Ceramide and Cholesterol Interactions in Phospholipid Membranes: A $^2$H NMR Study

by
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Abstract

Sphingolipids constitute a significant fraction of cellular plasma membrane lipid content. Among sphingolipids, ceramide levels are usually very low. However, in some cell processes like apoptosis, cell membrane ceramide levels increase markedly due to activation of enzymes like sphingomyelinase. This increase can change the physical state of the membrane by promoting molecular order and inducing solid ordered ($S_o$) phase domains. This effect has been observed in a previous $^2$H NMR study on membranes consisting of palmitoyl sphingomyelin (PSM) and palmitoyl ceramide (PCer). Cholesterol (Chol), is also present at high concentrations in mammalian plasma membranes and has a favorable interaction with sphingomyelin (SM), together forming domains in the liquid ordered ($L_o$) phase in model membranes. There are reports that Chol is able to displace ceramide (Cer) in SM bilayers and abolish the $S_o$ phase domains formed by SM:Cer. This ability of Chol appears to be concentration dependent; in membranes with low Chol and high Cer contents, $S_o$ phase domains hypothesized to be rich in Cer coexist with the continuous fluid phase of the membrane.

Here, first we study the effect of increasing PCer concentration in PSM:Chol bilayers, using $^2$H NMR. Chol:PCer mol ratios were 3:1, 3:2 and 3:3, at a fixed 7:3 PSM:cholesterol mol ratio. Both PSM and PCer were monitored, in separate samples, for changes in their physical state by introducing a perdeuterated palmitoyl chain in either molecule. Second, we investigate the effect of replacing PSM with DPPC to test the influence on membrane phase behavior of replacing sphingosine with a palmitoylated glycerol backbone. Third, we explore the effect of adding an unsaturated lipid present at a high level in plasma membranes, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), to the PSM:Chol:PCer 7:3:3 lipid mixtures. This was done to study an approximate cell membrane outer leaflet mimetic lipid mixture.

We found that PCer induces highly stable $S_o$ phase domains in PSM:Chol, DPPC:Chol and POPC:PSM:Chol bilayers. This effect is most pronounced in bilayers with Chol:PCer 1:1 molar ratios, and below 40 °C. PCer is more effective in ordering PSM:Chol bilayers than analogous bilayers composed of DPPC:Chol.
Keywords: Deuterium nuclear magnetic resonance (2H NMR) spectroscopy; Sphingolipids; Ceramide; Phospholipids; Cholesterol; Solid-ordered (S₀) domains; Liquid-ordered phase (L₀)
To

Nafiseh

And

Melody
Acknowledgements

First of all, I want to thank my wonderful supervisor, Dr. Jenifer Thewalt. I was inspired by her wealth of knowledge, everlasting positive attitude, patience, and caring nature. Thanks for all your support and also your NMR, lipids and life lessons. Next, I thank Dr. Martin Zuckermann, a member of my supervisory committee. Martin has always been encouraging and showed incredible interest in my work. I am thankful for all his invaluable suggestions and questions. Thanks to Dr. Michael Hayden for his positive feedback during my committee meetings and also thoroughly reading my thesis, his comments and suggestions significantly improved the quality of this thesis. I had the honour to collaborate with two distinguished scientists in the field of lipid research, Dr. Félix Goñi and Dr. Alicia Alonso from the University of the Basque Country. They supported planning the projects and writing up research manuscripts with their insight and depth of knowledge. Also, they provided partial financial support in purchasing lipids. I am grateful for all the work done by two members of Dr. Jenifer Thewalt’s lab. First, Dr. Sherry Leung, who taught me all the experimental and analysis techniques I needed to transform to the world of lipids and $^2$H NMR. She also significantly contributed to planning the projects. Second, Tejas Phaterpekar was a great help in preparing samples and running experiments related to the DPPC:Chol:PCer project. He helped me in interpreting and documenting $^1$H NMR spectra as well. I also thank all other members of Dr. Jenifer Thewalt's lab for their contributions to the present work and also for providing the friendliest lab atmosphere, thank you Dr. Miranda Schmidt, Dr. Mehran Shaghaghi, Bashe Bashe, Joanne Mercer and Iulia Bodnariuc.

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<th>Acronym</th>
<th>Description</th>
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<tr>
<td>$M_1$</td>
<td>First moment or average spectral width</td>
</tr>
<tr>
<td>$^1$H NMR</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>$^2$H NMR</td>
<td>Deuterium nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Cer</td>
<td>Ceramide</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>ddw</td>
<td>Deuterium depleted water</td>
</tr>
<tr>
<td>DilC18</td>
<td>1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine or Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>DPPC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>DSC</td>
<td>Electric field gradient</td>
</tr>
<tr>
<td>DSC</td>
<td>Electron paramagnetic resonance spectroscopy</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
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<tr>
<td>FID</td>
<td>Free induction decay</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>GP</td>
<td>General polarization</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>GPL</td>
<td>Glycerophospholipid</td>
</tr>
<tr>
<td>GUV</td>
<td>Giant unilamellar vesicle</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid crystalline</td>
</tr>
<tr>
<td>L_d</td>
<td>Liquid disordered</td>
</tr>
<tr>
<td>L_o</td>
<td>Liquid ordered</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>L_o</td>
<td>Liquid disordered phase</td>
</tr>
<tr>
<td>L_\beta</td>
<td>Gel phase</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicle</td>
</tr>
<tr>
<td>MβC</td>
<td>Methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>NAP</td>
<td>Naphtho[2,3-\a]pyrene</td>
</tr>
<tr>
<td>NBD-DPPE</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxa-diazol-4yl)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCer</td>
<td>Palmitoyl ceramide</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine or Palmitoyloleoylphosphatidylcholine</td>
</tr>
<tr>
<td>PSM</td>
<td>Palmitoylsphingomyelin</td>
</tr>
<tr>
<td>Pβ</td>
<td>Ripple phase</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>Rho-DOPE</td>
<td>N-rhodamine-dipalmitoyl-phosphatidylethanolamine</td>
</tr>
<tr>
<td>RT</td>
<td>Repetition time</td>
</tr>
<tr>
<td>SL</td>
<td>Sphingolipid</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>S₀</td>
<td>Solid ordered</td>
</tr>
<tr>
<td>SS NMR</td>
<td>Solid State Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>SSB</td>
<td>Supported planar bilayer</td>
</tr>
<tr>
<td>SSM</td>
<td>Stearoyl sphingomyelin</td>
</tr>
<tr>
<td>T₁</td>
<td>Spin lattice relaxation time</td>
</tr>
<tr>
<td>Tₘ</td>
<td>Main transition temperature</td>
</tr>
<tr>
<td>t-Pna</td>
<td>trans-Parinaric-Acid</td>
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Chapter 1.

Introduction

1.1. Biological membranes

Biological membranes, in cells and subcellular organisms, are structures that form a barrier between the inside and the outside of the cell or the cell’s organelles. These barriers are selectively permeable so that molecules such as nutrients are transported to the inside of the membrane and unwanted molecules such as metabolic wastes are transported to the extracellular medium. The selective permeability of the plasma membrane is essential for maintaining proper pH and concentration gradients that are, in turn, essential for metabolic reactions in cells. Several other roles have been described for biological membranes, including but not limited to providing substrates for energy transformation and biosynthesis and maintenance of the flow of information from the cell surroundings to within the cell. Lipids and proteins are the molecules that constitute the biomembranes. The basic structure of biomembranes is the so-called “lipid bilayer.” The lipid bilayer consists of two monomolecular sheets of lipid in which the polar headgroups are oriented toward the surfaces, and their nonpolar moieties are directed to the inside of the bilayer. Proteins are embedded in the lipid bilayer, and their orientation in the membrane depends on their structure and more specifically the polarity of the different parts of their structures. Why the lipid bilayer is formed and how the lipids and proteins are organized in biomembranes are the questions that will be answered next.

1.1.1. Hydrophobic effect and formation of bilayers

The generally accepted definition of a “lipid” is a biomolecule that can only sparingly be dissolved in water, but which can be easily dissolved in organic solvents. This is due to the mainly hydrophobic nature of lipids. However, even mainly hydrophobic lipids, such as cholesterol (Chol) and ceramides, have a small hydroxyl polar moiety. Therefore, in terms of polarity, membrane-forming lipids have a dual nature. Molecules that have both polar and nonpolar moieties are called amphiphilic molecules. Because of the amphiphilicity of lipids, when they are mixed with water, they spontaneously aggregate
into structures with polar and nonpolar regions. Depending on lipid type and preparation conditions, these structures have different physical characteristics. In Figure 1-1, some of these structures are shown. For example, micelles are lipid aggregates with a hydrophobic core consisting of lipid chains, and a polar surface region consisting of the polar headgroups of lipids in contact with water molecules. If lipid molecules are spread out at the surface of a buffer, a lipid monolayer, with the lipid tails oriented toward the air side of the buffer/air interface. Planar bilayers are created in aqueous solution when supported by a surface like mica, glass or silicon oxide. Vesicles are composed of lipid bilayer(s) with an aqueous core. Since in the present work, we prepared one type of vesicle known as multilamellar vesicle (MLV), the physical shapes and preparation conditions of this type of vesicle will be discussed further both in this chapter and Chapter 3. Next, we will discuss the organization of the membrane compartments, i.e. membrane lipids and proteins, in the lipid bilayer of biomembranes.

**Figure 1-1**  Examples of some lipid aggregates. When lipids are mixed with water, depending on their type, structure and experimental conditions, they form different lipid aggregates due to the hydrophobic effect [1]. In this figure, the polar headgroup of lipids are represented by a sphere and their hydrophobic parts are shown as one or two tails. Reprinted with permission from American Society for Cell Biology.
1.1.2. Fluid mosaic model and membrane structure organization

Singer and Nicolson proposed the fluid mosaic model for membrane organization in 1972 [2]. Briefly, this model suggests that, throughout lipid bilayer, lipids provide a sea so that proteins are randomly distributed and float in it. So the term “fluid” refers to the fluidity, i.e. mobility, of lipids and proteins, and the term “mosaic” refers to the proteins and lipids that are scattered across the lipid bilayer. Despite the fact that this model, to some extent still, describes the big picture correctly, advances in the field of membrane organization suggest strongly that this model needs to be revisited (for a review by Nicolson in 2014 see [3]). For example, it has been found that not only are there varieties of lipid and protein ratios and compositions of the biomembranes, but also the compositions of their inner and outer leaflets are different. The latter is known as the “lipid asymmetry.” Also, since 1972, several new findings pointed out that lipids and proteins are not mixed uniformly in the membrane. In addition, now we know that a lipid bilayer is not a two-dimensional isotropic fluid in which movement and composition are homogeneous in the lateral plane of the bilayer. Several factors both inside and outside of the membrane have been found to affect the membrane components’ organization. Along the transverse plane of the bilayer, interactions of membrane proteins or lipids with proteins on the periphery of the membrane (peripheral membrane proteins) induce short-lived complexes in the membrane.

In eukaryotic and some prokaryotic cells, the cytoskeleton, which is an actin or actin-like network, can compartmentalize the membrane. This was found by comparing the diffusion behavior of lipids and proteins in model membranes (which are composed only of certain lipids and proteins) vs biomembranes. In homogeneous model membranes, the diffusion of cellular membrane lipids and proteins are only restricted by collisions with other lipids and proteins, which makes them undergo a simple free Brownian motion. But, the diffusion of membrane components is found to be more complex than free Brownian motion. This was found to be the effect of the cytoskeleton, even on the lipids and proteins on the outer leaflet of the plasma membrane. Single-molecule tracking studies by Kusumi and his co-workers revealed that the cytoskeleton compartmentalizes the membrane (for a review by Kusumi see [4]). Kusumi proposed the “picket and fence” model for membrane organization affected by the cytoskeleton. Briefly, based on the picket and fence model, the membrane skeleton causes the formation of compartments with sizes about 30-200 nm in diameter; these compartments are the fences in his model which corral the
membrane molecules. On the other hand, some of the membrane proteins are anchored to the membrane skeleton, and they act as pickets in the membrane. Kusumi calls them pickets because as these proteins are attached to the membrane skeleton, hydrodynamic friction and steric hindrance caused by these proteins restrict the free diffusion of lipids and other membrane proteins. Based on Kusumi’s model, within each compartment, the movement of membrane components follows the free Brownian motion but, when a membrane molecule reaches the boundary of its related compartment, it can undergo the so-called “hop” diffusion to the adjacent compartment. There is not complete agreement on the diffusion coefficient of lipids and proteins in the membrane since different values have been obtained using different methods (see Table I in reference [4]). However, as an example to show the effect of the cytoskeleton on diffusion coefficients of lipids and protein in the membrane, Schwille and co-workers reported results obtained in a model membrane fluorescence correlation spectroscopy (FCS) study [5]. They found that in the absence of actin cytoskeleton, the diffusion coefficients of the lipids and protein in their studied membrane, were $9.9 \pm 0.6 \mu m^2/s$ and $5.5 \pm 0.9 \mu m^2/s$ respectively. These values became $4.8 \pm 0.4 \mu m^2/s$ and $0.7 \pm 0.1 \mu m^2/s$ for lipids and the protein upon coupling the membrane to an actin cytoskeleton.

Of particular relevance to the present study, two important factors were overlooked in the fluid mosaic model; First, the presence of lateral domains that result from preferential interactions of certain types of lipids and proteins. Second, the variety of lipids and their ability to induce different types of phase in the membrane. Regarding the formation of lateral domains, one outstanding example is the idea of “raft” domains which was proposed by Simons and Ikonen in 1997 [6]. The original definition of a raft was a domain in a biological membrane that is enriched in sphingolipids (SLs), cholesterol (Chol), glycosylphosphatidylinositol (GPI)-anchored proteins and some other proteins. Since 1997, results obtained from several studies and advances in the biophysical techniques shed light on the characteristics of rafts. More will be discussed on the raft domains later in the present chapter while explaining the cholesterol-sphingolipid interaction. There are several pieces of evidence for the existence of lateral domains in biological membranes (for a review see [7]).

The variety of lipids present in biomembranes is stunning. Cells use about 5% of their genes to synthesize more than 100 species of lipids [8]. Lipids have been found with a variety of headgroups, degrees of saturation, acyl chain types, chain lengths, and even
general structures (like sterols vs phospholipids). In addition, different biomembranes have very different lipid compositions. These two points highlight the fact that the lipids are not just amphiphilic molecules employed to provide structural roles in the membrane. Lipids have been found to induce different phases in membranes, and also each type of lipid has been found to interact with particular types of proteins. For example, annular lipids are lipids that form a shell around certain proteins; these lipids and proteins have strong interactions due to the coupling of their polar and non-polar regions (for a review see [9]). Also, lipids have been found to play important roles in signaling events in biomembranes. For example, it has been found that an increase in the level of a lipid, ceramide (Cer), in processes like apoptosis, contributes to cellular signaling through microdomain formation [10]–[12]. Also, it has been found that accumulation of Cer in the cell membrane antagonizes insulin signaling [13].

![Figure 1-2 An updated picture of the plasma membrane. Different colors for the headgroup of lipids were chosen to show lipid heterogeneity, lipid asymmetry and preferential interactions of certain lipids and proteins. Below the membrane is the cytoplasm and above is the extracellular fluid. Picture is taken from [14] and Reprinted with permission from Wiley online library.](image)

To summarize this section, an updated illustration of the fluid mosaic model for the biomembrane is shown in Figure 1-2. It should be noted that the fluidity of the membrane cannot be shown in a static picture, however, the cell membrane is a dynamic structure and all the lipids and proteins, although they have different diffusion coefficients, contribute to it. Moreover, the presence of cholesterol has been neglected in Figure 1-2.
However, Figure 1-2 still represents most aspects of the advancements on the fluid mosaic model and membrane organization. In Figure 1-2, different colors for the headgroup of lipids are used to indicate lipid heterogeneity and show lipid asymmetry. To show annular lipids in Figure 1-2, around each protein there are specific types of lipids, that are identified with different colors. The cytoskeleton is shown below the membrane in the cytoplasm region. Domains enriched only in lipids are also shown. In the next section, the structures of the types of lipids relevant to the studies in this thesis will be discussed.

1.2. Lipid structures: glycerophospholipids, sphingolipids and sterols

Membrane lipids can be categorized into three classes; glycerophospholipids (GPLs), sphingolipids (SLs) and sterols. Although the main focus of this study is the interactions between SLs and cholesterol, two types of glycerophospholipids have also been used in the prepared model membranes. Figure 1-3 summarizes the structures of lipids used in this thesis. Palmitoyl ceramide (PCer) and palmitoyl sphingomyelin (PSM) are examples of SLs, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-glycero-phosphatidylcholine (POPC) are examples of GPLs and cholesterol is an example of a sterol. The common structure amongst SLs is the sphingoid base, which is identified by a box drawn on the structure of PCer in Figure 1-3. The sphingoid base can have varying chain lengths. In SLs, an acyl chain is attached to the amide group of the sphingoid base; in the case of PCer and PSM, the acyl chain is a palmitoyl chain. Ceramides are in general the simplest SLs because they have a small hydroxyl group as their headgroup. Sphingomyelins are classified as SLs that have a phosphocholine (PC) polar headgroup. Other possible headgroups for SLs are sugars or oligosaccharides.

GPLs share a glycerol backbone in their chemical structure. The glycerol backbone is identified by a circle drawn on the structure of DPPC in Figure 1-3. Two fatty acid chains are attached to the glycerol backbone of glycerol. In the case of DPPC, the two acyl chains are the same: palmitoyl chains. In the case of POPC, one chain is palmitoyl, and the other chain is oleoyl. It should be noted that DPPC is not a typical cell membrane lipid (the
reason for using it will be explained later). Common GPL headgroups are PC or phosphoethanolamine (PE). DPPC and POPC both have a PC headgroup.

![Graphical representation of lipids](https://example.com/lipid_structures.png)

Figure 1-3: Structures of lipids used to prepare model membranes for this study. Lipid structures of DPPC POPC as examples of GPLs, PCer and PSM as examples of SLs and cholesterol, as an example of a sterol. The sphingosine backbone in PCer and the glycerol backbone in DPPC are boxed and circled in red respectively. There is a kink in the C2 position of the palmitoyl chain of PSM, PCer, POPC and DPPC which is not shown here. Readers are referred to [15] to see the molecular structures with a kink in the C2 position.

The common structure among sterols are the fused rings shown in the case of Chol in Figure 1-3. There are other types of sterols in biomembranes like ergosterol in yeast and fungi, but Chol is the major sterol found in mammalian membranes. Maintaining membrane fluidity is one of the most important roles of Chol in the cell membrane. In the next section, different bilayer phases that are induced in the membrane by the lipids introduced in Figure 1-3 will be discussed.
1.3. Lipid bilayer phases

The predominant phases found for lipids in bilayers are solid ordered (S₀) (also called a gel or Lβ' or Lβ), liquid ordered (L₀) and liquid disordered (Lₐ). These phases are different from each other since the physical behaviour of lipids is different in each phase. For example, at room temperature, a pure S₀ phase is formed when all the lipids present in the bilayer have fully saturated acyl chains, so that lateral diffusion and rotation about the lipid long axis are slow, i.e. "solid", and the rate of trans-gauche isomerization is so slow that most of the time the chains are in the all-trans configuration, i.e. "ordered." This phase is the most viscous phase among the aforementioned three phases. There is another lamellar phase characterized for lipid bilayers known as the ripple phase or Pβ'. In terms of dynamics, the ripple phase is similar to the S₀ phase, however, structurally there is a one dimensional undulation in the surface of lipid bilayers in the ripple phase [16]. In the Lₐ phase, maximum fluidity is achieved in the membrane through the presence of a high concentration of unsaturated lipids and/or when the saturated chains have enough thermal energy so that a very high rate of trans-gauche isomerization is attained. Depending on temperature, a membrane consisting only of a single saturated lipid could be in the S₀ or Lₐ phase. Below the S₀-Lₐ transition temperature (main transition or Tₘ), the phase of the membrane is S₀ and above Tₘ it is Lₐ. The lipid lateral diffusion coefficient is of order $10^{-8}$ cm$^2$/s in the Lₐ phase; it is reduced by a factor of $10^3$ for the S₀ phase [17]. The L₀ phase has physical properties intermediate between S₀ and Lₐ phases. It has only been observed in membranes containing sterols and is induced in membranes by preferential interactions of sterols with saturated lipids. For instance, in a ternary mixture of unsaturated lipids, fully saturated lipids, and Chol, the latter two tend to phase separate from the unsaturated lipid in the Lₐ phase and form domains in the L₀ phase [18]. L₀ is also called an ordered phase, since the number of gauche bonds is very low and trans-gauche isomerization rates ($\sim 10^{11}$ s$^{-1}$) are much lower than in the Lₐ phase ($\sim 10^{12}$ s$^{-1}$) [19]. In this thesis, the term liquid crystalline (LC) is used as an umbrella term to refer to both liquid phases, i.e. L₀ and Lₐ. Later we will show that lipids in the L₀ phase induced by Chol, show intermediate order between their S₀ and Lₐ phases in the absence of Chol.

The results of several studies show that when lipids are chosen to reflect the lipid content of biomembranes, multiple phases can be observed. This is of particular importance in the framework of the new insights to the fluid mosaic models that were
mentioned earlier. Domains with coexisting phases have also been observed in vivo (for a recent review see [20]). One recent example is the observation of lateral domains smaller than 40 nm in vivo in the gram-positive bacterium *B. subtilis* [21].

Next the two types of lipid domains, L_o domains rich in Chol and S_o domains induced by ceramides, their biological importance and reasons why they deserve to be studied will be discussed. This following discussion is centered on the significance of SLs in inducing lateral domains in membranes.

1.4. Sphingolipids and their roles in the formation of lateral domains in membranes

As the result of lipid asymmetry, some lipids are almost unique to only one leaflet in the cell membrane. SLs are a primary component of the outer leaflet of the plasma membrane [22]. Over the past two decades, there has been a considerable interest in studying the relationships between the structure and the functions of SLs in the membrane. For instance, the lipid raft hypothesis, which entails the lateral segregation of SLs, cholesterol (Chol) and some membrane proteins, is still one of the most heated topics in membrane research [6]. There are several studies suggesting the importance of SLs in cardiovascular disease, hypertension and type 2 diabetes [23]. SLs also have active roles in some cellular processes like programmed cell death (apoptosis) and proliferation both of which involve intracellular signal transduction. To point out the significance of SLs in lateral domain formation in the membrane, the interactions of two SLs, sphingomyelin and ceramide, and also SLs with Chol in membranes will be discussed briefly.

1.4.1. Sphingomyelin and ceramide interactions: S_o phase domains

Ceramide (Cer) is a simple SL which is the precursor of more complex SLs in their metabolic pathways. Structurally, Cer consists of a fatty acid chain that could have varying length, naturally ranging from 14-26 carbons, and degree of saturation (saturated or monounsaturated) attached to the amino group of a sphingoid base [24]. Ceramides are SLs that typically occur at very low membrane concentrations (less than 1 mol%), but under some cell processes like apoptosis, their levels can increase to 12 mol% [25]. For instance in apoptosis, sphingomyelinase (SMase) cleaves the phosphocholine headgroup of sphingomyelin (SM), converting it to Cer in the outer leaflet of the plasma membrane.
Formation of Cer in the cell membrane through the process of apoptosis has inspired researchers to study the consequences of increased ceramide concentration on the cell membrane and in model membranes. Investigating the changes in the physical behavior, and more specifically, the phase behavior of the membrane upon an increase in the Cer level in model membranes is the primary motivation for the present work.

Over the past 20 years, several studies on the effect of mixing Cer with other membrane lipids in model systems have been carried out (for recent reviews see [27]–[29]). All of these studies suggest that both the Cer structure and lipid composition of the membrane need to be considered in determining the effect of Cer on the membrane. Because of their highly hydrophobic properties, even small concentrations of ceramide can induce formation of S₀ phase domains in the membrane [30]–[32]. This ability has been observed in detail in model membranes containing PCer and PSM using a combination of ²H NMR and differential scanning calorimetry (DSC) [33]. The authors found that even small amounts of PCer (2-3 mol%) were enough to induce S₀ phase domains in PSM bilayers and that those domains were more stable than the S₀ phase domains formed by pure PSM. Cer enriched S₀ domains have been also observed in mixtures containing a variety of acyl chain length and degrees of saturation, like binary mixtures of egg yolk-SM:egg yolk-Cer, and bovine brain-Cer:bovine brain-SM [32], [34], [35]. These Cer-enriched S₀ domains were found to coexist with SM enriched S₀ and SM enriched L₀ phases below and above the S₀-L₀ transition temperature of SM respectively.

The capability of ceramides to self-assemble into domains up to micrometer size has been observed in the so-called “ceramide-rich platforms.” These platforms re-organize the receptors and signaling molecules into clusters within the cell membrane to amplify transmembrane signaling processes [36]. The formation of these highly ordered domains is due to the very hydrophobic nature of the ceramides and also could be the result of their ability to form hydrogen bonds via the hydroxyl group [37]. In an effort to study the effect of the NH and OH groups of the sphingosine backbone of Cer, Slotte and co-workers replaced these two groups separately and also simultaneously with a methyl group and studied the effect of incorporating them into a SM membrane [37]. The authors concluded that even with these replacements, Cer is able to generate S₀ domains, but the stability of these domains is substantially reduced in the absence of the NH group. This could be due to the ability of this group to form H-bonds with other lipids, especially other SLs.
The $S_\circ$ phase formed by ceramide has been reported to behave differently than those formed by other lipids like DPPC and PSM [27], [38], [39]. In studies using fluorescent probes to determine phase behavior of model membranes, probes that were partitioned into the $S_\circ$ phase generated by other lipids were unable to partition into the $S_\circ$ phase domains thought to be induced by ceramide [27], [40]–[42]. Due to this effect, $S_\circ$-$S_\circ$ phase immiscibility has been found in some model membranes. As an example, when PCer was mixed with POPC, at temperatures below the $S_\circ$ to $L_d$ phase transition of POPC, two distinct $S_\circ$ phases were observed; one rich in PCer and the other rich in POPC [43]. This effect has also been observed in other model membranes containing different lipid compositions (for a review see [27]). Above a certain threshold, the presence of ceramide in the membrane makes the vesicle unstable and ceramides can form crystals and leave the membrane. This threshold has been reported to be about 33 mol% in PSM:PCer monolayers [32]. However, in PSM:PCer MLVs, no sign of PCer crystals was observed up to 40 mol% PCer [33]. Increases in ceramide concentration can also lead to the formation of non-lamellar phases, like inverted hexagonal phases, and to enhanced membrane permeability (for a recent review article see [29]).

When POPC was added to binary mixtures of SL and Cer, $S_\circ$ domains hypothesized to be enriched in Cer were observed. Examples were: POPC:egg-SM:PCer [44], and POPC:PSM:PCer [39], where, in both cases, the hypothesized Cer-enriched $S_\circ$ domains were observed in coexistence with the fluid phase of POPC and depending on the lipid composition, with the SM enriched $S_\circ$ domains. However, in all of these studies, the presence of Cer in the so-called "ceramide-enriched" domains were not directly observed. For example, in [39], the observed long lifetime decay of trans-parinaric acid ($t$-PnA), as the fluorescent probe in PSM:POPC MLVs, upon addition of PCer, was interpreted as the sign that Cer-enriched $S_\circ$ domains had formed.

1.4.2. Sphingolipid and cholesterol interactions: $L_\circ$ or $S_\circ$ domains?

One of the most important theories on the formation of lipid lateral domains is the lipid raft hypothesis. Based on this theory, favorable interactions between SLs, Chol and some proteins in the membrane leads to packing of these molecules in domains in the cell membrane [6]. The original evidence found in support of this theory came from experiments in which the cells were treated with detergents like Triton X-100 at 4 °C [45]. SLs, Chol and GPI-anchored proteins were insoluble in Triton X-100. Thus they were
named detergent resistant membranes (DRMs) and were interpreted as membrane raft domains. However, it was found later that Triton X-100 promotes domain formation suggesting that specific molecules accumulate in DRMs because of their preferential interaction with Triton X-100 and not because they reside in raft domains [46]. Over time, results obtained from several studies and advances in a biophysical techniques shed light on raft characteristics. The most recent definition of a raft is a small (nanoscale), dynamic domains enriched with Chol, SLs and some proteins that are phase separated from the rest of the membrane, which form domains in the L₀ phase, and through lipid-protein and protein-protein interactions can join to form larger clusters [47]. Both old and new definitions of rafts focus on phase separated SL and Chol domains due to the preferential interactions of these two types of lipid.

It should be noted that the existence of lipid rafts in cell membranes is still under debate (for the most recent review see [48]). It has been hypothesized that the putative raft domains are very small (tens of nanometers [49]–[51]) and transient (tens of microseconds [52]–[56]) which makes them undetectable by current experimental methods (for a review on the limitations of each experimental technique in detecting rafts see [7]).

Regardless of the existence of raft domains, even the lipid content of the plasma membranes of mammalian cells point to the importance of Chol and SL interactions. As mentioned earlier, while SLs constitute a significant proportion of lipid contents of the plasma membrane, they almost exclusively reside in the outer leaflet [22]. Chol content of cell membranes is relatively high, and in some cases, like the human erythrocyte, it can reach up to 48 mol% [7], [57]. Therefore, from the perspective of the cell membranes’ outer leaflet, Chol and SL interactions are very important and need to be well characterized. Several model membrane studies have clearly pointed out the co-localization of saturated SM, as a common type of SL, and Chol in L₀ domains [41], [58], [59]. The preferential interaction between Chol and SM has been explained through the ability of Chol to occupy the space between the acyl chain and the polar head group of SM and also the possibility of hydrogen bonding between OH of Chol and the NH and OH group of SLs [60]. Notably, it is known from the partial phase diagram of Chol and PSM that even 10 mol% Chol is enough to induce Sₒ+Lₒ and Lₒ+Lₒ domains at temperatures below and above the phase transition of PSM, respectively [59]. In the same report, it was
described that at Chol concentrations above ~32 mol\%, the phase of the PSM:Chol binary mixture is $L_0$ from 25 to 60 °C.

**Sphingomyelin, cholesterol and ceramide interactions**

Considering the effect of Cer and Chol on the SM in membranes, what would be the physical state of the membrane in the presence of all three lipids? Since both Chol and ceramides are mainly hydrophobic molecules with relatively small polar headgroups, in bilayers, they need to be shielded against water by the polar headgroups of other lipids in the membrane (umbrella model) [61]. Because SM has a large, strongly polar headgroup, in binary mixtures of SM:Chol and SM:Cer, both Chol, and Cer show a high affinity toward SM, and at physiological temperatures, they form their own favored phases ($L_0$ in the case of SM:Chol and $S_0$ in the case of SM:Cer bilayers). Considering the changes to lipid composition due to SMase activation in early stages of apoptosis, Cer is most likely generated in SM-rich areas. Because of highly favorable interactions between Chol and SM, the interactions among the three lipids are important in determining cell fate.

There have been reports on the concentration-dependent displacement effect of both ceramide and Chol (with respect to each other) in phospholipid bilayers [42], [62]–[65] that could be important in various events of cell physiology [29], [58]. These data suggest that the stability of Chol-rich $L_0$ phase and ceramide-rich $S_0$ phase domains in the membrane depends on the relative concentration of the two lipids [29]. Hence, there could be a critical balance between Chol and ceramide concentrations that regulates signaling processes in the cell membrane.

A model membrane approach is the strategy to study the lipid mixtures of interest in this thesis. Next, the advantages and limitations of using model membranes in analyzing biomembrane components will be discussed.

### 1.5. Model membrane studies: advantage and limitations

Biological membranes are very complex systems. Their compositions differ greatly depending on the type of cell and organelles to which they belong and also their structural and functional roles. Physicists and chemists have taken a reductionist approach and have studied membrane components in model systems. Roles, dynamics and organizations of biomembrane components have been investigated in several model systems containing...
a numbers of biomembrane lipids and/or proteins. These studies have shed light on lipid-lipid and lipid-protein interactions within the membrane and also their interactions with membrane-bound proteins. In fact, most of the advances in the field of membrane organization have originated in model membrane studies (for a review see [66]). Despite the advances in designating novel model membrane systems [67], there are limitations in generalizing the results obtained in model membranes to biomembranes. First, most of the model membrane studies have been done on pure lipid systems ignoring proteins, which form a significant part of the membrane. As a rough estimate, for each membrane protein, there are 50 lipid molecules present in the plasma membrane (PM) [2], [49]. Lipids can bind to these proteins, and their order and movement can be affected [68]–[70]. Second, in biomembranes, there is always a flow of lipids into and out of the membrane and also lipid “flip-flop” between the two leaflets of the bilayer [71]–[74]. These lipid flows are not present in model membranes which could be another factor that plays a role in membrane protein and lipid sorting. Third, it was found that a high density of proteins in L_o domains in a model membrane destabilized L_o-L_d phase separations through steric pressure arising from protein-protein collisions [75]. As a result, lipids and proteins distributed homogeneously in the membrane. Finally, as proposed by Kusumi’s “pickets and fences” model, membrane skeleton (MSK) can compartmentalize the cell membrane through the formation of corrals [76]. Therefore, the phase behavior of lipids in model membranes could differ from cell membranes as they are much more complex system than artificial membranes. To add to this complexity, phase coexistence observed in model membranes are formed in thermodynamic equilibrium, while most of the interactions in biomembranes happen in non-equilibrium conditions [77]–[79]. These are all important factors that need to be considered for generalizing the results obtained in model membranes, including the present work, to lipid phase behavior in biological membranes.

1.6. The aim of this thesis

Busto et al. described lamellar S_o phases of ternary lipid composition containing saturated phospholipids, PCer and Chol [80]. In particular, the PSM:Chol:PCer (7:3:3 mol ratio) mixture was characterized by a variety of physical techniques, and found to present properties intermediate between those of the SM:Chol L_o and the SM:Cer S_o phases. In this thesis we intend, using ^2H NMR, to address the question of the physical state of model membranes containing a fixed 7:3 mol ratio of PSM:Chol and increasing amounts of PCer.
Some of the lipid compositions studied here were previously examined by other biophysical techniques [65], [80]–[82]. Regardless of the probe used and the types of bilayers (MLVs, giant unilamellar vesicles (GUVs), etc.), the authors hypothesized that the observed S₀ domains were enriched in Cer. Here, using ²H NMR, we aim to definitively determine whether or not the S₀ domains are enriched in Cer. The main advantage of ²H NMR is that the phase of a single lipid is unambiguously determined from its spectrum. In addition, the effect of replacing PSM (or PSM-d31) with DPPC (or DPPC-d31) in the membranes studied is considered. The latter study is aimed at explaining the predominance of saturated SM over saturated PC in the plasma membrane and to elaborate more on the effect of sphingoid base vs glycerol backbone on the physical state of the membranes.

In another effort, the physical states of SM and Cer are investigated in bilayers having a lipid composition approximating that of the outer leaflet of the plasma membrane. To this end, first, the effect of the addition of POPC as an unsaturated PC was monitored. Second, the Chol content was elevated to the same level as POPC and SLs (i.e. SM+Cer), and its effects on the phase behavior of SM and Cer were studied.

1.7. Structure of this thesis

The flow of the rest of this thesis is as follows; the next chapter is dedicated to the theory of deuterium (²H) nuclear magnetic resonance (NMR) and the quadrupolar echo which was the NMR technique used to record data presented in this thesis. In the third chapter, the methods used to prepare model membranes, parameters used in the ²H NMR experiments, and also the methods used to analyze the obtained spectra are explained.

The results of the phase behaviour studies on the PSM:Chol:PCer and DPPC:Chol:PCer ternary mixtures are presented in Chapter 4. Chapter 5 is dedicated to the results and discussion of a study of the quaternary mixtures of POPC:PSM:Chol:PCer with the emphasis on the phase behaviour of the SLs in the mixture. In both chapters, the results are followed by a detailed discussion on the obtained results including comparison to relevant data published by other research groups.
In the final chapter, a conclusion summarizing the results obtained in this thesis is provided. Suggestions for future experiments that will complement our view of the phase behaviour of the lipid mixtures studied here are also given in the last chapter.
Chapter 2. Solid state $^2$H NMR theory and applications in studying membrane lipids dynamics

Protons ($^1$H) are the most abundant nuclei in biological systems. $^1$H NMR of biological systems has revealed valuable information. However, although the proton has a high magnetic moment, it is a spin $\frac{1}{2}$ particle, and therefore does not have a quadrupole moment. Having a quadrupole moment is important for probing the orientation dependent interactions with the electric field gradient (EFG) present in the environment of the nuclei of interest in an NMR experiment. A less abundant isotope of hydrogen, deuterium ($^2$H), on the other hand, is a spin 1 system and possesses a quadrupole moment. It can potentially replace the proton in biological molecules without changing the biochemistry of the system of interest. Membrane lipids have fatty acyl (hydrocarbon) chains, and these have been extensively studied by $^2$H NMR. In principle protons in these fatty acyl chains can be replaced by deuterons and then the whole molecule can be incorporated into a biological membrane or a model membrane to study the dynamics of the fatty acyl chains. The power of using $^2$H NMR to study membrane lipids is that different phase(s) can be identified by the fact that they give rise to different $^2$H NMR line shapes. This will be explained in detail in chapters 4 and 5.

In this chapter, the Hamiltonian for solid state (SS) $^2$H NMR spectroscopy is introduced, and the effect of the quadrupolar (or in some references, solid) echo on the evolution of the spin system is investigated using the density operator treatment in operator space. In this thesis, $^2$H and D are used interchangeably to refer to deuterium.

2.1. Interactions in $^2$H NMR

Since the strength of the dipolar coupling, chemical shift anisotropy and J- coupling are negligible in $^2$H NMR [83], the most important interactions are the Zeeman and quadrupolar coupling interactions. In this case the general Hamiltonian will be:

$$
\mathcal{H} = \mathcal{H}_Z + \mathcal{H}_Q
$$

(2.1)
where $\mathcal{H}_Z$ characterizes the interactions of the $^2$H nuclei with the static magnetic field and $\mathcal{H}_Q$ describes the interaction between the deuteron’s charge distribution and the EFG. The Zeeman interaction is, by far, the strongest interaction in any magnetic resonance experiment. The full form of the nuclear Zeeman interaction is:

$$\mathcal{H}_z = -\vec{\mu} \cdot \vec{B}_0 = -\gamma \hbar I_z B_0$$  \hspace{1cm} (2.2)

where $\vec{\mu}$ is the magnetic dipole moment vector $\gamma \hbar \vec{I}$. Here $B_0$ is the strength of the static magnetic field and by convention, it is assumed to be in the z-direction in the laboratory frame of reference. $I_z$ is the component of $\vec{I}$, the spin angular momentum, parallel to the external field. $\gamma$ is the gyromagnetic ratio of $^2$H and $\hbar$ is the Planck constant divided by $2\pi$.

At equilibrium, the Zeeman interaction splits the 3-fold degeneracy of $^2$H nuclear energies, because from equation (2.2) the energy levels are:

$$E_m = -\gamma \hbar m B_0 = -\hbar \omega_0 m$$  \hspace{1cm} (2.3)

where $\omega_0 = \gamma B_0$ and for the deuterium nucleus the nuclear spin quantum number $m = +1, 0, -1$. If the Zeeman effect is the only interaction in the system, then based on the transition rule $\Delta m = \pm 1$, there are two allowed transitions with the same frequency. These transitions give one peak in the frequency spectrum as shown in Figure 2-1.

All nuclei with spin angular momentum greater than $\frac{1}{2}$ possess a quadrupole moment, $Q$, and this results from the non-spherical distribution of charges in the rest frame of the nucleus. Due to the electric charge distribution of the atom or molecule containing the nucleus, the EFG, at the nucleus interacts with $Q$. Following [84], the energy $E$ of a nuclear charge distribution $\rho(\vec{r})$ in an electric potential $V(\vec{r})$ is given by:

$$E = \int \rho(\vec{r})V(\vec{r})d\tau.$$  \hspace{1cm} (2.4)

This integral is taken over the nuclear volume. A Taylor’s series expansion of $V(\vec{r})$ about $\vec{r} = 0$ gives us:
where \( x_i \) is the \( i^{th} \) Cartesian coordinate \((x_1 = x, x_2 = y \text{ and } x_3 = z)\). If \( V_i \equiv \frac{\partial V}{\partial x_i}|_{\vec{r}=0} \), and
\[ V_{ij} \equiv \frac{\partial^2 V}{\partial x_i \partial x_j}|_{\vec{r}=0}, \text{ then the energy becomes:} \]
\[
E = V(0) \int \rho(\vec{r})d\tau + \sum_i V_i \int x_i \rho(\vec{r})d\tau + \frac{1}{2} \sum_{i,j} V_{ij} \int x_i x_j \rho(\vec{r})d\tau + \ldots
\]
(2.6)

\[
= E^{(0)} + E^{(1)} + E^{(2)} + \ldots.
\]

The first term in equation (2.6) is the energy of a point charge in an electric field potential of \( V(0) \). The second term is related to the nuclear dipole moment and its interaction with the electric field \( V_i \). This term vanishes for nuclear states with definite parity [84]. The third term represents the interaction between the quadrupole moment of the nucleus and the EFG. \( V_{ij} \) is the electric field gradient tensor at the nucleus \((\vec{r} = 0)\), which is symmetric.
\(V_{ij} = V_{ji}\). If we ignore s orbital electrons (which do not contribute to an EFG, because of the spherical symmetry of these shells), the EFG tensor is traceless because Laplace’s equation must hold at the nucleus:

\[
\nabla^2 V = \sum_i V_{ii} = 0
\]  

(2.7)

Therefore, the EFG tensor has only five independent terms, which can be written as:

\[
V_{ij} = \begin{pmatrix}
V_{11} & V_{12} & V_{13} \\
V_{12} & V_{22} & V_{23} \\
V_{13} & V_{23} & V_{33}
\end{pmatrix}.
\]  

(2.8)

This matrix will be diagonal in the principal axis system of the EFG, and the diagonal form of the matrix can be achieved by applying a coordinate transformation matrix \(R\):

\[
V^P = RV R^{-1} = \begin{pmatrix}
V_{11}^P & 0 & 0 \\
0 & V_{22}^P & 0 \\
0 & 0 & V_{33}^P
\end{pmatrix}.
\]  

(2.9)

Because the transformation does not change the trace of a matrix, \(V^P\) has only two independent components.

The nuclear quadrupole moment is also a second rank tensor, defined by:

\[
Q_{ij} = \int \left( 3x_i x_j - \delta_{ij} r^2 \right) \rho(\vec{r}) d\tau ,
\]  

(2.10)

where \(i, j = x, y \) and \(z\) and the integral is again over the nuclear volume. The quadrupole moment \(Q\) is equal to \(Q_{zz}\) and for the deuterium nucleus \(Q = 2.875 \times 10^{-27} \text{cm}^2\). With these definitions the electric quadrupole interaction energy, \(E^{(2)}\), becomes:

\[
E^{(2)} = \frac{1}{6} \sum_{i,j} V_{ij} Q_{ij}.
\]  

(2.11)

By using the Wigner-Eckart theorem \([84]\), the electric quadrupole Hamiltonian in the laboratory frame of reference is written as:
\[ \mathcal{H}_Q = \frac{eQ}{4I(2I-1)} \left[ V_0(3I_Z^2 - I^2) + V_{\pm 1}(I_{\pm I} + I_{\pm I}) + V_{\pm 2}I_{\pm I}^2 \right], \]  

(2.12)

where \( e \) is the elementary charge, \( I_{\pm} = I_x \pm iI_y \) are the spin angular momentum raising and lowering operators, and \( V_0, V_{\pm 1}, \) and \( V_{\pm 2} \) are defined as:

\[
\begin{align*}
V_0 &= V_{zz} \\
V_{\pm 1} &= V_{xz} \pm iV_{yz} \\
V_{\pm 2} &= \frac{1}{2}(V_{xx} - V_{yy} \pm 2iV_{xy}).
\end{align*}
\]

(2.13)

In the principal axes frame of reference, the quadrupolar Hamiltonian has a simple form since \( V_{ij} = V_{ii}\delta_{ij} \). If the X,Y and Z assignment of the principle axes\(^1\) are such that \( |V_{zz}| \geq |V_{yy}| \geq |V_{xx}| \), and by introducing the principal value of the EFG, \( eq \), where \( q \) depends on the chemical environment of the deuteron, and asymmetry (or biaxiality) parameters respectively as:

\[
\begin{align*}
\text{eq} &= V_{zz} \\
\eta &= \frac{|V_{xx} - V_{yy}|}{V_{zz}},
\end{align*}
\]

(2.14)

where \( 0 \leq \eta \leq 1 \), then the quadrupolar Hamiltonian in the principal axis frame of the EFG reduces to:

\[ \mathcal{H}_Q = \frac{eQ}{4I(2I-1)}V_{zz}[(3I_Z^2 - I^2) + \eta(I_X^2 - I_Y^2)]. \]

(2.15)

---

\(^1\) X, Y and Z are the Cartesian coordinates of the principal axes frame of reference such that \( V_{ij} = V_{ii}\delta_{ij} \) and hence, \( V_{XY} = V_{YX} = V_{XZ} = V_{ZX} = V_{YZ} = V_{ZY} = 0 \). On the other hand, \( x,y \) and \( z \) are an arbitrary choice of Cartesian coordinates that were introduced in equation (2.5).
As mentioned earlier, the Zeeman interaction is the strongest interaction in NMR, for instance, for the 7T magnet in our lab, its strength in frequency units is 46.8 MHz while the maximum strength of the quadrupolar interaction is approximately 250 kHz [85]. Therefore, the quadrupolar interaction can be treated as a perturbation compared to the Zeeman interaction. Also, the term related to the asymmetry parameter in the Hamiltonian can be neglected [86]. For example, in our experiments this parameter for carbon-deuterium (C-D) bonds is less than 0.05. Thus, the electric field is approximated to be axially symmetric and $V_{zz}$ is parallel to the C-D bond axis. Using first order perturbation theory, the energy levels of the Hamiltonian in equation (2.1) in the laboratory reference frame have the following form [87]:

$$E = -\gamma \hbar m B_0 + \frac{eQ}{4I(2I-1)}V_{zz}[3m^2 - I(I + 1)].$$  \hspace{1cm} (2.16)

For spin 1 nuclei, $m = +1, 0, -1$, and the three energy levels become:

$$E_{+1} = -\gamma \hbar B_0 + \frac{1}{4}eQV_{zz}$$

$$E_0 = -\frac{1}{2}eQV_{zz}$$  \hspace{1cm} (2.17)

$$E_{-1} = \gamma \hbar B_0 + \frac{1}{4}eQV_{zz}.$$  

There are two transitions allowed by the selection rule $\Delta m = \pm 1$, which are:

$$h\nu_+ = E_{-1} - E_0 = \gamma \hbar B_0 + \frac{3}{4}eQV_{zz}$$  \hspace{1cm} (2.18)

$$h\nu_- = E_0 - E_{+1} = \gamma \hbar B_0 - \frac{3}{4}eQV_{zz}.$$

If the spectrum is plotted so that the Larmor frequency is in the center as shown in Figure 2-1, then the two lines arising from $\nu_+$ and $\nu_-$ will appear symmetric and the frequency separation between the two (i.e. the quadrupolar splitting) is equal to:
\[ \Delta v_Q = v_+ - v_- = \frac{3 eQ}{2 \hbar} V_{zz}. \]  

(2.19)

The principal axis system of the EFG tensor does not necessarily coincide with the laboratory frame of reference. To fully understand the shape of a \(^2\)H NMR spectrum, it is necessary to transform the EFG tensor to the laboratory frame where the spin operators \(I_x, I_y, \text{ and } I_z\) are quantized. This can be achieved by rotations through Euler angles \(\alpha, \beta, \gamma\) [88]. The best choice for this transformation is the spherical coordinate system [86], [88], where the EFG tensor is expressed in terms of its irreducible components in the principal axis frame as follows:

\[
V^P_{(2,0)} = V_{zz}
\]

\[
V^P_{(2,\pm 1)} = 0
\]

\[
V^P_{(2,\pm 2)} = \frac{1}{\sqrt{6}} (V_{xx} - V_{yy}),
\]

(2.20)

where the superscript \(P\) in \(V^P_{(2,m)}\) indicates that the EFG tensor is represented in its principal axis frame, \(2\) refers for the fact that \(V^P_{(2,m)}\) is a spherical tensor of rank \(2\), and \(m\) refers to the components of \(V^P_{(2,m)}\). The transformation from this frame to the laboratory frame involves the Wigner rotation matrices \(D^{(2)}_{m m'}(\alpha, \beta, \gamma)\), where \(m\) and \(m'\) refer to the laboratory and principal axes frames respectively. This transformation is given by [88]:

\[
V_{(m,m')} = \sum_{m = -2}^{2} D^{(2)}_{m m'}(\alpha, \beta, \gamma) V^P_{(2,m)}.\]

(2.21)

If \(\eta \approx 0\), then \(V^P_{(2,\pm 2)} = 0\) and only \(V^P_{(2,0)} = V_{zz}\) needs to be transferred to the laboratory frame which yields [87]:
\[ V_{(2,0)} = V_{zz} = \sum_{m=-2}^{2} D_{m0}^{(2)}(\alpha, \beta, 0) V_{(2,m)}^p \]

\[ = V_{zz} \left[ \frac{1}{2} (3 \cos^2 \beta - 1) \right]. \quad (2.22) \]

By substituting this equation into equation (2.19), the quadrupolar splitting in the laboratory frame becomes angle dependent:

\[ \Delta v_Q(\beta) = \frac{3}{2} \frac{e^2 qQ}{\hbar} \left[ \frac{1}{2} (3 \cos^2 \beta - 1) \right]. \quad (2.23) \]

where \( e^2 qQ/\hbar \) is known as the static quadrupolar coupling constant and is equal to 168 kHz for C-D bonds. If one does not ignore the asymmetry parameter, the full form of the quadrupolar splitting is [83]:

\[ \Delta v_Q(\alpha, \beta) = \frac{3}{2} \frac{e^2 qQ}{\hbar} \left[ \frac{1}{2} (3 \cos^2 \beta - 1) + \frac{1}{2} \eta \sin^2 \beta \cos 2\alpha \right]. \quad (2.24) \]

where \( \beta \) and \( \alpha \) represent the polar and azimuthal angle that the Z-axis of the EFG reference frame makes with the direction of the static magnetic field (z-axis of laboratory frame). More specifically, if the C-D bonds in a sample are oriented in a single direction, \( \beta \) is the angle between the external magnetic field and the C–D bond as shown in Figure 2-2.

If the sample is not oriented, like the MLVs used in our experiments, then the distribution of the deuterium nuclei and as a result the C-D bond angles are random. If there are \( N \) deuterium nuclei in the sample, by the assumption that these nuclei are uniformly distributed over the surface of a sphere with radius \( r \), the surface area density of nuclei is \( N/4\pi r^2 \). Hence, \( dN \) which is the number of nuclei oriented between \( \theta \) and \( \theta + d\theta \) with respect to \( \vec{B}_0 \) is expressed as:
\[ dN = \frac{N}{4\pi r^2} 2\pi r^2 \sin \beta \, d\beta = \frac{N}{2} \sin \beta \, d\beta, \quad (2.25) \]

which gives rise to the following probability density for orientations of the C-D bonds:

\[ p(\beta) = \frac{\sin \beta}{2}, \quad (2.26) \]

From this equation, it is clear that \( \beta = 90^\circ \) is the most probable orientation and \( \beta = 0 \) is the least probable orientation. Thus, a typical SS \(^2\)H NMR spectrum consists of a superposition of doublets, separated by \( \Delta \nu_Q(\alpha, \beta) \) that are weighted by \( p(\beta) \). The lineshape that arises from these contributions is known as the "Pake doublet" [89].

The motion of molecules is another factor that affects the shape of spectra in \(^2\)H NMR experiments. From equation (2.24), the quadrupolar interaction depends on the orientation of C-D bonds with respect to the static magnetic field and hence is an anisotropic interaction. If the anisotropic motions of C-D bonds are much faster than the NMR timescale, \( \sim 10^{-6} \) s, they will affect the quadrupolar splitting observed in the Pake doublet, and only an average of the orientation of the C-D bond will be detectable. The quadrupolar splitting in the presence of axially symmetric motion is given by [90]:

\[ \Delta \nu_Q(\alpha, \beta) = \frac{3 e^2 q Q}{2 \hbar} |S_{CD}| \left[ \frac{1}{2} (3 \cos^2 \beta_n - 1) + \frac{1}{2} \eta \sin^2 \beta_n \cos 2\alpha \right], \quad (2.27) \]

where \( \beta_n \) is the angle between the external magnetic field and the director axis of the membrane lipids. In equation (2.27), \( S_{CD} \) is the so-called "order parameter" and is given by:

\[ S_{CD} = \frac{\langle 3 \cos^2 \theta_{CD} - 1 \rangle}{2}, \quad (2.28) \]

where \( \theta_{CD} \) is the angle between the C-D bond at any carbon position and the director axis of the lipid molecule (acyl chain axis of symmetry) and the angular brackets denote a time average. In Figure 2-3, the orientation of a lipid molecule with respect to the membrane
and the static magnetic field is shown, and more specifically the positions of the angles $\theta_{CD}$ and $\beta_n$ are shown.

From equation (2.27), if $\beta_n = 0$ and in the case of negligible asymmetry parameter (which is the case in our experiments), the magnitude of the order parameter $|S_{CD}|$ can be derived. The technique to transform the quadrupolar splitting for an unoriented sample (contributions from different $\beta_n$) to its oriented counterpart ($\beta_n = 0$), is called dePaking and will be explained in the next chapter. Note that if $\beta_n = 54.74\,^\circ$ the quadrupolar splitting is zero for samples with a negligible asymmetry parameter.
Figure 2-3 Illustration of the angles involved in the orientation dependence of the quadrupolar splitting in the presence of axially symmetric motion. $\beta_n$ represents the angle between the external magnetic field and the axis of symmetry of motion (the director axis of the lipid, $\hat{n}$) while $\theta_{CD}$ is the angle between the C–D bond and the director axis.

2.2. Quadrupole Echo: Density Operator Treatment in Operator Space

The signal in Solid-State NMR is very weak compared to the solution state due to the lack of rapid tumbling of molecules present in the latter. In solution-state NMR, a 90° pulse is applied to a sample in thermal equilibrium and the resulting signal, which is known as a “free induction decay” (FID), is Fourier transformed to get an NMR spectrum. In solid-state NMR, the FID after the 90° pulse decays very fast due to rapidly dephasing spins. Moreover, there is a finite receiver dead-time in the electronics which results in losing the first few points of the FID and leads to a distorted spectrum. The “quadrupole echo” was invented to overcome this problem by detecting the signal long after the radio frequency (RF) pulse by the formation of an echo using a pulse sequence. The pulse sequence used is $(90^\circ)_y - \tau - (90^\circ)_x - \tau - \text{echo}$, which is shown in Figure 2-4.

In this section, a summary of the physics describing the quadrupole echo is presented using the density operator treatment in operator space. The treatment presented here follows the same procedure shown in chapter 3 of [85]. In what follows, as in the previous section, all interactions except for Zeeman and quadrupolar coupling are ignored as they are small compared to these interactions. Moreover, all the calculations
presented here are made in the frame rotating at the Larmor frequency about the direction of the static magnetic field (rotating reference frame). The other simplification in this treatment is that the effect of RF pulses are assumed to be a pure rotation, and therefore when the pulses are applied, the dominant Hamiltonian would be due to the RF pulse, i.e., during the pulse quadrupolar coupling and relaxation effects are negligible. So, in the treatment that is followed, relaxation is ignored, however, a full treatment of the system under the influence of finite pulse durations is given in [91].

Figure 2-4 Quadrupolar echo pulse sequence. This pulse sequence is composed of two 90° RF pulses in the transverse plane of rotating reference frame which are 90° out of phase with respect to each other, i.e., if one is rotation about the x-axis, the other one is rotation about the y-axis.

2.2.1. Properties of the Density Operator

Density operator formalism is useful in the theoretical description of a non-interacting spin system [83], [91], [92]. Considering a set of N basis functions, \{\ket{n}\}, that span the configuration space with \langle n | m \rangle = \delta_{mn} (\delta_{mn} \text{ is the Kronecker delta}), and \sum_n \langle n | n \rangle = 1, the state vector \( \psi \) can be expanded as follows:

\[
\psi = \sum_n c_n \ket{n}.
\]  

(2.29)

If \( \psi \) is time-dependent, then the coefficients, \( c_n \), are time-dependent since the basis vectors \( \ket{n} \) are fixed in space. The expectation value of an observable, \( M \) is then defined as:
\[ \langle M \rangle = \langle \psi | M | \psi \rangle = \sum_{n,m} c_m^* c_n \langle m | M | n \rangle. \] (2.30)

Now, if operator \( \rho \) is defined so that its matrix elements are given by:

\[ \rho_{kl} = |l\rangle\langle k|. \] (2.31)

then:

\[ \langle \psi | \rho_{kl} | \psi \rangle = \sum_{n,m} c_m^* c_n \langle m | \rho | n \rangle \]

\[ = \sum_{n,m} c_m^* c_n \langle m | l \rangle \langle l | k \rangle \langle k | n \rangle \] (2.32)

\[ = c_l^* c_k \]

\[ = \langle k | \rho | l \rangle = \rho_{kl}. \]

From equation (2.30),

\[ \langle M \rangle = \langle \psi | M | \psi \rangle = \sum_{n,m} c_m^* c_n \langle m | M | n \rangle \]

\[ = \sum_{n,m} \rho_{nm} \langle m | M | n \rangle \]

\[ = \sum_{n,m} \langle n | \rho | m \rangle \langle m | M | n \rangle \] (2.33)

\[ = \sum_n \langle n | \rho M | n \rangle \]

\[ = Tr\{\rho M\}. \]

Therefore, the expectation value of any observable can be derived from equation (2.30). If the state vector is a function of time, then the time evolution of \( \rho \), now identified as the density operator, can be obtained from the Liouville-von Neumann equation [84]:
\[
\frac{d\rho}{dt} = \frac{i}{\hbar} [\rho, \mathcal{H}].
\] (2.34)

If the Hamiltonian \( \mathcal{H} \) is time-independent, then:

\[
\rho(t) = \exp\left(-\frac{i}{\hbar} \mathcal{H} t\right) \rho(0) \exp\left(\frac{i}{\hbar} \mathcal{H} t\right),
\] (2.35)

is a solution to the Liouville-von Neumann equation which can be used in equation to derive the expectation value of any observable at any time. To do this, the equilibrium form of the density operator at \( \rho(0) \) needs to be derived. If the basis set \( \{ |n\rangle \} \) represents the eigenfunctions of the time-independent Hamiltonian, \( \mathcal{H} \), then the matrix elements of the density operator at time "\( t \)" are:

\[
\langle k | \rho(t) | m \rangle = \langle k | \exp\left(-\frac{i}{\hbar} \mathcal{H} t\right) \rho(0) \exp\left(\frac{i}{\hbar} \mathcal{H} t\right) | m \rangle
\]

\[
= e^{-i(E_m - E_k)t} \langle k | \rho(0) | m \rangle.
\] (2.36)

Since we are interested in the diagonal elements of the density operator for calculating expectation values, from this equation we notice that all diagonal elements are constant over time.

Considering the spin system to be at thermal equilibrium with the lattice at temperature \( T \), and \( \psi \) in equation (2.29) being the state vector of the system, then the populations of states \( \{ |n\rangle \} \) follow the Boltzmann distribution:

\[
|c_n|^2 = \frac{e^{-E_n/kT}}{\sum_m e^{-E_m/kT}} = \frac{1}{Z} e^{-E_n/kT},
\] (2.37)

where \( k \) is the Boltzmann constant and \( Z \) is the partition function of the system. From equation (2.32), we can see that the populations are exactly the same as the diagonal elements of the matrix representation of the density operator (density matrix), therefore at equilibrium:
\[ \rho_{nn} = |c_n|^2 = \frac{1}{Z} e^{-E_n/kT}, \quad (2.38) \]

which can be written in operator form as:

\[ \rho_0 = \frac{1}{Z} e^{-\mathcal{H}/kT} \]
\[ Z = \text{Tr} \left\{ e^{-\mathcal{H}/kT} \right\} , \quad (2.39) \]

where \( \mathcal{H} \) is the Hamiltonian of the system at \( t = 0 \).

In pulsed NMR experiments, the system is usually allowed to reach equilibrium in the presence of a strong static magnetic field. Therefore the choice of the basis set is the eigenfunctions of the Zeeman Hamiltonian in equation (2.2). Once the RF pulse is applied, the Hamiltonian of the system will change, and the evolution of the spin system under the influence of the new Hamiltonian will be described by the Liouville-von Neumann equation. However, by introducing the operator space, the procedure of deriving the time evolution of the spin system can be significantly simplified.

### 2.2.2. Operator Space

As mentioned in the previous section, the complete set of basis vectors \( \{|n\rangle\} \) span the vector space so that every space vector can be expressed in terms of linear combinations of these basis vectors. By analogy, an operator space can be defined so that it is spanned by a complete set of operators \( \{|Q_k\rangle\} \), where the “double ket” notation is used to distinguish these operators from the basis vectors in vector space. Following [85], the orthonormality condition is defined as:

\[ \langle Q_k | Q_l \rangle = \text{Tr} \{ Q_k^\dagger Q_l \} = \delta_{kl} \]
\[ (2.40) \]

The unit operator, \( \varepsilon \), is one of the operators in this basis set, and by applying the orthonormality condition for any \( Q_i \neq \varepsilon \):
\[ Tr\{Q_i \mathcal{E}\} = Tr\{Q_i\} = 0. \]  
\[ (2.41) \]

Therefore, all operators except for the unit operator are traceless. Operators for spin \( I = 1 \), have nine elements as the operator space has \((2I + 1)^2 = 9\) elements. For instance, since \( Tr\{\mathcal{E}\} = 1 \), \( \mathcal{E} \) in its matrix form for spin \( I = 1 \) is:

\[
\mathcal{E} = \frac{1}{\sqrt{3}} \begin{bmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{bmatrix}.
\]
\[ (2.42) \]

By analogy to expanding the space vector in vector space, any operator can be expanded using the complete set of basis operators. Thus the density operator can also be expanded:

\[
\llbracket \rho \rrbracket = \sum_q c_q(t) \llbracket Q_q \rrbracket.
\]
\[ (2.43) \]

The expectation value of observables can be expressed in this new formalism as well:

\[
< M > = Tr\{\rho M\} = Tr\left\{ M \sum_q c_q(t) \llbracket Q_q \rrbracket \right\} \\
= \sum_q c_q(t) Tr\{\llbracket MQ_q \rrbracket\} \]
\[ (2.44) \]

In the final line of equation (2.44) the “double ket” notation is dropped for simplicity, and from here on we use script characters like \( Q \), for the basis set of the operator space.

Starting from the Liouville-von Neumann equation and substituting the density operator in the form expressed in equation (2.43), we get:
\[
\frac{d\rho}{dt} = \frac{i}{\hbar} [\rho, \mathcal{H}]
\]
\[
= \frac{i}{\hbar} \left[ \sum_q c_q(t) Q_q, \mathcal{H} \right]
\]
\[
= \frac{i}{\hbar} \sum_q c_q(t) [Q_q, \mathcal{H}].
\]

By multiplying this equation from the left by \( Q_p \) and taking the trace, we get an equation of motion for \( c_q(t) \):

\[
\frac{dc_p(t)}{dt} = \frac{i}{\hbar} \sum_q c_q(t) \text{Tr}\{Q_q[\mathcal{H}, Q_p]\}.
\]

Therefore, if the commutator of the Hamiltonian with operators that span the operator space is known all the equations of motions can be derived. We first need to identify these operators for a spin \( I = 1 \) system.

The best choice for the basis set in operator space depends on the Hamiltonian. In pulsed NMR, the Hamiltonian of the system changes, and depends on the state of the experiment. For instance, when applying an RF pulse with angular frequency \( \omega_1 \) along the \( x \) axis in the reference frame rotating at the Larmor frequency \( \omega_0 \), the Hamiltonian of the system in the rotating reference frame is:

\[
\mathcal{H}_1 = -\hbar \omega_1 I_x.
\]

Before and after the pulse, the dominant term in the Hamiltonian of the system is due to the \(^2\text{H} \) quadrupolar interaction. Considering the spin \( I = 1 \) system and in an axially symmetric case, we obtain the following expression from equation (2.14) and (2.15):
\[ \mathcal{H}_Q = \frac{e^2 q Q}{4l(2l-1)} [(3l_z^2 - l^2)] \]

\[ = \hbar \frac{\omega_Q}{3} (3l_z^2 - 2), \]

(2.48)

where \( \omega_Q = \frac{3 e^2 q Q}{4 \hbar} [(3 \cos^2 \beta - 1)] \). For spin \( I = 1 \), there are nine operators and with these Hamiltonians, following [85], the choice for the basis set is:

\[ Q_1 = \frac{1}{\sqrt{2}} I_x \]

\[ Q_2 = \frac{1}{\sqrt{2}} I_y \]

\[ Q_3 = \frac{1}{\sqrt{2}} I_z \]

\[ Q_4 = \frac{1}{\sqrt{6}} (3l_z^2 - 2) \]

\[ Q_5 = \frac{1}{\sqrt{2}} (I_x I_z + I_z I_x) \]

\[ Q_6 = \frac{1}{\sqrt{2}} (I_y I_z + I_z I_y) \]

\[ Q_7 = \frac{1}{\sqrt{2}} (I_x^2 - I_y^2) \]

\[ Q_8 = \frac{1}{\sqrt{2}} (I_x I_y + I_y I_x) \]

\[ Q_9 = \epsilon. \]

With these operators, the Hamiltonian for the RF pulse, the quadrupolar interaction and the total Hamiltonian in the Laboratory frame (with the RF off) are respectively:
\[ \mathcal{H}_1 = -\sqrt{2} \hbar \omega_1 Q_1 \]

\[ \mathcal{H}_Q = \left( \frac{2}{3} \right)^\frac{1}{2} \hbar \omega_Q Q_4 \]  

(2.50)

\[ \mathcal{H}_0 = \mathcal{H}_z + \mathcal{H}_Q = -\sqrt{2} \hbar \omega_0 Q_3 + \left( \frac{2}{3} \right)^\frac{1}{2} \hbar \omega_Q Q_4. \]

Now from equation (2.39), we can derive an expression for the equilibrium density operator in terms of the basis set in operator space. The exponential in that equation can be expanded using Taylor’s series, and by applying the high-temperature approximation, the density operator will have the following form:

\[ \rho_0 \approx \frac{1 - \mathcal{H}_0}{T \mathcal{H}_0}. \]  

(2.51)

With \( T \mathcal{H}_0 \mathcal{H}_0 = 0 \) and \( T \mathcal{H}_0 = 3 \), and neglecting the unit matrix in the numerator\(^2\), \( \rho_0 \) is approximated by:

\[ \rho_0 \approx -\frac{\mathcal{H}_0}{3kT}. \]  

(2.52)

From this equation, the net polarization of the system at equilibrium can be calculated. At equilibrium, there is only longitudinal magnetization, and it is proportional to:

\[ \langle I_z \rangle = T \mathcal{H}_0 = -\frac{1}{3kT} T \mathcal{H}_0 \mathcal{H}_0 = \frac{2\hbar \omega_0}{3kT}. \]  

(2.53)

\(^2\) Because it does not contribute to calculations in pulsed NMR since it commutes with all Hamiltonians and therefore does not have any effect on the equation of motions for \( c_q(t) \), which we will see it is necessary to calculate the expectation value of the spin polarization.
This is known as “Zeeman polarization”, and the quadrupole echo is designed so that it transfers the Zeeman polarization into the transverse plane and recovers it in the form of an echo long after the pulses to resolve the problem of the receiver dead-time.

2.2.3. Evolution of the density operator under the quadrupole echo in operator space

If the spin system is at equilibrium at time $t = 0$, the spin Hamiltonian is $\mathcal{H}_0$, and since the quadrupolar interaction is a small perturbation on the Zeeman interaction, the initial density operator can be assumed to have only one nonzero element, $Q_3$ (see equation (2.50)). Then, the initial form of the density operator is:

$$\rho(t = 0) = \sum_q c_q(0)Q_q$$

$$= c_3(0)Q_3$$

$$= \frac{\sqrt{2} \hbar \omega_0}{3kT} Q_3.$$  \hspace{1cm} (2.54)

The first RF pulse is applied at $t = 0$, with strength $\omega_1$ along the x-axis for a time duration $t_{\omega_1}$ as shown in Figure 2-4. During this time, the Hamiltonian in the rotating reference frame is $\mathcal{H}_1$ introduced in equation (2.50). Then the time evolution of $c_3$ from equation (2.46) is:

$$\frac{dc_3(t)}{dt} = \frac{i}{\hbar} \sum_q c_q(t) Tr\{Q_q[\mathcal{H}_1, Q_3]\},$$  \hspace{1cm} (2.55)

and since:

$$[\mathcal{H}_1, Q_3] = -\sqrt{2} \hbar \omega_1 [Q_1, Q_3]$$

$$= i \hbar \omega_1 Q_2,$$  \hspace{1cm} (2.56)

the equation of motion for $c_3(t)$ is given by:
\[
\frac{dc_3(t)}{dt} = -\omega_1 c_2(t).
\] (2.57)

Thus, equations (2.56) and (2.57) show that during the first pulse the z and y components of the magnetization are coupled (remember that \( Q_2 = \frac{1}{\sqrt{2}} I_y \)). With the same procedure the equation of motion for \( c_2(t) \) can be derived, which gives us:

\[
\frac{dc_2(t)}{dt} = \omega_1 c_3(t).
\] (2.58)

Equations (2.57) and (2.58) are coupled linear differential equations and give us the following solutions:

\[
c_2(t) = A_1 \cos \omega_1 t + B_1 \sin \omega_1 t
\]
\[
c_3(t) = A_2 \cos \omega_1 t + B_2 \sin \omega_1 t.
\] (2.59)

By applying the initial conditions, \( c_3(t = 0) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \) and \( c_2(t = 0) = 0 \), the final form of \( c_3 \) and \( c_2 \) at the end of the first pulse \( (t = t_{\omega_1}) \) are:

\[
c_2(t_{\omega_1}) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \sin \omega_1 t_{\omega_1}
\]
\[
c_3(t_{\omega_1}) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \cos \omega_1 t_{\omega_1}.
\] (2.60)

If this pulse is a 90° pulse i.e. \( \omega_1 t_{\omega_1} = \frac{\pi}{2} \), then at the end of the first pulse, only \( c_2 \) remains non-zero and the Zeeman polarization has been transferred into the y-axis of the transverse plane.

After the first pulse is turned off, the spin system evolves under the influence of the quadrupolar Hamiltonian for a time \( \tau \). Starting with the initial condition \( c_2(t_{\omega_1}) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \),
using equation (2.46) and following the same procedure for deriving $c_2$ and $c_3$, at $t = \tau$, we obtain\(^3\):

$$c_2(\tau) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \cos \omega_Q \tau$$

$$c_5(\tau) = -\frac{\sqrt{2} \hbar \omega_0}{3kT} \sin \omega_Q \tau.$$  \hfill (2.61)

When the second pulse, which is a 90° rotation about the $y$-axis with the strength of $\omega_2$, is applied, the spin system evolves under the following Hamiltonian for a time $t_{\omega_2}$:

$$\mathcal{H}_2 = -\hbar \omega_2 I_y = -\sqrt{2} \hbar \omega_2 Q_2.$$  \hfill (2.62)

Following the same procedure as before with initial conditions given by equation (2.61), and with $\omega_2 t_{\omega_2} = \frac{\pi}{2}$ for this pulse, the coefficients after this pulse are:

$$c_2(t_{\omega_2}) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \cos \omega_Q \tau$$

$$c_5(t_{\omega_2}) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \sin \omega_Q \tau.$$  \hfill (2.63)

Note that $c_2$ has not changed under the influence of the second pulse since $[\mathcal{H}_2, Q_2] = 0$ and thus $\frac{dc_2(t)}{dt} = 0$ in equation (2.46). After the second pulse, the Hamiltonian is again the quadrupolar interaction Hamiltonian. Following the same analysis as before, the non-zero coefficients at time $t'$ after the second pulse are:

\(^3\) Remember that from equation (2.43), $|p \ggq \sum_q c_q(t) |Q_q \ggq$, and from equation (2.49), $Q_5 = \frac{1}{\sqrt{2}} (I_x I_z + I_y I_x)$. 

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Neglecting the duration of each pulse \( t_{\omega_1} \) and \( t_{\omega_2} \), at time \( t = t' + \tau \), these coefficients become:

\[
c_2(t) = \frac{\sqrt{2} \hbar \omega_0}{3kT} \cos(\omega Q [t - 2\tau])
\]

\[
c_5(t) = -\frac{\sqrt{2} \hbar \omega_0}{3kT} \cos(\omega Q [t - 2\tau]).
\]

Now the final form of the density operator is:

\[
\rho = c_2(t) Q_2 + c_5(t) Q_5.
\]

Since \( Q_2 = \frac{1}{\sqrt{2}} I_y \), if the detection channel is along the y-axis in the rotating frame, the quadrupolar echo signal can be detected:

\[
\langle I_y(t) \rangle = \sqrt{2} Tr \{Q_2 \rho \} = \sqrt{2} c_2(t) = \frac{2\hbar \omega_0}{3kT} \cos(\omega Q [t - 2\tau]).
\]

At \( t = 2\tau \), the signal has a maximum which is exactly equal to the Zeeman polarization introduced in equation (2.53). Therefore, by applying the quadrupole pulse sequence, the Zeeman polarization is recovered (neglecting relaxation) and the problem of receiver dead time is solved.
Chapter 3.

Materials and Methods

In this thesis, a perdeuterated palmitoyl chain was used as the probe to monitor the phase of a single lipid using $^2$H NMR. A nondeuterated palmitoyl chain contains 31 protons (see Figure 1-3), but in its perdeuterated analogue, these are replaced by 31 deuterons. The lipids used in this study are commercially available synthetic lipids. N-palmitoyl-D-erythro-sphingosylphosphorylcholine (PSM), N-palmitoyl-d31-D-erythro-sphingosylphosphorylcholine (PSM-d31), 1-palmitoyl-2-palmitoyl-sn-glycero-phosphocholine (DPPC), 1-palmitoyl-2-palmitoyl-d31-sn-glycero-phosphocholine (DPPC-d31), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). N-palmitoyl-D-erythro-sphingosine (PCer) and N-palmitoyl-d31-D-erythro-sphingosine (PCer-d31) were obtained from Northern Lipids Inc. (Vancouver, BC). Deuterium depleted water was obtained from Sigma-Aldrich Canada (Oakville, ON). In total 18 samples in the form of MLVs were prepared. These MLVs comprise the following mixtures with indicated molar ratios:

Pure PSM-d31, PSM-d31:Chol:PCer 7:3:0, 7:3:1, 7:3:2 and 7:3:3;

PSM:Chol:PCer-d31 7:3:2 and 7:3:3;

Pure DPPC-d31, DPPC-d31:Chol:PCer 7:3:0, 7:3:1, 7:3:2 and 7:3:3;

DPPC:Chol:PCer-d31 7:3:2 and 7:3:3;

POPC:PSM-d31:Chol:PCer 10:7:3:3 and 10:7:10:3;


3.1. Multilamellar Vesicle (MLV) Preparation

MLVs are types of liposome that contain bilayers stacked in an onion-like structure. Hundreds of lipid bilayers are involved in this “onion” and water fills the inter-layer spaces. A simple representation of MLVs containing only one type of lipid is shown in Figure 3-1.
The thickness of the bilayers are of order 5 nm, and the overall size of MLVs have been reported to be between 0.5 and 10 μm [93]–[97].

Figure 3-1  Cartoon representation of a MLV. The MLV’s onion-like structure, with each layer being a lipid bilayer and with water molecules filling the interlayer space. Adapted from [98] with permission from Elsevier.

The protocol for preparing the MLVs used in this study involved the following steps:

1. An appropriate amount of each lipid was obtained by weighing them on a scale with a precision of 0.001 mg (1 μg).

2. To produce a homogeneous lipid mixture, they were dissolved in a mixture of nonpolar and polar solvents. Benzene:methanol 8:2 (v:v) was used as the solvent for preparing PSM-d31, PSM-d31:Chol:PCer 7:3:0, 7:3:1, 7:3:2, 7:3:3 MLVs. The lipids dissolved in benzene:methanol 8:2 (v:v) were frozen to liquid nitrogen temperature and were lyophilized until dry. Lyophilization removes the solvent molecules by sublimation. The total amount of solvent (benzene:methanol) used was 2 mL, and about 20 hours of time was required for the lyophilizer to remove the solvent molecules.
PSM:Chol:PCer-d31 7:3:2 could not be dissolved in 2 mL of benzene:methanol 8:2. Since lyophilization does not work with lower ratios of benzene:methanol (for example, benzene:methanol 7:3), we used chloroform:methanol 1:1 (v:v) as the solvent to dissolve the rest of the samples. In cases where chloroform:methanol 1:1 (v:v) was used as the solvent, the solvent was first dried under a stream of nitrogen gas, and then the sample was frozen to liquid nitrogen temperature and was put in the lyophilizer for 3 hours to remove possible residual solvent molecules.

3. The dried lipid mixture was then hydrated with deuterium depleted water (ddw). It is known that the amount of water can affect the physical state of lipids [99]. To ensure that all lipids were dissolved in an excess of water, 700-800 µL of ddw was used to hydrate the dried lipids. Ddw was transferred using a Rainin P1000 micropipette with the accuracy of ± 0.8% of the desired volume.

4. To form MLVs, the mixture of lipids and ddw undergo at least 5 cycles of freeze-thaw-vortex. All MLVs were frozen in liquid nitrogen and thawed at either 60 °C for pure PSM-d31 and DPPC-d31 MLVs, or at 90 °C for the rest of the MLVs. For freezing, the sample in a glass scintillation vial was dipped in liquid nitrogen. For thawing, the sample in the glass scintillation vial was dipped into a water bath of the appropriate temperature.

5. 700-750 µL of MLVs was then transferred from the glass scintillation vial to an NMR tube. The maximum capacity of the NMR tubes used in this study was either 700 or 750 µL. The NMR tube was sealed using Teflon tape and Parafilm to avoid dehydration of MLVs. Due to the high cost of lipids used in this study, after data acquisition with each MLV sample, the lipids were recovered and an appropriate amount of lipids were added to the recovered lipids to prepare the next MLV sample. After each round of ²H NMR experiments were done, the recovery process started. For the recovery, samples were pipetted out from the NMR tube and transferred to a scintillation vial. In order to recover as much lipid as possible, the sample in the NMR tube was diluted using nano-pure water. Then the sample in the scintillation vial was frozen in liquid nitrogen and was lyophilized overnight to remove the solvent (water in this case). Then the dried sample was weighed, and an appropriate amount of lipid was added to prepare the next sample following steps 2-5 described above.
3.1.1. Checking the sample composition using $^1$H NMR

The concentration of most of the MLV suspensions was checked using $^1$H solution NMR (at 400 MHz) in deuterated chloroform (CDCl$_3$). Signals from protons in different chemical environments contribute to the peaks in a solution state $^1$H NMR spectrum. The following peaks were used in the peak assignments for lipids present in each sample: the methyl singlet peak at 0.74 ppm and the H-6 vinylic proton peak at 5.36 ppm for cholesterol; the γ-(CH$_3$)$_3$ singlet peak at 3.24 ppm for DPPC; the γ-(CH$_3$)$_3$ singlet peak at 3.24 ppm and the vinylic multiplets found in the range of 5.4-5.8 ppm for sphingomyelin; the NH doublet peak at 6.2 ppm [100], and the vinylic multiplets found in the range of 5.4-5.8 ppm for ceramide. The actual and nominal percentage of the examined MLVs are presented in Table 3-1.

Table 3-1 The nominal vs actual lipid molar percentage in the MLVs that were analyzed using $^1$H NMR. Uncertainties were estimated as follows. Two integration values were obtained for each of the peaks mentioned in the text: one with the smallest and the other with the largest ppm intervals that could be considered to encompass the whole area under the peak. Then the average and the difference of these two values was considered as the integration and the associated error values for each peak respectively.

<table>
<thead>
<tr>
<th>MLVs</th>
<th>Nominal Percentage</th>
<th>Actual Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSM-d31:Chol:PCer 7:3:3</td>
<td>53.8: 23.1: 23.1</td>
<td>55.3± 4.4: 24.8± 1.6: 19.9± 4.4</td>
</tr>
<tr>
<td>POPC:PSM-d31:Chol:PCer 10:7:3:3</td>
<td>43.5: 30.4: 13.0: 13.0</td>
<td>41.1±2.0: 37.0± 4.8: 11.7± 0.6: 10.2± 0.5</td>
</tr>
<tr>
<td>POPC:PSM-d31:Chol:PCer 10:7:10:3</td>
<td>33.3: 23.3: 33.3: 10.0</td>
<td>35.8± 1.9: 23.7± 4.31: 32.5± 1.6: 8.0± 0.4</td>
</tr>
<tr>
<td>PSM:Chol:PCer-d31 7:3:2</td>
<td>58.3: 25.0: 16.7</td>
<td>58.5± 1.7: 26.7± 0.7: 14.8± 1.5</td>
</tr>
<tr>
<td>PSM:Chol:PCer-d31 7:3:3</td>
<td>53.8: 23.1: 23.1</td>
<td>47.4± 1.7: 23.2± 1.9: 29.4± 1.8</td>
</tr>
<tr>
<td>POPC:PSM:Chol:PCer-d31 10:7:3:3</td>
<td>43.5: 30.4: 13.0: 13.0</td>
<td>46.8± 2.0: 27.7± 2.6: 13.2± 0.4: 12.3± 0.4</td>
</tr>
<tr>
<td>POPC:PSM:Chol:PCer-d31 10:7:10:3</td>
<td>33.3: 23.3: 33.3: 10.0</td>
<td>32.9± 0.9: 21.9± 2.1: 35.4± 0.9 : 9.7± 0.2</td>
</tr>
<tr>
<td>DPPC:Chol:PCer-d31 7:3:2</td>
<td>58.3: 25.0: 16.7</td>
<td>60.5± 0.7: 24.5± 0.6: 15.0± 0.1</td>
</tr>
<tr>
<td>DPPC:Chol:PCer-d31 7:3:3</td>
<td>53.8: 23.1: 23.1</td>
<td>55.1± 1.4: 22.2± 0.7: 22.7± 0.5</td>
</tr>
<tr>
<td>DPPC-d31:Chol:PCer 7:3:1</td>
<td>63.6: 27.2: 9.1</td>
<td>64.6± 0.7: 27.6± 0.4: 7.6± 0.1</td>
</tr>
<tr>
<td>DPPC-d31:Chol:PCer 7:3:2</td>
<td>58.3: 25.0: 16.7</td>
<td>60.3± 0.7: 25.55± 0.7: 14.15± 0.1</td>
</tr>
<tr>
<td>DPPC-d31:Chol:PCer 7:3:3</td>
<td>53.8: 23.1: 23.1</td>
<td>53.9± 0.8:23.3± 0.4:22.8± 0.4</td>
</tr>
</tbody>
</table>
3.2. \(^2\text{H} \text{NMR}\)

\(^2\text{H} \text{NMR} \) experiments were performed with a 7.0 T Oxford Magnet (Oxford Magnet Technology, Witney, Oxon, UK) and a Tecmag Scout spectrometer (Tecmag Inc., Houston, Texas) at 46.8 MHz using the quadrupolar echo technique explained in the previous chapter and in [101]. Since the receiver electronics have a dead-time (approximately 10 µs) and cannot detect the full FID, the quadrupolar echo is necessary to detect the echo long after the receiver dead-time to achieve a nondistorted spectrum. A two-pulse sequence, both 90° pulses, and 90° out of phase is used in quadrupolar echo. In NMR language, this is \((90°)^{\gamma} - \tau - (90°)^{\chi} - \tau - \text{echo}\), where the duration of each pulse was 3.95 µs and \(\tau = 40 \mu\text{s}\) in the experiments performed in this study. The dwell time which is the time between successive points of the FID was 2 µs.

3.2.1. Repetition Time

The repetition time (RT) is the time between successive scans. Ideally, a short RT is desirable since it allows taking more data in a given amount of time. The drawback of a very short RT (tens of milliseconds) is that if it is less than about 5 times the spin-lattice relaxation time (50 ms for methylenes in the \(S_o, L_o\) and \(L_d\) phases) of the spin system, the net magnetization does not fully relax to an equilibrium state and results in signal loss [102]. The spin-lattice relaxation time for lipids in \(S_o\) and liquid crystalline phases is relatively short, but it is longer in solid crystalline phases where there is a higher restriction on the motion of the lipid chains [102]. Since the spectra obtained in this study were representative of the \(S_o\) phase or the liquid crystalline phase (or the superposition of both) a RT of 300 ms was used to acquire all spectra. However, to check for the presence of a solid crystalline phase, the MLVs with high PCer or PCer-d31 were also examined by increasing RT to 3 s. The spectral shape and the echo heights of the spectra taken with short (300 ms) and long (3 s) RT were the same indicating no sign of solid crystalline phase formation.

3.2.2. Quadrature Detection

In an NMR experiment, the offset of the signal from a reference frequency (typically the Larmor frequency) is detected, and hence, there are positive and negative frequencies getting into the receiver. In our \(^2\text{H} \text{NMR} \) experiments, two phase sensitive detectors (PSD),
which are 90° out of phase, are used to distinguish between positive and negative frequencies. This is called quadrature detection, and its final outcome is two modulated signals called “Real” and “Imaginary” signals. These two signals are then phase adjusted so that the real signal is entirely absorptive. The linear combination of these two signals is then Fourier transformed to give the ²H NMR spectrum.

3.2.3. Phase Cycling

To average out the non-ideal conditions in NMR experiments like imperfections in the 90° pulses, a set of 8 experiments was performed in which the phase is varied. There are four axes in the transverse plane (x, -x, y, -y) and 8 permutations are possible for applying the quadrupolar echo experiment (two 90° out of phase pulses): (x,y), (y,-x), (-x,-y), (-y,x), (x,-y), (-x,y) and (y,x). This method of data acquisition is called an 8-CYCLically Ordered Phase Sequence (8-CYCLOPS) and the real and imaginary signals from each acquisition are added up to build up the final phase-cycled spectrum [103].

3.2.4. Number of Scans

The signal to noise ratio (SNR) is directly proportional to the square root of the number of scans, SNR \( \propto \sqrt{\text{number of scans}} \). Typically for a sample that contains 40 mg of perdeuterated palmitoyl chain, we acquire 10000 scans which takes about 55 minutes per temperature setting. As mentioned in 3.1, samples were recovered after each experiment and an appropriate amount of lipid was added to prepare the next sample in a sequence. In this process, which requires lots of weighing and transferring lipids, some of the lipids, especially perdeuterated lipids which contribute to the NMR signal were lost. In order to compensate for lipid loss and obtain almost the same SNR for all spectra, the number of scans was increased as needed. In general, the number of scans varied from 10,000 to 80,000 scans.

3.2.5. Heating Procedure

After MLVs were prepared according to the protocol outlined in section 3.10, the data acquisition started. Waiting time of at least 20 minutes was used to let the MLVs equilibrate at each temperature then the data were acquired. The starting temperature for PSM-d31 MLVs was 22 °C and data were collected in 1 °C intervals from 21 to 38 °C.
From 38-42 °C, spectra were recorded every 0.1 °C to carefully map the main transition ($T_m$) of PSM-d31 MLVs at 40 °C. Then, from 42 to 50 °C, data were again recorded every 1 °C for PSM-d31 MLVs. Data were collected at 1 °C intervals from 21 to 45 °C and at 5 °C intervals from 45 to 70 °C for all other MLVs. The uncertainty in temperature measurements was 0.1 °C. After the experiment was done for all temperatures, one more spectrum for the lowest temperature was taken to check for any sample degradation effect. No difference was found between the original spectra taken at the lowest temperature and the ones taken after cooling the sample back down from the highest temperature to the lowest temperature.

3.3. Data Analysis

3.3.1. Average Spectral Width

The $^2$H NMR spectral line shapes of the lipids in each of the lamellar phases are different. One method to quantitatively compare these spectra is by calculating the average spectral width. To this end, the first moment of each spectrum, $M_1$, is calculated according to the following equation:

$$M_1 = \frac{\sum_{\omega=-x}^{x} |\omega| I(\omega)}{\sum_{\omega=-x}^{x} I(\omega)},$$

(3.1)

where $\omega$ is the offset from the Larmor frequency, $I(\omega)$ is the signal intensity at the frequency $\omega$ and $x$ was chosen so that the full spectrum resides between $(x, -x)$. The $^2$H NMR spectra are centred about the Larmor frequency and each pair of deuterons give rise to a Pake doublet which are extended both in the positive and negative frequency shifts of the spectrum. With the definition given for the average spectral width, the average of the first moment for both the left and the right hand side of the spectra is calculated. The error in $M_1$ is calculated by taking the first moments of the positive and negative frequency shifts subtracted from $M_1$ derived from equation (3.1). Since the $^2$H NMR spectra are highly symmetric, this error is less than 0.02% and the error bars are smaller than the symbols in each graph of $M_1$ presented in this thesis.
3.3.2. Order Parameter

Lipid chains in bilayers experience restrictions on their motion. Depending on the location of the carbon atom in the chain, this restricted motion is different. For instance, carbons closer to the headgroup of the bilayer have less freedom of motion compared to the carbons close to the middle of the bilayer\(^4\). \(^2\)H NMR is sensitive to the motion of the C-D bonds, and therefore the local “rigidity” of the C-D bonds is reflected in the spectrum of the deuterated lipid. The definition of the order parameter for each C-D bond was given in equation (2.28) as follows:

\[
S_{CD} = \frac{\langle 3\cos^2 \theta_{CD} - 1 \rangle}{2}
\]  

(2.28)

where \(\theta_{CD}\) is the angle between C–D bond at any carbon position and the director axis of the lipid molecule (acyl chain axis of symmetry) and the angular brackets denote a time average. In an all trans configuration of a lipid chain undergoing axially symmetric rotation, the order parameter reaches its maximum magnitude of 0.5. For a fully disordered lipid chain, the order parameter is 0. If, the lipid bilayers are oriented so that \(\beta_n = 0^\circ\) for all lipid molecules (oriented bilayers) and the quadrupolar splitting for the C-D bonds is well resolved, the magnitude of the order parameter \(|S_{CD}|\) can be calculated from the quadrupolar splitting:

\[
\Delta v_Q = \frac{3 e^2 qQ}{2h} |S_{CD}| = \chi |S_{CD}|
\]  

(3.2)

where \(\chi = 252\) kHz for the C-D bonds.

However, in the case of MLVs \(\beta_n\) is not zero and in order to use equation (3.2), the powder doublet spectrum needs to be transformed to a spectrum that represents the quadrupolar splitting in an oriented bilayer with \(\beta_n = 0^\circ\). The mathematical technique to deconvolute the powder spectrum to its \(\beta_n = 0^\circ\) counterpart is called “de-Pake-ing” (dePaking). In this study, a previously established procedure for dePaking was followed

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\(^4\) Due to the strong electrostatic interactions between the polar headgroups of the lipids.

\(^5\) \(\beta_n\) is the angle between the director axis of the bilayer and the external magnetic field.
Briefly, the assumption is that all orientations of the director axes of lipids are equally possible in an MLV (random distribution of orientations) and the powder spectrum results from the superposition of contributions from deuterium nuclear spins with a random distribution of orientations. DePaking is only possible when the powder spectrum is a superposition of Pake doublets. Therefore, it cannot be performed on the spectra representing the $S_0$ phase or superposition of an $S_0$ phase and a liquid crystalline phase.

**C-D bond peak assignments on dePaked Spectra**

If the deuterons are in the same chemical environment, they contribute equally to the area of the same peak in the $^2$H NMR spectrum, and they are considered to be magnetically equivalent. While this assumption is correct for most of the deuterons in the lipid chains, it is not true for the deuterons attached to C2 in the palmitoyl chain of the PSM-d31, PCer-d31 and the palmitoyl chain attached to the sn-2 chain of the DPPC-d31.$^6$. The reason for this non-equivalency is that these carbons are within the “kinked” conformation region of the palmitoyl chain which imposes a change in the order of these carbons. With the exception of non-equivalent deuterons, the assumption for the peak assignment to the carbons in the palmitoyl chain in the dePaked spectra is that the peak farthest away from the center of the spectrum comes from the carbon closest to the headgroup of the lipid (C3). As one moves away from the headgroup, the quadrupolar splitting (and therefore the order parameter) decreases. This assumption which was introduced by M. Lafleur et al. as a “smoothed orientational order parameter profile” [105] is not entirely correct. For example, in a $^2$H NMR study where the stearoyl chain of stearoyl-SM (SSM) was selectively deuterated at positions of 2, 3, 4, 6, 8, 10, 12, 14, 16 or 18, each sample contained SSM with only one deuterated carbon. The authors found that C4-C12 have almost the same order parameter in pure SSM bilayers [106]. They also found that in the presence of Chol (in SSM:Chol 1:1 bilayers) C6-C12 have higher order parameters than C2-C4 deuterons and that the highest order parameter occurs in the middle of the acyl chain for C8-C10. However, in the case of kinked palmitoyl chains from C4 to C16, the assumption that the order parameter decreases monotonically explains the general physical behavior of lipids in bilayers. In this thesis, the “smoothed order parameter profile” was used to assign peaks on the dePaked spectra to the carbons (or deuterons) of the perdeuterated palmitoyl chain presented in the related MLVs. An

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$^6$ Also, deuterons attached to C3 in PSM-d31 have been found to be inequivalent, although they are less non-equivalent than C2 deuterons.
example of peak assignment via this method is shown in Figure 3-2, where the powder and dePaked spectrum of PSM-d31 MLVs at 50 °C are shown along with the assignment of peaks in the dePaked spectrum.

![Figure 3-2](image)

Figure 3-2 The powder and dePaked spectra of PSM-d31 MLVs at 50 °C. The approximate location of the peaks arising from deuterons attached to carbons in the perdeuterated palmitoyl chain is shown. Note that the deuterons attached to C2 and C3 are non-equivalent and their peaks’ locations are shown with an * after the carbon number. In peak assignments, the data presented from selectively deuterated lipids were followed [107]. The blue stars are approximate locations of the limits of the integral for calculation of the area under the peaks. The green lines are the locations of each of the center of the signals from the deuterons of C2-C15 found from the peak assignment procedure explained in the text.

Since the signal from methyl deuterons does not fully relax within the 300 ms RT, the full signal from these deuterons is not captured. We think that the Pake doublet may be distorted if there is any orientation dependence to spin-lattice relaxation and therefore, the methyl splittings were measured directly from the powder spectrum and not from the dePaked spectrum. These peaks are the ones closer to 0 kHz in the powder spectrum as shown with the red arrow in Figure 3-2. The methyl peaks for PSM-d31 MLVs in Figure 3-2 are doublet peaks, which, to the best of our knowledge, is due to the fact that commercially available synthetic sphingomyelins are racemic and therefore, our PSM-d31 contain a fraction of L-threo-sphingosylphosphorylcholine in addition to D-erythro-sphingosylphosphorylcholine [108].

Each deuteron contributes equal intensity to the dePaked spectrum, hence, to determine the positions of methylene deuterons, the area of the rest of the overlapping
peaks are divided by the number of methylene deuterons (28 in the case of a perdeuterated palmitoyl chain). For negative frequencies, the upper and lower limits of integration used to calculate the area under the methylene peaks are indicated by blue asterisks in Figure 3-2. The area slice, which is the spectral area assigned to each deuteron, is then calculated. The center of each area slice represents the quadrupolar splitting associated with a deuteron on the perdeuterated acyl chain. These are indicated by the green lines in Figure 3-2. In the case of PSM-d31, for each carbon in the chain from C4-C15, which have equivalent deuterons attached to them, the splittings of the two associated deuterons were averaged and used as the negative shift quadrupolar splittings. The same procedure was carried out for the positive frequency shifts. Then the average of negative and positive shifts was taken as the quadrupolar splitting to be used in equation (3.2) to deduce the order parameter of the associated carbon.

To assign the peaks to the inequivalent deuterons attached to the “kinked” carbons, e.g. C2 and sometimes C3, for pure PSM-d31 or DPPC-d31 MLVs, data from ²H NMR experiments on the selectively deuterated palmitoyl chain of PSM or DPPC were used [107], [109]–[111]. For other MLV compositions, first, the total area under the peaks divided by the associated number of deuterons was calculated to identify peaks with contributions from an odd number of deuterons. For example, in the area slicing procedure for the dePaked spectrum of PSM-d31:Chol 7:3 at 50 °C, the bottom spectrum in Figure 3-3, the 16-20 kHz and the 30-35 kHz peak were found to come from one deuteron and three deuterons, respectively. Second, the dePaked spectra of the MLVs at 50, 55, 60, 65 and 70 °C were plotted, and the position of peaks as the temperature increases was monitored. The quadrupolar splittings of the inequivalent deuterons attached to C2 (or C3) have different temperature dependencies than the other methylene deuterons on the palmitoyl chain. This is due to their different average orientations with respect to the director axis of the membrane [112]. An example of this procedure is shown in Figure 3-3.
Figure 3-3  Peak assignment procedure for inequivalent deuterons from the dePaked spectra of PSM-d31:Chol 7:3 MLVs. (A) The dePaked spectra of PSM-d31:Chol 7:3 MLVs at 50, 55, 60, 65 and 70 °C. The approximate locations of the two deuterons attached to C2 (C2-d1 and C2-d2) and the average locations of the deuterons attached to C14 and C15 are shown. Since the spectra are highly symmetric, only half of the spectra are plotted. (B) Quadrupolar splittings of the indicated deuterons in (A) against temperature. The lines are just guides to the eyes and are not fitting curves.
Since the C2-d2 and the two C15 deuterons contribute to the area under the same peak in the dePaked spectrum at 50 °C, (see the bottom spectrum in Figure 3-3A), they are indistinguishable. Therefore, from the three centers of the area slices for this peak, the middle slice was assigned to the quadrupolar splittings of C2-d2 and C15 deuterons, and the error bars are extended to the location of the other two slices for both data points in Figure 3-3B. As it is clear from these two graphs, the peaks that are assigned to C2-d1 and C2-d2 are more resistant to increases in temperature than those assigned to C14 and C15. This procedure was repeated for the rest of the MLVs (except for pure PSM-d31 and pure DPPC-d31) to locate the peaks arising from deuterons that are attached to C2. This procedure was carried out for both positive and negative frequency shifts, and the average splitting was used to calculate the order parameter for the C2 (and C3 for PSM-d31) deuterons in each MLV.
Chapter 4.

Ternary MLVs composed of phospholipids, cholesterol and ceramide

In this chapter, the results of analysis of the spectra for MLVs containing PSM-d31, PSM-d31:Chol:PCer 7:3:0, 7:3:2, 7:3:3 and PSM:Chol:PCer-d31 7:3:2 and 7:3:3 are presented. In addition, the effect of replacing PSM (or PSM-d31) with DPPC (or DPPC-d31) in MLVs with the same molar ratio is thoroughly investigated. In the discussion section a detailed comparison of these results with those from other research groups is presented. Since analyzing the line shapes of the ²H NMR spectra is the main tool in determining MLV lipid phases, the results section begins by introducing the line shape characteristics of each phase. Then the spectra at 22, 40 and 50 °C are plotted for all MLVs. The reason for choosing these temperatures is as follows: 22 °C is the temperature at which most of the data available for comparison to the ones presented in this thesis are reported; 40 °C is the transition temperature of the main phospholipids (either DPPC or PSM) in our MLV samples; 50 °C is the temperature at which we found that all of the MLVs are in an LC phase, which enabled us to perform the dePaking analysis and obtain order parameter profiles.

4.1. Results

4.1.1. ²H NMR spectroscopy: Line shape analysis

The main advantage of using ²H NMR to investigate the phase of a specific phospholipid in MLVs is that the phase of the lipid can be identified from the line shapes of its spectrum.
Figure 4-1 Different $^2$H NMR line shapes for different lipid phases. Typical spectra for liquid-disordered ($L_d$), liquid-ordered ($L_o$) and solid-ordered ($S_o$) phases. The $S_o$ and $L_d$ spectra are obtained from pure PSM-d31 MLVs at 22 and 50 °C. The $L_o$ spectrum was obtained from the PSM-d31:Chol 7:3 MLVs at 35 °C. For more information on the arrows please refer to the text.

Figure 4-1 shows typical spectra for the $S_o$, $L_o$, and $L_d$ phases. In the $L_d$ spectrum, the green arrows show the "shoulder" and the red arrows show the "edge" regions. The $L_o$ spectrum is much wider than the $L_d$ spectrum, with a broader shoulder. Also, by comparing the middle (the region between the two edges) of the $L_d$ and $L_o$ spectra, it is evident that more peaks are resolved in the $L_d$ spectrum. For a membrane in the $S_o$ phase, the spectrum features a sloping shoulder as shown in the lowest spectrum in Figure 4-1 (purple arrows). When $S_o$+$L_o$ phase coexistence occurs, both the shoulder and the edge of the spectrum are more sloped compared to what is observed in a pure $L_o$ spectrum. Note that since $^2$H NMR spectra of lipids in both the ripple and gel phases are difficult to distinguish, the term $S_o$ used here encompasses both of these ordered phases.

4.1.2. Spectra at 22 °C

The spectra of all fourteen MLVs at 22 °C are shown in Figure 4-2. The spectra for the pure phospholipids PSM-d31 and DPPC-d31 show that these lipids are in the $S_o$ phase at 22 °C. By examining the spectral lineshapes in the presence of 30 mol% cholesterol, it is apparent that both PSM-d31 and DPPC-d31 are predominantly in the $L_o$ phase. However, the slightly sloping shoulders (between ~30 and 60 kHz) and edges (between ~26 and 30 kHz) of these spectra indicate that some of the phospholipids are in the $S_o$ phase. The phase diagrams of PSM-d31:Chol and DPPC-d31:Chol MLVs has been
determined from 25 °C to 60 °C (not 22 °C), but the $S_0$ + $L_0$ phase boundary (approximately 30 % Chol) at 25 °C slopes toward higher Chol concentrations as the temperature is decreased [113]. Therefore, the $S_0$ + $L_0$ phase coexistence observed here agrees qualitatively with the phase diagrams of PSM-d31:Chol and DPPC-d31:Chol MLVs in [113].

![Spectra for ternary MLVs at 22 °C. A: From bottom to top are the spectra for pure PSM-d31 and PSM-d31:Chol:PCer MLVs with molar ratios of 7:3:0, 7:3:1, 7:3:2 and 7:3:3 (red spectra). The blue spectra that are overlaid against the top two red spectra represent PSM:Chol:PCer-d31 MLVs with the molar ratios of 7:3:2 and 7:3:3. B: From bottom to top the spectra compared to pure DPPC-d31 and DPPC-d31:Chol:PCer MLVs with molar ratios of 7:3:0, 7:3:1, 7:3:2 and 7:3:3 (green spectra). The purple spectra represent DPPC:Chol:PCer-d31 MLVs with the molar ratios of 7:3:2 and 7:3:3. The spectra of 7:3:2 and 7:3:3 containing MLVs were overlaid so that the pairwise comparison between the spectra becomes visually more evident. The signal at 0 kHz for PSM:Chol:PCer-d31 7:3:2 and 7:3:3 spectra (blue spectra) arises from DOH in the sample.](image)

Low concentrations of PCer do not markedly affect the spectra at 22 °C. Qualitatively, the spectra of PSM-d31:Chol:PCer and DPPC-d31:Chol:PCer 7:3:1 MLVs are very similar to those from binary mixtures of phospholipid:Chol 7:3. However, addition of 20 or 30 mol% PCer to the 7:3:0 MLVs makes the shoulder and edges of the spectra significantly more sloped compared with the spectra of 7:3:0 and 7:3:1 MLVs. This implies that in the presence of 20 or 30 mol% added PCer, more PSM-d31 and DPPC-d31 are in the $S_0$ phase than is the case for 7:3:0 and 7:3:1 MLVs.
Finally, the top two spectra in each column of Figure 4-2 clearly show $S_0+L_0$ phase coexistence. These spectra represent PCer-d31 in PSM:Chol:PCer-d31 and DPPC:Chol:PCer-d31 7:3:2 and 7:3:3 MLVs. Although the spectra of PSM-d31 and DPPC-d31 in 7:3:2 and 7:3:3 MLVs also show $S_0+L_0$ phase coexistence, the $S_0$ phase features are more prominent in all MLVs containing PCer-d31. Examining the signal between ~ 26 to 30 kHz, we expect to see an approximately vertical "edge" in a typical $L_0$ spectrum and a sloping shoulder for a $S_0$ phase spectrum. When comparing the edges of the spectra for PCer-d31 in both 7:3:3 MLVs (top spectra in Figure 4-2), it is apparent that the spectrum of MLVs containing PSM (top left spectrum) has less "edge" contribution than the spectrum of the corresponding MLVs containing DPPC (top right spectrum). Thus, a greater proportion of PCer-d31 is in the $S_0$ phase in MLVs containing PSM relative to those containing DPPC. Quantitative fractions of the perdeuterated lipid present in either $S_0$ or $L_0$ phases could not be measured. This is because $S_0$ and $L_0$ spectra are similar in width and also have similar relaxation times, and therefore cannot easily be separated. The spectral subtraction technique [114] cannot be used here since it would require subtracting two spectra along a given tie line in a known phase diagram. The phase diagram of the ternary MLVs studied here has not yet been determined.

4.1.3. Spectra at 40 °C

The spectra for all of the MLVs at 40 °C are shown in Figure 4-3. PSM-d31 is in the transition from $S_0$ to $L_0$, and its spectrum exhibits a clear superposition of spectra from the $S_0$ and $L_0$ phases. The spectrum of DPPC-d31 mainly reflects the $S_0$ phase, with only a small amount of evidence of the $L_0$ phase (a more vertical shoulder compared to DPPC-d31 spectrum at 22 °C; a liquid crystalline methyl group signal at about +/- 4 kHz). The addition of 30 mol% cholesterol induces the $L_0$ phase in MLVs of both PSM-d31 and DPPC-d31, and adding 10 mol% PCer does not change this. However, upon addition of 20 or 30 mol% PCer to either 7:3:0 MLV system, subtle changes in the spectral edges and shoulders suggest that a fraction of either DPPC-d31 or PSM-d31 is in the $S_0$ phase. This effect is slightly more pronounced for PSM-d31:Chol than for DPPC-d31:Chol, indicating that PCer has a larger ordering effect on PSM-d31 than on DPPC-d31. The shoulders and the edges of the spectra of PCer-d31 in both 7:3:2 and 7:3:3 MLVs are somewhat more sloped compared to DPPC-d31 and PSM-d31 in the analogous MLVs. Hence, close to physiological temperatures, a fraction of PCer-d31 is still in the $S_0$ phase. As was the case
at 22 °C, the PSM:Chol:PCer-d31 7:3:3 spectrum has stronger $S_0$ phase features than the DPPC:Chol:PCer-d31 spectrum.

Figure 4-3 $^2$H NMR spectra of ternary MLVs at 40 °C. (A) PSM-d31:Chol:PCer MLVs (red) and PSM:Chol:PCer-d31 MLVs (blue) and (B) DPPC-d31:Chol:PCer MLVs (green) and DPPC:Chol:PCer-d31 (purple).

4.1.4. Spectra at 50 °C

Figure 4-4 shows that, at 50 °C, PSM-d31 and DPPC-d31 MLVs are in the $L_d$ phase, meaning that the transition from the $S_0$ phase to the $L_d$ phase is complete. Upon addition of PCer (or PCer-d31) to the binary 7:3:0 MLVs, a slight increase in the width of the spectra, in both Figure 4-4A and Figure 4-4B, was observed. However, all the binary and ternary MLVs display $L_0$ spectra. The MLVs with compositions PSM:Chol:PCer 7:3:2 and 7:3:3 in Figure 4-4A have very similar spectra independent of whether or not the PSM or PCer is deuterated. This confirms that sphingomyelin and ceramide are well mixed in these MLVs. Minor differences in the positions of some peaks will be explored later in the dePaking analyses. Looking at the corresponding MLVs in Figure 4-4B, we observe that DPPC:Chol:PCer-d31 7:3:2 and 7:3:3 have slightly larger spectral widths than DPPC-d31:Chol:PCer 7:3:2 and 7:3:3, respectively. Quantitative analyses of the spectral widths of all recorded spectra will be illustrated next.
4.1.5. Average spectral width analyses

Further insight into the effect of ceramide on PSM or DPPC can be gained from examining the temperature dependence of the average width of the spectrum ($M_1$) in more detail (Figure 4-5). Upon heating from room temperature to 50 °C, the PSM-d31 MLVs undergo a sharp transition from the $S_0$ phase to the $L_d$ phase, with $T_m= 40$ °C, indicated by a sudden reduction in $M_1$. No pre-transition from gel to ripple phase was observed for PSM-d31, in agreement with several previously published studies [33], [65], [113]. However, using low angle x-ray spectroscopy (LAXS), it has been reported recently that if aligned bilayers of PSM are incubated at 3 °C for 4.5 days, then heated at 12 °C/hour, they show a pre-transition at about 24 °C [115]. We did not pre-cool our samples for an extended period (see Materials and Methods). Thus, it is probable that these PSM-d31 MLVs are in the ripple phase below 40 °C.

The $S_0$ to $L_d$ transition at 40 °C is eliminated by the addition of 30 mol% cholesterol, and $L_0$ spectra are observed for PSM-d31:Chol 7:3 MLVs above 27 °C. Below 38 °C, the $M_1$ values for PSM-d31:Chol 7:3 are lower than those for PSM-d31; whereas they are higher above this temperature. This confirms that Chol regulates the fluidity of saturated lipids in model membranes. The addition of 10 mol% PCer to PSM-d31:Chol 7:3 results in
increased values of $M_1$ between 34 °C and 65 °C, but does not affect $M_1$ between 21-33 °C. In contrast, the addition of 20 mol% PCer to the PSM-d31:Chol 7:3 causes a significant increase in $M_1$ below 35 °C, due to an increased contribution from the S₀ phase (see Figure 4-2). At temperatures equal or greater than 35 °C, the increase in $M_1$ is similar to that found for the addition of 10 mol% PCer. Increasing the PCer content further to PSM-d31:Chol:PCer 7:3:3 results in slightly larger $M_1$ values below 35 °C and significantly larger $M_1$ values above 35 °C. As discussed earlier, S₀ phase domains were found in MLVs composed of PSM-d31:Chol:PCer 7:3:2 and 7:3:3, and these give rise to the observed high values of $M_1$ below 35 °C. Above 35 °C the larger $M_1$ values observed for PSM-d31:Chol:PCer 7:3:3, compared to 7:3:2, are due to the enhanced chain order of PSM-d31 molecules in the L₀ phase in the presence of 30 mol% added PCer.

The behavior of $M_1$ values upon an increase in temperature depends on which lipid is deuterium-labelled. For example, below 40 °C, the $M_1$ values of PCer-d31 in PSM:Chol:PCer-d31 7:3:2 and 7:3:3 MLVs (Figure 4-5A) are substantially higher than the analogous MLVs containing PSM-d31. This is due to the presence of a higher proportion of PCer-d31 than PSM-d31 in the S₀ phase (see Figure 4-2). When PCer-d31 is mainly in the L₀ phase, above 50 °C, the $M_1$ values of MLVs containing 20 or 30 mol% added PCer-d31 are more similar to those of PSM-d31:Chol:PCer 7:3:2 and 7:3:3.

The temperature dependence of $M_1$ for MLVs containing DPPC-d31 instead of PSM-d31 is shown in Figure 4-5B. The change in the slope of $M_1$ for DPPC-d31 observed at ~ 32 °C can be assigned to the pre-transition of DPPC-d31 from the gel phase to the ripple phase [116]. Below 32 °C, the $M_1$ values for DPPC-d31 are higher than those of PSM-d31. At about 41 °C, DPPC-d31, like PSM-d31, undergoes a sharp S₀ to L₀ transition. Above 40 °C, the value of $M_1$ for PSM-d31 is higher than that of DPPC-d31. The binary PSM-d31:Chol 7:3 spectra have higher $M_1$ values at 50 °C and above those of DPPC-d31:Chol 7:3 MLVs, consistent with previous observations [113]. However, the increase in $M_1$ upon addition of Chol to either PSM-d31 or DPPC-d31 is very similar. For example, at 50 °C, the $M_1$ values for PSM-d31 and DPPC-d31 are $0.58 \times 10^5$ s⁻¹ and $0.50 \times 10^5$ s⁻¹ while those for PSM-d31:Chol and DPPC-d31:Chol 7:3 are $1.00 \times 10^5$ s⁻¹ and $0.94 \times 10^5$ s⁻¹ respectively.
Figure 4-5  Top: $M_1$ vs. temperature for MLVs containing the indicated molar ratios. (A) PSM-d31:Chol:PCer MLVs (red) and PSM:Chol:PCer-d31 MLVs (blue) and (B) DPPC-d31:Chol:PCer MLVs (green) and DPPC:Chol:PCer-d31 (purple). Bottom: the same figure is shown as the top figure where the $M_1$ values of the pure PSM-d31 and pure DPPC-d31 are removed to show the differences between the $M_1$ values corresponding to binary and ternary MLVs.

How does added PCer affect the spectra of DPPC-d31:Chol 7:3? Above 35 °C, the temperature dependence of $M_1$ for DPPC-d31:Chol 7:3 is very similar to that of PSM-d31:Chol 7:3 as PCer is incorporated. On the other hand, below 35 °C, PCer does not
appreciably change $M_1$ in DPPC-d31:Chol 7:3. Assessing instead the effect of PCer-d31 on DPPC:Chol 7:3, increasing the amount of added PCer-d31 from 20 mol % to 30 mol % above 35 °C results in an ~3% increase to $M_1$. Below 35 °C, adding 20 mol% PCer-d31 to DPPC:Chol 7:3 MLVs results in spectra having $M_1$ values that are nearly identical to those observed for DPPC-d31:Chol:PCer 7:3:2 MLVs. However, below about 30 °C, the addition of 30 mol% PCer-d31 to DPPC:Chol 7:3 leads to spectra with a significant $S_0$ component and consequently markedly higher $M_1$ values. Generally, above 35 °C, PCer addition to either DPPC-d31:Chol 7:3 or PSM-d31:Chol 7:3 has a similar effect on the temperature dependence of $M_1$ (Figure 4-5A and B). This is also true for PCer-d31 addition to either DPPC:Chol 7:3 or PSM:Chol 7:3. For example, above 35 °C, the maximum difference between the $M_1$ values of DPPC:Chol:PCer-d31 7:3:3 and PSM:Chol:PCer-d31 7:3:3 is 5% at 65 °C and 70 °C. However, below 35 °C, the addition of either PCer or PCer-d31 to the phospholipid:cholesterol 7:3 binary mixtures has a significantly different effect on the temperature dependence of $M_1$. Adding 20 or 30 mol% PCer dramatically increases the $M_1$ of the spectra of PSM-d31:Chol 7:3 MLVs, but has nearly no effect on those of DPPC-d31:Chol 7:3 MLVs. Adding 20 or 30 mol% PCer-d31 to either PSM:Chol 7:3 or DPPC:Chol 7:3 results in clearly “distinct” changes to the temperature dependence of $M_1$. At 27 °C and below, the spectra of PSM:Chol:PCer-d31 7:3:2 and 7:3:3 have much larger $M_1$ values than those of the spectra of DPPC:Chol:PCer-d31 7:3:2 and 7:3:3. This reflects the fact that a substantially higher proportion of PCer-d31 is in the $S_0$ phase in MLVs contain PSM.

Generally, the $M_1$ traces for binary and ternary MLVs containing either PSM or PSM-d31 plotted in Figure 4-5A, seem to be converging above 35 °C. On the other hand, the $M_1$ traces of the corresponding MLVs containing either DPPC or DPPC-d31 in Figure 4-5B are similar at 35 °C but become different at higher temperatures. For instance, at 35 °C, the maximum difference between the value of $M_1$ for binary and ternary MLVs is 0.14 × 10^5 s^{-1} in Figure 4-5A but 0.07 × 10^5 s^{-1} in Figure 4-5B. At 50 °C, these values are 0.09 × 10^5 s^{-1} and 0.16 × 10^5 s^{-1} in Figure 4-5A and Figure 4-5B respectively.
4.1.6. DePaked spectra and smoothed order parameter profiles

Figure 4-6 $^2$H NMR dePaked spectra of the ternary MLVs. (A) PSM-d31:Chol:PCer MLVs (red) and PSM:Chol:PCer-d31 MLVs (blue) and (B) DPPC-d31:Chol:PCer MLVs (green) and DPPC:Chol:PCer-d31 (purple) at 50 °C. The brown arrows represent the signal from the deuterons on C2 of the deuterated palmitoyl chains in PSM-d31 and DPPC-d31 MLVs [33–36]. The black arrow indicates the peak representing one of the deuterons on C3 of the deuterated palmitoyl chain in PSM-d31 MLVs [107]. The latter peak is not resolved in the DPPC-d31 spectrum. The reason for the different behavior of the deuterons attached to C2 and C3 is the kink in the palmitoyl chain at these carbons [117].

At 50 °C, the spectra of all the MLVs with 20 mol% or 30 mol% added PCer or PCer-d31 have very comparable $M_1$ values (Figure 4-5). A detailed comparison of these spectra follows. In Figure 4-6, the dePaked spectra of all the MLVs at 50 °C are shown. Addition of PCer and Chol to PSM-d31 (Figure 4-6A) makes quadrupolar splittings larger, indicating that the palmitoyl chains are more ordered. An exception to this general observation is that the splitting of the 19 kHz peak on the PSM-d31 spectrum, which is assigned to one of the C2 deuterons [111], gets narrower upon addition of Chol and PCer. This effect has been observed before for PSM-d31:Chol binary mixtures [113]. By comparing the dePaked spectra of PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:2 (and 7:3:3), we observe that the width of the corresponding spectra and the positions of
the methyl group signals are remarkably similar. However, there are some subtle
differences that become apparent when order parameter profiles are calculated (see
below). As shown in Figure 4-6B, the DPPC-d31 quadrupolar splittings also get larger
upon addition of cholesterol and PCer, as was observed for PSM-d31 in Figure 4-6A.
When comparing the spectrum of DPPC-d31:Chol:PCer and DPPC:Chol:PCer-d31 in
7:3:2 (and 7:3:3) MLVs in Figure 4-6B, we find that they are very similar to the analogous
spectra (from MLVs containing PSM or PSM-d31) in Figure 4-6A. Quantitative
comparisons of dePaked spectra can be accomplished by examining the order parameter
profiles (Figure 4-7).

Figure 4-7  Order parameter profiles, |$S_{CD}$| values for each carbons/deuterons, at 50 °C obtained from
the dePaked spectra of the ternary MLVs. (A) PSM-d31:Chol:PCer (red) and PSM:Chol:PCer-d31 (blue); (B)
DPPC-d31:Chol:PCer (green) and DPPC:Chol:PCer-d31 (purple). The quadrupolar splittings for C2-d1 at 55
°C are used in the order parameter profiles of PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 MLVs and
also PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:2 MLVs (see Figure 4-8. The rest of the peaks are
assigned as explained in details in Chapter 3.

The smoothed order parameter profiles\(^7\) at 50 °C for the carbons along the
palmitoyl chain of the deuterated lipid in MLVs having each studied composition are shown
in Figure 4-7. The peaks were assigned as explained in Chapter 3. In Figure 4-7, we

\(^7\) For the definition of smoothed order parameter profiles see section 3.3.2.
observe that Chol orders all PSM-d31 and DPPC-d31 deuterons with the exception of deuterons attached to C2.

Upon addition of 10 - 30 mol% PCer to PSM-d31:Chol 7:3, the order parameter of carbons in the palmitoyl chain of PSM-d31 increases slightly. Examining the effect of the addition of 30 mol% Chol to either of the pure phospholipid MLVs using the order parameter profiles in Figure 4-7A and Figure 4-7B shows that Chol increases the order parameter of PSM-d31 and DPPC-d31 by approximately the same amount, especially in carbons close to the headgroup. For example, Chol increases the order parameter at C4 in both DPPC-d31 and PSM-d31 by ~ 0.16, whereas it increases the order parameter at C15 by 0.12 in DPPC-d31 and by 0.15 in PSM-d31. Generally, this confirms the results obtained from analysis of $M_1$ (which is directly proportional to the average order parameter). There, it was shown that differences in the values of $M_1$ of the spectra at 50 °C, upon addition of 30 mol% Chol are very similar. Comparing the order parameter profiles of MLVs containing PCer (or PCer-d31) in Figure 4-7B with those in Figure 4-7A reveals that PCer is more effective at ordering DPPC-d31 than PSM-d31. The order parameter profile data show that DPPC-d31 is consistently less ordered in DPPC-d31:Chol:PCer 7:3:0 and 7:3:1 compared to 7:3:2 and 7:3:3. The increase in order for a given chain position in DPPC-d31:Chol 7:3 is approximately linear up to 20 mol% added PCer, but no other significant increase is observed when 30 mol% PCer is added.

In general, based on the results of order parameter analysis, it can be stated that the palmitoyl chain in PSM-d31 is more ordered than the sn-2 palmitoyl chain of DPPC-d31 in pure phospholipid 7:3:0 and 7:3:1 MLVs. When PCer is present in higher concentrations, the order parameters of the deuterated lipids are very similar. The order parameters of 20 or 30 mol% PCer-d31 added to either PSM:Chol 7:3 or DPPC:Chol 7:3 are also very similar, both to each other and to the order parameters of PSM-d31 or DPPC-d31 in the analogous MLVs. So, at 50 °C, the order parameter profiles of all MLVs containing 20 or 30 mol% added PCer (or PCer-d31) are nearly indistinguishable.
4.1.7. Pairwise comparison of the dePaked spectra of the 7:3:3 MLVs

Figure 4-8  Pairwise comparision of the dePaked spectra of PSM-d31 and PCer-d31 in 7:3:3 MLVs. Depaked spectra Top: DePaked spectra for PSM-d31 in MLVs of PSM-d31:Chol:PCer and PCer-d31 in PSM:Chol:PCer-d31 7:3:3 at the indicated temperatures. The text related to each arrow, i.e. C2 and C15, is color coded as the same as the spectra. The C2-d1 peaks for both MLVs at 50 °C are very broad and cannot be distinguished as separate peaks. Since the position of the peak for this deuteron is almost identical at the temperatures shown, the quadrupolar splittings for C2-d1 at 55 °C were used in the order parameter profiles presented in Figure 4-7. Bottom: Smoothed order parameter profiles of the dePaked spectra at 50 °C.

In Figure 4-8 to Figure 4-10, pairwise comparisons of the dePaked spectra of MLVs containing 30 mol% added PCer or PCer-d31 are plotted at 50, 55, 60, 65 and 70 °C. The dePaked spectra of MLVs composed of PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 are compared in Figure 4-8. Both sphingolipids display very similar spectra from 50-70 °C, indicating that they are well mixed in the presence of Chol. Notably, the quadrupolar splittings are very similar at 50, 55 and 60 °C. The exception is one of the C2 deuterons (C2-d1) as indicated by arrows. For each of the SLs, the position of the C2-d1 peak is
almost static as the temperature increases from 50 to 70 °C. Also, C14 and C15 deuterons are slightly more ordered in PSM-d31 than PCer-d31 at these temperatures. At 65 and 70 °C, the quadrupolar splittings of the C3 to C16 deuterons in PSM-d31 are slightly larger than those of PCer-d31. This could be due to the presence of a higher amount of Chol in the L0 phase of PSM-d31 than that of PCer-d31 (see Table 3-1). However, since on average the increase in the order parameter of PSM-d31 relative to PCer-d31 at 70 °C is only ~6%, we propose that even at 70 °C, SM and Cer are well mixed in PSM:Chol:Cer 7:3:3 MLVs.

Figure 4-9  Pairwise comparision of the dePaked spectra of DPPC-d31 and PCer-d31 in 7:3:3 MLVs. Top: DePaked spectra for DPPC-d31 in MLVs of DPPC-d31:Chol:PCer and PCer-d31 in DPPC:Chol:PCer-d31 7:3:3 at the indicated temperatures. In general, the quadrupolar splittings are larger for PCer-d31. The position of one of the C2 deuterons are indicated by the arrows. The text related to each arrow is color coded as the same as the spectra. Bottom: Smoothed order parameter profiles of the dePaked spectra at 50 °C.
The same comparison is shown for DPPC-d31:Chol:PCer versus DPPC:Chol:PCer-d31 in Figure 4-9. At 55 °C and above, DPPC-d31 is less ordered than PCer-d31. However, this is not evidence of inhomogeneous mixing, since the lipid backbone is known to affect chain order [118]. In a binary mixture of a sphingolipid and a glycerophospholipid with a known phase diagram, such as POPC:PCer, the average order in the plateau region (C4-C6) of the palmitoyl chain can differ by more than 12% even in a homogeneous liquid crystalline region of the phase diagram [43]. The maximum difference in plateau order observed in Figure 4-9 is 11.4%, so we propose that the lipids are likely to be well mixed.

![DePaked spectra comparison](image)

**Figure 4-10** Pairwise comparison of the dePaked spectra of PCer-d31 in 7:3:3 MLVs containing either PSM or DPPC. Top: DePaked spectra for PCer-d31 in MLVs of PSM:Chol:PCer-d31 and in DPPC:Chol:PCer-d31 7:3:3 at the indicated temperatures. Bottom: The smoothed order parameter profiles at 50 °C. The spectra are very similar at 50 °C, but as the temperature increases, PCer-d31 in PSM-containing MLVs has higher quadrupolar splittings.
The dePaked spectra of PSM:Chol:PCer-d31 and DPPC:Chol:PCer-d31 7:3:3 MLVs are plotted in Figure 4-10. From this figure, it is apparent that the spectra at 50 °C are essentially identical, and the order parameter profiles at 50 °C confirms this. As the temperature increases, PCer-d31 is less disordered in PSM-containing MLVs than those containing DPPC. A similar conclusion can be reached by comparing slopes in the high-temperature region of Figure 4-5: it is apparent that the sphingolipid membrane is relatively insensitive to increasing temperature compared to the analogous DPPC-containing membrane.

Finally, the dePaked spectra of PSM-d31 and DPPC-d31 in both the 7:3:3 MLVs are shown in Figure 4-11. At 50 °C, both the dePaked spectra and the order parameter profiles show that PSM-d31 has larger quadrupolar splittings compared DPPC-d31. As
the temperature increases, the quadrupolar splittings of PSM-d31 decrease significantly compared to those of DPPC-d31 (except for C2-d1). One possible reason for this behavior could be the presence of a higher Chol concentration in the PSM-d31:Chol:PCer MLVs compared to DPPC-d31:Chol:PCer MLVs. In fact, our $^1$H NMR analyses of the actual lipid ratios showed that there is a slight difference between the nominal and actual ratios in these two samples of MLVs (Table 3-1). The phospholipid to Chol ratio was found to be 2.29 and 2.33 for the PSM-d31 and DPPC-d31 containing MLVs respectively. Therefore, the slightly higher Chol level in PSM-d31 containing 7:3:3 MLVs is one of the reasons that the PSM-d31 spectra in Figure 4-11 are less sensitive to increases in temperature. The other reason for the larger quadrupolar splittings of PSM-d31 in Figure 4-11, is the effect of the backbone in ordering the chains as mentioned earlier.

4.2. Discussion

We have thoroughly examined the influence of increasing ceramide concentrations on membranes composed of either PSM:Chol or DPPC:Chol. In the absence of Chol and ceramide, both DPPC-d31 and PSM-d31 undergo a sharp transition from the $S_0$ phase to the $L_0$ phase near 40 °C. We confirmed that the addition of 30 mol% Chol to either DPPC-d31 or PSM-d31 abolishes this phase transition as has been previously documented by $^2$H NMR and DSC [33].

Our results show that ceramide is able to form $S_0$ phases in DPPC and PSM bilayers in the presence of Chol at low temperatures. Adding PCer (or PCer-d31) to the 7:3 binary mixture of phospholipid:Chol, $S_0$ phase formation was observed in both DPPC (or DPPC-d31) and PSM (or PSM-d31) MLVs. The reason that PCer-d31 "prefers" the $S_0$ phase at lower temperatures could be related to its having a smaller ratio of headgroup size to chain cross sectional area compared to PSM. At lower temperatures, PSM chains have more freedom compared to those of PCer, therefore making the $S_0$ phase less favorable for PSM, but more favorable for PCer. At higher temperatures, PCer chains have enough thermal energy to compensate for the fact that the small headgroup does not provide space for chain motion and the $S_0$ phase is no longer observed. Below 35 °C, all ternary mixtures show a superposition of $S_0$ and $L_0$ phase spectra. Thus, the $S_0$ phases induced by addition of PCer (or PCer-d31) coexist with the $L_0$ phase induced by addition of Chol to either phospholipid. As shown in Figure 4-2, the spectra of PCer-d31 in PSM:Chol:PCer-d31 and DPPC:Chol:PCer-d31 7:3:3 MLVs at 22 °C show lineshapes
dominated by an S₀ phase spectrum. At the same temperature, PSM-d31 and DPPC-d31 in both PSM-d31:Chol:PCer and DPPC-d31:Chol:PCer 7:3:3 are primarily in the L₀ phase. Since the predominant lipid in all ternary MLVs is the phospholipid, the surface area of L₀ phase domains should be greater than that of the S₀ domains.

One reason for the observation of higher contributions from S₀ phases at higher concentrations of PCer (or PCer-d31) is the dilution of Chol. For example, 7:3:1, 7:3:2 and 7:3:3 MLVs contain 27, 25 and 23 mol% Chol respectively. It should also be noted that a substantial amount of S₀ phase is present in the binary mixture of either PSM-d31:Chol and DPPC-d31:Chol containing 23 mol% Chol [113].

Our results are consistent with previously published data on lipid mixtures with similar molar ratios. For example, as shown in Figure 4-12, confocal microscopy of giant unilamellar vesicles (GUVs) containing PSM:Chol:PCer and DPPC:Chol:PCer 7:3:3 (molar ratios) that are stained with naphtho[2,3-a]pyrene (NAP) clearly show two phases at 22 °C [80]. Since there are six rings in the molecular structure of NAP, it tends to accumulate in the Chol rich domains in the bilayers [80]. Thus, combining the results of NAP-stained GUVs and ²H NMR at 22 °C, it can be concluded that the probe partitions into domains that are rich in PSM and Chol, i.e. the L₀ phase. A comparison of the ²H NMR and GUV fluorescence data yields some information about the composition of the observed S₀ phases.
From $^2$H NMR, we know that they are rich in ceramide, while their dark nature in NAP-labelled GUVs indicates that they are Chol depleted. In the same article, both GUVs (PSM:Chol:PCer and DPPC:Chol:PCer 7:3:3) were examined with another probe, DiIC-18 (middle GUVs in Figure 4-12). The results for both DiIC-18 stained GUVs show an almost homogenous single phase GUV [80]. This is due to the fact that DiIC-18 partitions into ordered domains, and therefore partitions nearly equally in L$_o$ and S$_o$ phase domains [80]. Thus, the combined results from NAP- and DiIC-18-stained GUVs are consistent with the results obtained from $^2$H NMR experiments at 22 °C reported here and suggest that ceramide induces an ordered phase separate from L$_o$ in these mixtures.
Figure 4-13  Differential scanning calorimetry (DSC) thermograms of PSM:Chol:PCer MLVs. From top to bottom are: PSM:Chol:PCer 7:3:0, 7:3:1, 7:3:2 and 7:3:3 MLVs [65]. Reprinted with permission from Elsevier.
The results of differential scanning calorimetry (DSC) experiments performed on PSM:Chol:PCer 7:3:0, 7:3:1, 7:3:2 and 7:3:3 MLVs are shown [65] in Figure 4-13. The DSC thermogram of PSM:Chol 7:3 consists of a very wide, nearly flat peak indicating that there is no abrupt phase transition between 10 and 90 °C. As PCer is incrementally added to the MLVs, this endotherm becomes somewhat sharper and the center of the peak shifts to higher temperatures (58 °C for PSM:Chol:PCer 7:3:3). Our 2H NMR results suggest that this peak becomes sharper because, at low temperatures, some S0 phase forms in the MLVs upon addition of PCer. However, when comparing DSC and 2H NMR data, it should be noted that each spectrum in 2H NMR experiments is taken when the system is in thermal equilibrium which is not the case in DSC measurements. This could be why a transition is seen in DSC thermograms but not in $M_1$ vs. temperature traces calculated from 2H NMR spectra (Figure 4-5).

Figure 4-14  The height images SPBs from contact mode AFM of PSM:Cho:PCer SSBs. The pictures are showing SSBs of: PSM (a), PSM:Chol 7:3 (b), PSM:PCer:Chol 7:3:3 (c), DPPC (d), DPPC:Chol 7:3 (e), and DPPC:PCer:Chol 7:3:3 (f). Scale bars are 1 μm [81]. The black areas are lacking bilayers. Reprinted with permission from American Chemical Society.
In an atomic force microscopy (AFM) imaging study, the contact mode height images of the supported planar bilayers (SPBs) of DPPC and PSM at 23 °C showed almost the same height for each SPB (Figure 4-14a and d) [81]. As shown in Figure 4-14b and e, upon addition of Chol, in PSM:Chol and DPPC:Chol 7:3, the height for each SPB was found to be the same, but an increase in the height of both SPBs was observed. This probably corresponds to the formation of L\textsubscript{o} phases in both kinds of binary MLVs observed in our \textsuperscript{3}H NMR. In the presence of PCer in PSM:Chol:PCer and DPPC:Chol:PCer 7:3:3 in Figure 4-14c and f, domains could not be detected by AFM, neither in imaging nor in force spectroscopy modes. It is possible that the L\textsubscript{o} and S\textsubscript{o} domains in the ternary mixture have similar heights, and cannot be resolved by AFM. This would still be compatible with S\textsubscript{o}+L\textsubscript{o} phase coexistence for PSM/DPPC-d31:Chol:PCer and PSM/DPPC:Chol:PCer-d31 7:3:3 MLVs as found in this report.
Chapter 5.

Cholesterol and Ceramide interactions in sphingomyelin and POPC bilayers

This chapter is dedicated to studies on the effect of the addition of POPC to the ternary MLVs of PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3. First, POPC was added so that its concentration would be equal to the total SL concentration: POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:3:3. The next step was to study a system resembling the outer leaflet of the plasma membrane as closely as possible, an equimolar mixture of PC, SL and Chol. This was achieved by increasing the Chol content of the MLVs to the same level as the SLs and POPC: POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:10:3.

5.1. Results

5.1.1. Spectral shape analyses

In this section, the $^2$H NMR powder spectra of the MLVs containing POPC will be shown, and their spectral shape will be monitored as the temperature increases. Then the spectra at three different temperatures, 22, 40 and 50 °C will be compared for the PSM-d31 and PCer-d31 containing 10:7:10:3 and 10:7:3:3 MLVs. These spectra will also be compared to the spectra of the PSM-d31 and PCer-d31 containing 7:3:3 MLVs shown in the previous chapter.

In Figure 5-1, the spectra of POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs are plotted at 21, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 °C. At temperatures below 30 °C, the spectra of POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs are mainly in the S₀ phase with some contributions from the Lα phase. As the temperature increases, POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs make a
transition to the L_d phase with the S_o phase being completely melted at 45 ºC. On the other hand, less S_o phase spectral shape signature is observed in the spectra of POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs at temperatures below 30 ºC. Therefore, PSM-d31 in these MLVs is mainly in the L_d phase with some contributions from the S_o phase at temperatures below 45 ºC. Like POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs, no
contributions from the S₀ phase were observed at 45 °C in the spectra of POPC:PSM-
d31:Chol:PCer 10:7:3:3 MLVs. Therefore, all the spectra at temperatures ≥ 45 °C are in
the L₀ phase for 10:7:3:3 MLVs containing PSM-d31 as well. The non-existence of the L₀
phase is consistent with the low Chol level (13 mol %) in the 10:7:3:3 MLVs containing
either PSM-d31 or PCer-d31.

![NMR Spectra](image)

**Figure 5-2** The ²H NMR spectra of POPC:PSM-d31:Chol:PCer (red) and POPC:PSM:Chol:PCer-d31
(blue) 10:7:10:3 at the indicated temperatures.

In Figure 5-2, the spectra of POPC:PSM-d31:Chol:PCer and
POPC:PSM:Chol:PCer-d31 10:7:10:3 MLVs are plotted at the same temperatures as in
Figure 5-1. All of the spectra in both data sets and at all of the temperatures shown
represent L₀ phase spectra. The spectra become narrower as the temperature increases, but no evidence for a L₄ phase is observed at temperatures as high as 70 °C. This is consistent with the high Chol concentration in both mixtures. Therefore, the phase of POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:10:3 MLVs is homogenously L₀ for all of the temperature recorded in our ²H NMR experiments. These data support the previously stated conclusion that high cholesterol content prevents S₀ formation and phase separations in compositionally complex membranes at physiological temperatures.

The spectral shapes of the quaternary MLVs will be further investigated through pairwise comparisons of the data for MLVs containing the same molar ratios of SLs, POPC and Chol at 22, 40 and 50 °C. Also the spectra for PSM-d31 and PCer-d31 containing 7:3:3 MLV are both plotted in the same graphs to clearly show effects related to the addition of POPC to these MLVs.

Figure 5-3 Overlaid ²H NMR spectra for POPC:PSM:Chol:PCer 0:7:3:3, 10:7:3:3 and 10:7:10:3 MLVs at 22 °C. From bottom to top are the spectra for POPC:PSM-d31:Chol:PCer MLVs with molar ratios of 0:7:3:3, 10:7:3:3 and 10:7:10:3 (red spectra). Also, from bottom to top are the spectra for POPC:PSM:Chol:PCer-d31 MLVs with the molar ratios of 0:7:3:3, 10:7:3:3 and 10:7:10:3 (blue spectra). The signal at 0 kHz for the PCer-d31 containing MLVs spectra (blue spectra) arises from DOH in the sample.

The spectra of the quaternary MLVs along with the ternary MLVs containing PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 at 22 °C are plotted in Figure 5-3. As discussed in the previous chapter, the spectra of both PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 result from a combination of S₀ + L₀ phases. MLVs containing PSM-d31 have more prominent L₀ features while those containing PCer-d31 have more
prominent $S_0$ features. As POPC is added to the ternary MLV, POPC:PSM-d31:Chol:PCer 10:7:3:3, $S_0$ features become weaker but do not disappear. On the other hand, when POPC is added to PSM:Chol:PCer 7:3:3, the PCer-d31 spectrum retains its stronger $S_0$ features over the $L_0$ features. Although the growth of an LC edge between ~20-30 kHz is observed for this spectrum, PCer-d31 is significantly more in the $S_0$ phase than the $L_0$ phase in POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. Therefore, the effect of the addition of POPC to both PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 MLVs is generally to make the membranes more fluid. However, this effect is more pronounced for PSM-d31 containing MLVs compared to PCer-d31 containing MLVs.

As the level of Chol increases, in the equimolar SLs, POPC and Chol MLVs, both PSM-d31 and PCer-d31 show typical $L_0$ spectra. This shows that increasing the Chol content of the MLVs from 13 mol% to 33 mol% abolishes the $S_0$ phase, and brings the phase of the membrane to $L_0$ phase even at temperatures as low as 22 °C.

![Overlaid 2H NMR spectra for POPC:PSM-d31:Chol:PCer 0:7:3:3, 10:7:7:3 and 10:7:10:3 MLVs (red spectra) and POPC:PSM:Chol:PCer-d31 0:7:3:3, 10:7:7:3 and 10:7:10:3 MLVs (blue spectra) at 40 °C.](image)

The spectra of MLVs at 40 °C are shown in Figure 5-4. In the previous chapter, it was found that while both PSM-d31 and PCer-d31 are in the $L_0$ phase in both 7:3:3 MLVs, fractions of PCer-d31 are in the $S_0$ at 40 °C. The spectrum of POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs shows a spectrum predominantly in the $L_0$ phase with some $S_0$ features.
The same argument applies to the spectrum of POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. However, the spectrum of 10:7:3:3 MLVs containing PCer-d31 has more $S_o$ phase features than that of 10:7:3:3 MLVs containing PSM-d31. Therefore, at 40 °C, more PCer-d31 than PSM-d31 molecules are in the $S_o$ phase in 10:7:3:3 MLVs. This is similar to what was observed in the absence of POPC, i.e. in the ternary 7:3:3 MLVs, which contained a ceramide-enriched $S_o$ phase (section 4.1.3).

By increasing the Chol level to the same as the POPC and SL levels, the spectra of both PSM-d31 and PCer-d31 in 10:7:10:3 MLVs show $L_o$ behavior. Hence, at 40 °C, in MLVs with equimolar POPC, SLs and Chol, none of the $S_o$ features is observed in the spectrum of either 10:7:10:3 MLVs containing PSM-d31 or PCer-d31.

![Overlaid $^2$H NMR spectra for POPC:PSM-d31:Chol:PCer-d31 0:7:3:3, 10:7:3:3 and 10:7:10:3 MLVs (red spectra) and POPC:PSM:Chol:PCer-d31 0:7:3:3, 10:7:3:3 and 10:7:10:3 MLVs (blue spectra) at 50 °C.](image)

The spectra of the same MLVs at 50 °C are plotted in Figure 5-5. In the absence of POPC, both ternary 7:3:3 MLVs are in the $L_o$ phase. With the addition of POPC to both PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 MLVs, the PSM-d31 and PCer-d31 spectra become typical $L_o$ spectra. Therefore, it seems the transition from $S_o$ to $L_o$ is completed at 50 °C for both PSM-d31 and PCer-d31 in 10:7:3:3 MLVs. Upon increasing the Chol level, in POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:10:3, the spectra for PSM-d31 and PCer-d31 both show evidence of pure $L_o$ behavior.
Therefore, increasing the Chol content from 13 mol% in 10:7:3:3 MLVs to 33 mol% in 10:7:10:3 MLVs, keeps the membrane in the LC phase at 50 °C but orders the membrane by changing the phase of the membrane from L_d to L_o.

5.1.2. Average spectral width analyses

The result of $M_1$ analyses of the spectra as a function of temperatures is plotted in Figure 5-6. It was shown in the previous chapter that at temperatures below 40 °C, PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 MLVs were in S_o+L_o phase coexistence, with higher contributions from L_o phase and S_o phase for PSM-d31 and PCer-d31, respectively. The spectra were found to be L_o-like for both PSM-d31 and PCer-d31 in 7:3:3 MLVs at temperatures of 50 °C and higher. The general observation in Figure 5-6 is that the addition of POPC to both PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3, lowers $M_1$ values at all temperatures and generally makes the membranes more fluid in all quaternary MLVs than 7:3:3 ternary MLVs.

In POPC:PSM-d31:Chol:PCer 10:7:3:3, adding POPC lowers $M_1$ values in a temperature dependent manner. At temperatures below 27 °C, there is an average 7.5% decrease in $M_1$, while for temperatures between 50-70 °C, $M_1$ decreases by 38% on average. This difference in the decrease of the $M_1$ values is due to the fact that at temperatures below 27 °C, a fraction of the PSM-d31 molecules in POPC:PSM-
d31:Chol:PCer 10:7:3:3 is still in the S₀ phase. As it was also observed through the shape analyses, these S₀ domains make the transition to the Lₐ phase, upon heating from room temperature to 45 °C, and become homogenous in the Lₐ phase at 50 °C and higher. Hence, the broad transition in the $M_1$ trace of POPC:PSM-d31:Chol:PCer 10:7:3:3 in Figure 5-6A is due to the transition of the PSM-d31 molecules from the S₀+L₀ to the Lₐ phase. The trend that is observed for $M_1$ in POPC:PSM-d31:Chol:PCer 10:7:3:3 is in agreement with the shape analyses results which suggested S₀+Lₐ phase coexistence at temperatures below 45 °C and mostly a Lₐ phase at temperatures higher than 50 °C.

As shown in Figure 5-6A, the observed transition in the $M_1$ trace of POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs is abolished by increasing the Chol content of MLVs in POPC:PSM-d31:Chol:PCer 10:7:10:3. Increasing the Chol content decreases the $M_1$ values of POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs below 32 °C and increases them above this temperature. This again confirms the role of Chol as the regulator of membranes fluidity as observed in the previous chapter. Also, the absence of a transition in $M_1$ values for POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs is in agreement with the evidence for pure L₀ spectra observed for these MLVs in the temperature range 21 to 70 °C.

The same behavior as is observed for $M_1$ values of PSM-d31 in Figure 5-6A is observed for PCer-d31 in the ternary and quaternary MLVs, as shown in Figure 5-6B. Addition of POPC to ternary MLVs composed of PSM:Chol:PCer-d31 7:3:3 lowers $M_1$ values. However, at temperatures below 28 °C, this decrease is very small, and on average, there is a 5% decrease in the $M_1$ values over the temperature range 21-28 °C. This is due to substantial fractions of PCer-d31 molecules being in the S₀ phase in both PSM:Chol:PCer-d31 7:3:3 and POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. Similar behavior was found using spectral shape analyses, where it was observed that PCer-d31 retains its mostly S₀ phase spectral features in PSM:Chol:PCer-d31 7:3:3 MLVS even in the presence of substantial POPC in POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs.

As the POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs are heated above room temperature, a transition is observed in their $M_1$ values. From shape analyses, it is observed that the S₀ phase, which is rich in PCer-d31, melts upon heating and completing the transition to the LC phase at 45 °C. Therefore, the observed transition in the $M_1$ trace of POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs in Figure 5-6B is due to the transition of
PCer-d31 molecules from $S_0+L_d$ coexistence to $L_d$ phase. Note that the $M_1$ values for POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs seem to be more sensitive to temperature changes relative to POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs. This is because our $^1$H NMR experiments for determining the actual lipid ratios suggest that the ratio of PSM-d31/Chol is 3.1 for POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs, while the ratio of PSM/Chol is 2.0 for POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. This partially explains the higher temperature sensitivity of 10:7:3:3 MLVs containing PCer-d31 compared to those containing PSM-d31. Therefore, based on our spectral shape and $M_1$ analyses, we suggest that PCer-d31 in POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs is mostly in the $S_0$ phase, with a small contribution from the LC phase, over the temperature range 21-28 $^\circ$C. The $S_0$ phase melts at 45 $^\circ$C. At 50 $^\circ$C and at higher temperatures, the dominant LC phase of the PCer-d31 molecules is $L_d$.

In the POPC:PSM:Chol:PCer-d31 10:7:10:3 MLVs, where the Chol level is brought to the same level as the SLs and POPC, no transition in $M_1$ values is observed. Below 40 $^\circ$C, $M_1$ values for POPC:PSM:Chol:PCer-d31 10:7:10:3 MLVs are lower and above 40 $^\circ$C, they are higher than those of POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs, respectively. This is also in agreement with the role of Chol as the regulator of membrane fluidity. Generally, at temperatures below 32 $^\circ$C, as Chol levels increase, the decrease in $M_1$ values of PCer-d31 containing MLVs is greater than that for PSM-d31 containing MLVs. This is due to the fact that from the spectral shape analyses, the spectra of PCer-d31 containing MLVs had more features typical of a $S_0$ phase spectrum (and less $L_0$ phase features). It could also be due to deviations from the nominal lipid ratios as explained earlier for the POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs. The $M_1$ values of PSM-d31 and PCer-d31 in 10:7:10:3 MLVs are very similar at all temperatures. This is in agreement with similarities found in their powder $^2$H NMR spectra, especially both being in the $L_0$ phase over the temperature range 21-70 $^\circ$C.

In summary, $M_1$ analyses are in complete agreement with the shape analyses. The most important results of both analyses are that higher fractions of PCer-d31 were found to be in the $S_0$ phase at temperatures below 40 $^\circ$C compared to PSM-d31 in the 10:7:3:3 MLVs. Moreover, it was found from both analyses, that in the presence of 33 mol% Chol, the other reason is the presence of a higher level of PCer-d31 in the $S_0$ phase observed for POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. Generally, lipids in the $S_0$ phase are more sensitive to temperature change than the same lipids in the $L_0$ phase.

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8 The other reason is the presence of a higher level of PCer-d31 in the $S_0$ phase observed for POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. Generally, lipids in the $S_0$ phase are more sensitive to temperature change than the same lipids in the $L_0$ phase.
no $S_0$ phase is present and the phase of the membranes are homogeneously $L_0$ phase at all recorded temperatures. Further insight into the phase behavior of the studied quaternary MLVs can be gained through dePaking the powder spectra and obtaining smoothed order parameter profiles.

### 5.1.3. DePaked spectra and smoothed order parameter profiles

As was shown in Figure 5-6, the $M_1$ values at temperatures $\geq 50$ °C are comparable for each pair of MLVs with the same molar ratios of POPC, SLs and Chol. To further analyze the spectra at higher temperatures, the dePaked spectra at 50 °C are shown in Figure 5-7 and a detailed comparison of these spectra follows. As discussed in the previous chapter, the dePaked spectra of PSM-d31:Chol:PCer and PSM:Chol:PCer-d31 7:3:3 MLVs are very similar and both represent spectra in the $L_0$ phase at 50 °C. As POPC is added to these MLVs, the quadrupolar splittings become significantly smaller. This is shown in the middle two spectra in Figure 5-7A and Figure 5-7B for POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs and POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs, respectively. The two spectra are very similar and have a spectral width comparable to a typical $L_0$ spectrum. As the Chol concentration increases and reaches the same level as those of SLs and POPC, the quadrupolar splittings become larger in both POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:10:3 MLVs (the two top spectra in
Figure 5-7A and Figure 5-7B, respectively). The two spectra are very similar and the quadrupolar splittings are comparable to a typical L₀ spectrum.

![Figure 5-7](image)

**Figure 5-7**

Order parameter profiles, $|S_{CD}|$ values for each carbon/deuteron, at 50 °C obtained from the dePaked spectra of for POPC:PSM:Chol:PCer 0:7:3:3, 10:7:3:3 and 10:7:10:3 MLVs. (A) PSM-d31:Chol:PCer 7:3:3 and POPC:PSM-d31:Chol:PCer 10:7:3:3 and 10:7:10:3 (red) and (B) PSM:Chol:PCer-d31 7:3:3 and POPC:PSM:Chol:PCer-d31 10:7:3:3 and 10:7:10:3 (blue).

Figure 5-8 shows the smoothed $S_{CD}$ profiles calculated from the dePaked spectra of Figure 5-7. Addition of POPC to the membranes at 50 °C decreases the order parameters of the carbons on both the deuterated PSM and the deuterated PCer, except for C2 and C16, and converts the phase from L₀ to L₄. The addition of POPC to 7:3:3 MLVs containing either PSM-d31 or PCer-d31 changes the phase from L₀ to L₄, and significantly decreases the order parameter as discussed earlier.

Increasing the Chol content to 33 mol% increases the order parameters of C3-C15 carbons. As was observed in the powder and dePaked spectral shape and $M_1$ analyses, this increase is the result of PSM-d31 and PCer-d31 being in the L₀ phase at 50 °C in the 10:7:10:3 MLVs. More detailed comparisons of the dePaked spectra of POPC containing MLVs at higher temperatures follows.
5.1.4. Pairwise comparisons of dePaked spectra

Figure 5-9  Pairwise comparision of the dePaked spectra of PSM-d31 and PCer-d31 in 10:7:3:3 MLVs. Top: DePaked spectra for PSM-d31 in MLVs of POPC:PSM-d31:Chol:PCer and PCer-d31 in POPC:PSM:Chol:PCer-d31 10:7:3:3 at the indicated temperatures. The arrows represent the position of one of the C2 deuterons (C2-d1). The text related to each arrow, i.e. C2, is color coded the same the spectra. Bottom: The smoothed order parameter profiles of the same MLVs at 50 °C.
In Figure 5-9, the dePaked spectra of POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs and the calculated smoothed order parameter profiles at 50 °C are shown. The quadrupolar splittings at 50 °C are very similar for both MLVs containing PSM-d31 and PCer-d31. However, PSM-d31 is more ordered than PCer-d31, especially at the carbon positions in the range C10-C15. This can also be seen from the order parameter profiles at the bottom of Figure 5-9. Generally, as the temperature increases, more peaks are resolved for both MLVs, and the quadrupolar splittings increase slightly for the MLVs containing PSM-d31. The higher order of POPC:PSM-d31:Chol:PCer 10:7:3:3 MLVs is due to the previously mentioned higher level of Chol molecules found in MLVs containing PSM-d31 from our ¹H NMR analyses (Table 3-1).

This explains the higher order and lower temperature sensitivity of PSM-d31 over PCer-d31 in 10:7:3:3 MLVs for the temperature range recorded. To confirm that all of the spectra are in the L^d phase, the order parameters can be compared to order parameters of systems with known phase diagrams such as PSM-d31:Chol MLVs [113]. The order parameters plotted in Figure 5-9 are in the same range as the order parameters reported for PSM-d31:Chol 91.5:8.5 MLVs at 47 °C which from the related phase diagram corresponds to the pure L^d phase [113]. Therefore, PSM-d31 and PCer-d31 in 10:7:3:3 MLVs at 50 °C and higher temperatures are in a homogenous L^d phase.

In Figure 5-10, the dePaked spectra of POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:10:3 MLVs and their calculated smoothed order parameter profiles at 50 °C are shown. The quadrupolar splittings of the two sets of spectra are very similar at all temperatures shown. At 50 °C, C3-C12 as well as C16 experience the same order, but C13-C15 carbons are slightly more ordered in MLVs containing PSM-d31. The order parameters in Figure 5-10 are comparable to the order parameter profiles of PSM-d31:Chol 77.5:22.5 MLVs at 47 °C, which was reported to be in the in L_o+L^d phase coexistence region of the PSM-d31:Chol phase diagram [113].
Figure 5-10  Pairwise comparison of the dePaked spectra of PSM-d31 and PCer-d31 in 10:7:10:3 MLVs.
Top: DePaked spectra for PSM-d31 in MLVs of POPC:PSM-d31:Chol:PCer and PCer-d31 in POPC:PSM:Chol:PCer-d31 10:7:10:3 at the indicated temperatures. The arrows represent the position of one of the C2 deuterons (C2-d1). The text related to each arrow, i.e. C2, is color coded as the same as the spectra.
Bottom: The smoothed order parameter profiles of the same MLVs at 50 °C.

On the other hand, they are comparable to the order parameter profiles of POPC-d31:Chol 55:45 MLVs at 30 °C reported in [119], which based on the later reported phase diagram of POPC-d31:Chol MLVs is in pure L₀ phase [120]. Also, the order parameter
profiles reported for bovine-brain-SM:Chol:Palmitic-Acid-d31 1:1:1 MLVs at 50 °C and pH=5.2 are comparable to that of presented in Figure 5-10 [121]. Therefore, there is a balance of evidence from the literature that indicates that the order parameter profiles of both 10:7:10:3 MLVs at 50 °C are consistent with typical L₀ spectra. This is in agreement with our line shape and M₁ analyses which indicated that first, the spectral width is comparable to pure L₀ spectra, and second, the absence of a transition in the temperature dependence of M₁. Therefore, we suggest that the phase of both 10:7:10:3 MLVs is pure L₀.

5.2. Discussion

We have continued our investigations on the effect of presence of high ceramide concentrations in model membranes. Here we added a low-melting PC to the SL containing MLVs to study a lipid composition that approximately resembles the lipid content of the outer leaflet of plasma membranes during apoptosis. To this end, first, POPC was added so that its level was equal to the SL concentration of the 7:3:3 ternary MLVs studied in the previous chapter, i.e. POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. Second, equimolar MLVs containing POPC, Chol and SLs were investigated using ²H NMR.

We have found that ceramide enriched S₀ phase domains at temperatures ≤ 40 °C, are still present in POPC:PSM-d31:Chol:PCer and POPC:PSM:Chol:PCer-d31 10:7:3:3 MLVs. Similar to what was observed in the ternary MLVs, the amount of S₀ phase is significantly higher in MLVs containing ceramide, and S₀ phase was more prominent when deuterated ceramide was used as the probe.

The M₁ analyses shown in Figure 5-6 suggest that this effect is more pronounced in the temperature range 21-28 °C. This shows that even in the presence of a significant amount of a low-melting temperature lipid (44 mol% POPC), the thermal energy of PCer-d31 cannot overcome the chain motional restriction imposed by the small head group area. The S₀ phases observed in 10:7:3:3 MLVs containing either PSM-d31 or PCer-d31 coexist with the Lₐ phase. The S₀ phase in both 10:7:3:3 MLVs is not completely melted until ~ 45 °C, indicating the stability of the S₀ phase even at physiological temperatures.
At 50 °C and above, both PSM-d31 and PCer-d31 in 10:7:3:3 MLVs are in the \( L_d \) phase. This is due to the presence of a high level, 44 mol%, of a lipid with one unsaturated chain (POPC) in the 10:7:3:3 MLVs. In addition, the dilution of Chol is a possible explanation for the lack of a \( L_o \) spectrum signature in 10:7:3:3 MLVs.

In the previous chapter, it was found that in the presence of 30 mol% Chol, both PSM-d31 and DPPC-d31 MLVs are mainly in the \( L_o \) phase, with possible \( S_o \) phase coexistence at low temperatures (below 27 °C). Here we find that in the presence of 33 mol% Chol, in 10:7:3:3 MLVs containing either PSM-d31 or PCer-d31, the \( L_o \) phase is the only observed phase over the temperature range 21-70 °C. No signatures of \( S_o \) and \( L_d \) phase were observed in any of our analyses in the high Chol content quaternary MLVs. Next, the results presented in this chapter will be compared to some other published data on membranes with lipid mixtures relevant to the ones presented here.

The detailed phase diagram of POPC:PSM:Chol has been determined by three research groups [122]–[124]. The phase diagrams presented in references [122] and [123] were obtained from fluorescent microscopy in 2003 and 2005 respectively. In order for lipid domains to be detected by optical microscopy, their sizes need to be in the range of \( \sim 300-500 \) nm [125], [126]. Also, later in 2006, it was found that the strong illumination in fluorescence microscopy induces artefacts that result in domain formation due to the presence of fluorophores [127]. Thus the accuracy of the original phase diagrams (in references [122] and [123]) has been questioned. In 2012, the phase diagram presented in reference [124] was obtained with spin label Electron Paramagnetic Resonance (EPR) in much more detail compared to [122] and [123]. More than 200 distinct compositions were studied using spin label EPR (in reference [124]) as opposed to fewer than 40 distinct compositions shown on the phase diagram in references [122] and [123]. In this technique, a nitroxide ring is incorporated into the acyl chain of PC lipids (not POPC) as the probe, and domain sizes greater than 1-2 nm can be detected [58], [128], [129].

The purpose of the study using spin label EPR was, first, to shine a light on discrepancies between the phase diagrams presented earlier in [122] and [123]. For example, in reference [123], at higher mol fractions of POPC, a uniform liquid phase was reported, while in reference [122], \( L_o + L_d \) phase coexistence was observed in these regions of the phase diagram. Moreover, at higher PSM concentrations, liquid-liquid phase coexistence was reported in reference [123]. In the same high PSM concentration regions,
pure \( L_0 \) or \( L_0+S_0 \) was observed in reference [122]. Second, EPR is more sensitive to lipid domain size, and utilizes a probe that has less influence on lipid packing than the probes used for microscopy. Therefore, for the purpose of comparing our results to the phase diagram of POPC:PSM:Chol, we picked the phase diagram obtained via spin label EPR, keeping in mind that the EPR probe can perturb chain mobility even at membrane concentrations as low as \( \sim 0.5 \) mol% [124], [129].

The phase diagram of POPC:PSM:Chol bilayers obtained from spin label EPR is shown in Figure 5-11 [124]. To compare our results to this phase diagram [124], we must decide how to treat the fourth lipid, PCer. We first calculated our POPC:PSM:Chol ratios neglecting PCer. Thus, POPC:PSM:Chol:PCer 10:7:3:3 and 10:7:10:3 become POPC:PSM:Chol 10:7:3 (\( \equiv 50:35:15 \)) and 10:7:10 (\( \equiv 37:26:37 \)), respectively. These points are marked in Figure 5-11 as blue and yellow squares. On the phase diagram, \( L_0+L_d \) phase coexistence was observed for POPC:PSM:Chol 10:7:3 at 23 °C, with approximately 33% \( L_0 \) and 67% \( L_d \). Our analysis shows that POPC:PSM:Chol:PCer 10:7:3:3 is in the \( S_o+L_d \) phase coexistence region with PCer-d31 being mainly in the \( S_o \) phase and PSM-d31 being in the \( L_d \) phase. For the POPC:PSM:Chol 10:7:10, only the \( L_0 \) phase was reported, in agreement with our observations.

Next, PCer and PSM were treated as a single entity, PSM, and the POPC:PSM:Chol ratios were recalculated. That is, POPC:PSM:Chol:PCer 10:7:3:3 and 10:7:10:3 become POPC:PSM:Chol 10:10:3 (\( \equiv 43.5:43.5:13 \)) and 10:10:10 (\( \equiv 33.3:33.3:33.3 \)) respectively. These points are plotted as red and green squares in Figure 5-11. This way, we can test whether or not the presence of more SLs in the bilayers causes the previous points (blue and yellow squares) to shift toward the \( S_0, L_0 \) or \( L_d \) region of the phase diagram. The red square in Figure 5-11 represents the result of this test for POPC:PSM:Chol 10:10:3. Compared with the blue square, the red square is shifted towards the \( S_0 \) region of the phase diagram, and lies very close to the boundary between the \( L_0+L_d \) and the \( S_0+L_0+L_d \) phase coexistence regions. Given that we treated PCer as PSM and, as the affinity of PCer to induce \( S_0 \) and \( L_d \) phases is known to be high, it is very likely that if PCer was present in the bilayers tested in [124], the red square would have been shifted to the \( S_0+L_d \) coexistence region. Therefore, the shift toward the \( S_0+L_d \) region of the phase diagram as the SL level increases is in very good agreement with the \( S_0+L_d \) phase coexistence observed in our experiments for POPC:PSM:Chol:PCer 10:7:3:3 at room temperature.
Figure 5-11  The phase diagram of POPC:PSM:Chol mixtures derived from spin label EPR. The colored squares are approximately the locations of the compositions relevant to those of the present work. They represent the following compositions; blue: POPC:PSM:Chol 10:7:3 (50:35:15), red: POPC:PSM:Chol 10:10:3 (43.5:43.5:13), yellow: POPC:PSM:Chol 10:7:10 (37:26:37) and green: POPC:PSM:Chol 10:10:10 (33.3:33.3:33.3). Reprinted from [124] with permission from Elsevier.

From the phase diagram in Figure 5-11, the suggested phase for the bilayers containing ≥ 30 mol% Chol is pure L₀. Notably, the green square in Figure 5-11 which represents equimolar POPC:PSM:Chol bilayers, is in the pure L₀ phase. This is again in complete agreement with the observed L₀ phase for POPC:PSM:Chol:PCer 10:7:10:3 MLVs in the present work.
Quaternary mixtures of POPC:PSM:Chol:PCer have also been studied using fluorescence spectroscopy, confocal and two-photon microscopy [130]. In that study, GUVs containing six distinct compositions of POPC:PSM:Chol:PCer - 80:13:0:7, 72:12:5:11, 60:13:14:13, 45:14:25:16, 34:14:33:19 and 25:14:40:21 - were examined with a multi-probe approach. Three examples of the results presented in [130] are discussed first, and then a comparison to the data presented in this thesis follows. First, confocal fluorescence microscopy of quaternary mixtures of POPC:PSM:Chol:PCer 80:13:0:7, 72:12:5:11, 60:13:14:13, 34:14:33:19 labeled with 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benoxa-diazol-4yl) (NBD-DPPE) and N-rhodamine-dipalmitoyl-phosphatidylethanolamine (Rho-DOPE) are shown in Figure 5-12. It is known that NBD-DPPE partitions into L_o phase domains while Rho-DOPE does not partition to these domains, but rather favors L_d phase domains [63]. Neither probe can partition into S_o phase domains, and hence the dark domains in Figure 5-12 represent the S_o phase. In the absence (0% Chol) or in the presence of an intermediate amount of Chol (5% or 14%), the phases of the GUVs shown in Figure 5-12A-C are S_o+L_d. In high Chol content GUVs, such as the case in Figure 5-12D, three distinct phases were observed. The red domain is in the L_d phase, green areas represent the L_o phase and the dark regions within the L_o phase represent the S_o phase.
As another example from the studies presented in [130], data from two-photon fluorescence microscopy experiments with GUVs labelled with Laurdan are shown in Figure 5-13. The results of the Laurdan GP analysis show that $S_\alpha + L_d$ phase coexistence exists below 15 mol% Chol (Figure 5-13A-C). Also, $S_\alpha + L_\alpha$ phase coexistence was observed in the presence of 33 mol% Chol, as illustrated in Figure 5-13D. No $L_d$ phase was observed in GUVs containing 33 mol% Chol when Laurdan was used as the probe. Hence, it seems the results obtained using Laurdan contradict those obtained using combined Rho-DOPE and NBD-DPPE in high Chol content GUVs. The authors explained this as being the result of strong photoselection by Laurdan when placed in highly ordered domains [130]. Laurdan is generally known as a probe that is equally soluble in different lipid phases [131]. However, the authors of reference [130] found that the phase behaviour of a variety of fluorescent probes, including Laurdan, depends not only on the type of phase but also on the composition of the phase. This implies that these probes treat, for instance, the $S_\alpha$ phase formed by pure DPPC GUVs differently from the $S_\alpha$ phase formed by PCer in the variety of binary, ternary and quaternary lipid mixtures that were studied.
Therefore, care must be taken when attempting to generalize the results obtained from fluorescence microscopy and spectroscopy experiments.

As a third example of the results presented in [130] that are pertinent to our work, Trans-parinaric (acid t-PnA) was incorporated into the previously mentioned six quaternary GUVs in order to monitor the intensity decay of the probe in time-resolved fluorescence measurements [130]. The fluorescence decay was then modelled as a sum of exponential decay functions and the lifetime associated with each component was extracted [130]. A long lifetime component found in the quaternary MLVs of POPC:PSM:Chol:P Cer 80:13:0:7, 72:12:5:11, 60:13:14:13, 45:14:25:16, 34:14:33:19 and 25:14:40:21 was not found in the lipid mixtures lacking PCer. This long lifetime component has also been observed in other studies [41], [63], and was attributed to PCer-enriched, highly ordered S	extsubscript{0} domains. In Figure 5-14, decay time of the long lifetime component is plotted as a function of the Chol/PCer ratio in the previously mentioned quaternary MLVs. In Figure 5-14, there is a peak close to Chol:PCer 1:1, but as the Chol:PCer ratio increases the lifetime of this component becomes significantly shorter. This is due to the

$\text{Figure 5-14} \quad \text{Decay time of the long lifetime component derived from time-resolved fluorescence measurement. Measurements were carried out on the quaternary MLVs of POPC:PSM:Chol:P Cer 80:13:0:7, 72:12:5:11, 60:13:14:13, 45:14:25:16, 34:14:33:19 and 25:14:40:21 labeled with t-PnA [130]. The x-axis is the ratio of Chol to PCer in the MLVs. Reprinted with permission from the publisher.}$
solubilization of the PCer-driven $S_0$ domains in the Chol driven $L_0$ domains in the membrane.

There is generally good agreement between our results and those reported in reference [130]. Close to room temperature, our low Chol containing quaternary MLVs, POPC:PSM-d31:Chol:PCer and POPC:PSM-d31:Chol:PCer 10:7:3:3, showed $L_d+S_0$ phase coexistence in agreement with the results of both the combined NBD-DPPE/Rho-DOPE and Laurdan fluorescence microscopy. In our high Chol content MLVs, POPC:PSM-d31:Chol:PCer and POPC:PSM-d31:Chol:PCer 10:7:3:3, the only observed phase was $L_0$. On the other hand, combined NBD-DPPE/Rho-DOPE fluorescence microscopy showed three phase coexistence, $S_0+L_0+L_d$, while Laurdan fluorescence microscopy showed $S_0+L_0$ phase coexistence. However, in both of the later cases, the $L_0$ phase is the predominant GUV phase. The PSM/PCer ratio in the high Chol content GUVs (33 mol%) studied using fluorescence microscopy is 0.74, while in our high Chol content MLVs this ratio is 2.33. A few points need to be considered when comparing our high Chol content MLV results with those containing 33 mol% Chol discussed in [130]. First, the presence of a higher level of PCer in [130] could induce domains in the $S_0$ phase, which might explains the observation of such domains in Figure 5-12D and Figure 5-13D. Second, POPC is found to be excluded from the $S_0$ phase enriched in PCer [43], so the $L_d$ domains evident in Figure 5-12 are likely rich in POPC and poor in PCer. We suggest that it is mainly the composition difference between our high Chol content MLVs and those studied in [130] that results in appearance of different phases ($S_0+L_0+L_d$ coexistence in [130] when GUVs labelled with NBD-DPPE/Rho-DOPE are used and pure $L_0$ when $^2$H NMR is used as in the present work). It should also be noted that in our study, only the phase behavior of PSM and PCer in the POPC:PSM:Chol:PCer MLVs were monitored using perdeuterated palmitoyl chains. To make a detailed comparison with the results presented in [130], information about the phase behavior of POPC and Chol would be needed from further $^2$H NMR experiments on POPC:PSM:Chol:PCer MLVs. Using that information, discrepancies between the combined NBD-DPPE/Rho-DOPE and Laurdan fluorescence microscopy results might be resolved. Currently, such experiments are undergoing in our laboratory.

The results from the decay of the long lifetime component of $l$-PnA shown in Figure 5-14 is in very good agreement with our results from ternary (previous chapter) and quaternary (this chapter) MLVs. Similar to what is observed in Figure 5-14, our quaternary
MLVs results suggest that in the presence of comparable amounts of Chol and PCer, in POPC:Chol:PSM-d31:PCer and POPC:Chol:PSM:PCer-d31 10:7:3:3, domains in the S₀ phase form especially in PCer-d31 containing MLVs. On the other hand, in POPC:Chol:PSM-d31:PCer and POPC:Chol:PSM:PCer-d31 10:7:10:3, where the Chol:PCer ratio is 3.33, S₀ phase domains dissolve in Chol driven L₀ domains. Generally, these observations again point to the idea of a possible critical balance between ceramides and Chol in cell membranes playing a role in the regulation of membrane fluidity as first proposed by the authors of [130] and then further investigated by other research groups (see [29] for a review).

Finally, as shown in Figure 5-15. AFM and fluorescence correlation spectroscopy (FCS) on quaternary mixtures containing stearoyl SM (SSM) and stearoyl Cer (SCer) in DOPC:SSM:Chol:SCer SPBs at ~23 °C show highly ordered domains enriched in SCer [132]. In SPBs containing DOPC:SSM:Chol:SCer 1:0.88:1:0.12 (4% SCer) Chol driven L₀ domains were observed in coexistence with the L₀ phase using both techniques. Upon increasing SCer and decreasing Chol levels, in DOPC:SSM:Chol:SCer 1:0.64:1:0.36 (12% SCer), a shrinkage of those domains was observed which was interpreted as
formation of SCer driven $S_0$ domains. These observations are in agreement with those presented in our work. In MLVs containing POPC:PSM-d31:Chol:PCer and POPC:PSM-d31:Chol:PCer 10:7:3:3 we observed highly ordered PCer driven domains in the $S_0$ phase similar to those observed for DOPC:SSM:Chol:SCer 1:0.64:1:0.36 SPBs in [132]. These domains are dissolved due to an increase in Chol content in POPC:PSM-d31:Chol:PCer as well as POPC:PSM-d31:Chol:PCer 10:7:10:3 MLVs and SPBs containing DOPC:SSM:Chol:SCer 1:0.88:1:0.12. Note that in AFM and FCS studies, the authors of [132] used a non-natural unsaturated lipid, DOPC, in their SSB preparations. DOPC, in comparison to POPC, which is a natural lipid, has one more unsaturated acyl chain and as a result favours the $L_d$ phase even more than POPC. This could be the reason why the extent of the $L_d$ phase in observed [132], is higher than what we observed in quaternary MLVs.
Chapter 6.

Conclusion, Biological Implications and Suggestions for Future Experiments

Here we have investigated the physical behavior of model membranes containing two major lipids involved in apoptosis. The sphingolipid, PSM, and PCer were studied using $^2$H NMR in ternary MLVs containing Chol, and also in quaternary MLVs containing POPC and Chol. For the ternary MLVs, the lipid molar ratios were initially chosen so that bilayers lacking PCer were formed and studied, and then the effect of the incremental addition of PCer to the membrane was examined. In quaternary MLVs, the effect of adding POPC was investigated in two stages, first with low Chol content and second with high Chol content bilayers. Also, we tried to shine a light on the question of why nature chose saturated sphingolipids over saturated glycerophospholipids in the plasma membrane, even though DPPC shares a very similar molecular structure and main transition temperature (i.e. physical behavior) with PSM. To this end, the effect of replacing PSM (or PSM-d31) with DPPC (or DPPC-d31) in all ternary MLVs was studied as well.

PCer induces formation of $S_0$ phase domains in ternary MLVs containing PSM and Chol, and also in quaternary MLVs containing POPC, PSM and Chol at temperatures below 40 °C. The observed $S_0$ domains are enriched in PCer while most of the PSM, depending on the Chol level, remains either in the $L_o$ phase or in the $L_d$ phase. This was proved by placing the probe, i.e. the perdeuterated palmitoyl chain, on either sphingolipid in two different MLV preparations having the same molar ratios of lipids, and performing $^2$H NMR spectroscopy. Generally, the results of our experiments are in agreement with what has been indirectly observed in similar lipid mixtures using other biophysical probes such as AFM, DSC, and fluorescence microscopy [65], [80], [81], [132], [133]. While the presence of ceramide-enriched $S_0$ domains was implied in these previous studies, here we have proven that whenever $S_0$ domains in the ternary or quaternary MLVs studied in this theses are observed, they are enriched in PCer. Based on these observations, we propose that, near physiological temperature, $S_0$ domains in SM and POPC membranes form in the presence of Chol only when the concentration of PCer is comparable to that of Chol. For example, we have shown that in MLVs containing PSM:Chol:PCer 7:3:1 and POPC:PSM:Chol:PCer 10:7:10:3, where the Chol:PCer ratio is 3 and 3.33 respectively,
Chol driven $L_0$ domains solubilize the PCer induced $S_0$ domains. On the other hand, we have shown that in MLVs containing PSM:Chol:PCer 7:3:2 and 7:3:3 and also in POPC:PSM:Chol:PCer 10:7:3:3, where the Chol/PCer ratio is 1.5 or 1, PCer is able to form $S_0$ domains.

One important question that could be addressed in future studies is whether, in the presence of ceramide, Chol partitions into both $L_0$ and $S_0$ phases or is concentrated mainly in the $L_0$ phase. Selectively labeled deuterated Chol, like Chol-d1 [134], could be used to determine the phase of Chol in MLVs. A second important question to pursue is the effect of increasing PCer levels in quaternary MLVs above those studied in our work, i.e. POPC:PSM:Chol:PCer 10:6:10:4, 10:5:10:5, etc. Notably, it is interesting to investigate whether Cer-induced $S_0$ domains form when the SL content of the MLVs is only PCer (POPC:Chol:PCer 1:1:1 MLVs). Determining the phase of POPC in the quaternary MLVs would be the next step of this research. Given the discrepancies reported in [130] and previously mentioned in Chapter 5, regarding the phase of POPC:PSM:Chol:PCer 34:14:33:19 GUVs inferred using Laurdan on the one hand, and Rho-DOPE and NBD-DPPE on the other hand, $^2$H NMR experiments can unambiguously determine the phase of each lipid in MLVs containing the same ratios of the reported GUVs.

Turning to the question of why nature chose saturated SLs over DPPC, our observations show that the ordering effect of an increase in PCer level in DPPC:Chol and PSM:Chol MLVs is temperature dependent. Comparing the extent of ordering, at 35 °C and below, we observed that ceramide is much more capable of inducing $S_0$ phase domains in MLVs containing PSM (or PSM-d31). From 35-50 °C, however, no significant differences were found upon increasing in ceramide content in MLVs containing either PSM (or PSM-d31) or DPPC (or DPPC-d31). We speculate that at low temperatures the interaction between PSM and PCer is stronger than that between DPPC and PCer so that cholesterol is excluded from PSM:PCer domains to a greater extent, allowing a higher proportion of $S_0$ phase to form. Presumably, above 35 °C, thermal motion negates the increased stability of the PSM:PCer interaction. It cannot be determined here whether the behaviour below 35 °C or between 35-50 °C is more biologically relevant. Therefore, the reason why nature chose saturated SL over saturated PC in biological membranes remains elusive.
The potential of Chol and Cer, together, to affect the lipid packing in membranes could have significant biological importance. Cells could utilize this potential by maintaining a critical balance between the local membrane concentrations of Cer and Chol to regulate membrane fluidity in signaling processes such as apoptosis. From a cancer therapeutic perspective, it has been reported that some cancer cells like breast cancer and prostate cancer cell lines contain higher levels of Chol than those of their healthy counterparts [135]. These tumor cells are highly prone to cell death upon Chol depletion by methyl-β-cyclodextrin (MβCD). Also, it has been shown that in several cancer cell lines, like Glioma, an increase in plasma membrane Cer significantly increases the sensitivity of tumor cells to chemotherapy [136], [137]. These reports integrated with our observations suggest that the Chol/Cer ratio in cell membranes is a determining factor in cell death and survival and that decreasing this ratio could be a good target in cancer therapy.
References


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