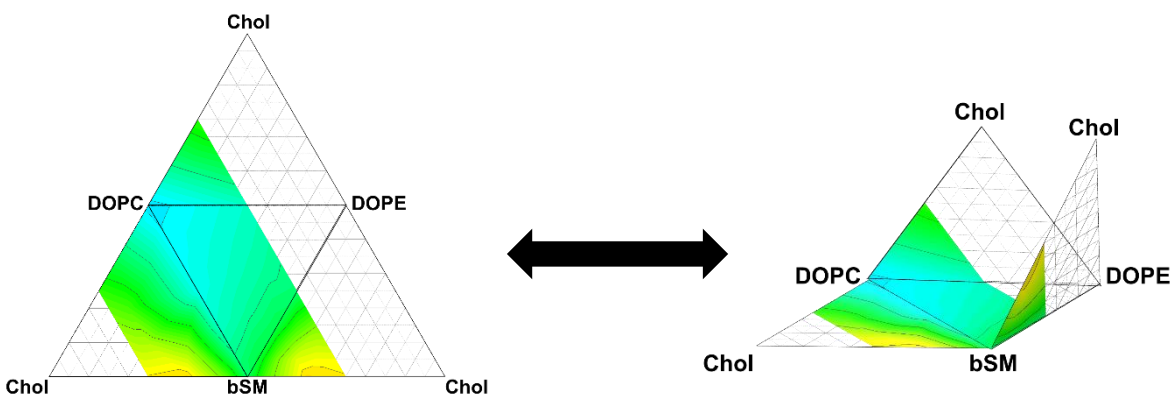
### 3.12. Summary of the 4 faces

| Face               | Previous Research                                                                                                                                                                                                                                                                                      | This Research                                                                                                                                                                                                                                   |
|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 (bSM/DOPC/Chol)  | <ol style="list-style-type: none"> <li>1. Lipid rafts from SM and Chol interaction (de Almeida et al., 2003b).</li> <li>2. Dominant heterogeneous phase (Nyholm et al., 2011).</li> <li>3. Order/disorder provided by Chol, disorder by DOPC (Nyholm et al., 2011; Schachter et al., 2022).</li> </ol> | <ol style="list-style-type: none"> <li>1. Confirmation of previous research key points such as certain ratios of bSM and Chol being the most ordered.</li> </ol>                                                                                |
| 2 (bSM/DOPC/DOPE)  | <ol style="list-style-type: none"> <li>1. Focus on DOPE as a helper lipid in homeostatic function (Mochizuki et al., 2013).</li> <li>2. Examination of phase behavior still unclear.</li> </ol>                                                                                                        | <ol style="list-style-type: none"> <li>1. DOPE induces a similar order/disorder effect at the interface as Chol to bSM membranes with temperature.</li> <li>2. DOPE provides slight order to DOPC membranes across all temperatures.</li> </ol> |
| 3 (bSM/DOPE/Chol)  | <ol style="list-style-type: none"> <li>1. Not yet well explored or researched.</li> </ol>                                                                                                                                                                                                              | <ol style="list-style-type: none"> <li>1. Compositions with high Chol remained relatively ordered or heterogeneous across temperature.</li> </ol>                                                                                               |
| 4 (DOPC/DOPE/Chol) | <ol style="list-style-type: none"> <li>1. Not yet well explored or researched.</li> </ol>                                                                                                                                                                                                              | <ol style="list-style-type: none"> <li>1. Heterogeneity moderately maintained across temperature primarily with increased inclusion of Chol.</li> </ol>                                                                                         |

**Table 3-1** Summary of important points addressed in previous papers regarding the examined lipid classes relevant to phase behavior vs findings in this work of the phase behavior of binary and ternary mixtures.

### 3.13. Tracking trends across multiple phase diagrams

Among the 4 examined ternary diagram faces of the proposed “flattened tetrahedron diagram,” the primary extensively studied ternary mixture in previous studies has been that of bSM/DOPC/Chol. Due to the plethora of unique interactions that were previously mentioned, the focus on bSM/DOPC/Chol is a known important area of research, however, when considering the brain as well as other biologically relevant membranes, it is important to include other dominant lipid classes. PE is another dominant lipid class that has not had its phase behavior as extensively studied, but due to its limitations as a “helper” lipid, PE phase diagrams are moreover restrictive

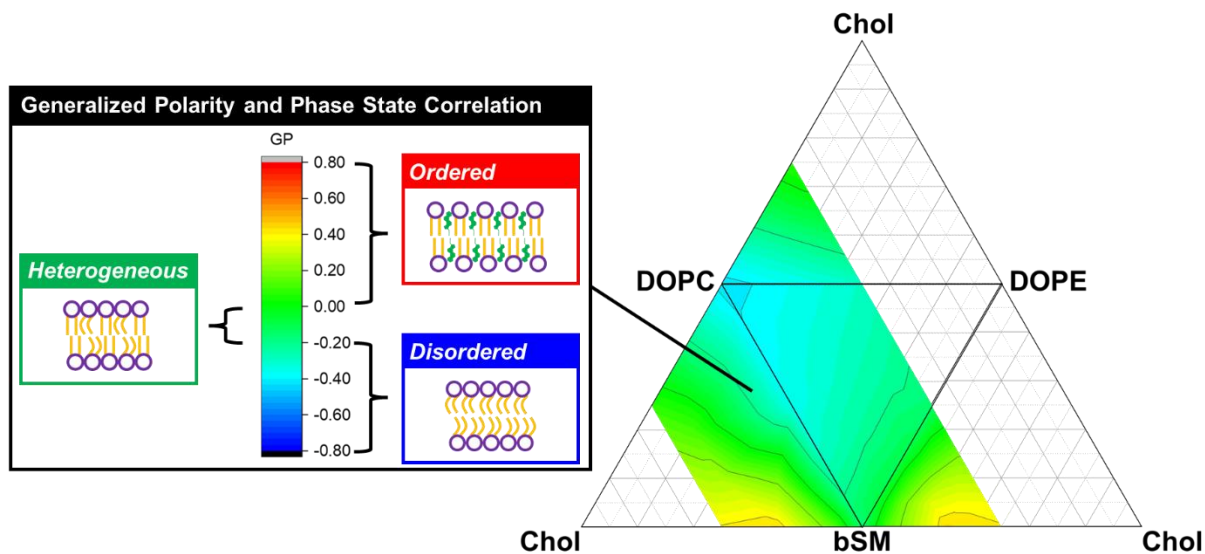


**Fig. 3-14 a)** Flattened tetrahedron diagram of the analyzed compositions at 35 °C. Contour lines represent the shifts in  $GP_{340}$  in 0.2 increments. **b)** Semi-folded version of the tetrahedron phase diagram.

when examined together with Chol. To obtain an overall conceptual grasp on the trends of all 4 lipid components, we have proposed adjoining 4 ternary phase diagrams at their axes to comprehensively display the trends across these diagrams (**Fig. 3-14**). This diagram depicts a smooth transition of  $GP_{340}$  values across its borders into the neighboring ternary diagram and is thereby a useful tool in tracking the trends of polarity across multiple phase diagrams in the 2D space.

Since many research projects employ ternary phase diagrams and are beginning to employ tetrahedral diagrams, the future use of this flattened tetrahedron phase diagram as a tool for examining phase trends is a significant aspect of lipid research. As future research of various ternary compositions is accumulated, the use of this flattened tetrahedron diagram is an important preliminary method of comparing characteristics that coincide with other related ternary diagrams. Tracking these trends may provide unique opportunities to contrast different regions of separate ternary phase diagrams for similar or different interactions in a less tedious and more consistent way. Ultimately, this flattened tetrahedron phase diagram serves as a preliminary work and may be “folded” to create the 3D tetrahedron phase diagrams that are beginning to arise in the fields of lipid research that illustrate membranes with 4 lipid components in their 3D interior.

#### 4. Summary



**Fig. 3-15** Summary of  $GP_{340}$  and phase state correlation depicted on the completed flattened tetrahedron phase diagram at 35 °C.

When considering biologically relevant lipid membranes, there exist many different classes of lipids that may compose a membrane. Specifically in brain lipidomic studies, alongside bSM, PC, and Chol, another abundant lipid class is PE. Singular ternary phase diagrams are limited in their approach due to the ability to only visualize the trends of up to 3 lipids, therefore it is necessary to begin the expansion of this concept to include more components. By constructing a flattened tetrahedron diagram with 4 unique ternary diagrams, DOPE was included in the overall phase analysis as an additional ternary membrane component in related ternary diagrams. Inclusion of DOPE in unary, binary, and ternary compositions was examined primarily with Laurdan to evaluate polarity. DPH was used supplementally at the borders of the ternary diagrams and suggests the order induced by DOPE may primarily occur in the interfacial region and not the deeper region of the membrane, most notably in bSM membranes. Incorporation of DOPE into bSM surprisingly revealed parallels to Chol inclusion with a similar disordering effect at lower temperatures and ordering at higher temperatures at the membrane interface. In DOPC membranes, DOPE induced order was observed but much more subtle across the measured temperature range. Using the proposed flattened tetrahedron diagram, the trends of the selected lipids were tracked across different ternary mixtures via adjoined ternary phase diagram borders, summarized in **Figure 3-15**. This work acts as an important preliminary step in expanding into a 3D tetrahedron phase diagram when the flattened diagram is folded, which ultimately allows for the analysis and characterization of more complex quaternary membrane compositions in future studies.

## Chapter 4

### **Tetrahedron phase diagram analysis of quaternary lipid membrane compositions using Laurdan and DPH fluorescence**

#### **1. Introduction**

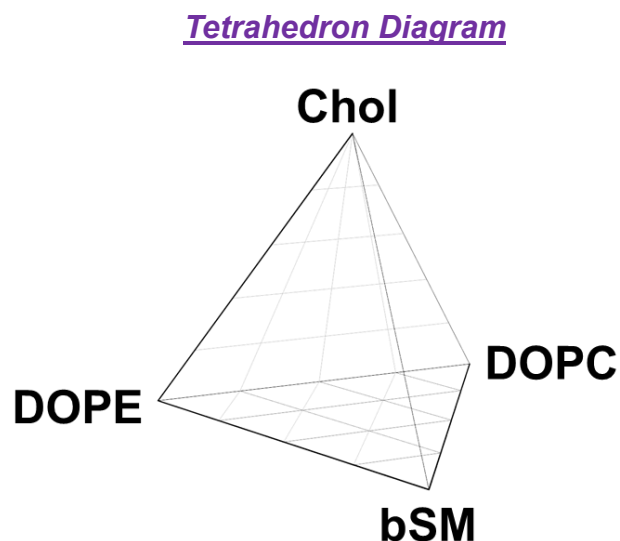
Various lipidomic studies of the brain have revealed the presence of various types of lipid species in the composition of brain lipid membranes and the potential connections of these lipids to brain pathology (Fahy et al., 2011; Orešič et al., 2008; Watson, 2006; Yoon et al., 2023). Some of the most prevalent lipid species within the brain lipidome include sphingomyelin (bSM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cholesterol (Chol) (Fitzner et al., 2020; O'Brien & Sampson, 1965). Although most lipids share a core structure of a hydrophilic phosphate head and hydrophobic fatty acid tails, there exist unique variations within these basic structures that ultimately result in differing molecular properties. Some of these lipid property variations include the transition temperature, viscosity, hydrophobicity, polarity, and fluidity among several others. Considering the existence of a variety of these lipid properties, lipid membranes will naturally be characterized by a combination of properties of the various lipid species composing them, of which several of these properties have possible links to brain pathology. Research on membrane fluidity and Chol inclusion has shown that removal of Chol from the membrane and increased fluidity can increase membrane perforation by the Alzheimer's disease (AD) affiliated amyloid- $\beta$  peptide (Fernández-Pérez et al., 2018). Additional research has indicated that in the AD brain there is a significant reduction in PE and an elevation in bSM (Pettegrew et al., 2001). Since these pathologies encompass multiple lipid species and prior

knowledge that naturally occurring lipid membranes generally have several lipid species that compose their overall structure, analysis of artificially constructed multi-component membranes is presumably one of the key intermediary steps in the thorough characterization of specific lipid species' properties, their impact on the overall structure of the membrane, and consequently the healthy metabolic functions of the brain.

Liposomes are one of the more well-established methods to study the lipid membrane in isolation due to the ease of their preparation and simplicity of their structure. Liposomes can take the form of small unilamellar vesicles (SUVs) and act as a biomimetic environment of the basic plasma membrane of a cell with the absence of interference by other cellular processes. Additionally, the ability to control the composition of liposomes also allows them to be utilized as a platform for membrane composition related analytical studies. Multi-component liposomes can easily be prepared in several different methods including traditional thin-film evaporation as well as emerging techniques such as microfluidics. Microfluidic liposome preparation is an alternative and emerging protocol that allows an efficient means of producing multi-component liposomes due to its speed and reliability and has thus received recent spotlight in producing liposomes, therefore it is the primary method used in this work (Choi et al., 2023).

Fluorescence spectroscopy is an established and reliable technique that is often utilized to analyze different properties of membranes by employing various fluorescent probes such as 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan) and 1,6-diphenyl-1,3,5-hexatriene (DPH) for polarity and fluidity, respectively. Laurdan, with its amphipathic structure, can insert into the membrane into the interfacial region between the polar heads and nonpolar tails of the lipids composing the membrane and thereafter detect polarity with its sensitivity to its surrounding solvent environment (Parasassi et al., 1990, 1991, 1998; Watanabe et al., 2019). Using excitation

at a specific wavelength, the Laurdan fluorophore moiety will exhibit a large dipole moment that is relaxed dependent on its surrounding solvent environment and conveyed via its emission spectra. This emission is then quantified using the generalized polarization ( $GP_{340}$ ), with a higher  $GP_{340}$  indicating a more ordered phase and a lower  $GP_{340}$  representing a more disordered phase. DPH, unlike Laurdan, will insert deeper into the hydrophobic core of the membrane and convey the fluidity via the movement of the membrane lipid hydrocarbon chains. When using vertically polarized light to excite the DPH at a specific wavelength, the parallel component will decrease while the perpendicular component will increase with higher fluidity due to the increased freedom of rotational movement that the DPH has to depolarize the polarized fluorescence. Thus, by quantifying the inverse of polarization ( $1/P$ ), the fluidity of the membrane may be determined with a large  $1/P$  indicating a higher fluidity and a lower  $1/P$  indicating a lower fluidity. In this way, both Laurdan and DPH play important roles in both the characterization of the membrane and the overall analysis of phase state.



**Fig. 4-1** Depiction of the tetrahedron phase diagram that will be used to characterize quaternary lipid membrane compositions.

Past membrane characterizing studies have extensively explored 2-3 lipid component membranes and defined their phase states via ternary phase diagram (de Almeida et al., 2003a; Feigenson, 2009; Nyholm et al., 2011; Thewalt & Bloom, 1992; Veatch & Keller, 2005). Ternary phase diagrams are useful in that they can integrate 3 different lipids at various ratios to display how altering the ratios of the component lipid species may impact the polarity, fluidity, and ultimately the phase of the analyzed membrane. Although these diagrams are important additions to the field of lipid research, one of the most obvious observed limitations is that only a maximum of 3 lipid components may be analyzed per diagram. Logically, this limitation has prompted past researchers to introduce a basic 3D tetrahedron phase diagram to incorporate a 4<sup>th</sup> lipid in the analysis of membranes, however this diagram has yet to be widely incorporated in current lipid membrane research (Konyakhina et al., 2013). This work has the primary aim of revealing the variations that may occur by introducing a 4<sup>th</sup> lipid component into the membrane where past research has primarily focused on ternary membrane analysis. Additionally, this work has the aim of emphasizing the usefulness of a 3D tetrahedron phase diagram that incorporates 4 separate ternary phase diagrams joined together at their edges that allows for the inclusion of this additional fourth lipid component (**Fig. 4-1**).

## **2. Experimental Section**

### **2.1. Materials**

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) lipids were purchased from NOF Corporation (Tokyo, Japan). Porcine brain sphingomyelin (bSM) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol), 6-dodecanoyl-2-dimethylamino naphthalene (Laurdan), and 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescent probe was purchased from Sigma-Aldrich (St. Louis, MO,

USA). Any other relevant chemicals were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

## **2.2. Fabrication of a microfluidic chip**

A microfluidic device was fabricated using standard photolithographic techniques as described in a previous study (Choi et al., 2023). Briefly, a negative photoresist (SU-82075) was spin-coated to achieve a thickness of 80  $\mu\text{m}$  on a silicon wafer, followed by a baking process at 60 °C for 3 minutes and 95 °C for 9 minutes. This photoresist was exposed to ultraviolet light at an intensity of 10  $\text{mJs}^{-1}$  using a mask aligner. After the exposure, a post-baking step was then conducted first at 60 °C for 2 minutes, then at 95 °C for 9 minutes. For eliminating any residual photoresist, the silicon wafer was developed for 5 minutes. Developed master mold was next used to cast PDMS (10:1 base to curing agent ratio), then degassed under vacuum. Polymer mixture was then cured in an oven at 70 °C for 2 hours and separated carefully from the mold. Patterned PDMS layer was then bonded to a flat PDMS sheet after plasma treatment, then the assembled chip was cured further at 80 °C in a convection oven for 8 hours. Dimensions of the PDMS microfluidic chip were 11 mm  $\times$  35 mm  $\times$  8 mm, with a cross-junction of two inlets and an outlet. Both the width and height of the channel were 80  $\mu\text{m}$ . This microchannel was plane type without any microstructures.

## **2.3. Preparation of liposomes via microfluidics**

Liposomes of quaternary compositions were prepared by mixing ultra-pure water (aqueous phase) and ethanol solution of lipids in a fabricated microfluidic device (Lee et al., 2025). Phases were adjusted using two syringe pumps MSP-1D (ASONE, Osaka, Japan). Total flow rate (TFR) = 10  $\text{mLh}^{-1}$ , an initial lipid concentration = 10 mM, and flow rate ratio (FRR) = 4 were adjusted. 3 mL of each prepared liposome sample were diluted to final lipid concentration = 1 mM and

placed in the 14000 MWCO dialysis membrane tubing and then dialyzed in 300 mL ultra-pure water overnight.

#### 2.4. Sample preparation for Laurdan and DPH fluorescence measurements

Laurdan dissolved in ethanol (100  $\mu\text{M}$ ) was added into liposomes in a 1:100 (Laurdan:lipid) ratio and diluted with UltraPure water to achieve the desired ratios. Separately, DPH dissolved in ethanol (100  $\mu\text{M}$ ) was added into liposomes in a 1:250 (DPH:lipid) ratio and diluted with UltraPure DI water to achieve the desired ratios. Each sample was then incubated for a minimum of 30 minutes within body temperature range (37  $^{\circ}\text{C}$ ) with minimal exposure to light to obtain samples ready for measurement. Each sample was then measured across a temperature range of 20 – 70  $^{\circ}\text{C}$  in increments of 5  $^{\circ}\text{C}$ .

#### 2.5. Evaluation of polarity via Laurdan and generalized polarization ( $GP_{340}$ )

Laurdan measurements were quantified using generalized polarization (**Eqn. 4-1**) where  $I_{440}$  represents the gel phase and  $I_{490}$  represents the liquid-crystalline phase. Temperature was plotted against  $GP_{340}$  at each incremental value (20 – 70  $^{\circ}\text{C}$ ) to reveal the impact of increasing temperature on the polarity of each measured lipid composition.

$$GP_{340} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (4-1)$$

#### 2.6. Evaluation of fluidity via DPH and $1/P$

DPH measurements were quantified using **Eqn. 4-2** and **Eqn. 4-3** where the inverse of polarization ( $1/P$ ) is taken to display a more linear relationship between fluidity and numeric value.  $I_{\parallel}$  indicates the emission intensity parallel to the vertically excited light and  $I_{\perp}$  represents the emission intensity perpendicular to the vertically excited light. Temperature was plotted against

1/P at each incremental value (20 – 70 °C) to reveal the impact of increasing temperature on the fluidity of each measured lipid composition.

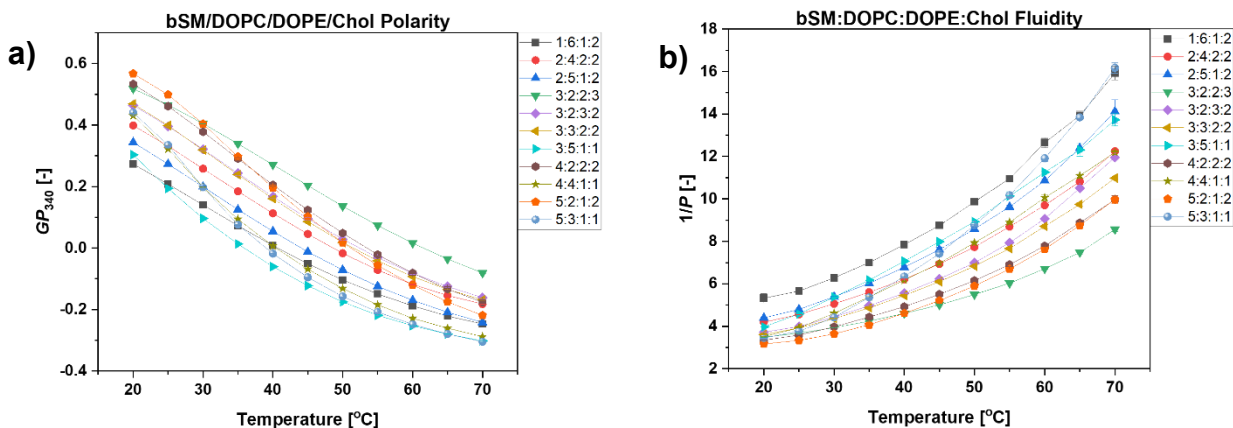
$$P = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + GI_{\perp}) \quad (4-2)$$

$$G = i_{\perp} / i_{\parallel} \quad (4-3)$$

## 2.7. Deconvolution of Laurdan fluorescence spectra

Each spectra was deconvoluted into 4 component peaks: Each component curve represented the solvent environment detected by Laurdan [Peak 1: non-polar ( $\epsilon_r < 5$ ), Peak 2: polar aprotic ( $5 < \epsilon_r < 20$ ), Peak 3: polar protic ( $10 < \epsilon_r < 20$ ), and Peak 4: polar protic ( $\epsilon_r > 20$ )] where  $\epsilon_r$  is the dielectric constant. Deconvolutions were performed with OriginPro's peak deconvolution application. Peaks were deconvoluted with the following parameters: second derivative, Gaussian, quadratic Savitzky-Golay smooth derivative, with upper bounds for the FWHM set at 60 for peaks 1-3. Area fit %, the theoretical area from negative to positive infinity of wavelength, was obtained for each deconvoluted peak, averaged, and plotted.

### 3. Results



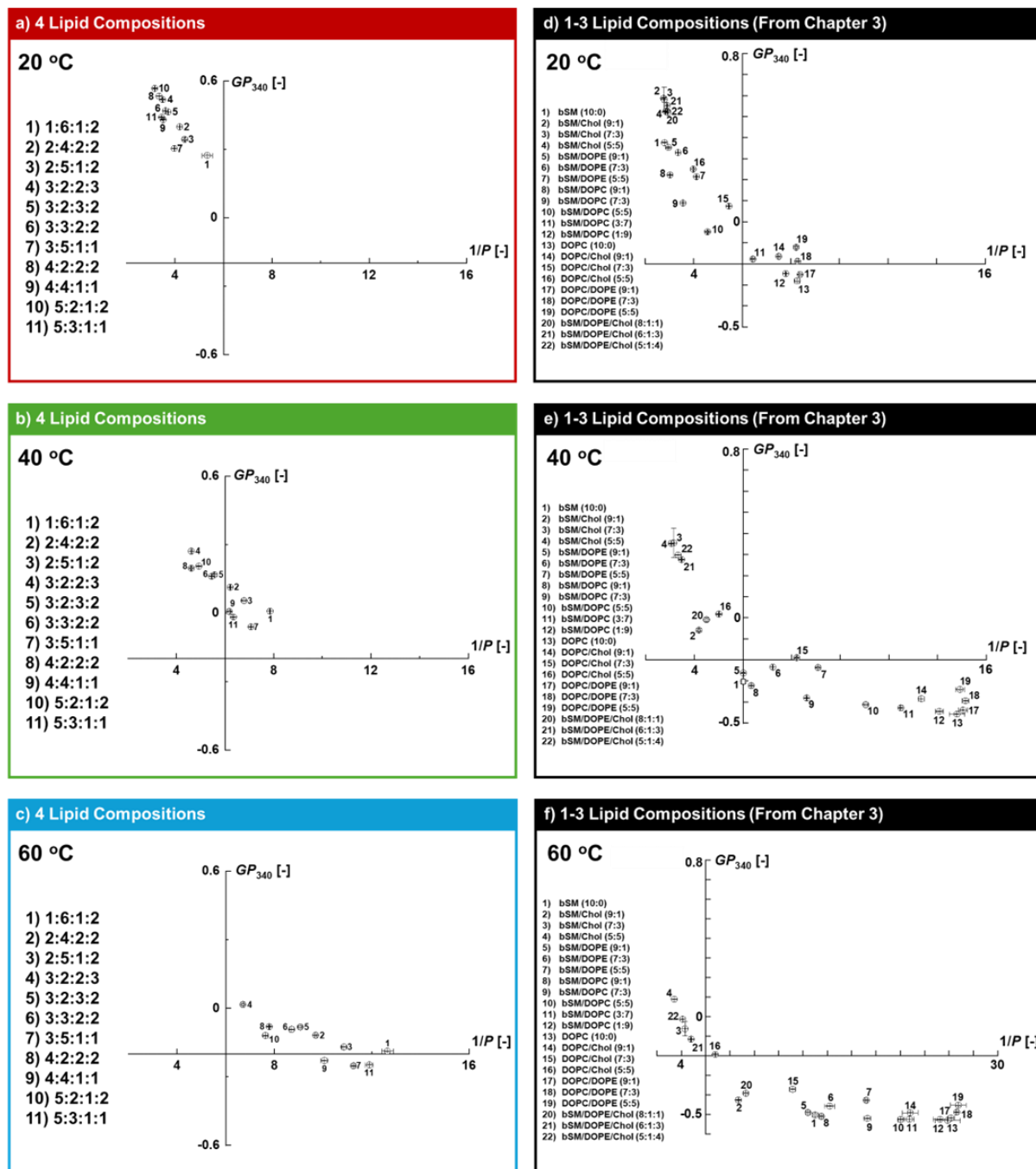
**Fig. 4-2** Properties of the SM/DOPC/DOPE/Chol membrane: **(a)** Polarity via  $GP_{340}$  given across the temperature range of (20 °C to 70 °C). **(b)** Fluidity via  $1/P$  given across the temperature range of (20 °C to 70 °C).

#### 3.1. Polarity ( $GP_{340}$ ) and fluidity ( $1/P$ ) of multi-component liposome systems across temperature

To utilize the proposed tetrahedron phase diagram, liposomes composed of the 4 different lipid components bSM, DOPC, DOPE, and Chol in varying ratios were analyzed across a temperature range (20 – 70 °C). This temperature range allowed us to deduce how the inclusion of different ratios of each of the 4 different lipids with varying properties influence the robustness in polarity and fluidity of the lipid membrane. Polarity was quantitatively analyzed by utilizing the generalized polarization ( $GP_{340}$ ) via Laurdan fluorescence at each 5 °C incremental value (**Fig. 4-2a & Eqn. 4-1**). Similarly, fluidity was analyzed quantitatively with the inverse of polarity ( $1/P$ ) via DPH fluorescence also at each 5 °C incremental value (**Fig. 4-2b, Eqn. 4-2, & Eqn. 4-3**). As the temperature is increased across the compositions the  $GP_{340}$  decreases while the  $1/P$  value increases, indicating that the membrane is increasing its polarity and fluidity, respectively. It is noted that the membrane composition with the highest Chol percentage of

bSM/DOPC/DOPE/Chol (3:2:2:3) is the most robust to change at higher temperatures, indicating the significance of higher amounts of Chol in the membrane in retention of polarity. General trends seemed to align with the usual expectation of the membrane, that increasing the temperature in the membranes will consequentially result in an increase in both the polarity and the fluidity of the membrane. Results in this section suggest that this expectation of temperature influence may be true to a certain extent even with the incremental increase of membrane complexity from unary, binary, ternary, to quaternary membranes, albeit there may be slight differences and variations due to the increased complexity of the membrane composition.

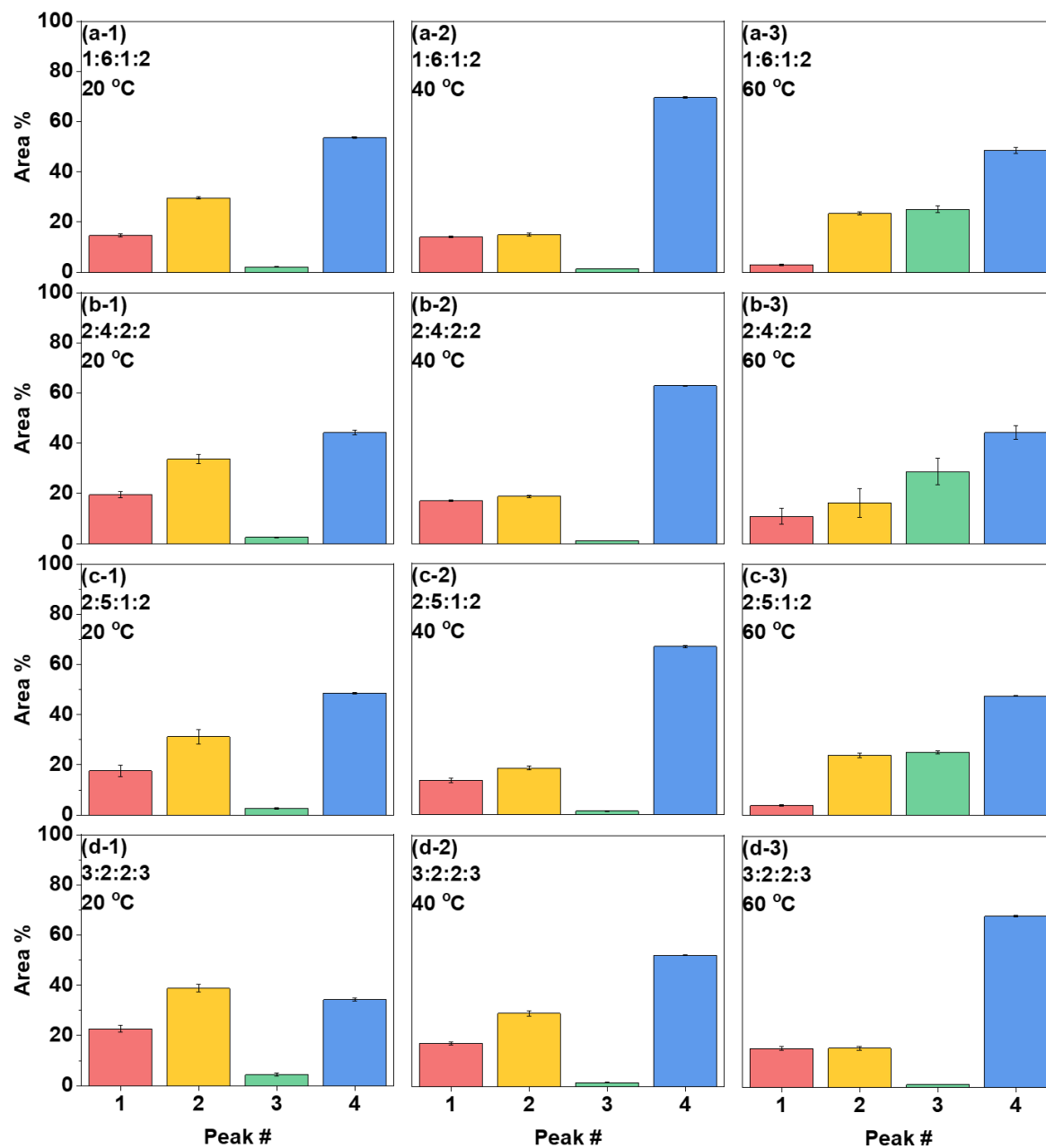
### 3.2. Cartesian diagrams of quaternary membrane compositions

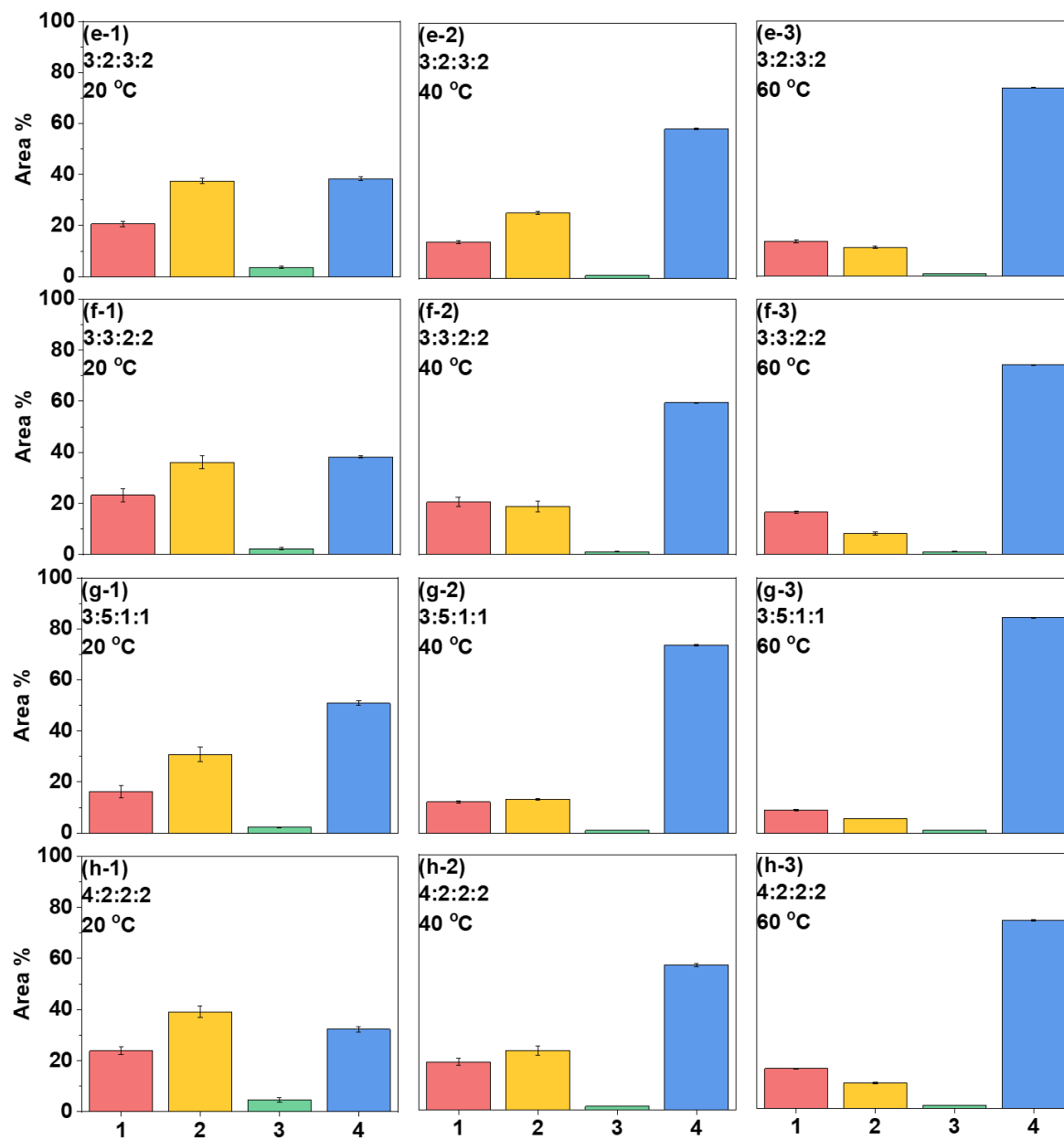


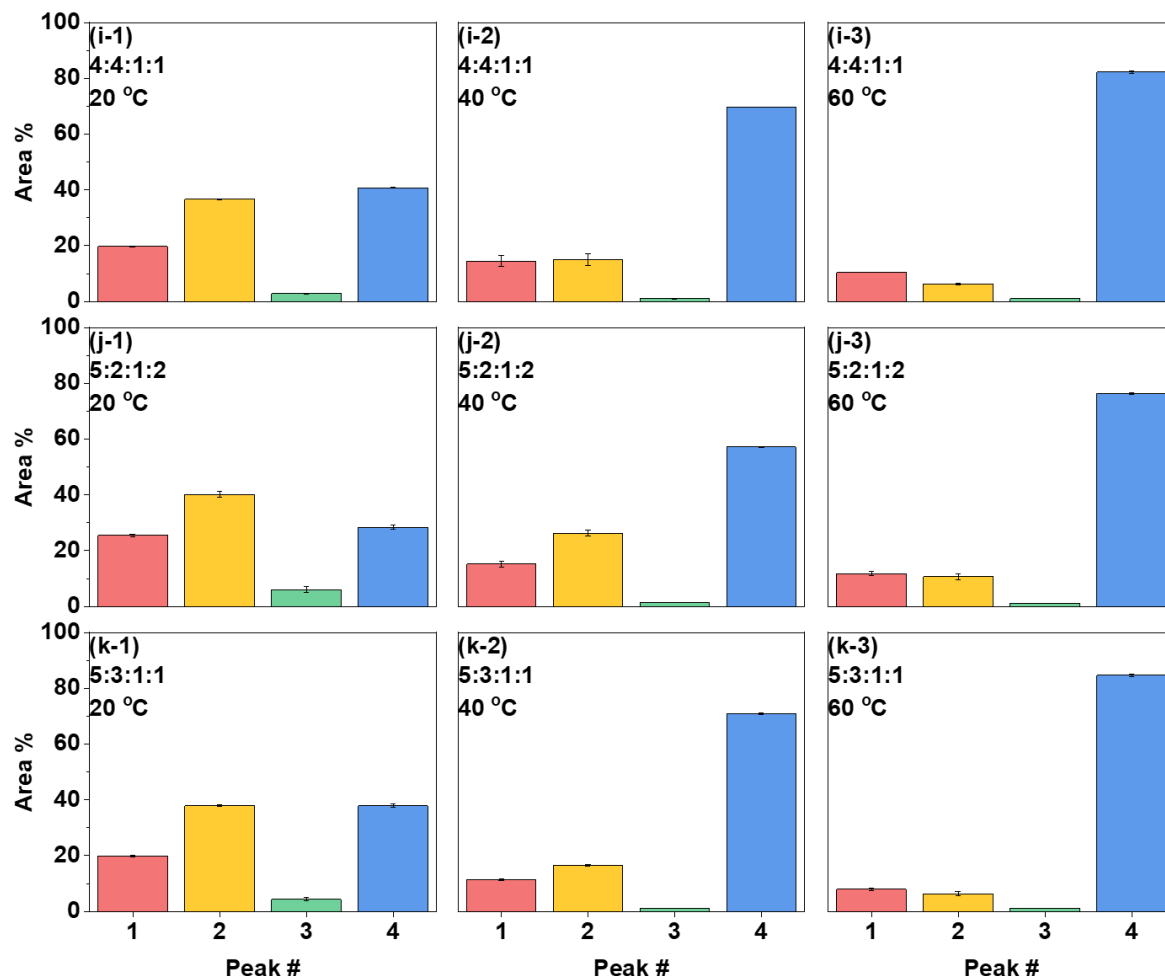
**Fig. 4-3** Cartesian diagrams of  $GP_{340}$  vs  $1/P$ : **a-c)** Quaternary compositions analyzed in this chapter and **d-f)** Comparative binary mixtures from chapter 3.

Cartesian diagrams were constructed for the quaternary mixtures according to previous protocol (Suga & Umakoshi, 2013). Quadrant I represents the heterogeneous phase, quadrant II the ordered phase, and quadrant IV the disordered phase. Comparison between unary, binary, ternary, and quaternary mixtures revealed a robustness in the ordered phase as membrane complexity was increased from unary, binary, ternary, to quaternary compositions (**Fig. 4-3**). Notably, for the quaternary compositions, the compositions with the least amount of Chol (10%) were the only compositions to enter quadrant IV by the high temperature (60 °C) mark. For comparative purposes, certain unary, binary, and ternary compositions are displayed from chapter 3 and reveal that amidst the most robustly ordered compositions at 60 °C are the binary compositions of bSM/Chol (7:3) and (5:5) in addition to the ternary compositions with a Chol % greater than 10%. Interestingly, the DOPC compositions, even with higher Chol %, seemed to ultimately transition into the disordered phase.

### 3.3. Deconvolution analysis of quaternary membrane compositions



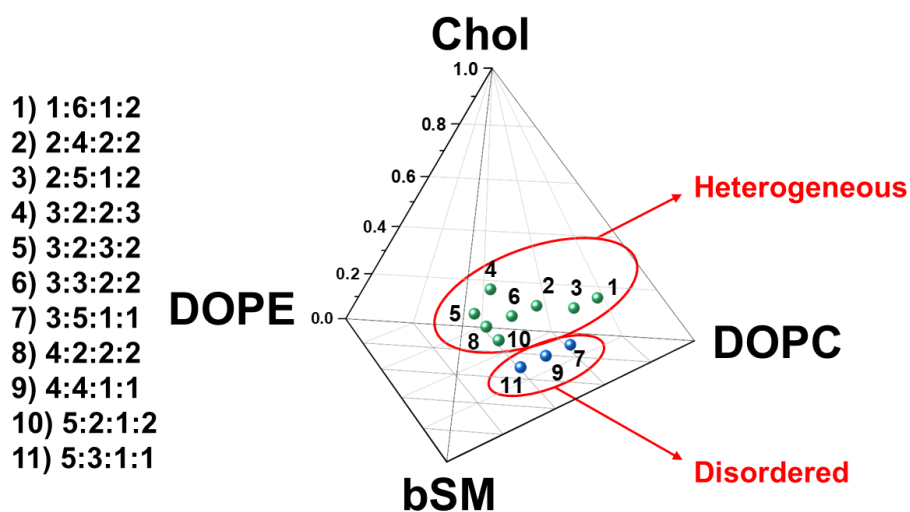




**Fig. 4-4** Deconvolution analysis of each analyzed quaternary composition (a-k) at 1) 20 °C, 2) 40 °C, and 3) 60 °C for SM/DOPC/DOPE/Chol.

Each quaternary composition Laurdan spectra was deconvoluted into 4 peaks to reveal the solvent environment of the Laurdan fluorescent probe according to each membrane composition. As a general trend increasing the temperature resulted in peak 4, the relaxed species and polar protic peak, increasing in dominance. Interestingly, across the majority of compositions, although peak 4 increased, there was a retention of either or both of peaks 1-2 as the second and third most dominant peaks. Only in compositions with a high % of DOPC was there a chance of peak 3 being similar or more dominant than peaks 1 or 2 (Fig. 4-4a-3, 4-4b-3, & 4-4c-3).

### 3.4. General Discussion



**Fig. 4-5** Tetrahedron diagram of the quaternary composition indicating both heterogeneity and disorder within at 60 °C.

Overall results heavily emphasized the heterogeneous phase when temperature was increased to at least the 60 °C mark for all the quaternary compositions analyzed aside from those with the lowest Chol (10%) (**Fig. 4-3c & 4-5**). When looking at  $GP_{340}$  and  $1/P$  data in isolation, it seems that all compositions followed a typical trend of increasing polarity and fluidity proportionally to temperature. However, when plotting  $GP_{340}$  and  $1/P$  against each other on the cartesian coordinate plane, results surprisingly showed robustness in staying in quadrant I (the heterogeneous phase) even at high temperatures such as 60 °C. This result was in heavy contrast to most of the unary/binary mixtures analyzed that readily went into quadrant IV (disordered phase) when the temperature was elevated to a similar level. Similarly, the ternary compositions with greater than 10% Chol were also robust in abstaining from quadrant IV, however, they remained in quadrant II (ordered phase). These results suggest that at quaternary mixtures,

complex interactions may occur between the 4 analyzed lipid species that retain these membrane compositions in the heterogeneous phase.

Further deconvolutions of the Laurdan spectra held a trend across the quaternary compositions of having an increasingly dominant peak 4, indicating a polar interfacial membrane environment, which coincided with temperature increase. However, the majority of these compositions also retained some level of peaks 1-2, which correspond to a non-polar and polar aprotic environment, respectively. Maintenance of peaks 1-2 while simultaneously having a dominant peak 4 supports the idea of phase coexistence or heterogeneity as there seems to be two different solvent environments that are represented in the deconvolution. Additionally, most of this robustness of the heterogeneous phase seems to be due to the interfacial region detected by Laurdan, as there are large positive shifts in  $1/P$  but maintenance of points in quadrant I, suggesting acyl chain regions are becoming more fluid but the  $GP_{340}$  value is being relatively stable with the varied temperature. These points at 60 °C are mapped in a tetrahedron phase diagram to display the variation of phases within (**Fig. 4-5**).

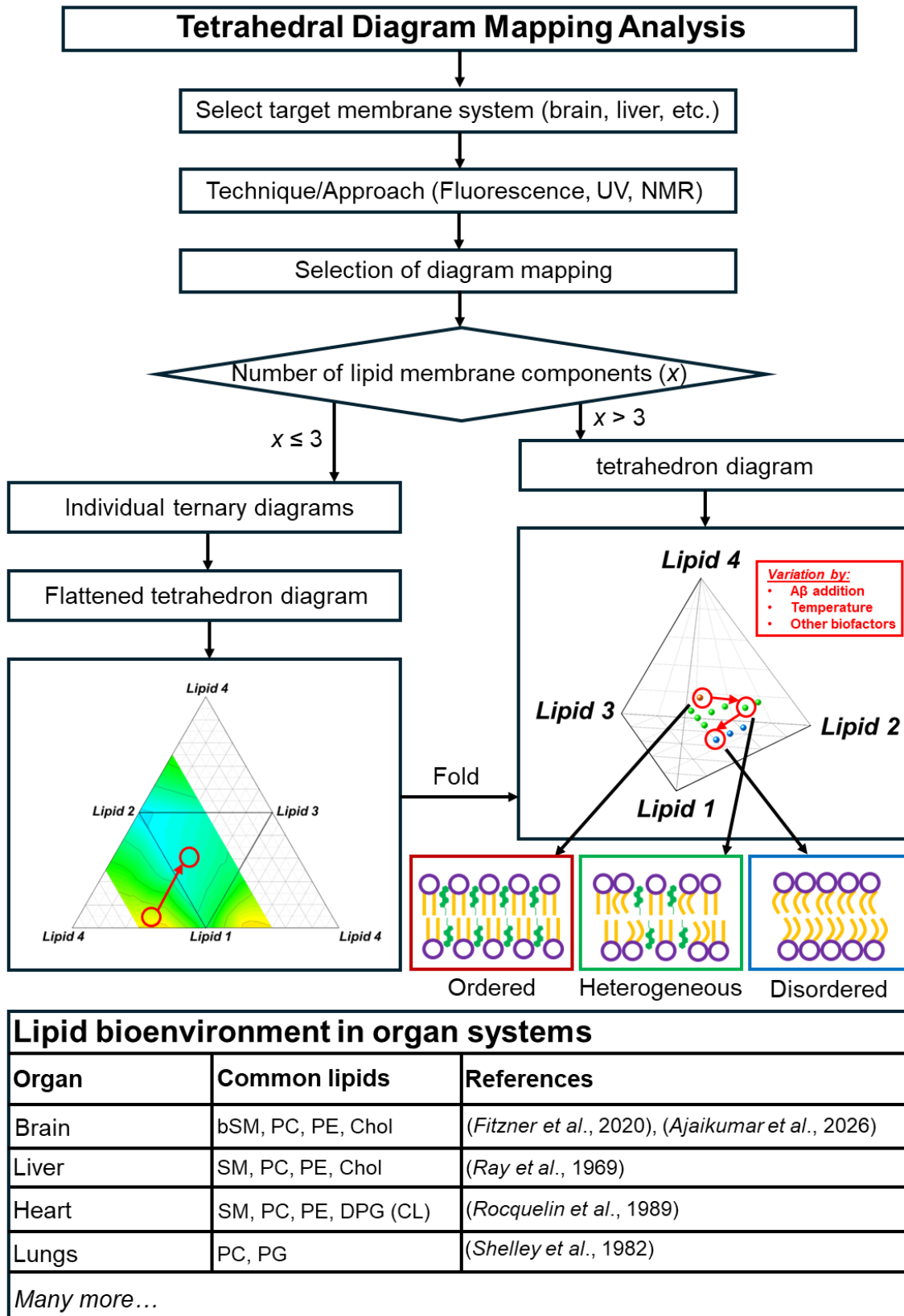
### **3.5. Establishing protocol for tetrahedral diagram mapping analysis**

Although there are several points that have been mapped into the tetrahedron diagram in this study, there exist many more combinations that can be plotted into a tetrahedron diagram of the brain. Additionally, many other organ systems such as the liver, heart, and lungs heavily rely on lipid composition for their anatomical structure and function (Ray et al., 1969; Rocquelin et al., 1989; Shelley et al., 1982). This chapter aims to propose tetrahedral diagram mapping analysis to assist in thorough characterization of increasingly complex membranes across various organ systems (**Fig. 4-7**). Ultimately, comprehensive tetrahedral mapping of the lipid bioenvironment of

different organ systems will open the door to lipid membrane therapy focused on biomedical research pertaining to the properties of the lipid membrane.

#### **4. Summary**

Overall, this data is highly indicative of quaternary membrane compositions holding robustness in heterogeneity at higher temperatures. Both cartesian diagram analysis and deconvolution analysis are indicative of the majority of compositions maintaining this heterogeneous phase at higher temperatures. Additionally, this data is able to reveal some insight into the interior of the tetrahedron diagram. Whereas the previous chapter emphasized there were distinct transitions across compositions at 35 °C, this chapter emphasized the interior is primarily in the heterogeneous phase, which provides insight into the possible status of the phase in increasingly complex membranes in a non-biological setting. This allows further hypotheses to be made about more complex membranes in an actual biological setting and is thereby a key intermediary work between the realms of research and application.



**Fig. 4-7** Schematic for tetrahedral diagram mapping analysis.

## Chapter 5

### Amyloid Beta (A $\beta$ ) Fibrillation Kinetics and its Impact on Membrane Polarity

#### 1. Introduction

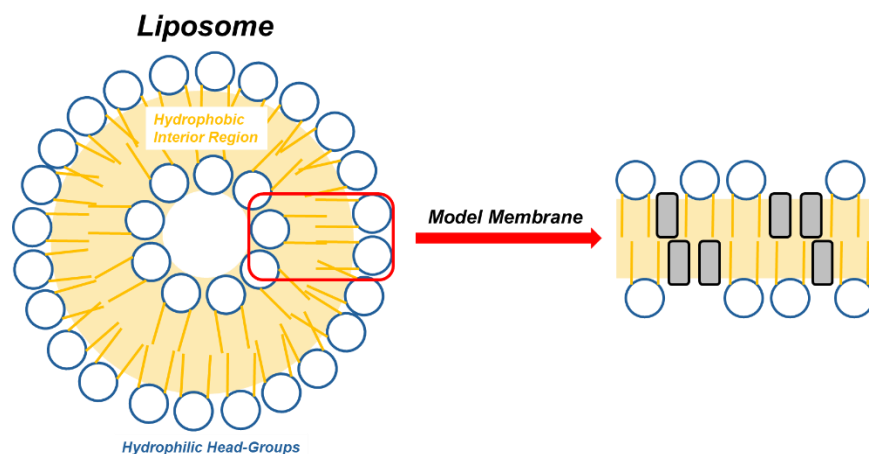
Since the publication of the Amyloid Cascade Hypothesis, amyloid beta (A $\beta$ ) peptide's fibrillation into toxic-insoluble plaques has often been made the focus of extensive research on neurodegenerative diseases such as Alzheimer's disease (Hardy and Higgins 1992; Koo et al. 1999; Chen et al. 2017a; Flagmeier et al. 2020; Hampel et al. 2021). A $\beta$  peptide is a product of the regulated cleavage of the transmembrane amyloid precursor protein (APP) by several secretases into peptide fragments of varying lengths 57; previous studies have noted that processing deficiencies in this cleavage pathway can be largely indicative of amyloid plaque formation (Esch et al., 1990; Sisodia, 1992). A $\beta$  kinetics describe the 3 different stages of growth, the monomer, oligomer, and matured fibril, which coincide with the nucleation, elongation, and maturation phases, respectively (Schmit et al., 2011). Cleaved monomer units undergo a nucleation process into oligomers, after which these oligomers deposit as the toxic fibrils (Arosio et al., 2015; Chen et al., 2017b). Although there are many possible routes of toxicity, one study has suggested that an aspect of aggregate toxicity may involve lipid mobility; related to this, certain studies have suggested that the aggregating oligomeric and prefibrillar states are more toxic than the mature amyloid fibrils; other studies have proposed that this fibrillation process itself may catalyze a secondary nucleation mechanism that promotes further fibrillation 60–63. Fibrillation ultimately accumulates into the toxic and insoluble extracellular amyloid plaques that are widely observed in Alzheimer's disease and other neurodegenerative diseases.

A $\beta$  fibrillation is characterized by the increasing abundance of  $\beta$ -sheet structures, therefore a large presence of these  $\beta$ -sheets is indicative of advanced fibrillation. One of the most used methods to detect these fibrils is by using Thioflavin T (ThT) fluorescence probe interactions with A $\beta$  (Naiki et al., 1989). ThT can detect these amyloid fibrils by a  $\beta$ -sheet interacting mechanism and produces a fluorescence signal that allows the quantification of fibrils (LeVine, 1995). By using ThT alongside the different model membranes used for this series of experiments, the influence of membrane composition on the temporal parameters of fibrillation on the membrane can be elucidated. Although research on amyloidosis and the lipid membrane remain a hot topic in recent years, deeper insights into membrane composition's interactions with A $\beta$  are yet to be explored.

Sphingolipids, unsaturated phospholipids, and cholesterol (Chol) are among the dominant lipids of the brain and are thereby the focus of this work (Fitzner et al., 2020). As a simple model of brain lipid systems, the ternary system of SM/DOPC/Chol has been studied, wherein 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and porcine brain SM are employed as models for unsaturated phospholipid and sphingolipids, respectively. Although DOPC itself is not a naturally occurring lipid, its wide employment and categorized phase behavior has made it the prime selection used for this work (Nyholm et al., 2011). At body temperature (37 °C) SM has just reached its transition temperature (35-38 °C) and is transitioning into a more fluid/disordered state while the already fluid/disordered DOPC with its much lower transition temperature (-17 °C) is becoming much more disordered (Meyer et al., 1999). Since both of these lipids are either already in or approaching a fluid phase state, incorporation of Chol into the membrane can alter the membrane into a more ordered state. SM in particular, is a lipid that has an amide moiety and has hydrogen bond interactions with its neighboring lipids, thereby analyzing interactions between SM

and A $\beta$  may reveal unique processes that alter the kinetics of A $\beta$  fibrillation. DOPC, although an artificial lipid, is a good component lipid in analyzing the interactions of A $\beta$  with a fluid lipid interface due to the previously established experiments. (Nyholm et al., 2011). Chol is another important component that has been implicated in influencing the start of the fibrillation behavior of A $\beta$ . (Banerjee et al., 2021).

Liposomes are a unique platform for analyzing events occurring on the membrane without perturbation by cellular processes that may occur during *in vivo* experiments (**Fig. 5-1**). Thus, to determine how disordered or ordered membranes have been influenced by the A $\beta$  fibrillation process, this work employs small unilamellar vesicle (SUV) liposomes with different compositions of SM, DOPC, and Chol to analyze any variation in fibrillation due to the membrane composition. By employing fluorescent probes such as Laurdan during the A $\beta$  fibrillation process, the lipid membrane properties may be quantitatively analyzed via fluorescence spectroscopy.



**Fig. 5-1** Basic overview of the liposome (lipid vesicle) and its lipid bilayer. Blue circles (lipid head group), yellow lines (lipid tail group), gray rectangles (Chol).

Membrane polarity and fluidity are correlated properties that may be analyzed with different fluorescence probes. In this study we specifically employ the Laurdan fluorescent probe that was originally developed for its solvatochromic property for detection of the solvent polarity (Weber and Farris 1979). Past research has employed Laurdan specifically for its use in

characterization of the membrane polarity (Parasassi et al. 1998). We thus use Laurdan in this work to determine the hydration state of the membrane's surrounding environment, which we can relate to the polarity of the membrane via generalized polarization.

## **2. Experimental Section**

### **2.1. Materials**

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from NOF corporation (Tokyo, Japan). Porcine brain sphingomyelin (SM) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Human Amyloid Beta 1-40 (A $\beta$ 40) was purchased as a synthetic product from the Peptide Institute (Osaka, Japan). All other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan).

### **2.2. Liposome Preparation**

To prepare the SUVs used in this work, stock of the designated lipid or Chol was first dissolved with chloroform (CHCl<sub>3</sub>). Each solution was then placed in a round bottom flask according to the molar ratios of lipid:Chol ranging from 10:0, 9:1, 7:3. And 5:5. Each lipid solution was then rotary evaporated under vacuum conditions until a clear lipid film was visible. This film was placed in a vacuum chamber for a minimum of 2 hours. Each film was then hydrated and underwent 5 cycles of freeze (-80 °C) and thaw (60 °C) for the homogenous formation of the lipid vesicles. These suspensions were then extruded via apparatus (Lipofast; Avestin Inc., Ottawa, ON, Canada) and a polycarbonate membrane pore (100 nm) to obtain the desired lipid vesicles.

### **2.3. Preparation of Human A $\beta$ (1-40) with Thioflavin T (ThT) Probe**

Aqueous ammonia (10%) was first diluted 100 times to achieve a 0.1% concentration solution. Diluted ammonia solution was added to stock human A $\beta$  (1-40) to obtain an A $\beta$  solution. Solutions of A $\beta$  (50  $\mu$ L, 200  $\mu$ M), liposome (12.5  $\mu$ L, 20 mM), ThT (10  $\mu$ L, 1 mM), NaCl (100  $\mu$ L, 1 M), and Tris-HCl buffer (827.5  $\mu$ L, 100 mM, pH 7.5) were mixed to achieve the final 1 mL volume, placed in a cuvette, sealed with parafilm, and placed in an incubator (37 °C) to replicate body temperature present during aggregation. Control solution replacing liposome with excess Tris buffer was then made to identify the impact of liposome presence.

### **2.4. Preparation of Human A $\beta$ (1-40) with Laurdan Probe**

Laurdan probe was inserted in a 1:100 ratio of Laurdan:lipid and each component for sample preparation was adjusted accordingly. Stock A $\beta$  (1-40) was dispersed in aqueous NH<sub>3</sub> (0.1%). Solutions of liposome (12.5  $\mu$ L, 20 mM), Laurdan (25  $\mu$ L, 100  $\mu$ M), NaCl (100  $\mu$ L, 1 M), and Tris-HCl buffer (812.5  $\mu$ L, 100 mM, pH 7.5) were mixed in an Eppendorf tube and incubated for a minimum of 30 minutes at 37 °C. Once the incubation period was completed, A $\beta$  (50  $\mu$ L, 200  $\mu$ M) was added to the solution.

### **2.5. Fluorescence Spectroscopy Measurements**

Each ThT sample was measured using a fluorescence spectrophotometer (FP-8500; JASCO, Tokyo, Japan) using the ThT excitation wavelength of 444 nm and emission wavelength of 460-600 nm and measured at room temperature (25 °C). Laurdan samples were measured at excitation wavelength 340 nm and emission wavelengths 400-600 nm. Measurements started at 0 hours (immediately following sample preparation) and subsequent measurements were taken

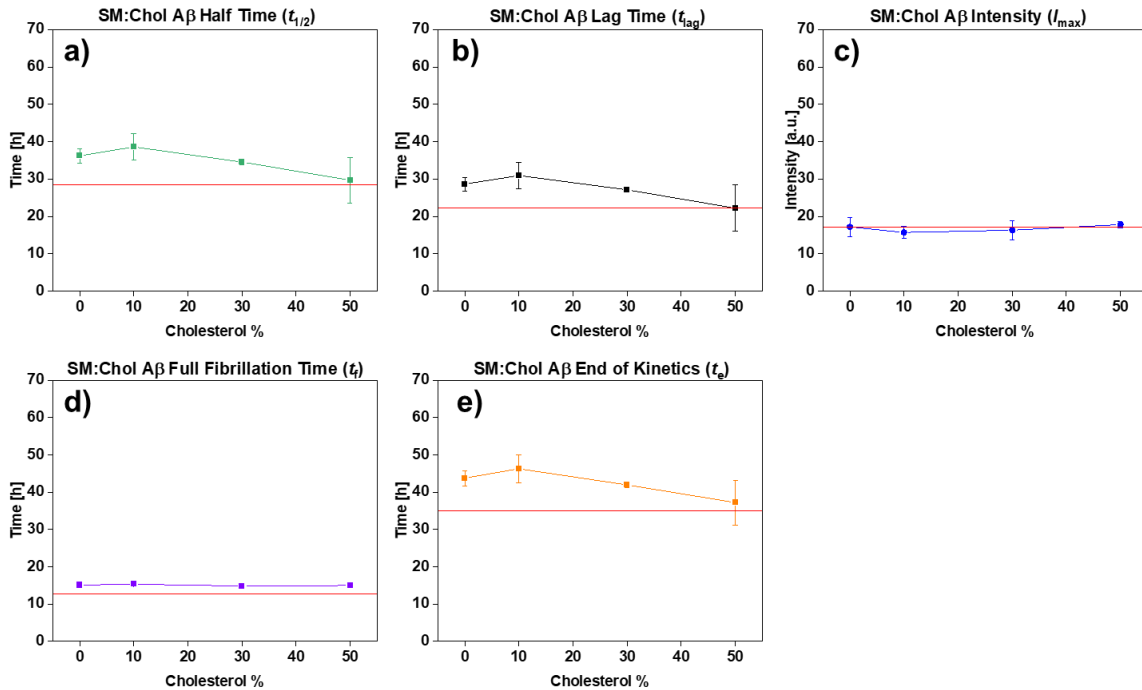
incrementally until 72-96 hours to identify the time course of the A $\beta$  fibrillation kinetics (via ThT) and membrane polarity variations (via Laurdan).

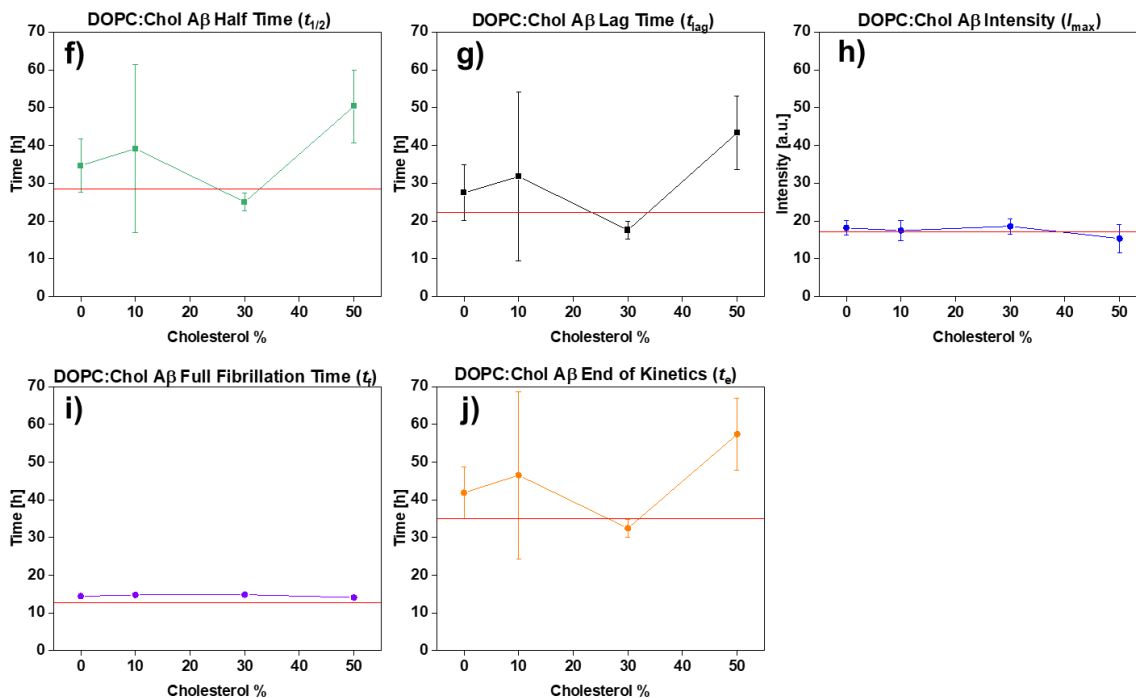
### 3. Results & Discussion

#### 3.1. Impact of Chol on the Fibrillation of A $\beta$ on SM or DOPC

|                  | $t_{1/2}$ | $t_{lag}$ | $t_f$ | $t_e$ | $I_{max}$ | $I_{min}$ | $dx$ |
|------------------|-----------|-----------|-------|-------|-----------|-----------|------|
| Control          | 28.59     | 22.26     | 12.67 | 34.93 | 17.2      | -1.1      | 3.6  |
| Error            | 6.52      | 6.44      | 0.18  | 6.59  | 1.49      | 0.02      | 0    |
| SM/Chol (10:0)   | 36.23     | 28.67     | 15.12 | 43.79 | 17.21     | 0.41      | 3.6  |
| Error            | 1.92      | 1.81      | 0.27  | 2.03  | 2.57      | 0.13      | 0    |
| SM/Chol (9:1)    | 38.63     | 30.96     | 15.34 | 46.3  | 15.72     | 0.48      | 3.6  |
| Error            | 3.62      | 3.47      | 0.31  | 3.77  | 1.64      | 0.12      | 0    |
| SM/Chol (7:3)    | 34.56     | 27.14     | 14.84 | 41.98 | 16.35     | 0.24      | 3.6  |
| Error            | 0.75      | 0.68      | 0.18  | 0.82  | 2.52      | 0.07      | 0    |
| SM/Chol (5:5)    | 29.72     | 22.23     | 14.99 | 37.22 | 17.83     | 0.36      | 3.6  |
| Error            | 6.12      | 6.21      | 0.18  | 6.03  | 0.82      | 0.13      | 0    |
| DOPC/Chol (10:0) | 34.68     | 27.46     | 14.43 | 41.89 | 18.19     | -0.01     | 3.6  |
| Error            | 7.1       | 7.34      | 0.49  | 6.86  | 1.85      | 0.29      | 0    |
| DOPC/Chol (9:1)  | 39.15     | 31.77     | 14.77 | 46.53 | 17.5      | 0.23      | 3.6  |
| Error            | 22.21     | 22.29     | 0.15  | 22.14 | 2.67      | 0.11      | 0    |
| DOPC/Chol (7:3)  | 25.04     | 17.61     | 14.86 | 32.47 | 18.61     | 0.29      | 3.6  |
| Error            | 2.35      | 2.36      | 0.05  | 2.33  | 2.05      | 0.01      | 0    |
| DOPC/Chol (5:5)  | 50.37     | 43.31     | 14.11 | 57.42 | 15.38     | -0.14     | 3.6  |
| Error            | 9.64      | 9.76      | 0.23  | 9.53  | 3.7       | 0.1       | 0    |

**Table 5-1** Key variables for A $\beta$  kinetics including: (1) Half-point of fibrillation ( $t_{1/2}$ ); (2) Lag time/nucleation ( $t_{lag}$ ); (3) Time from the start to the end of elongation ( $t_f$ ); (4) Time from the start to the end of the whole A $\beta$  kinetic process ( $t_e$ ); (5) Maximum intensity ( $I_{max}$ ); (6) Minimum intensity ( $I_{min}$ ); an (7) Time constant ( $dx$ ).





**Fig. 5-2** Kinetic parameters for Aβ fibrillation taken from **Table 5-1** with each horizontal red line representing the control (no lipid membrane) for each respective parameter (control error not shown). **(a)** SM/Chol  $t_{1/2}$  (time vs Chol %) [h]. **(b)** SM/Chol  $t_{lag}$  (time vs Chol %) [h]. **(c)** SM/Chol  $I_{max}$  (intensity vs Chol %) [a.u.]. **(d)** SM/Chol  $t_f$  (time vs Chol %) [h]. **(e)** SM/Chol  $t_e$  (time vs Chol %) [h]. **(f)** DOPC/Chol  $t_{1/2}$  (time vs Chol %) [h]. **(g)** DOPC/Chol  $t_{lag}$  (time vs Chol %) [h]. **(h)** DOPC/Chol  $I_{max}$  (intensity vs Chol %) [a.u.]. **(i)** DOPC/Chol  $t_f$  (time vs Chol %) [h]. **(j)** DOPC/Chol  $t_e$  (time vs Chol %) [h].

To compare the influence of Chol composition on Aβ fibrillation, porcine brain sphingomyelin (SM) (phase transition temperature: 38 °C; rigid at 37 °C) and 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (phase transition temperature: -17 °C; fluid at 37 °C) were used for preparing liposomes. Chol was incorporated in 4 different lipid:Chol molar ratios (10:0, 9:1, 7:3, and 5:5) to investigate how the gradually increasing amounts of Chol influences Aβ fibrillation.

Referencing **Table 5-1** and **Fig. 5-2**, time for half completion of fibrillation ( $t_{1/2}$ ) was  $28.6 \pm 6.5$  h for Aβ<sub>40</sub> without a membrane.  $t_{1/2}$  values were noticeably elevated when a membrane was included, except for the 7:3 DOPC/Chol composition (**Fig. 5-2f**). Similarly, the systems including membranes, except for 5:5 SM/Chol (**Fig. 5-2b**) and 7:3 DOPC/Chol (**Fig. 5-2g**), resulted in

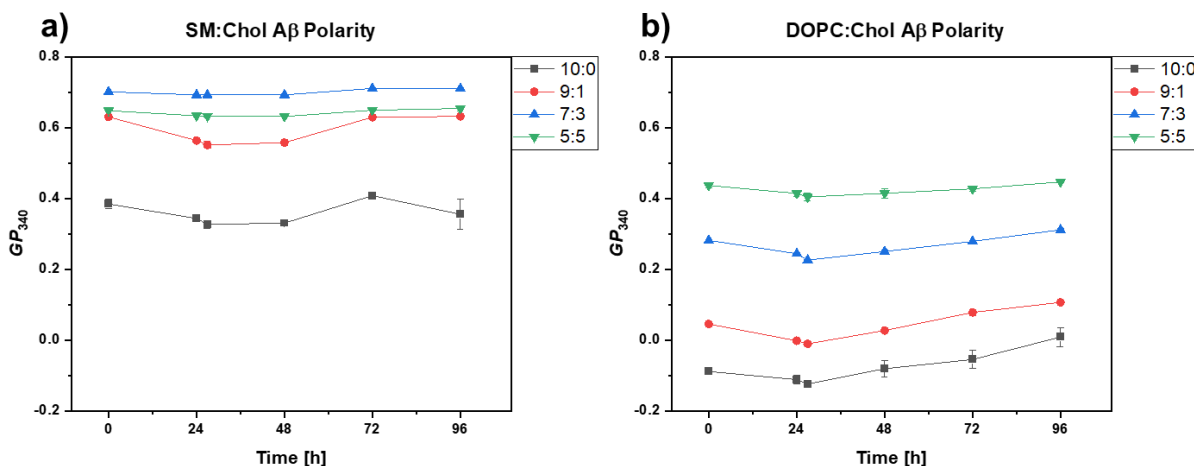
noticeably larger  $t_{lag}$  values compared to control with no liposomes. Lastly, time for fibrillation completion ( $t_f$ ) was relatively the same among membranes tested in this study but were all slightly larger than the control.  $t_f$  ignores  $t_{lag}$  so a separate variable  $t_e$  was also made to include the sum of  $t_f$  and  $t_{lag}$  (**Eqn. 5-4, 5-5, & 5-6**). This  $t_e$  is larger for all membrane compositions, except for 7:3 DOPC/Chol (**Fig. 5-2j**), compared to the control. Notably, 7:3 DOPC/Chol membranes have a possibility of reducing  $t_{lag}$  while 5:5 DOPC/Chol can be depicted as having a much higher  $t_{lag}$ . This result is in contrast to the SM/Chol compositions as only the 5:5 SM/Chol membranes have a possibility of having a lower  $t_{lag}$  than the A $\beta$  with no membranes. These results are thus indicative of the membrane being an overall speed enhancer of A $\beta$  fibrillation kinetics for DOPC membrane compositions of 30% or less Chol and SM compositions of around 50% Chol. This result is supported by past research that has also suggested that the membrane and Chol have potential to act as catalysts for A $\beta$  fibrillation (Banerjee et al., 2020; Habchi et al., 2018). In the case of these experiments, it seems that when there is enough Chol included to favor a liquid-ordered ( $l_o$ ) phase (Nyholm et al., 2011), there is visible enhancement to certain temporal parameters.

### 3.2. Laurdan Polarity Analysis of A $\beta$ Fibrillation on the SM and DOPC Membrane

After examining the influence of membrane composition on A $\beta$  fibrillation, the aim shifted to reveal effect of A $\beta$  fibrillation on the polarity of the membrane via use of the Laurdan fluorescence probe. For the same compositions of SM/Chol and DOPC/Chol analyzed above, the fluorescence spectra of Laurdan in the presence of A $\beta$ 40 was examined and the generalized polarization ( $GP$ ) was calculated with the given equation (below) where  $I_{440}$  and  $I_{490}$  represent the intensity values at wavelengths 440 nm (gel) and 490 nm (liquid-crystalline), respectively (Parasassi et al., 1990, 1991). Measurements were taken from the 0h to 96h mark to examine

polarity differences that may coincide with the key kinetics of the A $\beta$  fibrillation process.  $GP_{340}$  values were then plotted against the time course of fibrillation (**Fig. 5-3**).

$$GP_{340} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (5-1)$$

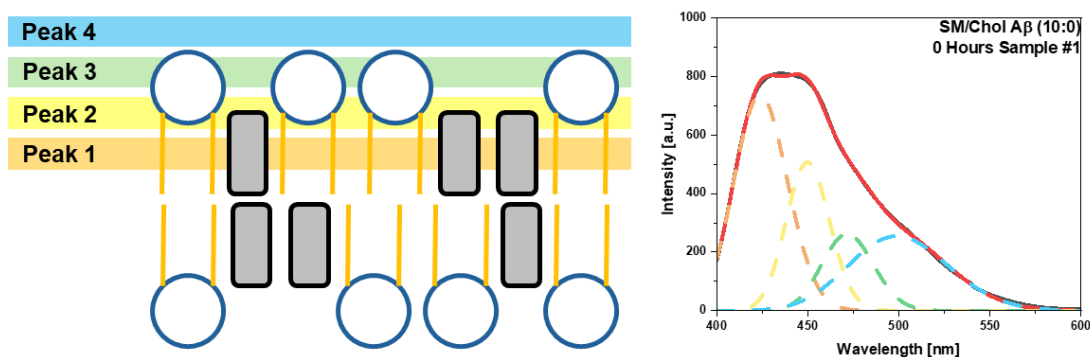


**Fig. 5-3** Polarity of each membrane composition depicted via  $GP_{340}$  values.  $GP_{340}$  and polarity are inversely correlated, indicating a lower  $GP_{340}$  value corresponding to higher polarity. **a)** Changes to the SM membrane polarity over the time course of fibrillation with different content of Chol. **b)** Changes to the DOPC membrane polarity over the time course of fibrillation with different content of Chol.

First, for SM/Chol there is a slight decrease in the  $GP_{340}$  value (increase in lipid membrane polarity) that possibly coincides with the nucleation phase ( $t_{lag}$ ) of A $\beta$  growth (**Fig. 5-3a and Table 5-1**). As the fibrillation approaches the end of kinetics time ( $t_e$ ), the majority  $GP_{340}$  values seems to return to the range of pre-fibrillation. These results overall indicate that the fibrillation process may overall increase membrane polarity during the start of kinetics, but once fibrillation is completed there is a drastic decrease in membrane polarity back to its pre-fibrillation values, suggesting the kinetic process causes the increase but the mature fibril results in the decrease. Since Laurdan is generally inserted into the membrane deeper than the superficial surface region, this change in polarity during the fibrillation process also suggests that during A $\beta$  fibrillation at the membrane surface, there is also an impact in the interior region of the lipid membrane.

For DOPC/Chol compositions there was also a similar decrease in the  $GP_{340}$  values after the 0 h initial mark (**Fig. 5-3b**). Unlike SM/Chol membranes, there seems to be a faster increase in  $GP_{340}$  after the initial decrease, most pronounced in the DOPC membrane compositions with lower amounts of Chol. In this instance, the Chol incorporation into the membrane seems to either inhibit a larger decrease in  $GP_{340}$  values post-fibrillation or alternatively help to retain the polarity increase that was brought forth by the A $\beta$  elongation process. Compared to the SM/Chol membranes, DOPC/Chol membranes ultimately appeared to be less robust to change and therefore experiences much more variation to their  $GP_{340}$  values with the addition of Chol to the membrane.

### 3.3. Deconvolution Analysis of SM/Chol and DOPC/Chol Membranes

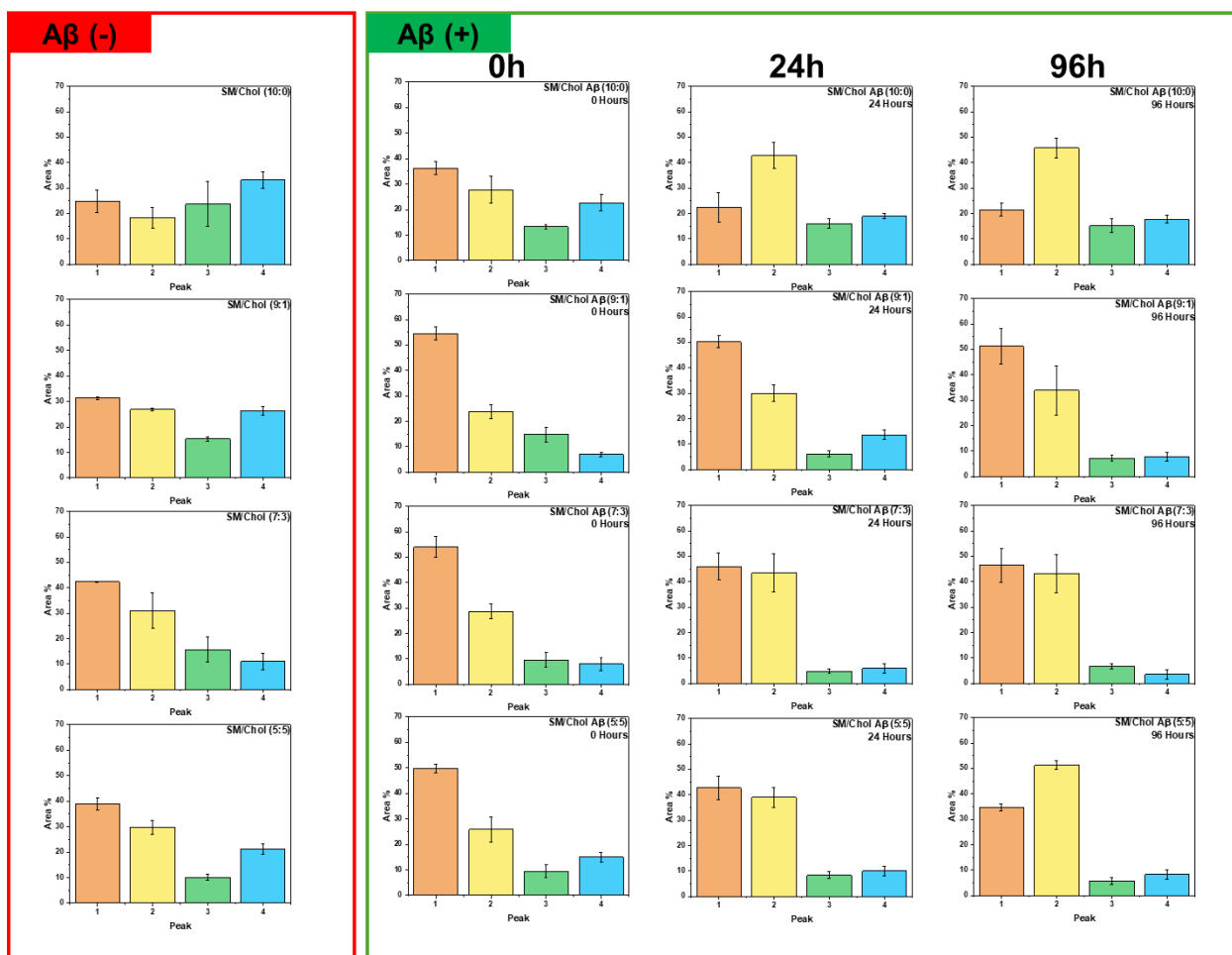


**Fig. 5-4** Schematic depiction of deconvolution analysis as performed in this work. Each peak corresponds to the respective region of the lipid membrane as follows: nonpolar (peak 1;  $\epsilon_r < 5$ ), polar aprotic (peak 2;  $5 < \epsilon_r < 20$ ), and polar protic (peak 3;  $\epsilon_r < 20$  and peak 4;  $\epsilon_r > 20$ ).

For a more in-depth analysis of the impact of A $\beta$  fibrillation on the polarity of both SM/Chol and DOPC/Chol, deconvolution analysis of each membrane was performed according to previous protocols using PeakFit software, where peak 1 was below 440 nm, peak 2 from 440-460 nm, peak 3 from 460-480 nm, and peak 4 at values 480 nm or above (Watanabe et al., 2019). Each membrane's Laurdan fluorescence spectra was deconvoluted into four component curves depicting the nonpolar (peak 1;  $\epsilon_r < 5$ ), polar aprotic (peak 2;  $5 < \epsilon_r < 20$ ), and polar protic (peak 3;  $10 < \epsilon_r < 20$  and peak 4;  $\epsilon_r > 20$ ) environment of the membrane, where  $\epsilon_r$  is the dielectric constant

(Fig. 5-4). Larger dielectric constants represent a higher polarity while small dielectric constants represent lower polarity. Thus peak 1 may be representative of a deeper layer of the membrane than peak 4, which is more representative of the area near the surface. Based on the peak area of each component, it was presumed which general region of the membrane may be contributing the most to the polarity of the overall membrane during the multiple stages of A $\beta$  kinetics. Obtained Laurdan spectra were deconvoluted as follows: (1) with no A $\beta$  present (2) A $\beta$  before fibrillation at 0 h (3) during the A $\beta$  fibrillation process at 24 h and (4) after the fibrillation ( $t_e$ ) at 96 h. These parameters enabled us to infer which possible region of the membrane (surface to deeper layer) was most heavily impacted by A $\beta$  fibrillation at each measured time slot (0 h to 96 h) of the kinetic process.

### 3.4. SM/Chol

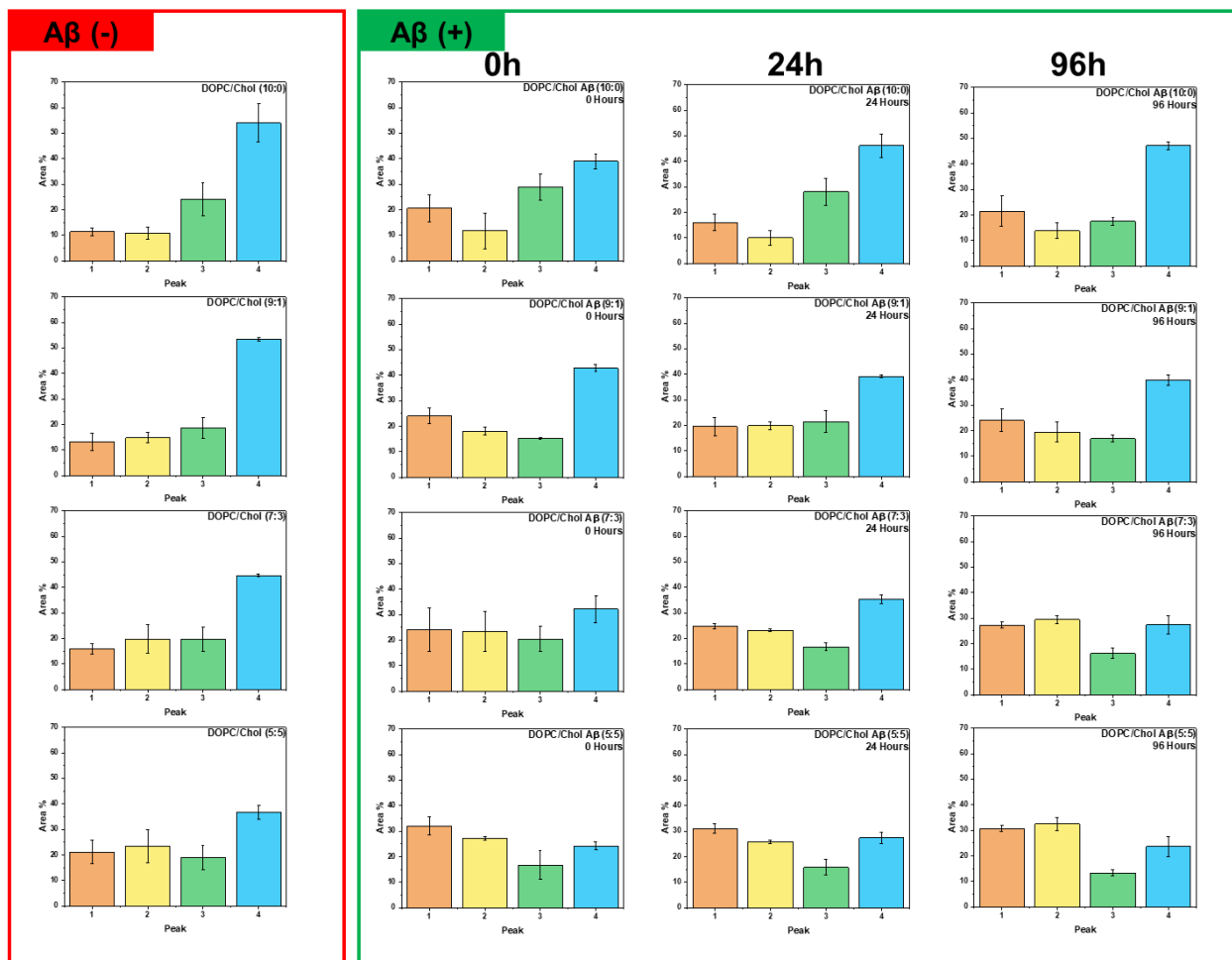


**Fig. 5-5** Area % of the deconvoluted  $GP_{340}$  spectra for SM/Chol without amyloid  $\beta$  ( $A\beta^-$ ) and with amyloid  $\beta$  ( $A\beta^+$ ). Samples with amyloid  $\beta$  were deconvoluted immediately upon introduction of  $A\beta$  (0h), 1 day after introduction of  $A\beta$  (24h), and at the estimated completion of fibrillation at 4 days (96h).

Firstly, for SM/Chol membrane compositions with no  $A\beta$  inclusion, there was a slight dominance of the polar peak 4 for 0% Chol, but as Chol was incrementally added to the membrane the nonpolar peak 1 gradually increased its dominance (**Fig. 5-5**). Although it seems contradictory that the expectedly more fluid SM/Chol (5:5) had a large dominance of the hydrophobic peak 1, this may be attributed to the creation of large  $l_o$  domains causing this effect (Samsonov et al., 2001). Upon addition of  $A\beta_{40}$  at 0 h there was an immediate dominance of peak 1 across all compositions, possibly due to the hydrophobic nature of the C-terminal of  $A\beta$  (Chen et al., 2017a). At the 24 h

mark, the most dominant components for all composition were peak 1 and peak 2, with peak 1 being larger across all composition except for the SM/Chol (10:0) membrane. Upon reaching 96 h of aggregation, peak 1 remained the dominant curve for all compositions except for SM/Chol (10:0) and SM/Chol (5:5), where peak 2 was markedly larger for these specific compositions. This indicates that for SM/Chol (9:1, 7:3, and 5:5) the major contributor to the  $GP_{340}$  values was the nonpolar region (peak 1) at possibly the deeper region of the membrane, while the SM/Chol (10:0) and partially the SM/Chol (5:5) composition had the polar aprotic region (peak 2) as the main contributor to its respective spectra. Such a large difference between peaks 1 and 2 in SM/Chol (10:0) and SM/Chol (9:1, 7:3, and 5:5) at 24 h and 96 h (excluding 5:5 at 96 h) suggests that Chol plays a large role in maintaining the deeper layered hydrophobic/non-polar nature of the membrane. Past research has suggested that Chol can decrease the hydrophobicity near the surface of the membrane, thereby causing a possible preference for the dominance of peak 1 in these results (Subczynski et al., 1994). Although slight shifts could be seen for peaks 3 and 4, these peaks and their changes throughout fibrillation remained relatively low or negligible compared to peaks 1 or 2.

### 3.5. DOPC/Chol

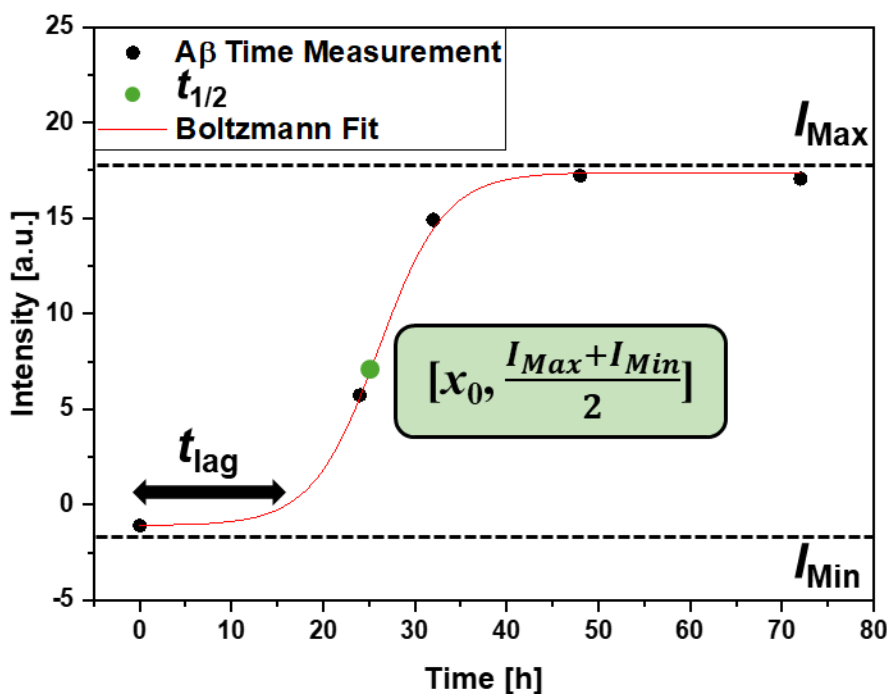


**Fig. 5-6** Area % of the deconvoluted  $GP_{340}$  spectra for DOPC/Chol without amyloid  $\beta$  ( $A\beta^-$ ) and with amyloid  $\beta$  ( $A\beta^+$ ). Samples with amyloid  $\beta$  were deconvoluted immediately upon introduction of  $A\beta$  (0h), 1 day after introduction of  $A\beta$  (24h), and at the estimated completion of fibrillation at 4 days (96h).

For DOPC/Chol deconvolutions (**Fig. 5-6**), unlike SM/Chol, the results without  $A\beta$  emphasized that peak 4 was a large contributor to the spectra across all compositions. As more Chol was added to the sample there was a noticeable increase in both peaks 1 and 2, however, peak 4 remained the dominant contributor. Upon addition of  $A\beta_{40}$  (0h), all compositions favor an increase in peak 1 contribution while peak 4 decreased. Notably, only in the 5:5 DOPC/Chol composition did peak 1 replace peak 4 as the most dominant component when  $A\beta$  was added. Once again, this effect may be attributed to the hydrophobic nature of the C-terminal  $A\beta$  (Chen et al.,

2017a). 24 h after addition of A $\beta$ 40, no major shifts in component-dominance were observed aside from slight shifts such as increased peak 3 in the 9:1 composition. When analyzed again at the 96 h mark, peak 4 was once again a dominant contributor. Also notable at 96 h is the larger presence of peak 2 in the 7:3 and 5:5 compositions, whereas at 24 h peak 1 was always a larger contributor. Overall, the deconvolution of the DOPC liposomes emphasize the polar nature of the membrane surface via peak 4 contribution but reveals additional variations in peaks 1 and 2 that suggest A $\beta$  aggregation possibly impacts the polarity in the deeper layers of the lipid membrane.

### 3.6. A $\beta$ Kinetic Analysis Parameters and Aggregation Over Time



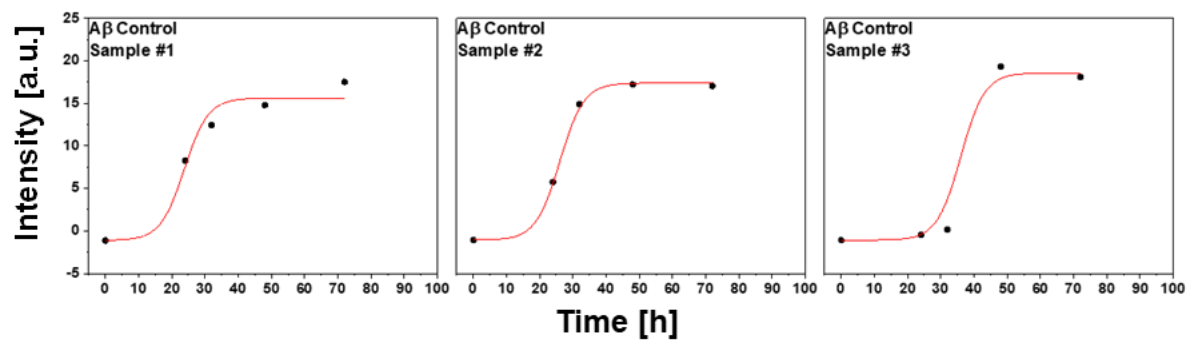
**Fig. 5-7** Fibrillation kinetics curve for A $\beta$  growth. Time measurement values were fitted according to the Boltzmann equation.  $I_{Max}$  represents the intensity at the end of fibrillation,  $I_{Min}$  represents the intensity before fibrillation begins,  $t_{1/2}$  represents the point where half the fibrillation was expected completed, and  $t_{lag}$  the nucleation phase setting up fibrillation.

When analyzing the fibrillation kinetics of A $\beta$  there exist several noteworthy events. Firstly, the “lag phase” or “nucleation phase” occurs, which encompasses the period at which the A $\beta$

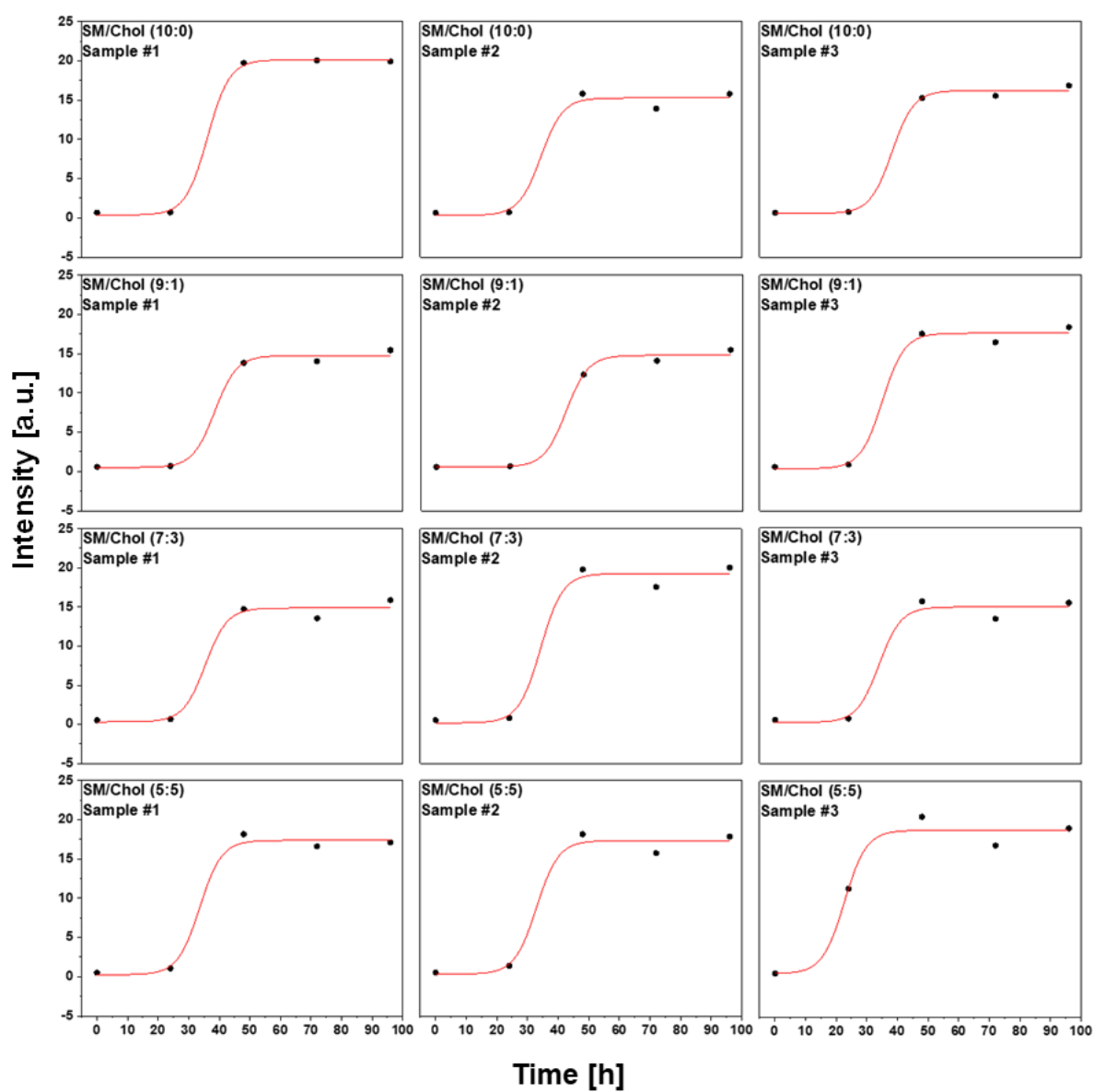
monomers build into oligomers and from oligomers into fibrils (Arosio et al., 2015). During this period of A $\beta$  growth, the fluorescence intensity given by ThT will be relatively low to nonexistent since there is not a vast presence of  $\beta$ -sheet structures in the monomeric units. This period is appropriately labeled as  $t_{lag}$  (lag time) in the amyloid beta kinetics curve (**Fig. 5-7**).

After the nucleation phase of the kinetics curve, there is a sudden large increase in the intensity of the ThT signal. One notable point during this growth period is  $t_{1/2}$  which corresponds to the time invested for half of the fibrillation to complete. By subtracting the lag time ( $t_{lag}$ ) from  $t_{1/2}$  and doubling the resulting value, the time for total fibrillation ( $t_f$ ) will be given by **Eqn. 5-5**. Further,  $t_{1/2}$  may be denoted on the kinetics curve at the point  $[t_{1/2}, \frac{I_{max}+I_{min}}{2}]$  with  $I_{max}$  being the maximum intensity marking completion of fibrillation and  $I_{min}$  being the intensity before fibrillation begins (**Fig. 5-7**).

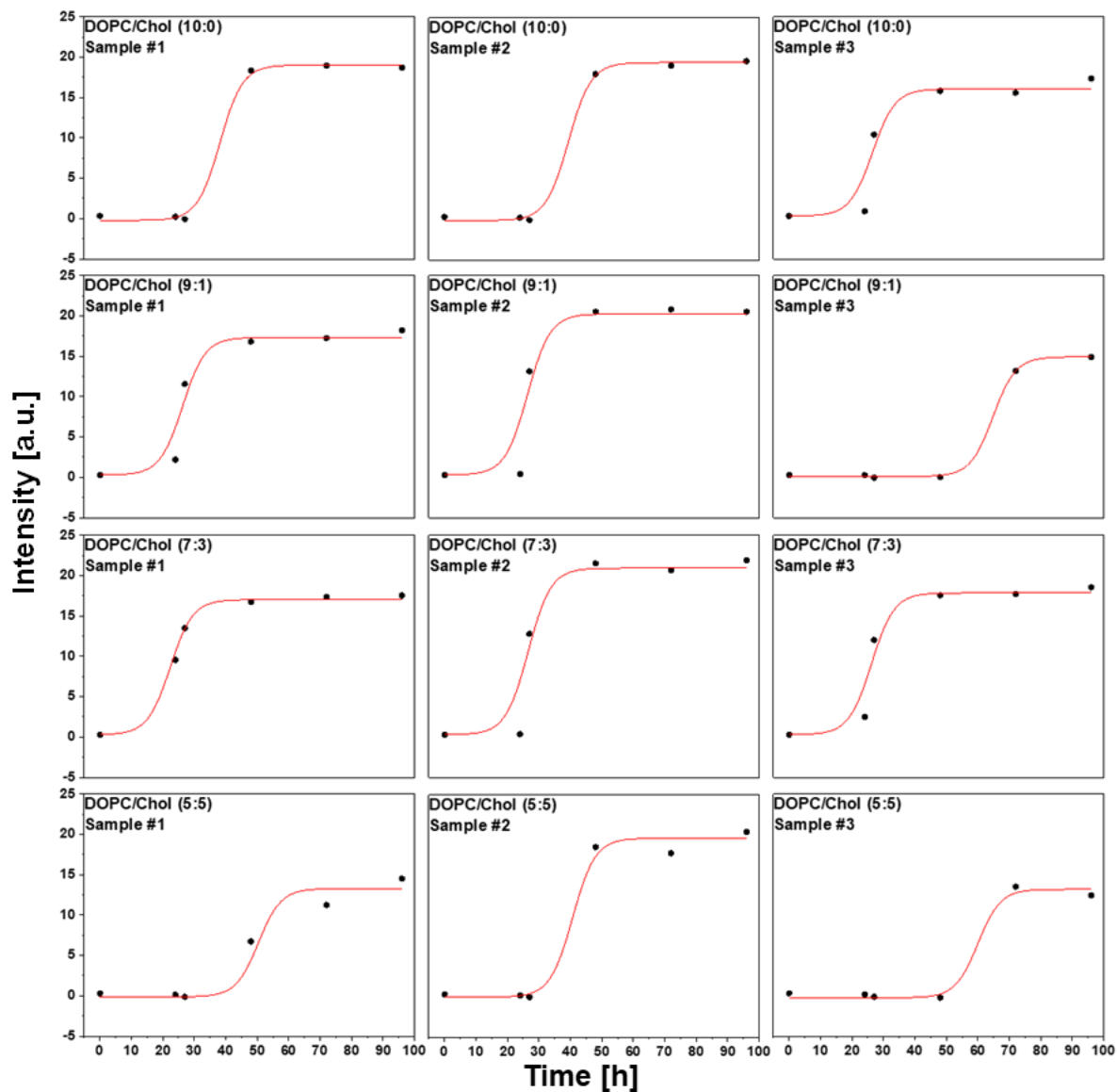
### a) A $\beta$ Control (No Lipid Membrane)



### b) SM/Chol



**c) DOPC/Chol**



**Fig. 5-8** Boltzmann fit curves for: **a)** A $\beta$  control (no membrane), **b)** A $\beta$  SM/Chol, and **c)** A $\beta$  DOPC/Chol.

Time course of fluorescence intensity values for ThT were analyzed (**Fig. 5-8**), via the Boltzmann equation (**Eqn. 5-2**). Over the time course of fibrillation kinetics that ended at the 72-96 h mark,  $t_{1/2}$  and  $I_{\max}$  values were used to calculate the slope of fibrillation kinetics ( $y_f$ ) (**Eqn. 5-3**),  $t_{\text{lag}}$  (**Eqn. 5-4**), and consequently the total time for fibrillation to complete ( $t_f$ ) (**Eqn. 5-5**); where  $A_2$  is  $I_{\max}$ ,  $A_1$  is  $I_{\min}$ ,  $dx$  is the time constant, and  $x_0$  is  $t_{1/2}$ .  $t_e$  (**Eqn. 5-6**) is the time for end of kinetics

and indicates that the entire fibrillation process has been completed and the maximum intensity ( $I_{\max}$ ) has been reached. These values are used to reveal any differences with the incremental increase in Chol and are summarized in **Fig. 5-7**.

$$y = \left( \frac{A_1 - A_2}{1 + e^{\frac{x-x_0}{dx}}} \right) + A_2 \quad (5-2)$$

$$y_f = \frac{A_2 - A_1}{4dx} \quad (5-3)$$

$$t_{lag} = \left( -\frac{1}{y_f} \right) \left[ \frac{A_1 + A_2}{2} - y_f(x_0) \right] \quad (5-4)$$

$$t_f = 2(t_{\frac{1}{2}} - t_{lag}) \quad (5-5)$$

$$t_e = t_{lag} + t_f \quad (5-6)$$

### 3.7. Impact of Membrane Composition on A $\beta$ Fibrillation

Various publications have reported that there are many different characteristics of a membrane that influence the fibrillation of A $\beta$  (Banerjee et al., 2020; Bucciantini et al., 2014; Matsuzaki, 2014). Results from this study have supported this notion by indicating that the key variables in A $\beta$  kinetics ( $t_{lag}$  and  $t_f$ ) can vary in the presence of membranes. Nucleation time ( $t_{lag}$ ) generally increased in liposome suspensions compared to control, most likely due to an initial interaction between A $\beta$  monomers and the membrane surface, slightly delaying the time that the monomeric units can interact and aggregate. Most of the liposome compositions used in this study that lead to variation in  $t_{lag}$  fall between the category of liquid-disordered ( $l_d$ ) and liquid-ordered ( $l_o$ ) (DOPC/Chol 10:0, 9:1, 7:3, 5:5) (SM/Chol 5:5); the remainder are in either solid ordered ( $s_o$ ) or solid ordered with some liquid ordered ( $s_o + l_o$ ) (SM/Chol 10:0, 9:1, 7:3) (Nyholm et al., 2011).

Membrane compositions that were in their fluid phases ( $l_o$  or  $l_d$ ) revealed potential to have a shortened nucleation time that fall within the margin for error. One hypothesized method for enhancement by fluid membranes is lipid incorporation into the fibril structure (Krausser et al., 2020). Specifically, compositions of DOPC/Chol membranes with 30 mol% or less Chol and SM/Chol (5:5) revealed a potential shortened nucleation (**Fig. 5-2f-j**). One possibility for this result could be that the fluid membranes were able to have more contact points with A $\beta$ 40 due to the more flexible nature of a soft membrane due to the lower bending moduli and thereby have a catalytic effect on nucleation (Takechi-Haraya et al., 2016). However, total fibrillation time ( $t_f$ ) remained a stagnant value across all membrane compositions, suggesting that although fibrillation could be initiated sooner on fluid membranes, the total time for aggregation to complete interestingly took a similar amount of time. One explanation noted in the past is that only membranes with certain characteristics, such as oxidized membranes, have notable enhancement of fibrillation kinetics (Shimanouchi et al., 2012). When comparing the SM and DOPC liposomes, both types of liposomes showed similar  $t_f$  values, which implicates the DOPC-enriched membrane as using its fluid quality to enhance primarily the nucleation but not the elongation or maturation of the fibril.

### 3.8. Polarity Changes Caused by A $\beta$ Fibrillation

For both SM/Chol and DOPC/Chol, after passing their lag times, there was a consistent increase in the polarity (decrease in  $GP_{340}$ ). When the A $\beta$  fibril elongation is presumed to be finished, there is then a noticeable decrease in the membrane polarity. These variations of polarity over time heavily indicate that the overlapping kinetic stages could be responsible (**Fig. 5-3**). This result can be verified as attributed to fibrils by overlapping points with the kinetics data in **Table 5-1**. For SM/Chol compositions the end of fibrillation ( $t_e$ ) range of 37-44 h coincides with the point

at which the  $GP_{340}$  value begins to increase back to or beyond its original value at 0 h. For DOPC/Chol compositions with the wider range of the end of fibrillation ( $t_e$ ) from 32-57 h, the kinetic parameters seem to also coincide with the  $GP_{340}$  values. DOPC/Chol liposomes showed a more gradual and steady increase of  $GP_{340}$  between the end of nucleation ( $t_{lag}$ ) to the end of kinetics ( $t_e$ ), and by 96 h the  $GP_{340}$  values became larger than those at the starting 0 h which was not as distinct for SM/Chol. These results once again suggest that the DOPC membrane was less robust to change and is more readily influenced by both the incorporation of Chol and the kinetics of A $\beta$ .

Although the  $GP_{340}$  results assume fibrils are only influencing the membrane at their usual contact points at the membrane surface, the deconvolution analysis implicates the fibrillation process impacting the interior region of the membrane as seen in the deconvolution analysis (**Fig. 5-5 & 5-6**). Past research has suggested that there is a two-step mechanism for disruption caused by A $\beta$  that includes: 1) disruption immediately after introduction of A $\beta$  and 2) subsequent fibril formation (Sciacca et al., 2012, 2018). This effect may be noticeable in both **Fig. 5-5** and **Fig. 5-6** as there is variation among deconvoluted peaks for 0 h samples that include A $\beta$  compared to those without.

#### 4. Summary

From the results obtained in this work, we may conclude that the inclusion of a membrane has possible impact on the lag time ( $t_{lag}$ ) of A $\beta$  and consequently the parameters that the lag time is affiliated with for calculations. However, the overall time for the elongation and fibrillation to start and complete revealed itself to be relatively stagnant due to the unchanging full fibrillation time ( $t_f$ ). From analyzing polarity of the membrane via  $GP_{340}$  there was a notable increase in polarity followed by a decrease in polarity that possibly coincides with the key points in the kinetic process. Deconvolution analysis was done to examine whether deeper layers of the membrane

were influenced by this polarity variation or not. Results revealed that this variation is present in both SM/Chol and DOPC/Chol. Hydrophobic/non-polar and interior polar regions of the membrane become more dominant as the fibrillation process occurs. For DOPC/Chol we noted that the polarity was less robust to change upon addition of A $\beta$ . Thus, we may conclude that the DOPC/Chol membrane has more impact on A $\beta$  fibrillation kinetics, however, the fibrillation itself has more impact on the characteristics of SM/Chol.

## **Chapter 6**

### **General Conclusions**

This study has ultimately provided insight into the examination of increasingly complex brain relevant lipid membranes using the primary technique of fluorescence spectroscopy. Additionally, this study has emphasized the need for incorporation of previously suggested techniques that have yet to be widely integrated into the realm of lipid membrane analysis. Specifically, the results obtained in this study have aimed to further establish the tetrahedron diagram as a useful mapping tool for the analysis of phase in complex biologically relevant lipid membranes.

Chapter 1 provided background information on the main goals of this study. Information was provided on the brain lipidome and the most dominant lipids associated with the brain (bSM, PC, PE, and Chol). Fluorescence spectroscopy was then introduced as the primary method of analysis used in the course of this study using fluorescent probes such as ThT, Laurdan, and DPH for analyzing different aspects of the membrane or associated molecules. Lastly, the necessity of techniques that broaden the scope of the traditional ternary phase diagram was briefly discussed as the overarching goal of this dissertation.

Chapter 2 introduced the concept of fluorescence analysis of the membrane using a simple unary and binary mixture of bSM and Chol. This mixture was chosen due to the widely studied complex interactions of bSM and Chol, allowing this mixture to be simple in quantity of components but complex in potential properties. Results emphasized that compositions with

Chol % of 30% and 50% had a robustness of remaining in an ordered phase. Deconvolution of these compositions revealed heterogeneity, suggesting that there is both order and disorder in the solvent environment, indicating that these compositions may be in the  $l_o$  phase. These results thus provide support for the existence of the  $l_o$  phase in bSM/Chol mixtures, a known precursor to lipid raft phenomena.

Chapter 3 expanded on the concepts introduced in the previous chapter by introducing ternary compositions to the study. 4 separate ternary diagrams that corresponded to the different variations of the 4 selected brain relevant lipids were constructed. Since PE is an often overlooked lipid compared to Chol in membrane analytical studies, this chapter hoped to reveal some of its properties and showed that DOPE acted in a similar manner to Chol in inducing disorder at lower temperatures and order at higher temperatures in the bSM membrane. In the DOPC membrane, DOPE primarily induced order. These concepts were illustrated by proposing the “flattened tetrahedron diagram” as an intermediary step between chapters to track trends across multiple separate ternary phase diagrams more readily.

Chapter 4 adapted the concepts examined in the previous chapters and applied them to more complex quaternary membrane compositions. Quaternary membrane compositions revealed themselves to be surprisingly robust in heterogeneity as the temperature was increased. Deconvolution analysis of these compositions revealed that Laurdan experienced both a disordered polar environment and a more ordered non-polar and polar aprotic environment, further solidifying the concept of heterogeneity. Finally, this chapter incorporated these quaternary compositions into the folded 3D tetrahedron diagram and revealed that even with the variation in phase on the faces, the interior of the tetrahedron was determined to be primarily heterogeneous. This chapter thereby provided insight into quaternary composition membranes and the utility of a tetrahedron diagram

in membrane analysis. Tetrahedron diagram mapping analysis was finally proposed for biologically relevant lipid membranes.

Chapter 5 then pivoted into the more biological sphere of membrane analysis by introducing the membrane to A $\beta$  peptide. Connections between membrane composition and A $\beta$  aggregation kinetics were analyzed and revealed that certain DOPC/Chol membranes influenced some temporal parameters of A $\beta$  aggregation more noticeably. Laurdan and deconvolution analysis revealed the A $\beta$  aggregation itself seemed to influence the properties of the bSM/Chol membrane more noticeably.

Lastly, chapter 6 has summarized the main points in each chapter of the study. Overall, this study hopes to provide insight into increasingly complex membrane properties. This study also hopes to provide support for the necessity in incorporating techniques that progress the current ternary phase diagram to adopt more complex membrane compositions via the tetrahedron diagram.

## Suggestions for Future Works

### 1. Examination of A $\beta$ interactions with more complex membranes

In chapter 5, this study focused on the simple unary and binary mixtures of bSM/Chol and DOPC/Chol for their interactions with A $\beta$ . However, since biologically relevant membranes are more complex, the interactions of A $\beta$  with ternary and quaternary membranes is an area of analysis that should still be expanded upon using the fluorescent methodology used in this study. Additionally, since DOPC is a synthetic lipid, one proposal would be to re-examine the effects on A $\beta$  aggregation kinetics for consistency with a naturally occurring biological lipid.

This research primarily focused on Laurdan fluorescence and deconvolution, however, DPH fluorescence, as shown in other chapters, is complimentary to Laurdan and may provide further insight into the phase of the membrane at each stage of A $\beta$  aggregation. Cartesian diagrams may be made and allow the order and disorder of the membrane to be elucidated.

This research also focused on only one type of A $\beta$ , the (1-40) variant, thus examination of other variants such as A $\beta$  (1-42) may prove useful in further establishing a trend for behavior of A $\beta$  kinetics with certain membrane compositions. Microscopy techniques to examine the appearance of fibrillation across variants and membrane compositions may also prove useful for revealing behaviors of A $\beta$ .

## **2. Expansion in tetrahedron diagram utility and more complex membranes**

This research served as preliminary work in further proving the usefulness in a tetrahedron diagram for analysis of a quaternary membrane composition. Specifically, there were only 4 primary lipids analyzed in this research project (bSM, DOPC, DOPE, and Chol). Thus, this tetrahedron diagram mapping analysis should be applied to various different biologically relevant membrane compositions to prove utility. Additionally, membrane compositions with restrictive ratios (DOPE and Chol) should be replaced with other lipid species to further obtain relevant points for the entirety of the interior of the tetrahedron diagram.

Although this research aimed to broaden the scope of lipid research from ternary to quaternary, this concept may be further expanded with increasingly complex membranes. For example, a 5 or 6 composition membrane may be examined with the same line of logic with their corresponding 3D geometric shapes. This may bring research closer to replicating more biologically relevant membranes for medical insight or other therapeutic applications of lipid membrane analysis.

## Nomenclatures

|                             |   |                                                            |        |
|-----------------------------|---|------------------------------------------------------------|--------|
| $T_m$                       | = | Phase transition temperature                               | [°C]   |
| $P$                         | = | Fluorescence polarization                                  | [-]    |
| $1/P$                       | = | Fluidity                                                   | [-]    |
| $G$                         | = | Correction factor                                          | [-]    |
| $GP_{340}$                  | = | Generalized polarization with excitation wavelength 340 nm | [-]    |
| $I_{440}$                   | = | Intensity at 440 nm                                        | [a.u.] |
| $I_{490}$                   | = | Intensity at 490 nm                                        | [a.u.] |
| $I_{  }$                    | = | Intensity parallel to the excited light                    | [a.u.] |
| $I_{\perp}$                 | = | Intensity perpendicular to the excited light               | [a.u.] |
| $t_{1/2}$                   | = | Half-time                                                  | [h]    |
| $t_{lag}$                   | = | Lag time                                                   | [h]    |
| $t_f$                       | = | Full fibrillation time                                     | [h]    |
| $t_e$                       | = | End of kinetics                                            | [h]    |
| $I_{max}$                   | = | Maximum intensity                                          | [a.u.] |
| $I_{min}$                   | = | Minimum intensity                                          | [a.u.] |
| $\epsilon$ and $\epsilon_r$ | = | Dielectric constant/relative permittivity                  | [-]    |

## List of Abbreviations

|           |                                               |
|-----------|-----------------------------------------------|
| bSM/SM    | Porcine brain sphingomyelin                   |
| DOPC      | 1,2-dioleoyl-sn-glycero-3-phosphocholine      |
| DOPE      | 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine |
| Chol      | Cholesterol                                   |
| SUV       | Small unilamellar vesicle                     |
| Laurdan   | 6-dodecanoyl-2-dimethylamino naphthalene      |
| DPH       | 1,6-diphenyl-1,3,5-hexatriene                 |
| ThT       | Thioflavin T                                  |
| A $\beta$ | Amyloid- $\beta$                              |
| AD        | Alzheimer's disease                           |
| $s_o$     | Solid-ordered/gel                             |
| $l_o$     | liquid-ordered                                |
| $l_d$     | liquid-disordered                             |

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## List of Publications

### [Papers]

1. Ajaikumar, A., Watanabe, N.M., Suga, K., Okamoto, Y., and Umakoshi, H. Amyloid beta (A $\beta$ ) fibrillation kinetics and its impact on membrane polarity. *J Bioenerg Biomembr* **57**, 1–10 (2025).
2. Ajaikumar, A. and Umakoshi, H. Fundamental characteristic analysis of sphingomyelin and cholesterol interactions via Laurdan and DPH fluorescence. (Membrane accepted) (2026).
3. Ajaikumar, A., Watanabe, N.M., Toshii, M., Okamoto, Y., and Umakoshi, H. Analysis of interfacial membrane polarity of brain-related lipids via flattened tetrahedron phase diagram. (Langmuir accepted) (2026).
4. Ajaikumar, A., Lee, J.H., Watanabe, N.M., and Umakoshi, H. Tetrahedron phase diagram analysis of quaternary lipid membrane compositions using Laurdan and DPH fluorescence. (In preparation).

### [Related Papers]

1. Lee, J.H., Lim, D.B., Aiba, H., Watanabe, N.M., Yoshimoto, N., Kwak, M.K., Ajaikumar, A., Jung, H.S., and Umakoshi, H. High-throughput membrane physicochemical profiling of multi-component liposome. (In preparation).

**[Conference/Symposium]**

- 1) Ajaikumar, A., Watanabe, N.M., Okamoto, Y., and Umakoshi, H. Impact of Amyloid- $\beta$  Fibrillation on Membrane Polarity. SCEJ 89<sup>th</sup> Annual Meeting, Osaka, Japan. (2024).
- 2) Ajaikumar, A., Watanabe, N.M., Toshii, M., Okamoto, Y., and Umakoshi, H. Comprehensive polarity analysis of common brain affiliated lipid membranes. 47<sup>th</sup> Annual Meeting of the Membrane Society of Japan, Tokyo, Japan. (2025).

## Acknowledgements

Arun Ajaikumar expresses his sincere gratitude to Professor Dr. Hiroshi Umakoshi (Division of Chemical Engineering, Graduate School of Engineering Science, The University of Osaka) for his constructive comments, feedback, encouragement, and patience throughout the course of this study. Arun Ajaikumar also wishes to express his gratitude to Professor Dr. Nobuyuki Matsubayashi and Professor Dr. Norikazu Nishiyama (Division of Chemical Engineering, Graduate School of Engineering Science, The University of Osaka) for their insightful questions and comments toward the contents of this dissertation. Arun Ajaikumar also wishes to sincerely thank Professor Dr. Nozomi Morishita Watanabe (Division of Chemical Engineering, Graduate School of Engineering Science, The University of Osaka) for her constructive feedback and continued encouragement throughout the entirety of this study. Additional gratitude is given to Professor Dr. Keishi Suga (Graduate School of Engineering, Tohoku University) for his feedback on several parts of this dissertation and collaboration on published work. Gratitude is also given to Professor Dr. Yukihiro Okamoto for various assistance throughout this study.

Sincere appreciation is also given to Professor Dr. Tonya Kuhl (The University of California, Davis) for her various constructive comments on this study. Additional appreciation is given to Professor Dr. Hosup Jung (Seoul National University) for provision of instruments used for completing parts of this study. Special thanks are also given to Ms. Keiko Fukumoto for her support throughout this study.

Arun Ajaikumar wishes to give special thanks to Junghu Lee and Miyu Toshii (Division of Chemical Engineering, Graduate School of Engineering Science, The University of Osaka) for their assistance in experimentation during this study. Special thanks are also given to all the past and present members of the B-ICE laboratory for their various assistance and kindness throughout the course of this study. Additional gratitude is given to Ms. Hiromi Ishikawa and Ms. Naoko Nakanishi (International Student Room, Graduate School of Engineering Science, The University of Osaka) for their various assistance and kindness throughout this study.

Arun Ajaikumar also expresses great appreciation for the JEES T. Banaji Scholarship, JASSO Scholarship, and Graduate School of Engineering Science Sigma Scholarship for financial support throughout this study.

Arun Ajaikumar would like to express the utmost gratitude to his parents Malathi Marimuthu and Ajaikumar Raju for their continued and unwavering support and patience throughout the entirety of his academic career.

Arun Ajaikumar would also like to express his sincere appreciation and gratitude to all his friends that continually encouraged and supported him throughout the course of his study.

Arun Ajaikumar would like to thank God for providing the opportunity, strength, and patience needed to graduate this doctoral course.