To my mother, and father
Cyra B. and Robert V. Mair
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This work is focused on determining the effect BMP has upon model membrane lipid morphology, and acyl chain dynamics, using magnetic resonance spectroscopy, in order to further understand BMP’s role in lipid catabolism and lysosomal storage disease.
CHAPTER 1
INTRODUCTION

Lipid Description and Classification

In general, lipids can be described as molecules that are composed of mainly hydrogen and carbon atoms, they can be as simple as alkanes or as complex as lipopolysaccharides(1). Lipid molecules isolated from biological sources are generally classified as either neutral storage lipids or zwitterionic/charged membrane lipids (Figure 1-1)(1-3). The chemical composition of storage lipids (triacylglycerides or triacylglycerols) includes a glycerol backbone with three fatty acid molecules linked via ester bonds to three reactive hydroxide groups on the glycerol backbone(3). Membrane lipid chemical structures contain either a glycerol or a sphingosine backbone with fatty acids linked to two of the reactive groups of the glycerol or to the reactive amine group of the sphingosine (Figure 1-2). The other reactive hydroxide moiety on each backbone is linked to either a saccharide or more commonly, linked to a phosphate group.

Most biologically relevant membrane lipids are amphipathic in nature, having both a polar and an apolar region(1-3). The polar portion of a lipid is usually referred to as the polar head group region, which is defined as the volume containing the substituted phosphate/saccharide, the glycerol or the sphingosine backbone, and the alpha carbons of the carboxylic acid chains. The apolar portion is defined as the acyl chain tail region(1, 2). Usually, the apolar tails are long hydrocarbon chains covalently bound via ester bonds to glycerol or amide bonds to sphingosine backbone moieties (Figure 1-2)(1, 2). According to Yeagle, a “long chain” is defined as a hydrocarbon chain of 12 or more carbon atoms(1).
**Typical Membrane Lipid Structure**

The most abundant eukaryotic membrane lipids are glycerophospholipids(1, 2), named for the glycerol backbone and the phosphate group. These particular lipids are derivatives of sn-glycero-3-phosphoric acid, in which the sn1 and sn2 positions commonly contain esterified, long chain, fatty acids(2). The stereospecific numbering (sn) of phospholipids is derived by drawing a Fischer projection of glycerol with the β-hydroxyl group on the left, the sn1 position is then located at the top of this projection while the sn3 position is located at the bottom. Typically, in lipids isolated from natural mammalian sources, the acyl chain located in the sn1 position is saturated and the acyl chain in the sn2 position contains at least one site of unsaturation or double bond(1). Chemical moieties attached to the phosphate, located at the sn3 position of membrane lipids, are referred to as head groups, the chemical identities of which give rise to the diverse functionality found in biologically important lipids. For example, in glycerophospholipids, the phosphate group has a negative charge, thus the chemical structure/identity of the head group imparts the characteristic charge or zwitterionic character, apparent size of the head group region, and propensity to hydrogen bond to other membrane lipid molecules. These head group moieties include, but are not limited to, glycerol, inositol, ethanolamine, serine, choline, and carbohydrates(1, 2).

**Lipid Self-Assembly and Organization**

Because of their amphphilic character, lipids with polar heads and apolar tails self-assemble according to their solvent environment. In aqueous environments, the entropic hydrophobic effect causes apolar acyl chain regions to organize in such a manner as to minimize the free energy of the system and exclude the maximum number of water molecules from the lipid assembly(1, 2). Several polymorphs/mesomorphs or macroscopic aggregation states are possible for solvated lipids, e.g. lamellar, hexagonal, micellar and inverted micellar (Figure 1-
The specific polymorphism formed can be influenced by many factors including structural features of the lipid, such as chemical composition of the head group region, length and degree of saturation of the acyl chains, and extrinsic parameters such as temperature, pressure, and degree of hydration. Each of these factors provides a means of controlling the packing density (interaction energy) of the individual lipid molecules within an aggregate, which in turn can have dramatic effects upon the physical and morphological properties of the assembly.

Under physiological conditions, the most common type of lipid organization found in living cells is a two-dimensional lamellar or bilayer structure. A bilayer is formed by stacking lipid molecules tail-to-tail bound on either side by polar head groups in a repeating pattern. Although the biologically relevant mesophase/polymer is lamellar, other types of mesophase structures can exist under certain circumstances, e.g. increasing the temperature or changing the hydration state of lamellar assemblies can result in the formation of a hexagonal aggregate structure. Lipids organized in a three-dimensional hexagonal arrangement either surround a cylindrical column of solvent with polar groups or organize with polar head groups facing out toward the bulk solvent phase. Micelles and inverted micelles are organized in a similar fashion as the hexagonal “phases” except the aggregates are spherical in shape. The specific assembly formed is influenced by the solvent environment and the apparent molecular shape of the lipids.

Molecules that occupy a molecular cross-sectional area resembling a cone, inverted cone, or cylinder are predicted to form hexagonal, micellar, or lamellar aggregates, respectively. A simple geometric model based on relative cross-sectional areas of head groups and acyl chains can be used to predict aggregation states for hydrated lipid molecules.
Lipid molecules with a head group cross-sectional area greater than the cross-sectional area of their acyl chains self-assemble into a micellar type structure. Those with head group area approximately equal to the acyl chains area prefer to assemble into a lamellar type structure, while lipids with head group area less than the area of the acyl chains assemble into a hexagonal type structure. An illustration of the geometric shapes and representative lipids of each category can be found in Figure 1-4. Sodium dodecyl sulfate (SDS) has a head group with a much larger cross-sectional area than its single acyl chain, thus hydrated SDS molecules will polymorph into micellar aggregates. A different situation arises for 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). The phosphocholine head group area and the cross-sectional area of the two palmitoyl chains are similar; therefore, DPPC is predicted to form a lamellar aggregate in an aqueous environment. Phosphatidic acid (PA) in an acidic environment has a small head group area with respect to the area occupied by the two acyl chains and forms a H_{II} aggregate.

Two broad categories of “phase” transitions can be defined for lipid polymorphs or aggregates: 1) those that result from changes in organization and packing of the acyl chains and mean volume occupied per lipid molecule, and 2) those that alter the mesophase or polymorphic structure of the lipid aggregate(1, 4). Aggregation state, or polymorphic transitions, can be altered in various ways including partitioning of a variety of molecules into the assembly, changing temperature, degree of hydration and lipid composition of the assembly, and varying ionic strength or pH of the solvent(1, 2, 4, 6), or via changes in extrinsic variables such as temperature and pressure. However, in biological systems, the pressure can often be assumed to remain constant; hence, most studies of membrane biophysics focus on the effects of changes in temperature, not pressure. A common phase transition in lamellar mesophases exploited within the membrane biophysics field is the lamellar gel to lamellar liquid transition(1, 2, 4). This
transition is illustrated in Figure 1-3. The gel phase (Figure 1-3 A) is characterized by closely packed molecules and a more extended acyl chain conformation. Whereas the liquid state (Figure 1-3 B) retains a two-dimensional order but lipid molecules are diffusing axially and the acyl chain region is less ordered (high probability of trans/gauche isomerization) when compared to the gel phase(4, 7). Factors such as solvent ionic strength, degree of acyl chain saturation, lipid composition (mixtures of lipid molecules) and addition of protein/peptides perturbants can all affect the temperature at which this thermotropic phase transition occurs(1, 2, 4). For example, at temperatures below the main transition temperature ($T_m$), fully hydrated DPPC exists in either a lamellar gel state with saturated acyl chains extended and highly ordered (very few gauche rotamers), or in a ripple phase ($P_{\beta'}$) with lipid molecules slightly tilted with respect to the bilayer normal(8) resulting in a decrease in bilayer thickness with respect to the lamellar gel state. The ripple phase is characterized by tilted and extended acyl chains that appear to have a symmetric rotational axis (defined in Chapter 3), thus the lipids are more disordered with respect to the gel phase but more ordered than the liquid lamellar phase(1). However, at temperatures above $T_m$ the chains become less extended, thus reducing the bilayer thickness to a greater extent, making the chains more disordered until a pure liquid crystalline phase ($L_{\alpha}$) is present. Also, fully hydrated, non-bilayer structures, e.g. $H_{\Pi}$, can be induced by further increasing the temperature(4, 9). An example of a lipid exhibiting a transition to the $H_{\Pi}$ state from either the lamellar liquid or lamellar gel state is phosphatidylethanolamine (PE)(4, 9).

**Characterization of Model Membrane Structural Properties**

X-ray diffraction, neutron scattering, solid state nuclear magnetic resonance (SSNMR), and electron paramagnetic resonance (EPR) are techniques commonly used to investigate the structure and dynamical properties of lipid assemblies(2, 10, 11). Although diffraction and
scattering methods provide a detailed description of the thermodynamic phase structure of lipid assemblies and bilayer thicknesses, these techniques measure only a static macroscopic structure with little information regarding molecular motion and require a periodic lattice (2, 12). A periodic lattice or an array of a large number of molecules arranged in a periodic structure typically occurs in very pure systems. However, biological membranes are inherently heterogeneous; thus, diffraction techniques are limited to purified systems in which a well organized lattice structure has been induced. In some cases, diffraction techniques can be used to measure the macroscopic structure in less organized systems but they lack the resolution obtainable by magnetic resonance and other spectroscopic techniques.

Investigations utilizing NMR and/or EPR provide detailed molecular level information regarding the degree of order at individual chemical bonds for particular molecules (10, 11, 13-16). The types of order/disorder commonly investigated using these spectroscopic techniques include a motionally averaged picture of phospholipid head group and acyl chain angular orientation with respect to the bilayer normal (10, 16, 17). Because all phospholipids (by definition) contain a phosphate group, the phosphorus atom provides a natural probe for $^{31}$P NMR investigation of average head group orientation. Advantages of this technique include 100% natural abundance of the $^{31}$P isotope and a good sensitivity due to a relatively large gyromagnetic ratio ($\gamma$ is about 40% of the $^1$H nucleus). It has been shown that solid-state $^{31}$P NMR chemical shift line shapes can be used to differentiate between lamellar, hexagonal ($H_{II}$), and isotropic polymorphic assemblies (4); however, line shape can only be consistent with a particular mesomorphic state. X-ray or cryogenic electron microscopy measurements are needed to fully confirm the aggregate morphology.
The degree of acyl chain organization of the bilayer interior can be investigated either by ²H NMR studies of lipids containing deuterated acyl chains or by EPR studies of lipid dispersions containing 0.5 – 1 mol% of a lipid labeled with a paramagnetic nitroxide spin- probe (Figure 1-5)(10, 11, 14, 18). A major disadvantage for both EPR and ²H NMR measurements is the need for a synthetic probe. Additionally, ²H has a γ value that is only 15% of the ¹H nucleus and requires large samples compared to ³¹P or EPR sample sizes due to its relatively low sensitivity. Despite these constraints, magnetic resonance techniques are invaluable for biophysical investigations of heterogeneous, non-periodic model and biological membranes. Thus, they are the primary tools employed in our pursuit to describe the conformation and acyl chain order of mixed lipids in a model membrane system.

**Bis(monoacylglycerol)phosphate**

Since first isolation from ovine lung homogenate in 1967, bis(monoacylglycero)phosphate (BMP) (Figure 1-6), also known as lysobisphosphatidic acid (LBPA), has been found to represent less than 1% of total phospholipid mass in most tissue and cell types(19, 20). Although a majority of mammalian cells contain a small amount of BMP, its concentration in late endosomes (LE) is elevated to near 15% of the total lipid content (21-23) and up to 70% of the total lipid content of internal membrane domains within the LE(23).

Endosomes are intercellular organelles that act as a staging area for sorting endocytosed material either back to the plasma membrane for recycling or to specialized organelles (lysosomes) for degradation(20, 24). Endosomes can be identified either by the presence of internal membranes, also known as multivesicular structures, inside the lumen of a limiting (boundary) membrane or by lipid composition(22, 25). Early endosomes have a limiting membrane with a lipid composition very similar to that of the plasma membrane, whereas late
endosomes are characterized by an absence of a significant amount of cholesterol and a relatively high concentration of BMP. In certain situations, multilamellar internal membrane structures (multivesicular bodies or MVBs) are present in late endosomes. A question that naturally arises is: “Do elevated BMP levels in these cellular structures play a significant role in controlling membrane organization?”

BMP is negatively charged and has an atypical sn-1-glycerophospho-sn-1’-glycerol (sn1:sn1’) stereoconfiguration with respect to sn-3-glycerophosphate structures exhibited by most other glycerophospholipids. This unique structure and negative charge is likely to have functional implications beyond its ability to resist degradation by most phospholipases due to their stereospecific recognition of the sn-3 stereoconfiguration.

Even though the sn1:sn1’ and sn3:sn1’ stereoisomers are different, similar thermotropic phase transition temperatures were measured by differential scanning calorimetry (DSC) for 1,3’ dimyristoyl sn1:sn1’ (40°C) and 1,3’ dimyristoyl sn3:sn1’ (42°C) BMP structural isomers, justifying the use of the non-natural stereoisoform as a first approximation for characterization purposes. However, it has been reported that sn-(3-hydroxyl-2-oleoyl)glycerol-1-phospho-sn-1’-(3’-hydroxy-2’-oleoyl)glycerol may be the biologically relevant isoform. It has also been shown by gas chromatography and mass spectrometry that the major fatty acid components of BMP, isolated from baby hamster kidney (BHK) cells, are oleic acids (91%).

Three structural isoforms are shown in Figure 1-6; the synthetic (S,R or 1,3’ diacyl sn3, sn1’) molecule was used in this work due to its commercial availability and is designated throughout this dissertation as either BMP, depending on the fatty acid substituents, or collectively BMP. Because each acyl chain of BMP contains an unsaturated site, this lipid may increase the overall bilayer disorder of model and endosomal membranes due to
intermolecular packing constraints(29). Increased lipid disorder is most likely not the only role BMP plays in modulating endosomal membrane morphology because other negatively charged lipids containing unsaturated chains, such as phosphatidylinositol (PI), would most likely have a similar effect on lipid order as BMP and thus affect a specific hydrolysis reaction in a similar manner. This has been shown, but only at concentrations of PI much greater than that found in vivo(30). Therefore, BMP’s unique geometric structure must also be an important factor in controlling endosomal morphology and molecular trafficking in late endosomal organelles.

According to literature, the geometric shape predicted for BMP is either a cone or inverted cone(25, 26, 29, 31, 32). If the former were true, BMP would most likely exist in an H_{II} aggregate or may induce H_{II} aggregation as the relative BMP concentration is increased in model or biological membranes. However, at pH 7.4 BMP_{18:1} forms multilamellar vesicles and lacks any three-dimensional structural changes according to fluorescence emission of pyrene labeled lipids incorporated into BMP multilamellar vesicles (MLVs)(29). Also, studies of sn-(3-hydroxyl-2-oleoyl)glycerol-1-phospho-sn-1’-(3’-hydroxy-2’-oleoyl)glycerol induces multilamellar structure formation, as visualized by fluorescence and cryogenic electron microscopy in the presence of a pH gradient in lipid mixtures having a similar composition to that found in late endosomes(26). Moreover, the small and wide angle X-ray diffraction patterns for both 1,3’ dimyristoyl sn3:sn1’ and 3,3’ dimyristoyl sn1:sn1’ BMP are consistent with a lamellar structure(28). There is not any indication that any isoform of BMP exists in or induces either a H_{II} or micellar structure in the current literature.

The electron microscopy and X-ray diffraction data described above provide a static description of the lipid aggregate macromolecular structure. This dissertation reports results obtained from morphological studies of BMP and BMP mixed with model membrane lipids
using magnetic resonance techniques to investigate the microscopic structure and dynamic structural properties of BMP and BMP mixed with model membrane lipids.

**Bis(monoacylglycerol)phosphate May Be Important to Ganglioside Catabolism**

Gangliosides and glycosphingolipids (GSLs) are components of eukaryotic plasma membranes and are involved in passing cellular signals from outside the cell to the cell interior(2, 33). The degradation of these particular lipid molecules occurs in acidic cellular compartments of lysosomes, specifically on the surface of intraendosomal and intralysosomal vesicular structures (an example of a GSL is shown in Figure 1-7)(20, 34).

A model for endocytosis and GSL digestion, proposed by Sandhoff and Kolter, can be seen in Figure 1-8(20, 34). Vesicles containing GSLs, which are destined for endosomal and lysosomal compartments, begin as either invaginations or clathrin coated pits formed in the plasma membrane(35). These vesicles fuse with early endosomes where some lipids and proteins are shuttled back to the plasma membrane for recycling (36, 37), while others are sorted from the limiting membrane of the early endosome and incorporated into intralysosomal structures(20). Early endosomes mature into late endosomes that transiently fuse with lysosomes where enzymatic digestion occurs(20). During this maturation process, the luminal pH of the endosome decreases and the protein and lipid composition of the intralysosomal structures change (20) becoming enriched in GSLs and BMP and depleted in cholesterol(20, 38).

Glycosphingolipid degradation occurs in the lysosome as a stepwise cleavage of monosaccharide units from the oligosaccharide head group of the GSLs until the recyclable biomolecule sphingosine is produced(20, 33, 34). *In vivo*, several accessory molecules are needed to degrade glycosphingolipids containing head groups of four or fewer sugar molecules: a water-soluble enzyme (the hydrolase), a sphingolipid activator protein (SAP), and possibly a membrane surface including anionic phospholipids like BMP or PI(20, 33, 34). However, it has
been demonstrated that the enzymatic cleavage reaction does occur in vitro with micellar ganglioside substrate in the absence of a membrane surface\cite{30}. SAPs are membrane binding cofactors believed to have variable specificity for both membrane lipids and enzymes\cite{20,34}. These cofactors are required for the enzymatic cleavage and are believed to either extract the ganglioside from the bilayer and present the lipid to the hydrolase for an aqueous reaction or to lift the ganglioside slightly from the bilayer surface for hydrolysis (a membrane associated reaction)\cite{20,33,34}.

Any dysfunction in the SAP, substrate, or hydrolytic enzyme, can lead to a particular form of lysosomal storage disease. There are many diseases associated with storage of lipids in both endosomes and lysosomes. These are classified by the non-degraded lipid or protein accumulated in either the endosome or lysosome\cite{20,33,34}. Subclasses or variants of the storage diseases are characterized by the particular molecule in which the defect occurred. For example, Tay-Sachs, or GM2 gangliosidosis, is caused by mutations of the gene encoding for the enzyme $\beta$-hexosaminidase A; GM1 gangliosidosis and Morquio Type B syndrome are caused by a mutation in the enzyme GM1 $\beta$-galactosidase\cite{20,33}.

It has already been shown that in the presence of BMP or PI in model membranes (POPC/CHOL/GM2/BMP mixed lipid composition) the rate of GM2 hydrolysis by $\beta$-hexosaminidase A in the presence of the sphingolipid activator protein GM2-activator protein (GM2AP) is more than two orders of magnitude faster than in BMP free liposomes\cite{30}. How BMP influences the hydrolysis rate is still unknown, but it is possible that BMP modulates surface charge, membrane order, vesicle size/shape, or any combination of these properties.

The following work presented in this dissertation focuses on model membrane morphological perturbations caused by BMP. It is likely that perturbations such as modulating
membrane surface charge, changing membrane packing parameters, and preferential interaction of BMP with GM2 are crucial for the interaction of the catalytic triad (β-hexosaminidase A, GM2, and GM2AP) to function properly. BMP may, under certain conditions, preferentially sequester various lipid substrates into small vesicular or multilamellar structures required for transportation to the lysosome, which might provide the proper topology needed for efficient enzymatic cleavage. In support of this hypothesis, there is not any evidence for microdomain (raft) formation when BMP is present in POPC bilayers at either acidic or neutral pH(29). Hence, it is likely that BMP may induce vesicle budding events, as opposed to microdomain formation.

**Biological Membranes**

Biological membranes are important cellular structures, because they create selective chemical and physical barriers between cells or organelles and their surroundings. Lipid composition and geometrical packing patterns, along with incorporation of proteins and other molecules into the lipid matrix, such as cholesterol, allow membranes to exhibit diverse mesophases, surface properties, and permeabilities(1, 2, 4, 31, 39). The aforementioned properties play a role in the ability of biological membranes to participate in small molecule, protein and lipid trafficking, membrane fusion, and to act as platforms for catalysis(1, 2, 40-42). Biological membranes also adopt a variety of shapes, for example red blood cell membranes resemble small biconcave discs, lysosomal membranes resemble hollow spheres, the endoplasmic reticulum and the golgi apparatus membranes have very convoluted surfaces. Differences in size and shape of biological membranes are likely to be related to function, and it is known that lipid interactions with proteins and other molecules are often needed to modulate membrane shape(1, 2).
Dissertation Outline

The work presented in this dissertation focuses on the effect of BMP on the morphology of model phosphoglycerol membranes. Magnetic resonance techniques were used to measure phosphate head group orientation with respect to the bilayer normal and the average angular excursion from the bilayer normal of either a nitroxide spin probe or a deuterated acyl chain as a function of temperature and/or BMP concentration. By using both SSNMR ($^2$H) and EPR, we were able to monitor lipid acyl chain dynamics (order/disorder) on two different time scales: slow motional fluctuations (~$10^5$ Hz) and rapid motional fluctuations (~$10^8$ Hz), respectively. $^{31}$P SSNMR measurements of chemical shift anisotropy can be correlated to head group order, mesophase, and changes in phase transition temperature for several lipid aggregates.

After analysis of data collected with each of the previously mentioned techniques, we were able to confirm the lamellar aggregation of pure BMP, and characterize some of the effects on head group and acyl chain order caused by incorporation of BMP into model membranes. General background information about lipids, lipid self-assembly, membrane characterization, and specific information about BMP is found in Chapter 1. Chapter 2 reports the materials and methods utilized throughout and experimental parameters needed to perform our investigations. An overview of basic magnetic resonance applications and expected spectroscopic signals obtained from membrane lipid dispersions are discussed in Chapter 3. The focus of Chapter 4 is the solubilization of model (PC), pure BMP and PC/BMP mixed membranes. Chapters 5 and 6 report results on characterization of overall perturbations of the acyl chain and head group regions of model membranes caused by BMP.

In the field of membrane biophysics, it is strongly desired to understand how proteins and lipid molecules alter the physical shapes of membrane structures within cells. A detailed understanding of how BMP modulates bilayer physical properties, at the molecular level, will
directly impact other research in the Fanucci group that is focusing on characterizing the membrane binding interactions of GM2AP with POPC and POPC:GM2 containing vesicles. Results from this work may also begin to explain why BMP alters the enzymatic rates of GM2 hydrolysis when it is incorporated into lipid vesicles. On a broader scale, the relatively high levels of BMP within the late endosome have been shown using fluorescence microscopy to lead to vesicle budding and multilamellar structure formation(26).

Figure 1-1. Lipid classification. Adapted from Lehninger, Principles of Biochemistry, 4th edition(3).
Figure 1-2. A) Anatomy of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and B) D-erythro-sphingosine-1-phosphate.
Figure 1-3. Cross-sectional representations of lipid polymorphic structures in aqueous environments: A) lamellar gel ($L_{\beta}'$); B) lamellar liquid crystalline ($L_{\alpha}$); C) hexagonal ($H_{II}$); D) hexagonal ($H_{I}$). Adapted from Gruner et al. Ann. Rev. Biophys. Chem. 1985(4).
<table>
<thead>
<tr>
<th>Lipid</th>
<th>Geometric Shape</th>
<th>Associated Mesophase</th>
</tr>
</thead>
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<td>Micellar</td>
</tr>
<tr>
<td>1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC)</td>
<td><img src="image" alt="Lamellar" /></td>
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</tr>
<tr>
<td>Phosphatidic Acid (pH &lt; 3)</td>
<td><img src="image" alt="Hexagonal (HII)" /></td>
<td>Hexagonal (HII)</td>
</tr>
</tbody>
</table>

Figure 1-4. Geometric shape approximations and lipid aggregates. Adapted from Gruner et al., Ann. Rev. Biophys. Chem. 1985(4).
Figure 1-5. Examples of labeled lipids: A) 1-Palmitoyl-2-Stearoyl-(5-DOXYL)-sn-Glycero-3-Phosphocholine; B) 1,2-Dipalmitoyl-D62-sn-Glycero-3-Phosphocholine.
Figure 1-6. BMP structural isomers: A) (S,R isomer) sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol (ammonium salt), (1,3' dioleoyl sn3:sn1’ or BMP18:1); B) (S,S isomer) sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol (ammonium salt) (3,3’ dioleoyl sn1:sn1’); C) (R,R isomer)sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol (ammonium salt) (1,1’ dioleoyl sn3:sn3’).

Figure 1-7. GM1 lipid: GalBeta1-3GalNAcBeta1-4(NeuAcAlpha2-3)GalBeta1-4GlcBeta1-1'-Cer (GM1 ganglioside).
Figure 1-8. Sandhoff-Kolter model for lysosomal membrane digestion and endocytosis; Adapted from Annu. Rev. Cell Dev. Biol., 2005(20).
CHAPTER 2
MATERIALS AND METHODS

Materials

The following lipids and lipid derivatives, dissolved in chloroform, were purchased from Avanti Polar Lipids (Alabaster AL, USA), stored at -20°C and used without further purification. DPPC (1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine), d62-DPPC (1,2-Dipalmitoyl-D62-sn-Glycero-3-Phosphocholine), POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine), d31-POPC (1-Palmitoyl(D31)-2-Oleoyl-sn-Glycero-3-Phosphocholine), BMP18:1 ((S,R Isomer)sn-(3-Oleoyl-2-Hydroxy)-Glycerol-1-Phospho-sn-3'-(1'-Oleoyl-2'-Hydroxy)-Glycerol, Ammonium Salt)), 5-DOXYL (1-Palmitoyl-2-Stearoyl-(5-DOXYL)-sn-Glycero-3-Phosphocholine), 10-DOXYL (1-Palmitoyl-2-Stearoyl-(5-DOXYL)-sn-Glycero-3-Phosphocholine), 16-DOXYL (1-Palmitoyl-2-Stearoyl-(5-DOXYL)-sn-Glycero-3-Phosphocholine). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, C₉H₁₈N₂O₄S), EDTA (ethylenediamine tetraacetic acid, C₁₀H₁₆N₂O₈), sodium citrate (Na₃C₆H₅O₇), and NaCl (sodium chloride) were purchased from Fisher Biotech (Pittsburgh, PA). CHCl₃ (chloroform), C₆H₁₂ (cyclohexane), CH₃OH (methanol), I₂ (iodine), PrCl₃ (praseodymium chloride), HCl (hydrochloric acid), NaOH (sodium hydroxide), H₂SO₄ (sulfuric acid), and NH₄OH (ammonium hydroxide) were obtained from Fisher Scientific (Pittsburgh, PA). Reagents for the phosphate assay were purchased as a kit (QuantiChrom™ Phosphate Assay Kit (DIPI-500) from BioAssay Systems (Hayward, CA).

Multilamellar Vesicle Preparation

Multilamellar vesicles (MLVs) for phosphorus (³¹P) and deuterium (²H ) experiments were prepared from mixtures of lipids dissolved in chloroform. Stock bottles containing lipids in chloroform were allowed to reach room temperature before opening. Prior to vesicle
preparation, glassware was cleaned with methanol and chloroform; flasks and syringes were rinsed at least once with HPLC grade methanol and washed three times with chloroform.

The desired amount of lipid stock solution was drawn into a clean, gastight, syringe (Hamilton) and transferred to an appropriate container. If a mixture of two or more lipids was prepared, the previous procedure was repeated for each component of the lipid mixture. After use, the headspace of all stock bottles were covered with argon or nitrogen gas, the cap was wrapped in Parafilm or Teflon tape and returned to the freezer. Mixtures were prepared in either round bottom or pear-shaped flasks (the tapered bottom of the pear-shaped flask is helpful during the hydration of the lipid film), and solvent was removed by rotary evaporation at room temperature using a water-cooled solvent trap. Dry lipid films (both single component and mixtures) were re-dissolved in 4:1 (v:v) co-solvent system of cyclohexane and methanol (HPLC grade) to ensure complete mixing of the component lipids prior to lyophilization. This dispersion was gently mixed and flash frozen by placing the flask into a container with liquid nitrogen (LN2). Frozen lipids were lyophilized, typically overnight and subsequently hydrated with an appropriate buffer (5:1 buffer:total lipid, mass:mass) in a 55°C oven for at least one hour, followed by gentle vortex mixing of the hydrated dispersion; however, samples containing mostly POPC were hydrated at room temperature (POPC has a main phase transition near 0°C)(43). General lipid handling and sample preparation procedures can be found at www.avantilipids.com.

For nuclear magnetic resonance (NMR) measurements, approximately 200 μL or enough of the hydrated MLV dispersion to fill the tube was used for 31P experiments. For 2H measurements ~ 750 μL was loaded into a custom cut (~ 38 mm long for 2H), 5mm outer diameter NMR tube. These tubes were either special ordered (Wilmad) or conventional tubes
were cut in our laboratory to fit inside the RF coil of the solid state probe. Prior to sample loading the NMR tubes were washed with sample buffer. Buffers were first prepared using nano-pure deionized water, and brought to the appropriate pH with either HCl or NaOH. Aliquots of each buffer were then flash frozen with N2 (l), lyophilized, and re-hydrated with an equivalent volume of deuterium depleted water (Cambrige Isotope Laboratories)(44). Total lipid concentration for NMR experiments was near 275 mM based on theoretical values. Either two or four mol% of d62-DPPC was used in lipid samples prepared for 2H measurements.

MLVs and large unilamellar vesicles (LUVs) for electron paramagnetic resonance (EPR) experiments were also prepared from mixtures of lipids dissolved in chloroform. However, due to small sample volumes used for EPR measurements, lipid mixtures were prepared in a glass culture tubes, and blown dry with a stream of nitrogen gas. Dry lipid films were placed under vacuum overnight then hydrated with an appropriate buffer in a 55°C oven for at least one hour. Mixtures containing mostly POPC were hydrated at room temperature. For hydrated MLVs, total lipid concentration was near 100 mM, (4-5 μL) were loaded into a 1.8 mm inner diameter (id.) borosilicate capillary tube purchased from Vitrocom. Typically, samples contained 1 mol% of the DOXYL spin label for EPR measurements.

LUVs were prepared by extrusion through polycarbonate membranes. Gas tight syringes were cleaned with chloroform, rinsed three times with methanol, and finally thoroughly rinsed with sample buffer. The mechanical extrusion device was assembled according to the manufacture’s instructions (www.avantilipids.com). A volume of sample buffer was first injected into the extrusion device to fill the void volume in the extrusion chamber. An appropriate amount of lipid solution was loaded into one syringe and inserted into the extrusion device and the solution was passed through a membrane (typically 100 nm pore size) a minimum
of 55 times, until the lipid suspension became transparent. One pass is defined as solution moving from the starting syringe across the polycarbonate membrane and back to the starting syringe.

**Phosphate Assay**

Both MLV and LUV suspensions were diluted to obtain a concentration near 30 ng/100 μL. Three 100 μL aliquots of each diluted sample were placed in separate labeled glass culture tubes. Specific quantities of a phosphorus standard purchased from BioAssay Systems, ranging between 0 and 100 nmol, were placed in separate tubes; each standard quantity was made in triplicate in order to estimate the error of the standards. A volume (0.45 mL) of 8.9 N sulfuric acid was added to each tube and the tubes were placed in an oven at 220°C for 25 minutes to decompose the lipid and liberate the phosphate. Sample tubes were cooled to room temperature, and 3.9 mL of 1 M NaOH was added to each tube (this reaction is exothermic). Tubes were again cooled to room temperature and vortex mixed. A volume of 800 μL was transferred from each tube to a standard, disposable, UV-vis cuvette and 200 μL of malachite green phosphate analysis solution (BioAssay Systems) was added to each cuvette. The contents of each cuvette was mixed by inversion, and allowed to equilibrate for 10 minutes. Cuvettes were placed in a UV-vis spectrometer and the absorbance values at 650 nm were recorded. Unknown sample absorbance values were compared to the calibration curve prepared from the absorbance values of the standard solutions. This procedure for determination of phosphorus content has been modified from the general procedure of Warner et. al., 1956(45).

**Thin Layer Chromatography**

Lipid integrity was verified by thin layer chromatography (TLC) before and after exposure to magnetic resonance radio frequency pulsing as the ester bonds in the lipids are somewhat
labile. A very small amount (~2 μL) of lipid dissolved in chloroform or hydrated lipid suspension was added to seven drops of chloroform and spotted on silica coated aluminum plates (Whatman) purchased from Fisher Scientific. TLC plates were placed in a chamber containing a mobile phase of 65:25:4 (by volume) chloroform:methanol:ammonium hydroxide (14 N) and the solvent front was allowed to migrate to approximately 75% of the plate height. For quantitative experiments, TLC plates were washed in the mobile phase, and air dried prior to spotting of the sample. The stationary phase was allowed to dry and the slides were exposed to I₂ vapor for visualization of the lipid fractions.

**Magnetic Resonance**

Wide line ³¹P NMR experiments were performed using a Tecmag spectrometer operating at a resonance frequency of 145.2 MHz. Spectra were acquired with a CP/MAS probe purchased from Doty Scientific, Inc., with variable temperature capability. A two pulse Hahn echo pulse sequence with CYCLOPS phase cycling was used with ¹H decoupling. Details of the pulse sequence include an echo pulse spacing of 40 μs, a 4 μs ³¹P pulse (π/2), a 5 s recycle delay, 5 μs dwell time, 1024 - 8192 time domain data points. A minimum of 2048 transients were averaged for each experiment.

²H experiments were performed on a Bruker Avance spectrometer operating at a resonance frequency of 61.4 MHz. Spectra were acquired with a high power, broad band, and high temperature probe manufactured by Bruker. A standard quadrupole echo pulse sequence was used with 3.2 μs excitation pulses, 40 μs pulse spacing, a 500 μs recycle delay, and a 4 μs dwell time. Typically 8192 time domain data points were collected and a minimum of 14400 transients were averaged per spectrum.
Paramagnetic resonance experiments were completed using a modified ER200 (Bruker) with an ER023M signal channel, an E032 field controller, SPEX data acquisition software and a loop gap resonator (Medical Advances, Milwaukee, WI). Typical EPR experimental parameters are a 100 G spectral width, 20 mW of average microwave power, and a 0.16 s time constant. The sample temperature was controlled by flowing either compressed air or nitrogen through a copper coil immersed in a circulating water bath and passing the gas over the sample tube.

**Data Processing**

Raw $^{31}$P free induction decays were base line corrected, zero filled to twice the number of data points collected in the time domain, left shifted (if required), Fourier transformed, apodized by exponential multiplication (100 Hz), and phase corrected using the NTNMR software provided by Tecmag.

Raw $^2$H free induction decays were base line corrected, zero filled to twice the number of data points collected in the time domain, left shifted (if required), apodized by exponential multiplication (100 Hz), Fourier transformed and phase corrected using a Matlab routine provided by the Long research group.

EPR line shapes were baseline and phase corrected and area normalized using Labview software written by Dr. Christian Altenbach (UCLA, laboratory of Dr. Wayne Hubbell). Second moments and peak-to-peak widths were also calculated by Labview software written by Dr. Altenbach.
CHAPTER 3
MAGNETIC RESONANCE APPLICATIONS IN MEMBRANE BIOPHYSICS

Hydrated Lipid Motions and Order

Lamellar biological and model membrane systems are often described as two-dimensional “fluids” where lipids, proteins, and other molecules are allowed to diffuse laterally within the boundaries of a two-dimensional matrix(1, 2). However, this dynamic description should also include many other motional degrees of freedom for individual lipid molecules including individual bond vibrations and rotations, trans/gauche isomerization of the acyl chains, molecular rotation about and wobble of the symmetric, molecular long axis perpendicular to the bilayer normal, and lipid exchange or “flip-flop” between the two monolayers of the bilayer structure. The time scales of these motions span more than fifteen orders of magnitude, from bond vibrations, on the order of femtoseconds to flip-flop motions on the order of seconds to minutes(1, 3). Figure 3-1 depicts a cross-section of a bilayer plane and illustrates three selected motions and their respective reorientation times. Lipid molecules in Figure 3-1 are represented by circles (polar head groups) and squiggle lines (apolar acyl chains). Figure 3-1 A illustrates the molecular rotation and wobble motion of a lipid molecule in the top monolayer of the membrane, B illustrates the molecular exchange that occurs during lateral diffusion, and C illustrates the flip-flop exchange of lipid molecules between the two monolayers.

Spectroscopic methods such as NMR and EPR can provide information regarding the aggregation state and the orientation of single bonds, or molecules within a membrane with respect to a reference coordinate system, usually the long axis of the lipid molecule, or the bilayer normal in lamellar systems(4-7). Average orientations are determined by mapping the experimental observables such as the principal components of the chemical shift, the quadrupole coupling, and the hyperfine tensor values onto the molecular frame. Specific details regarding
these tensors and the spectroscopic signals obtained for each method are discussed later in this monograph.

Experimental signals acquired by these techniques are related to the time averaged reorientation of the previously mentioned tensor values and are usually converted to order parameters ($S_i$) used to describe average tensor orientations with respect to a reference coordinate system in the static limit. In general, order parameters for unoriented systems in a uniaxial magnetic field are expressed as second order Legendre polynomials: $S = \frac{1}{2} (3 \cos^2(\theta) - 1)$, and are functions of the average angle ($\theta$) between the molecular frame and the reference frame (the magnetic field). Unfortunately, the experimental observables for membranes that are not mechanically oriented with respect to the magnetic field provide a total order parameter that is the product of two order parameters, one describing the orientation of the molecule or bond with respect to the bilayer normal and the other describing the orientation of the bilayer normal with respect to the magnetic field. Figure 3-2 shows the order parameter of a specific molecular site (i) as a function of the angular orientation of that site with respect to the bilayer normal. According to the mathematical expression for order parameters, the maximum occurs at an angle of 0° and is assigned a value of +1, while the minimum occurs at an angle of 90° and is assigned a value of $-\frac{1}{2}$.

Two important pieces of information must be kept in mind when evaluating or reporting order parameters: 1) A small order parameter value is not necessarily low because of rapid, random motional averaging; for example, it may be low because the tensor is oriented at the magic angle (54.7°), which leads to an intrinsic order value of zero, and 2) Different spectroscopic techniques are sensitive to different time scales of tensor reorientations. For example, NMR reports motions reorienting faster than 10 μs (such as trans/gauche
isomerization) as an averaged resonance weighted by the number of individual conformations represented. Techniques such as infrared (IR) spectroscopy, however will report the same motions as a superposition of individual resonances. Therefore, one should always keep in mind the time bases of the experimental methods when comparing order parameters.

**Nitroxide Spin-Probes**

Stable free radical spin probes have been used for many years to investigate the dynamics of model and biological membranes assemblies\(^4, 8-15\). A variety of hydrophobic and amphiphilic organic spin-probes have been employed such as, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), paramagnetic oxazolidine ring labeled fatty acids, and paramagnetic oxazolidine ring labeled lipids\(^13-15\). The structures of selected spin-labels are shown in Figure 3-3; A depicts a hydrophobic nitroxide spin-label (TEMPO), B) depicts the ring structure incorporated into the acyl chain regions of the fatty acid and the labeled lipid shown in C and D, while C and D are amphiphilic nitroxide spin-labels.

**Description of Nitroxide Spin-Label Order Parameter in Hydrated Lipid Bilayer Assemblies Obtained by Electron Paramagnetic Resonance**

Electron paramagnetic resonance (EPR) spectra of spin \(\frac{1}{2}\) molecules, such as nitroxide radicals, trapped as impurities in crystals with fixed orientation can be described by the basic spin Hamiltonian of McConnell and McFarland (Eqn. 3-1), neglecting proton hyperfine interactions and including only the \(g\) (electron screening tensor) and \(T\) (hyperfine coupling tensor).\(^13\) The hyperfine tensor is a result of coupling between the electron angular momentum operator (\(S\)) and the nuclear angular momentum operator (\(I\)). For a nitroxide radical (\(S = 1/2\) and \(I = 1\)), and the hyperfine interaction will give rise to three absorption lines, thus the EPR derivative signal has three peaks. The exact details of the EPR transitions and line shapes are discussed later. \(\beta\) and \(\beta_N\) are the Bohr magnetons for the electron and nucleus respectively, \(g\)
is the electron screening tensor (analogous to the NMR chemical shift), $T$ is the hyperfine coupling tensor, $S$ and $I$ are spin angular momentum operators, and $\hbar$ is Planck’s constant.

$$H_T = |\beta| S \cdot g \cdot H_e + \hbar S \cdot T \cdot I - g_N \beta_N I \cdot H_N$$  \hspace{1cm} (3.1)

An energy level diagram illustrating electronic Zeeman and hyperfine interactions of a representative spin-probe in an external field along with the resulting derivative EPR spectrum is illustrated in Figure 3-4. When an unpaired electron is placed in an external field ($B_0$) the degeneracy of electron spin angular momentum is lifted, which is referred to as the electronic Zeeman interaction. For a nitroxide radical, the electron spin angular momentum and nuclear spin angular momentum are coupled through a hyperfine interaction tensor ($T$), due to the interaction of the electron angular momentum and the $^{14}\text{N}$ nucleus with a magnetic moment, splitting each electronic spin state into $(2I + 1)$ states. The hyperfine interaction is not dependent upon field strength, but has an anisotropic spatial dependence that determines the energy splitting (spacing between peaks in derivative mode) of the three allowed transitions (see Figure 3-5). Selection rules are ($\Delta m_e = +/- 1$) and ($\Delta m_I = 0$) for a one photon process. The resulting energy transitions can be probed with microwaves oscillating at the correct frequency.

However, as discussed earlier in this chapter, self-assembled, spin-labeled fatty acids and phospholipids in an aqueous environment are neither solution nor static structures but undergo rapid, anisotropic molecular motions about the molecular long axis. This motion must be described by a time dependent equation(4, 13). This time dependent Hamiltonian can be separated into two parts: 1) a time-independent, effective Hamiltonian ($H'_T$) (Eqn. 3-2) and 2) a Hamiltonian that is a function of time. As long as the time dependent fluctuations are sufficiently averaged; $H'_T$ will adequately describe the system(4, 13).

$$H_T = |\beta| S \cdot g \cdot H_e + \hbar S \cdot T' \cdot I - g_N \beta_N I \cdot H_N$$  \hspace{1cm} (3.2)
Unfortunately, experimental powder line shapes of hydrated lipids only yield information about molecular orientation with respect to the laboratory reference frame and must be related to the molecular axis system using an oriented reference molecule. The reference molecule used for understanding spin-labeled lipids was spin-labeled 5-α-cholestan-3-one (Figure 3-5), with \( g \) values \((g_x, g_y, g_z) (2.0089, 2.0058, 2.0021 \pm 0.001)\) and \( T \) values \((T_x, T_y, T_z) (5.8, 5.8, 30.8 \pm 0.5 \text{ G})\) calculated by Hubbell\(\text{(13, 16)}\). However, if \( g' \) and \( T' \) tensors in Eqn. 3-2 are appropriate time averages of \( g \) and \( T \) from Eqn. 3-1, the energies and thus the two effective tensors, can be related to calculated tensor values of the trapped radical through the averaged squared directed cosine projected onto the principal axis system of the trapped nitroxide\(\text{(4, 13)}\). Equations 3-3 and 3-4 show the energy obtained from the effective Hamiltonian in the laboratory frame and in terms of the basic Hamiltonian, respectively. From Eqn. 3-3 and 3-4, using the relation \( \alpha^2 + \beta^2 + \gamma^2 = 1 \), and since \( T_{xx} \) and \( T_{yy} \) are equal for 4,4-dimethyloxazolidine-3-oxyl,\(\text{(13)}\) the value of \( T'_{zz} \) can be derived and is shown in Eqn. 3-5. In Eqn. 3-5, \( a \) is the isotropic component of the hyperfine tensor \((a = 1/3 \text{ Tr}(T) = 1/3 \text{ Tr}(T'))\) and \( S \) is the order parameter. It can be shown that \( S \) can be related to calculated values of \( T_{xx} \) and \( T_{zz} \) and experimental values of \( T'_y \) and \( T'_z \) as seen in Eqn. 3-6. Because the hyperfine interaction is dependent on solvent polarity the order parameter requires a slight correction which can be seen in equation 3-7\(\text{(9, 13)}\).

\[
E = |\beta| \left[ g_{zz} S_z \cdot H_z + h T_{zz} S_z \cdot I_z - g_N \beta_N I_z \cdot H_z \right] \quad (3.3)
\]

\[
E = |\beta| \left( \alpha^2 g_{xx} + \beta^2 g_{yy} + \gamma^2 g_{zz} \right) S_z \cdot H_z + h \left( \alpha^2 T_{xx} + \beta^2 T_{yy} + \gamma^2 T_{zz} \right) S_z \cdot I_z - g_N \beta_N I_z \cdot H_z \quad (3.4)
\]

\[
T'_{zz} = \alpha^2 T_{xx} + \beta^2 T_{yy} + \gamma^2 T_{zz} = a + \frac{2}{3} (T_{zz} - T_{xx}) \quad S
\]

\[
S = \frac{1}{2} (3 \gamma^2 - 1) = \frac{T'_y - T'_z}{T_{zz} - T_{xx}} \quad (3.6)
\]
Orientation of the Nitroxide Spin-Label in Hydrated Lipid Bilayer Assemblies and Expected EPR Lineshapes

Referring to Figure 3-3 C and D and recalling the orientation of individual hydrated lipid molecules in a bilayer aggregate, one can see that the nitrogen-oxygen bond is oriented parallel to the bilayer surface, making the orbital designated \( p_z \) perpendicular to the bilayer surface and parallel to the acyl chain long axis. The electron spin angular momentum is most strongly coupled to the \(^{14}\text{N} \) nuclear angular momentum in the direction of \( p_z \) and the hyperfine coupling is minimal in the plane perpendicular to this orbital; therefore, the largest component of the hyperfine coupling tensor is \( T_{zz} \) and \( T_{zz} > T_{xx} = T_{yy} \)\(^{(4, 17)}\). This is again illustrated in Figure 3-6 where the \( z \)-axis represents the nitrogen \( p_z \) orbital and the \( x \)-axis represents the N-O bond vector\(^{(4, 17)}\). Even though X-band (9 – 10 GHz) EPR is dominated by the hyperfine interaction, it is important to note that the \( g \) tensor is also anisotropic with the largest value in the direction of the N-O bond (because \( g \) is a first order function of field it becomes significant in high field EPR)\(^{(17)}\). Figure 3-7 illustrates two representative line shapes: spectrum A represents an axially symmetric, nitroxide label with restricted motion (broad spectral lines) and visible splitting of the low and high field lines, while spectrum B) represents an axially symmetric, nitroxide label without restricted motion (narrow spectral lines).

The central resonance line is invariant to changes in the hyperfine interaction\(^{(17)}\); therefore, the central peak-to-peak line width \( (\Delta H_{pp} \text{ Figure 3-7 B}) \) is also a good approximation of relative mobility of the spin-label. There are two other methods commonly used to determine mobility of nitroxide spin labels: normalized fractional intensity \( (f_i) \), and second moment \( (M^2) \) analysis\(^{(4, 13, 18)}\). Normalized fractional intensity, as a function of a dependent variable (e.g.
temperature), tracks the change in intensity (I) of a specific spectral position with respect to an intensity designated as final (I₀) divided by the difference in an intensity designated initial (Iᵢ) and the final intensity (I₀) (Eqn. 3-8)(19). This is an adequate method for comparing mobility within a set of similar experiments, but for small changes in spectral intensity the level of noise must be near zero.

\[ f_i = \frac{I-I_f}{I_i-I_f} \quad (3.8) \]

The coupling strength or spitting of the z component of \( T' \) (T' \(_{zz'}\)) measured for a specific nitroxide labeled site yields information about the relative order of an aggregated lipid system in the vicinity of that site. This coupling strength is directly related to angular deviations (\( \gamma \)) of the spin-probe (pz orbital) from a plane perpendicular to the bilayer normal, and indirectly related to local environmental order. These angular excursions for the bilayer normal are mainly caused by trans\( \rightarrow \)gauche isomerizations in the local vicinity of the probe(5, 13, 20). The probabilities of these isomerizations for free polymer chains and lipids in an assembly are a function of the total internal energy of the hydrocarbon chain in a specific conformation(5, 20). However, due to interaction with other molecules the total internal energy for assemblies must include both intramolecular and intermolecular energies(5, 20). Examples of interaction energies that can be included in the intermolecular group include van der Waals interactions, electrostatic repulsions and thermodynamic energies from hydrophobic forces. Considerations must also be made for restricted motion near the head groups (conical boundary condition)(5, 20). Moreover, in assembled aggregates the isomerization probability (chain order) is a function of depth (or location) in the hydrophobic domain of the aggregate establishing an average, site-dependent order(13, 20). Although the EPR active spin-labeled lipids may perturb lipid aggregate
structures they still report at least qualitative information concerning the order/disorder of the hydrated lipid aggregates as they would exist without the spin-label.

**Solid State $^{31}$P and $^2$H NMR of Hydrated Lipid Aggregates**

The following general discussion of NMR will focus on two specific nuclei: $^2$H and $^{31}$P. $^2$H is an I = 1 nucleus with a dominant quadrupolar coupling interaction, and $^{31}$P is an I = $\frac{1}{2}$ nucleus with a dominant anisotropic chemical shift (CSA) interaction, under the condition of full proton decoupling. There are two allowed transitions in the radio frequency energy range for I = 1 ($^2$H) nuclei and a single allowed transition for I = $\frac{1}{2}$ ($^{31}$P) nuclei; both transitions obey the selection rule $\Delta m_I = \pm 1$. An energy diagram illustrating the quadrupolar splitting and allowed transitions for an I = 1 nucleus can be seen in Figure 3-8. Application of a strong external magnetic field lifts the degeneracy of the nuclear spin angular momentum of an I =1 nucleus; therefore, three (2I + 1) nuclear Zeeman states, designated +1, 0, and -1 are present. Further perturbations of these nuclear Zeeman states are caused by an interaction of the nuclear spin angular momentum with the electric field gradient (EFG) at the quadrupolar nucleus. Energies for these transitions are given in Figure 3-8, where $e_q$ is the electric field gradient, $Q$ is the quadrupole moment, $e$ is the elementary charge, $\theta$ is the angle of the principle axis of the efg with respect to the applied field, $\phi$ is the angle in the x,y plane, and $\eta$ is the asymmetry parameter($^5, 21, 22$). The value of $\eta$ is defined as $(|\nu_{XX}| - |\nu_{YY}|)/|\nu_{ZZ}|($$^5, 21$), where $\nu_{ii}$ are diagonal elements of the electric field tensor $V$.

Lipid MLVs in an aqueous environment have all possible spatial orientations with respect to the applied field, and yield the classic Pake powder spectrum (Figure 3-9), which is narrowed and axially symmetric ($\eta = 0$) because of rapid rotational motion around the long axis of the lipid molecule($^5, 7, 21, 23$). There are three distinct features of the Pake-doublet when all possible
values of $\theta$ and $\phi$ are considered: steps ($\Delta \nu_{ZZ}$), shoulders ($\Delta \nu_{YY}$), and singularities ($\Delta \nu_{XX}$).

Figure 3-10 shows the average angular orientation of the elements of both the electric field tensor ($V$) and chemical shift tensor $\sigma$ with respect to the applied field ($B_0$) (5). Also note that the quadrupolar splitting is invariant with respect to the applied field. The difference in frequency units between each location ($\nu^Z = \pm \frac{3}{4} \chi_0 (3 \cos^2 \theta - 1 - \eta (1 - \cos^2 \theta) \cos 2\phi)$) of the two maxima of a specific Pake doublet defines the averaged quadrupolar coupling constant and the orientation (order parameter $S_{CD}$) of a specific deuteron with respect to the applied field (5, 21). The value of the coupling constant (5, 21) can be compared to the static limit and related to angular deviations and local order as discussed in the previous section for EPR probes.

**Magnetic Resonance Line Shapes and Order in Hydrated Lipids**

Spectroscopic information such as line shape and residual coupling strengths obtained from the natural $^{31}\text{P}$ and virtually non-perturbing $^2\text{H}$ NMR experiments allow for indirect aggregated structural assignments and determination of relative order/disorder in the head group and acyl chain regions of the aggregates. $^2\text{H}$ and $^{31}\text{P}$ spectra of lipid aggregates have characteristic line shapes, which can be associated with aggregation states and degree of order of hydrated lipids. As discussed previously, order/disorder is defined as the degree of motional averaging relative to the static limit.

Typical $^{31}\text{P}$ and $^2\text{H}$ line shapes and their corresponding aggregate types for hydrated lipids are shown in Figure 3-11. Each carbon atom within the acyl chain region is deuterated and the resulting $^2\text{H}$ spectrum will be a superposition of quadrupole splittings from each site, but for clarity only one site is shown. Visual inspection of $^{31}\text{P}$ line shapes yields information about aggregation type: lamellar gel phase spectra have a broad, asymmetric chemical shift anisotropy (CSA) line shape; lamellar liquid crystalline spectra have a narrowed axially symmetric CSA.
lineshape; very small MLVs, micelles, and cubic phases yield isotropic peaks; as more motional averaging narrows the NMR spectral width(3, 21-23, 25). It is important to note that $^{31}$P powder NMR cannot differentiate between aggregates with signals that appear isotropic, because the chemical shift of each of these aggregation states is the isotropic value $\sigma_{\text{iso}}$, see Figure 3-11.

Hexagonal phases have a $^{31}$P line shape that is opposite in sign and half the numerical span value of the corresponding lamellar line shape, because the CSA is averaged in a second dimension, with respect to lamellar liquid crystalline spectra.(26) $^2$H spectra for the lamellar gel phase are broad and unresolved; similar to the span of $^{31}$P spectra, the quadrupole splitting of the hexagonal aggregate is also half as broad as the lamellar liquid phase(5).

The NMR data collected from hydrated lipid aggregates not only afford aggregate structural assignments but also allow for comparisons of the relative order/disorder among different individual locations, albeit on a different time scale, to those obtained from EPR measurements. Comparisons of relative order will be used to determine the morphological effects of incorporating the negatively charged, atypically shaped BMP molecule into model membranes.

![Figure 3-1. Selected lipid motions and associated correlation times in hydrated lamellar structures: A) rotational diffusion and long axis wobble; B) lateral diffusion; C) flip-flop.](image)

\[\tau \sim 100 \text{ ps} - 10 \text{ ns}\]

\[\tau \sim 100 \text{ ns} - 1 \text{ ms}\]

\[\tau \sim \text{ seconds} - \text{ minutes}\]
Figure 3-2. An order parameter as a function of the angular displacement of the plane containing a specific carbon and two deuterium atoms from the bilayer normal.
Figure 3-3. Common organic radical spin-labels: A) 2,2,6,6-tetramethylpiperidine-1-oxyl; B) 4,4-dimethyloxazolidine-3-oxyl; C) (5-DOXYL) steric acid; D) 1-palmitoyl-2-stearoyl-(5-DOXYL)-sn-glycero-3-phosphocholine.
Figure 3-4. Energy level diagram illustrating the electronic Zeeman and electron-nuclear hyperfine interactions and the resulting derivative of EPR transitions for a spin-probe such as, TEMPO in solution.

Figure 3-5. 4,4-dimethyloxazolidine-3-oxyl labeled 5-α-cholestan-3-one.
Figure 3-6. Spatial dependence of the coupling strength of the anisotropic hyperfine interaction; where the z-axis represents the nitrogen p$_z$ orbital and the xy-axis represents the N-O bond plane. The individual couplings can be obtained by rotating the uniaxial magnetic field so that it is coincident with each axis.
Figure 3-7. Theoretical nitroxide label EPR line shapes: A) Representation of partially immobilized environment; B) Representation of an isotropic solution environment.

\[ \Delta E = \gamma hB_x \]

A)

\[ \Delta E = \gamma hB_x + \frac{3e^2qQ(3\cos^2\theta - 1 - \eta(1 - \cos^2\theta)(\cos2\phi))}{8} \]

B)

\[ \Delta E = \gamma hB_x - \frac{3e^2qQ(3\cos^2\theta - 1 - \eta(1 - \cos^2\theta)(\cos2\phi))}{8} \]

Figure 3-8. A) Energy level diagram illustrating the nuclear Zeeman and quadrupolar coupling interaction of $^2$H in an applied field $B_0$. B) Energy level diagram for the $^1$H decoupled chemical shift of $^{31}$P in an applied field $B_0$. 

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Figure 3-9. Theoretical quadrupolar echo powder spectrum of a single deuterium labeled site ($\eta \neq 0$).

\[ \Delta v_{XX} = \Delta v_{YY} = \frac{1}{2} \Delta v_Q (1 + \eta) \]

\[ \Delta v_{ZZ} = \frac{1}{2} \Delta v_Q (1 - \eta) \]

\[ \sigma_{zz} = \sigma_{11} \sin^2 \theta \cos^2 \phi + \sigma_{22} \sin^2 \theta \sin^2 \phi + \sigma_{33} \cos^2 \theta \]

Figure 3-10. Graphical representation of A) CSA and B) EFG the angular orientations with respect to the applied field ($B_o$). This figure has been adapted from Santos(24).
Figure 3-11. Theoretical powder spectra of various lipid aggregates: A) axially symmetric $^{31}$P and B) a single axially symmetric $^2$H labeled site.
CHAPTER 4
MONITORING MODEL BILAYER SOLUBILIZATION BY DETERGENT MOLECULES USING EPR SPECTROSCOPY

Model Membrane Solubilization

The underlying question addressed within this dissertation is the investigation of the structure of BMP vesicles and the effect this lipid has upon model membrane morphology of POPC/BMP or DPPC/BMP mixed vesicles. Structure shall be defined as head group and acyl chain packing, as well as the morphology of lipid aggregation states. Using the geometrical shape approximations discussed in Chapter 1, BMP is assumed to have a polar region (defined as the phosphate group and both glycerol moieties) that occupies a larger cross-sectional area than the apolar, acyl chain region (refer to Figure 1-6 or Figure 4-1 B). According to the simple geometrical packing model (described in Chapter 1), we can predict that hydrated BMP lipids may form micellar or highly curved vesicular types of assemblies. Our first hypothesis, based on simple geometric constraints alone, is that BMP could have detergent-like properties similar to sodium dodecyl sulfate (SDS or the DS\(^{-}\) anion), such as micellar aggregation and the ability to solubilize lipid bilayer membrane aggregates. The structures of SDS and the S,R isomer of BMP are shown in Figure 4-1.

According to literature, there are three stages for pure membrane solubilization by detergent molecules\((1, 2)\): 1) detergent monomer molecules bind membranes and partition into the membrane structure by inserting their acyl chain into the hydrophobic region of the lamellar structure, thus increasing the overall size of the aggregate structure; 2) membranes become saturated with detergent molecules and a two phase equilibrium exists between saturated membranes and detergent/lipid mixed micelles; and 3) bilayer structures become fully solubilized and lipid molecules are incorporated into detergent micelles (termed mixed micelles).
The molecular species and or aggregates present in each stage of the solubilization process are as follows: 1) detergent monomer, and lipid/detergent membrane aggregate; 2) detergent monomer, saturated lipid/detergent membrane aggregate, and detergent/lipid mixed micelles; 3) detergent monomer and detergent/lipid mixed micelles.

Both micelle formation and membrane solubilization can be investigated using cw-EPR spectroscopy with positional isomers of spin-labeled lipids (Figure 4-2). If BMP does form micellar structures, the fast rotational correlation time of the assembly will motionally average (narrow) the line shapes of the DOXYL spin-probes incorporated into the micelles (3) when compared to the line shapes obtained from relatively immobilized labels in bilayer aggregates(4).

Typical EPR experimental conditions utilize as little as 0.5 mol% of a spin labeled fatty acid or spin labeled phospholipid to probe structure and dynamics in model membrane systems(5). Again, motional averaging will narrow the overall EPR line shapes, compared to the static powder pattern. Spectral features such as the peak-to-peak width of the central resonance line ($\Delta H_{pp}$), the normalized spectral fractional intensity ($f_I$), order parameter ($S_i$), and second moment ($M^2$) can be used to compare the degree of motional averaging and relative order of these spin-probes incorporated into lipid assemblies. The values of these parameters can then be compared among those obtained from probes incorporated into mixed micellar, lipid/SDS aggregates, mixed lipid/SDS bilayer structures, and single component, bilayer mesostructures of model membrane lipids made of POPC and DPPC. Similarly, the effect of BMP upon the organization, morphology and acyl chain dynamics of POPC and DPPC bilayers can be investigated using the same strategy, by precisely incorporating 1 mol% of the label into the assemblies and varying the position of the DOXYL spin-label along the acyl chain of the phospholipid.
The solubilization of POPC by SDS has been studied in detail by light scattering, NMR and isothermal titration calorimetry (ITC)\(^2, 6, 7\). Critical values used to map phase boundaries are obtained by calculating the points of inflection from either right angle light scattering intensity or normalized heats of reaction (ITC) as a function of detergent concentration\(^2\). These same critical values and phase boundaries can also be determined by monitoring the isotropic \(^{31}\)P NMR signal intensity from lipids in mixed micellar aggregates\(^6\). From these types of data, Seelig and coworkers constructed a phase diagram with SDS concentration \(C_D^o\) as the dependent variable plotted as a function of total lipid concentration \(C_L^o\). The linear phase boundaries described by Eqns. 4-1 and 4-2 as determined by Seelig and coworkers, have been reproduced in Figure 4-3\(^6\). The approximate boundaries between the saturated bilayer and the mixed micelle/bilayer coexistence (Eqn. 4-1) and the mixed micelle/bilayer coexistence and the mixed micellar regions (Eqn. 4-2) are indicated by solid black lines. The intercept of each line corresponds to the minimum concentration of detergent needed to either saturate or completely solubilize the POPC vesicles at 56°C\(^6\). According to this phase diagram for SDS partitioning and micellization of POPC membranes, we sampled the bilayer, micelle/bilayer coexistence, and micellar regions in our EPR investigation. In Figure 4-3 open circles represent various POPC vesicle samples prepared with the 10-DOXYL labeled lipid in the presence of SDS, and closed squares represent various SDS POPC vesicle samples prepared with the 5-DOXYL labeled lipid in the presence of SDS. It should be noted that our EPR experiments were carried out at room temperature, whereas the Seelig investigations were performed at 56°C. However, given that POPC is in the L\(_\alpha\) phase above 0°C, we anticipate that the experimentally determined phase boundaries would show little temperature dependence

\[
C_D^o = 0.283C_L^o + 2.2 \quad (4.1)
\]
Characterization of the EPR Line Shapes of Spin-probes Located in Bilayer Aggregates in the Presence of an Anionic Detergent

Control experiments were performed that characterized the EPR line shapes of DOXYL labeled lipids during partitioning of the anionic detergents SDS into lamellar POPC model membranes and consequent micellization of these membranes. EPR spectra were collected for DOXYL lipids (1 mol%) incorporated into POPC large unilamellar vesicles (LUVs) that were made by extrusion through polycarbonate membranes with a 100 nm pore size. POPC and POPC/BMP LUVs were prepared according to the procedure in Chapter 2 and hydrated in a buffer containing 20 mM HEPES, 100 mM NaCl and 0.02% NaN₃, at pH 7.4 or 5 mM HEPES, 100 mM NaCl, 0.1mM EDTA, at pH 7.4. The concentration of the DOXYL labeled lipid was 1 mol% of the total lipid fraction for all samples unless otherwise indicated. Samples containing SDS were allowed to equilibrate for at least 30 minutes prior to measurements. EPR measurements were collected using a 100 G spectral width with 20 mW of microwave power and a 0.16 s time constant, at room temperature unless otherwise indicated.

In order to characterize the effects that detergent partitioning and subsequent micellization has upon the EPR line shape of DOXYL labels in model POPC membranes, SDS was titrated into POPC vesicles containing the DOXYL spin-probe. Two sites within the bilayer structures were examined, carbon positions 5 and 10 on the sn2, steric acid chain of the labeled lipid (Figure 4-2 A and B respectively). Changing the position of the nitroxide probe provides information about acyl chain order both near the lipid head group and well within the hydrophobic region of the molecular assemblies.

As detergent is incorporated into a membrane-like structure, the bilayer packing is disrupted, thus changing the interactions between lipid molecules and consequently the micro-

\[ C_D^* = 2.2C_I^* + 1.69 \]
environment of the spin-probe. The order parameter of each labeled lipid is expected to decrease as the system moves through each stage of the solubilization process \((1 \rightarrow 2 \rightarrow 3)\)(8, 9). This change in order should be apparent in EPR line-shapes as the residual anisotropic, hyperfine coupling strength is affected by local micro-environment(4, 5, 10-14).

Both 5 and 10-DOXYL labeled lipids report observable changes, such as, a smaller order parameter and a smaller fractional intensity as the concentration of SDS is increased, which is consistent with SDS partitioning into the bilayer and subsequent micellization. These changes in acyl chain order can be characterized by any of four numerical values: the second moment, the peak-to-peak central line width, the normalized fractional intensity or the order parameter values(4, 11, 15). These values have been defined previously in Chapter 3. Values of the second moment, order parameters \(S_i\), normalized fractional intensity \(f_i\), and \(\Delta H_{pp}\) obtained from EPR line shapes for the lipid solubilization experiments are listed in Tables 4-1 and 4-2. Figures 4-4 and 4-5 display EPR line shapes as a function of detergent concentration for nitroxide labeled positions 5 and 10 respectively. From these spectra, it is easy to see the line shape changes upon addition of SDS, and that the DOXYL label in position 5 is more sensitive to membrane micellization.

The EPR line shape shown in Figures 4-4 (spectrum a) is for 5-DOXYL labeled lipid in POPC LUVs with no SDS and shows a typical anisotropic powder-like pattern expected for a spin-probe with restricted motion intercalated in a membrane structure(4, 11). This line shape reflects the most immobilized spin-label/lipid motion in this series as indicated by the largest splitting of the hyperfine interaction tensor component \(T^{\omega}_{ij}\), the largest value of the second moment, the largest fractional intensity, and the largest order parameter \((26.4 \text{ G}, 202 \text{ G}^2, 1, \text{ and } 0.68 \text{ respectively})\). Moreover, the order parameter for spectrum a corresponds to motional
averaging with an angular deviation of 28° from the bilayer normal. Spectrum d shows a line shape that is the most mobile for the series with values of the hyperfine splitting, second moment, fractional intensity, and order parameter determined to be 20.7 G, 198 G², 0.0, and 0.40 respectively, corresponding to motions leading to an average angular deviation of 39° from the bilayer normal. Assuming the boundaries of the phase diagram in Figure 4-3, this line shape can be understood in terms of solubilization of the POPC bilayer by SDS resulting in the formation of mixed micelles. Hence some of the 5-DOXYL lipid is now in a micellar environment and the line shape parameters reflect the increased correlation time of the smaller spherical micelle compared to the POPC vesicle. The values of angular deviation from the bilayer normal (θ) were calculated using Eqn. 3-6 along with the order parameter obtained from Eqn. 3-7. Also note the variable used to represent the average orientation with respect to the bilayer normal is changed from γ to θ. Spectra b and c in Figures 4-4 and 4-5 are EPR line shapes obtained from the region on the phase diagram in Figure 4-3 between the two phase boundaries but are not simple superpositions of line shapes obtained from labeled lipids in saturated lamellar bilayer aggregates and those located mixed SDS/lipid micelles. This may be because spectrum d in Figure 4-4 was obtained from a location very near the phase boundary, therefore the label is reporting motion from both mixed micelles and saturated lamellar structural environments.

For tracking changes in acyl chain order it is most useful, at least for this investigation, to compare spectral parameters such as the fractional intensities, the peak-to-peak widths, and the order parameters, because the changes in second moment appear to be extremely sensitive to baseline correction errors. Figures 4-6 and 4-7 show plots of each of these values as a function of the ratio of SDS to lipid concentrations (C_{SDS}/C_{POPC}) for samples incorporating a 5 and 10-DOXYL labeled lipid, respectively. Recall, a less ordered location in the bilayer, such as
position 10, has a narrower line shape because the degree of motional averaging is greater for
spin probes closer to the center of the bilayer (a larger probability for trans/gauche isomerization,
(16) thus more disorder). Unfortunately, number of data points collected for the spin-labeled
lipids, incorporated into POPC LUVs, is too small to draw any quantitative conclusions with
regards to utilizing EPR line shapes of incorporated spin probes to define the phase boundaries
for the solubilization of POPC LUVs by SDS. However, we have measured the characteristic
line shapes and corresponding trends in fractional intensity, peak-to-peak line width, second
moment, and order parameters, for two spin probe positions during the solubilization of POPC
bilayers by SDS detergent.

**Hydrated Bis(monoacylglycerol)phosphate Assemblies Solubilized by Sodium Dodecyl
Sulfate**

In order to initially characterize the effects that detergent partitioning and subsequent
micellization has upon the EPR line shape of DOXYL labels in BMP aggregates and compare
them to the results obtained for POPC LUVs, SDS was titrated into extruded BMP structures
containing the 5-DOXYL PC. Due to the relative monetary expense of purchasing BMP and the
exploratory nature of our investigation only a single site within the aggregated structure was
examined, carbon position 5 on the sn2, steric acid chain of the labeled lipid.

The EPR spectrum (Figure 4-8 spectrum a) for 99.5% BMP_{18:1} mesostructures (extruded
through 100nm diameter membranes) doped 0.5% 5-DOXYL spin-labeled lipid in an aqueous
environment, and in the absence of SDS is broad ($T_{||} = 24.6$ G and $T_\perp = 9.0$ G) and has similar
structural features as the line shapes obtained from the lamellar POPC assemblies in seen in
Figure 4-4 ($T_{||} = 26.4$ G and $T_\perp = 8.8$ G). Spectra (Figure 4-8 b – i) are line shapes illustrating the
effect of SDS partitioning and solubilization of BMP aggregates. These spectra show a similar
trend in spectral line narrowing previously seen in POPC lamellar assemblies as SDS
concentration is increased. The similar line shapes and spectral parameters (in the absence of SDS) indicate that the acyl chain environment in BMP dispersions is similar to that in POPC LUVs. In addition, the trend in variation of the line shape parameters as SDS solubilization occurs for BMP is analogous to those observed upon SDS solubilization of POPC LUVs. The second moment, $\Delta H_{pp}$, normalized fractional intensity, order parameter, and angular deviation values obtained from analysis of these line shapes of 5-DOXYL PC in BMP as a function of SDS concentration are listed in Table 4-1.

Figure 4-9 shows a comparison of the values of peak-to-peak width, second moment, order parameter, and normalized fractional intensity as a function of SDS/Lipid concentration ratio for BMP and POPC aggregates obtained using the 5-DOXYL labeled lipid. Again, it is most instructive to examine the trends in $\Delta H_{pp}$, $S_i$, and $f_I$ because the second moment value is very sensitive to baseline correction errors. By visual inspection it is clear that each of these parameters shows an increase in disorder as SDS concentration increases, indicating both partitioning and solubilization of the lipid structures by SDS. The order parameter for pure BMP (0.62) is lower than pure POPC (0.68) and could be explained by BMP’s second unsaturated chain; this would probably result in a larger volume requirement per lipid molecule and reduce steric constraints for isomerization. The values used to track partitioning and solubilization of the BMP aggregates follow the same general trend as POPC LUVs. The similarity of these trends may be another positive indication that BMP assembles into a lamellar aggregate with acyl chain packing and dynamics similar to those in POPC lamellar structures.

Since more EPR data was collected for the BMP solubilization study than for the POPC solubilization study it may be possible to make more quantitative statements concerning the sensitivity of spin-probes to the solubilization process. For example, according to Blume and
coworkers a limiting ratio of ~1.5 SDS molecules to 1 POPC molecule corresponds to the saturation point of POPC vesicles and onset of solubilization at 65°C(2). A similar ratio is detected by our EPR investigation of BMP aggregates; clearly indicated by the discontinuity between concentration ratios 1.3 and 1.7 of plots C and D of Figure 4-9. This is not in agreement with the value of 0.28 reported earlier in equation 4-2 reported by Seelig for data collected at 56°C. However, in the low concentration range (Figure 3C of (6)) light scattering data at 20°C and ITC data at 56°C report a much steeper slope corresponding to a ratio of approximately 1.4 or 1.5(2). This ratio is in good agreement with our experimental value of ~1.5 SDS molecules to 1 POPC molecule. There is another possible discontinuity present in our EPR data for the region between concentration ratios 2.5 and 3.5 corresponding to the beginning of solubilization of the aggregate structure; this discontinuity is more obvious in Figure 4-9 plot D. Seelig and Blume report ratios of 2.2 and 2.7 SDS molecules per lipid molecule needed to initiate solubilization of POPC membranes at 56°C and 65°C, respectively(2, 6).

According to the previous observations and the assumption that BMP has a similar solubilization diagram as POPC, we believe we have sampled each region of the phase diagram for the solubilization of BMP by SDS detergent, and have obtained line shapes that are characteristic of each region(3, 9). Therefore, the spectra for BMP dispersions found in Figure 4-8 are assigned to the following regions of a solubilization diagram a lamellar BMP, b-d SDS monomer/SDS partitioned into BMP bilayers, e-g SDS monomer/SDS saturated BMP bilayers/mixed SDS BMP micelles, and h-i SDS monomer/mixed SDS BMP micelles.

Given that our EPR results for BMP solubilization behavior are consistent with those obtained by other techniques for bilayer forming POPC; the acyl chain packing in BMP dispersions likely adopts a similar lamellar structure. Hence, these data are not consistent with a
model of BMP$_{18:1}$ as a micellar aggregate. This conclusion is in accordance with light scattering, electron microscopy and fluorescence results indicating BMP$_{18:1}$ forms MLV assemblies(17), and X-ray and molecular dynamics data obtained by Kobayashi showing BMP$_{14:0}$ also forms a stable lamellar aggregate(18).

Figure 4-1. Model membrane perturbants: A) Sodium dodecyl sulfate; B) (S,R isomer) sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol (ammonium salt).

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Figure 4-1. Model membrane perturbants: A) Sodium dodecyl sulfate; B) (S,R isomer) sn-(3-oleoyl-2-hydroxy)-glycerol-1-phospho-sn-3'-(1'-oleoyl-2'-hydroxy)-glycerol (ammonium salt).
Figure 4-2. Positional isomers of spin-labeled lipids: A) 1-palmitoyl-2-stearoyl-(5-DOXYL)-sn-glycero-3-phosphocholine (5-DOXYL); B) 1-palmitoyl-2-stearoyl-(10-DOXYL)-sn-glycero-3-phosphocholine (10-DOXYL).
Figure 4-3. Phase Diagram for SDS and POPC LUVS at 56°C reproduced from linear regression analysis by Seelig and coworkers. Solid lines represent phase boundaries: the symbol (■) indicates samples made with POPC LUVs containing 5-DOXYL labeled lipid and (○) indicates samples made with POPC LUVs containing 10-DOXYL labeled lipid to collect representative spectra for specific regions of the phase diagram in our solubilization experiments.
Figure 4-4. cw-EPR spectra of POPC LUVs with 5-DOXYL spin probe (1 mol%) at room temperature in 20mM HEPES, 100mM NaCl, 0.02% NaN₃ at pH 7.4: a) 27 mM POPC 0mM SDS; b) 22 mM POPC 6mM SDS; c) 11 mM POPC 18mM SDS; d) 7 mM POPC 22mM SDS.

Figure 4-5. cw-EPR spectra of POPC LUVs with 10-DOXYL spin probe (1 mol%) at room temperature in 20mM HEPES, 100mM NaCl, 0.02% NaN₃ at pH 7.4: a) 27 mM POPC 0mM SDS; b) 19 mM POPC 8mM SDS; c) 10 mM POPC 19mM SDS; d) 8 mM POPC 21mM SDS.
Figure 4-6. Various spectral parameters of 5-DOXYL labeled lipid incorporated into POPC LUVs as a function of SDS/Lipid concentration ratio at room temperature in 20mM HEPES, 100mM NaCl and 0.02% NaN₃ and pH 7.4: A) Peak-to-peak width of central derivative line; B) Second spectral moment; C) Order parameter; D) Average normalized fractional intensity. Order parameter error bars are estimated by assuming a 1 G error in the difference between the parallel and perpendicular components of the hyperfine tensor.
Figure 4-7. Various spectral parameters of 10-DOXYL labeled lipid incorporated into POPC LUVs as a function of SDS/Lipid concentration ratio at room temperature in 20mM HEPES, 100mM NaCl and 0.02% NaN₃ and pH 7.4: A) Peak-to-peak width of central derivative line; B) Second spectral moment; C) Order parameter; D) Average normalized fractional intensity. Order parameter error bars are estimated by assuming a 1 G error in the difference between the parallel and perpendicular components of the hyperfine tensor.
Figure 4-8. cw-EPR spectra of BMP with 5-DOXYL spin probe (1 mol%) at room temperature in 5 mM HEPES, 100 mM NaCl, and pH 7.4; a) 40mM total lipid 0mM SDS; b) 40mM total lipid 17mM SDS; c) 40mM total lipid 35mM SDS; d) 40mM total lipid 52mM SDS; e) 40mM total lipid 69mM SDS; f) 40mM total lipid 87mM SDS; g) 40mM total lipid 104mM SDS; h) 40mM total lipid 139mM SDS; i) 20mM total lipid 104mM SDS.
Figure 4-9. Various spectral parameters of 5-DOXYL labeled lipid incorporated into POPC LUVs (■) and BMP aggregates (□) as a function of SDS/Lipid concentration ratio at room temperature in 20mM HEPES, 100mM NaCl, 0.02% NaN₃ at pH 7.4 for POPC LUVs and 5 mM HEPES,100 mM NaCl, at pH 7.4 for BMP aggregates: A) Peak-to-peak width of central derivative line; B) Second spectral moment; C) Order parameter; D) Average normalized fractional intensity. Order parameter error bars are estimated by assuming a 1 G error in the difference between the parallel and perpendicular components of the hyperfine tensor.
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<th>[SDS] mM</th>
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CHAPTER 5
PERTURBATIONS OF LAMELLAR LIQUID CRYSTALLINE ORDER BY BIS(MONOACYLGLYCEROL)PHOSPHATE

**Solid State Phosphorus-31 NMR Investigation of Hydrated Bis(monoacylglycerol)phosphate Aggregation State**

Solid state $^{31}$P NMR experiments were performed at 145.2 MHz using a two pulse Hahn echo sequence with full proton decoupling on single component BMP$_{18:1}$ MLV samples in an aqueous environment. The $^{31}$P NMR measurements allowed us to investigate the head group order and the mesophase structure of hydrated BMP MLV dispersions. Visual inspection of the line shapes of phosphorus NMR spectra provides information about head group orientation and information needed to discriminate among lamellar, H$_{II}$, and isotropic aggregation states(16, 59, 66). Both changes in the span of the chemical shift anisotropy and in the overall line shapes are governed by individual molecular motions, local environment of phosphorus nuclei, and overall aggregate tumbling rate, each of which affect the orientation of the $^{31}$P chemical shift tensor in the phosphate head group with respect to the applied field(16, 67).

The $^{31}$P NMR spectrum of the BMP$_{18:1}$ MLV dispersion shown in Figure 5-1 is narrow ($\Delta\sigma = -11.6$ ppm) and has an overall line shape characteristic of a lamellar mesophase. This finding is in agreement with our previous conclusion in Chapter 4 that BMP forms a stable lamellar structure. This being said, BMP would also then be expected to form stable lamellar structures when mixed with model lipids such as POPC and DPPC. All of the EPR and NMR evidence presented thus far indicates that BMP adopts a lamellar aggregate structure and thus should not exhibit detergent behavior. However, the effect BMP has on model membrane morphology or any possible role BMP may play in late endosomal lipid trafficking or in the hydrolysis of GSLs has yet to be addressed.
In this section of the dissertation, EPR results are presented regarding the interaction of BMP with model membrane lipids in the L$_{α}$ phase. POPC and BMP LUVs of mixed composition were prepared with POPC, varying amounts of BMP$_{18:1}$ or BMP$_{14:0}$ and 1 mol% of spin-labeled lipid (5-DOXYL or 10-DOXYL). These experiments parallel the previous EPR investigations reported in Chapter 4 with single component, lipid LUVs and SDS detergent. EPR spectra shown in Figure 5-2 obtained from POPC/BMP mixed LUVs have the typical anisotropic powder patterns seen previously in Chapter 4. However, the distinct lines shape changes seen in EPR spectra for the 5-DOXYL labeled lipid that occurs when SDS partitions into lamellar aggregates are not detected in POPC/BMP mixed vesicles, indicating that BMP does not solubilize POPC membranes.

To track the changes that occur in the EPR line shapes of the doxyl labeled lipids as a function of BMP concentration, values of $\Delta H_{pp}$ and the order parameter ($S_i$) were determined from the EPR line shapes. These data are plotted as a function of the concentration ratio of BMP to POPC ($C_{BMP}/C_{POPC}$) in Figures 5-3 and 5-4, respectively, and values are listed in Table 5-1. Figure 5-3 shows that the peak-to-peak width of the central derivative line and the order parameter values are constant for both the 5 and 10-DOXYL labeled lipids in POPC/BMP$_{14:0}$ mixed LUVs for concentrations up to 20 mol% BMP$_{14:0}$. Figure 5-4 shows the values of $\Delta H_{pp}$ and order parameter for both the 5 and 10-DOXYL labeled lipids in POPC/BMP$_{18:1}$ mixed LUVs are also constant, within experimental error, over the same concentration range. It is also important to note the average value of the order parameters for the 5 and 10-DOXYL positions are very similar for both BMP$_{18:1}$ and BMP$_{14:0}$. Moreover, these results indicate that mixed composition
LUVs of POPC/BMP (less than 20 mol % BMP) have the same degree of acyl chain order as pure POPC (within experimental error) at both label positions regardless of BMP’s degree of acyl chain saturation or chain length. These results further support our previous claim that BMP does not have classic detergent properties, and indicates BMP does not perturb the acyl chain order of POPC LUVs, at least not on the time scale observed by EPR.

**31P NMR of 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine /Bis(monoacylglycerol)phosphate mixed MLVs**

The conclusions drawn in the previous section indicate that incorporation of BMP into POPC vesicles does not alter the order or packing of the acyl chains as can be observed on the EPR timescale. Here, the effects of BMP on head group orientation were investigated using solid-state $^{31}$P NMR to characterize the interaction of BMP with POPC in hydrated MLVs. Solid state Phosphorus-31 NMR experiments obtained at 145.2 MHz using a two pulse Hahn echo sequence with full proton decoupling were performed on mixed POPC/BMP$_{18:1}$ MLV samples in an aqueous environment to investigate the head group order and mesophase structure. POPC and POPC doped with BMP$_{18:1}$ MLV samples were prepared according to the procedure for LUV preparation but were not extruded (see Chapter 2). All $^{31}$P spectra were aligned so that isotropic value of either axially symmetric fully hydrated POPC MLVs or axially symmetric fully hydrated DPPC MLVs was assigned to 0 ppm, see Figure 3-11.

Recalling Figure 3-11, $^{31}$P spectra for hydrated lamellar aggregates have characteristic powder averaged chemical shift line shapes. The solid state $^{31}$P NMR spectrum of the hydrated single lipid component POPC MLVs at 37°C is shown in Figure 5-5. This spectrum exhibits a classic axially symmetric lamellar line shape with a span of $-46 \pm 1$ ppm, which is in excellent agreement with the reported literature value ($- 46 \pm 1$ ppm)\(^{(68-70)}\). Line shape simulation via software (dmfit) \(^{(71)}\) did not offer any advantage over direct measurement from the experimental
line shapes in estimation of $^{31}$P chemical shift span, therefore all reported span values were measured directly from the experimental data.

According to the model of glycerophospholipid orientation with respect to the normal of a bilayer for DPPC and DPPE; the order parameter of the C$_1$C$_2$ bond vector ($S_{(c1)-(c2)}$ from (72)) obtained from $^2$H NMR ($C_1$ and $C_2$ are glycerol carbons enumerated using the sn naming system see Figure 1-2), $S_{C1C2}$ is 0.66 and defines the tilt angle of the C$_1$C$_2$ axis with respect to the bilayer normal.(72, 73) This wobble freedom reduces the span of the strict axially symmetric powder average for $^{31}$P ($\Delta \sigma \sim -124$ ppm) to a maximum of -82 ppm for hydrated MLVs (16) ($\Delta \sigma = (\sigma_{11} - \sigma_{33})S_{C1C2} = -82$ ppm). However, the experimental value (-46 ppm) is clearly narrower indicating an additional angular offset of the CSA axis from the bilayer normal. The experimental principal value $\sigma_{||}$, is $\sim -30$ ppm for pure POPC (the trace of the CSA is invariant to motion so $\sigma_{\perp} = 16$ ppm) and yields an angle of about 33$^\circ$ from the normal of the bilayer. Again the order parameters are represented as second order Legendre polynomials ($S = \frac{1}{2} (3 \cos^2(\theta) - 1)$). Both of the previous values are in good agreement with current literature; Lorigan and coworkers measured $\sigma_{||} = 30 \pm 1$ ppm and $\sigma_{\perp} = 15 \pm 1$ ppm from oriented POPC MLVs(70), and an angle with respect to the bilayer normal of $\sim 30^\circ$ has been reported for hydrated dipalmitoyllecithin(8, 9).

Figure 5-6 shows $^{31}$P line shapes for various mixtures of POPC/BMP MLVs. Two important observations about mixed POPC/BMP$_{18:1}$ MLVs can be made by visual inspection of the line shapes: 1) the CSA span of the POPC line shape decreases as the negatively charged BMP concentration increases when compared to the span of single component POPC (spectrum a 2) the spectra of the mixed lipids do not appear to be a simple superposition of the single component POPC and single component BMP CSA line shapes. Each of the previous
observations, more clearly illustrated in Figure 5-7, indicates the lipids are interacting (mixing) in the same lamellar structures.

According to work by Seelig and Scherer, the orientation that the phosphate/choline group has with respect to the bilayer normal is sensitive to membrane surface charge(68, 74). Quadrupolar splitting of the α and β carbons located on the choline head group of deuterium labeled POPC and the CSA span of POPC were measured as a function of positive charged dioctadecyldimethylammonium-bromide (2C_{18}N2C_{1}+Br-) and negative charged sodium didodecyl phosphate (2C_{12}PO_{4}Na+) amphiphilic molecules incorporated into POPC MLVs. A linear relationship relating CSA span and mole fraction of 2C_{12}PO_{4}Na+ was determined to an amphiphile mole fraction (χ_b) of 0.3, (Δσ = - 45.6 + 18.7χ_b ppm) and a plateau value of CSA span ~ -36 ppm at high amphiphile concentrations(68). Increased disorder in the phosphate/choline region, indicated by a decrease in the value of the ^{31}P CSA span, implies a larger angular deviation from the bilayer normal(68).

Figure 5-8 shows the change in span of the POPC rich line shape of POPC/BMP mixed MLVs up to a mole fraction BMP of 0.3 (closed squares) and the best linear fit of that data (Δσ = - 45.6 + 16.0χ_{BMP} (dashed line)), however the limiting span value observed by Seelig and Scherer is not obvious in our data due to the noise level near σ_{11}. Line shape simulations may be helpful in determining the limiting span in this case. Comparison of the two regression lines indicates that the phosphate groups of MLVs containing POPC/BMP are interacting and ordered in a similar fashion to the MLVs with the model anionic amphiphile used by Seelig and Scherer. At a mole fraction BMP of 0.3 both regressions predicts an angle of inclination to the bilayer normal of ~36° and a value of Δσ of -40 ppm, in good agreement with our experimental values (θ = 35° and Δσ = –41 ppm)(63). These results indicate that BMP, when mixed with the model
membrane lipid POPC up to 30 mol% imparts a surface charge similar to that of $2\text{C}_2\text{PO}_4\text{Na}^+$ and has a similar effect on the average orientation of the head group with the bilayer normal.

As for the very narrow span (-11.6 ppm) observed for pure BMP structures there are at least two possible explanations: 1) BMP forms LUVs that have a small diameter (highly curved surface), or 2) the phosphorus has a large tilt angle with respect to the normal of the bilayer. Molecular dynamics (MD) simulations indicate that BMP$_{14:0}$ has a bilayer thickness on the order of 4.2 nm and a $20^\circ$ tilt angle with respect to the bilayer normal(28). According to the MD it seems possible that the lamellar structures formed by single component BMP may have a small diameter and thus a narrowed CSA span. This claim should be further substantiated by dynamic light scattering experiments.

![Chemical Shift Spectrum](image)

Figure 5-1. $^{31}$P NMR chemical shift spectrum of BMP$_{18:1}$ MLVs: in 5 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4, at T = 37 °C. The CSA is referenced to the isotropic value of axially symmetric, hydrated POPC MLVs.
Figure 5-2. cw-EPR spectra of POPC/BMP mixed LUVs containing 1 mol% of either 5 or 10-DOXYL spin probe in 5 mM HEPES, 100 mM NaCl, at pH 7.4; A) and C) a) 23 mM POPC 0 mM BMP\textsubscript{14:0}; b) 23 mM POPC 0.2 mM BMP\textsubscript{14:0}; c) 22 mM POPC 1 mM BMP\textsubscript{14:0}; d) 21 mM POPC 2 mM BMP\textsubscript{14:0}; e) 19 mM POPC 5 mM BMP\textsubscript{14:0}; B) and D) a) 20 mM total lipid 0 mM BMP\textsubscript{18:1}; b) 23 mM POPC 0.2 mM BMP\textsubscript{18:1}; c) 22 mM POPC 1 mM BMP\textsubscript{18:1}; d) 21 mM POPC 2 mM BMP\textsubscript{18:1}; e) 19 mM POPC 5 mM BMP\textsubscript{18:1}. 
Figure 5-3. $\Delta H_{pp}$ and $S_i$ of 5 (■) and 10-DOXYL (○) labeled lipid (1 mol%) incorporated into POPC/BMP14:0 mixed LUVs as a function of BMP14:0/Lipid concentration ratio at room temperature in 5mM HEPES, 100mM NaCl and pH 7.4: A) Peak-to-peak width of central derivative line; B) Order parameter. Order parameter error bars are estimated by assuming a 1 G error in the difference between the parallel and perpendicular components of the hyperfine tensor. The error in $\Delta H_{pp}$ is ± 0.2 G based on three independent measurements of single component POPC LUVs.

Figure 5-4. $\Delta H_{pp}$ and $S_i$ of 5 (■) and 10-DOXYL (○) labeled lipid (1mol%) incorporated into POPC/BMP18:1 mixed LUVs as a function of BMP18:1/Lipid concentration ratio at room temperature in 5mM HEPES, 100mM NaCl and pH 7.4: A) Peak-to-peak width of central derivative line; B) Order parameter. Order parameter error bars are estimated by assuming a 1 G error in the difference between the parallel and perpendicular components of the hyperfine tensor. The error in $\Delta H_{pp}$ is ± 0.2 G three independent measurements of single component POPC LUVs.
Figure 5-5. $^{31}$P NMR chemical shift of POPC MLVs in 5 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4, at $T = 37^\circ C$. The CSA is aligned according to the isotropic value of fully hydrated POPC MLVs.
Figure 5-6. $^{31}$P NMR spectra of POPC/BMP18:1 MLVs in 5 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4, at $T = 37^\circ$C. A) a) 278 mM POPC 0 mM BMP; b) 276 mM POPC 1.8 mM BMP; c) 275 mM POPC 2.8 mM BMP; d) 270 mM POPC 8.3 mM BMP; e) 264 mM POPC 13.7 mM BMP f) 249 mM POPC 27.8 mM BMP; g) 224 mM POPC 52.3 mM BMP; h) 192 mM POPC 82 mM BMP; i) 139 mM POPC 133 mM BMP j) 53.8 mM POPC 215 mM BMP; k) POPC 0 mM 42 mM BMP B) shows the same spectra as A) but in reverse order.
Figure 5-7. $^{31}$P NMR spectra of POPC/BMP18:1 MLVs in 5 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4, at T = 37 °C. A) 278 mM POPC 0 mM BMP (solid line) and 192 mM POPC 82 mM BMP (dashed line); B) 192 mM POPC 82 mM BMP (dashed line) and a linear combination of two line shapes with a contribution of 70% pure POPC line shape and 30% pure BMP line shape.
Figure 5-8. CSA span of (POPC/BMP MLVs) as a function of BMP mole fraction (■) and linear fit (Δσ = -45.6 + 16.0χ_{BMP} (dashed line)).
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Table 5-2  Parameters defining order of the 10-DOXYL nitroxide spin-probe in lipid aggregates at room temperature.

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Table 5-3  Values of CSA span for POPC/BMP mixed MLVs at room temperature.

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CHAPTER 6
PERTURBATIONS OF THERMOTROPIC PHASE TRANSITIONS DUE TO
BIS(MONOACYLGLYCEROL)PHOSPHATE

Thermotropic Phase Behavior of 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine
/Bis(monoacylglycerol)phosphate MLVs Investigated by $^2$H NMR

Incorporation of a lipid deuterated in the acyl chain region allows order/disorder to be measured along the length of the apolar region of a MLV assembly. We chose perdeuterated DPPC for this investigation in order to simultaneously probe the effects of BMP on the molecular order at each carbon position along the acyl chain region as well as its effects on thermotropic phase transition temperatures of DPPC(17). Investigations of a thermotropic phase transition are not practical using POPC because the main phase transition of POPC occurs at 0°C and is near the freezing point of water, which makes this type of measurement difficult(43). The following experimental $^2$H NMR data affords information concerning the order of the acyl chain region and is analogous to that obtained from EPR measurements with the n-DOXYL labeled lipids discussed earlier. However, $^2$H NMR operates on a much slower time scale, thus allowing more averaging by the lipid motions. Also, recall that substitution of deuterium for hydrogen atoms in the acyl chain region is only a small structural perturbation when compared to the large nitrooxide labels used for EPR measurements.

$^2$H NMR line shapes as a function of temperature were obtained for DPPC MLVs and DPPC doped with BMP$_{18:1}$ (5 mol%) MLVs in low ionic strength buffer ($I_m < 0.05$ m) and are shown in Figures 6-1 and 6-2. The main phase transition ($T_m$) for DPPC MLVs occurs at ~41.5°C as determined by DSC measurements(29); however, this transition is depressed to ~37.7°C for perdeuterated DPPC MLVs, also determined by DSC(75). A comparison of the line shapes in Figure 6-1 A and B shows evidence that incorporation of BMP into the lamellar structure increases the onset temperature of a thermotropic phase transition relative to single
component DPPC MLVs. This is easily visualized in spectra at 35 – 37 °C in Figure 6-1 A and B. NMR line shapes for MLVs containing BMP are broad and unresolved at 35 and 36°C, indicative of a lamellar gel phase but the spectrum of single component DPPC has already started to narrow and shows resolved terminal methyl peaks (the Pake doublet with the smallest quadrupole splitting) at 36°C, indicating more motional freedom.

At temperatures above the main phase transition of single component DPPC, ²H line shapes (Figure 6-1 B) obtained from mixed DPPC/BMP (5 mol%) MLVs show a broadening of the terminal methyl signal relative to that of DPPC MLVs. Initially we were inclined to interpret this broadening as an indication of restricted mobility and evidence of an interdigitated phase. However, results obtained from EPR experiments, with 16-DOXYL labeled lipid (discussed in the next section), did not confirm our hypothesis and the cause of the broad, unresolved peaks and a molecular level understanding of the structure or dynamics that leads to the broadening of the terminal methyl signal remain unknown.

²H NMR measurements were also performed on DPPC and DPPC/BMP mixed MLVs at near biological ionic strength (I_m ~ 100 m), and the results show quite different effects of BMP on the onset of a thermotropic transition temperature. When the number of counter ions in the buffer is much greater than the number of negative BMP molecules (~15000:1) a decrease in the phase transition temperature is observed when compared to BMP effects in low ionic strength buffer and when compared to single component DPPC in near biological ionic strength buffer. The former can be visualized by comparing the spectra at 35°C of Figure 6-2 A and B. It is clear that for MLVs doped with BMP under physiological buffer conditions a significantly larger portion of the acyl chains are disordered when compared to single component DPPC at 35°C.
These effects on acyl chain order can be seen more clearly by comparing the order parameter \( S_{\text{total}} = S_{\text{mol}} * S_{\text{LD}} \) values (Tables 6-1 and 6-2) for the terminal methyl groups as a function of temperature or the residual quadrupolar splittings as a function of temperature in Figure 6-3. Referring to Chapter 3 the quadrupolar splitting, \( \Delta \nu \), is defined for axially symmetric motion about the lipid’s long molecular axis as \( \Delta \nu = \frac{3}{2} \chi_{Q} S_{\text{total}} \), and \( \chi_{Q} \) is 167 kHz.\(^{(76)}\) \( S_{\text{mol}} \) is assumed to be positive and equal to \(-3S_{\text{CD}}\) and \( S_{\text{CD}} \) is \( \frac{3 \cos^{2}(\theta_{\text{PD}})-1}{2} \).\(^{(46, 77)}\) The angle \( \theta_{\text{PD}} \) describes the orientation angle of the C-D bond vector with respect to the director axis, and \( S_{\text{LD}} \) takes the same functional form as \( S_{\text{CD}} \) but describes the orientation angle \( \theta_{\text{LD}} \) of the director with respect to the applied magnetic field. Computational methods are available to “dePake” or approximate \( S_{\text{LD}} \)\(^{(46, 78, 79)}\) but the signal to noise level of our data is not high enough to obtain this value. However, \( S_{\text{total}} \) should be sufficient to show trends related to the relative order of a specific site in the bilayer.

Figure 6-3 show plots of the terminal methyl order parameter and residual methyl splittings for MLV dispersions of DPPC (filled symbols) and for DPPC/BMP (5 mol\%) (open symbols) Data were collected for temperatures ranging from 35 to 43\(^{\circ}\)C, but order parameters cannot be determined for those spectra in the gel phase. A discontinuity is observed in the trend of order parameter values and residual quadrupolar splittings when plotted as a function of temperature for the terminal methyl peaks in single component DPPC dispersions. This discontinuity occurs for temperatures between 37 and 38\(^{\circ}\)C in Figure 6-3 A and B, and its value coincides nicely with the detected phase transition temperature from the gel to the \( L_{\alpha} \) phase of perdeuterated DPPC in 50 mM phosphate buffer observed by Davis, which were measured by both DSC and \(^{2}\)H NMR.\(^{(75)}\) A comparison of the temperature dependent residual quadrupolar splittings between
single component DPPC and DPPC/BMP mixed MLVs obtained in low salt buffer (5 mM Na\(^+\), Figure 6-3 A) shows that BMP does not affect the order in the acyl chain region at temperatures above 37°C. Additionally, the main phase transition temperature for DPPC/BMP (5 mol%) mixed MLVs is elevated in comparison to that of single component DPPC in low salt buffer.

The order parameter and residual quadrupolar splitting profiles for MLVs in near biological ionic strength buffer (105 mM Na\(^+\), Figure 6-3 B) show that in the presence of BMP the phase transition is significantly depressed but the overall order is similar to single component DPPC at temperatures above 37 °C. Additionally, the terminal methyl group signal is not broadened, thus the broadening of the terminal methyl intensity previously observed at low ionic strength is not an effect of BMP alone. In order to determine the onset temperature of the phase transition for DPPC/BMP (5 mol%) we must collect data for several temperatures below 35 °C.

Overall the total order parameter and residual quadrupolar splitting results lead to values for thermotropic phase transitions that are consistent with literature values; however, the \(^2\)H data should be recollected to obtain higher quality line shapes, and to cover larger temperature range. This will not only afford a better estimation of phase transition temperatures and experimental error but the spectra can be “dePaked” and an order parameter that is independent of orientation with respect to the magnetic field can be assigned to each site along the acyl chains. Some of this work has already begun by others in the Fanucci research group, and the broadening of the terminal methyl signal at low ionic strength has been reproduced but the cause of the broadening is still unexplained.

**Thermotropic Phase Behavior of 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine/Bis(monoacylglycerol)phosphate MLVs Investigated by EPR**

The effects of BMP\textsubscript{18:1} on thermotropic phase transitions of lamellar assemblies was also monitored by EPR spectroscopy with the n-DOXYL labeled lipids incorporated into a model
membrane phospholipid containing fully saturated acyl chains, (DPPC). The lipid containing a single unsaturated acyl chain (POPC) could not be used for these experiments because the main phase transition is near 0°C(43). Given that water freezes near this temperature, DPPC was a more appropriate model lipid for this investigation. 16-DOXYL spin-labeled lipids were used to obtain EPR data parallel to the previous 2H NMR study regarding changes in acyl chain order near the center of the bilayer.

The EPR spectra of DPPC MLVs (containing 1% 16 Doxyl PC) at two different buffer ionic strengths are shown in Figures 6-4 (low with 5 mM Na+) and 6-5 (near biological ionic strength with 105 mM Na+), and demonstrate the temperature dependent order profile of the nitroxide label located at carbon position 16 of the steric acid chain of the label in DPPC MLVs. The broadest splitting of the parallel component of the hyperfine interaction is observed when the hydrated lipids are in the gel phase, indicating the most ordered environment (largest order parameter) of the series. As the temperature is increased, the lipid molecules obtain more kinetic energy, allowing for more motional freedom, which results in a narrowing of the EPR lineshape. Plots of spectral parameters such as the fractional intensity, the peak-to-peak width, and the order parameter as a function of temperature are expected to give characteristic sigmoidal shaped profiles for smooth thermotropic phase transitions(80-83). The phase transition temperature is defined as the inflection point of the sigmoidal shaped line.

A comparison of the temperature dependence of the peak-to-peak width, the second moment, and the order parameter values (Tables 6-3 to 6-4) for DPPC MLVs (1% 16-DOXYL) at low ionic strength (5 mM Na+) and DPPC MLVs (1% 16-DOXYL) at near biological ionic strength (105mM Na+) are shown in Figure 6-6. It is clear that the acyl chain order is almost identical for all temperatures regardless of ionic strength, including the thermotropic phase
transition occurring near 35°C. This transition is assigned as the pre-transition of single component DPPC MLVs, because the breadth of this transition is much larger than the breadth (0.5°C) of the main phase transition obtained by Davis (75, 84). Figure 6-6 D and E show the first derivative of the sigmoidal fit used to determine the inflection point and thus the transition temperature, and the sigmoidal fit overlain on the plot of peak-to-peak line width, respectively. The values of the pre-transition temperatures calculated from each of the parameters defining bilayer order are listed in Table 6-5. Additional data should be collected in the range between 25 and 35°C to verify the shape of the sigmoidal curve and the transitions temperatures.

EPR spectra for 16-DOXYL PC (1 mol%) incorporated into DPPC/BMP18:1 MLV dispersions prepared in a buffer with low ionic strength (Figure 6-7) have similar shapes as those seen previously for this spin probe in single component DPPC dispersions. In additions, values of the peak-to-peak line width, the second moment, and the order parameters are similar to those obtained for single component DPPC. From these data we can conclude that little to no change in order of the acyl chains near the center of the bilayer is caused by BMP18:1 in low ionic strength buffer.

Analysis of the EPR line shapes from 16-DOXYL in DPPC/BMP18:1 MLVs prepared in buffer that mimics biological ionic strength show a lowering (compared to DPPC) of the pre-transition temperature when compared to the value obtained for MLVs in low ionic strength buffer. Specifically, this conclusion can been seen by comparing the line shapes between 35°C and 37°C in Figures 6-7 and 6-8, and it is more clearly evident in the plots of ΔHpp, second moment, and S₁ values as a function of temperature (Tables 6-6 and 6-7) in Figure 6-9. Clearly, this thermotropic phase transition is altered for DPPC/BMP (5 mol%) mixed MLVs in near biological ionic strength buffer with respect to DPPC/BMP (5 mol%) mixed MLVs in low ionic
strength buffer (Figure 6-9 C). This possible depression of a thermotropic transition is consistent with the result determined earlier in this chapter by $^2$H measurements under similar conditions.

The degree of order is similar at 25 °C for the MLVs containing BMP (5 mol%) in near biological ionic strength buffer as can be seen by comparing the peak-to-peak width values in Figure 6-10 C, indicating a similar packing arrangement of the acyl chains in the gel phase. However, a comparison of the peak-to-peak width and order parameter values in Figure 6-10 A, B, and D indicate that DPPC/BMP (5 mol%) mixed MLVs in near biological ionic strength buffer are less ordered than those in low ionic strength buffer. Clearly, experiments should be repeated at 25°C and data should be obtained in the temperature region between 25 and 35°C in order to verify either observation. At temperatures above the chain melting transition ($T_m$) of single component DPPC (~ 41.5°C) (75, 84) the order parameters are identical within experimental error. This result is consistent with the observation that the order parameters for 5 and 10-DOXYL labels in POPC/BMP (5 mol%) mixed MLVs in the $L_\alpha$ phase are not affected by inclusion of BMP into the MLVs.

1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine /Bis(monoacylglycerol)phosphate $^{31}$P NMR

Additional information concerning the effects that BMP has on lipid molecular order can be obtained by examining changes in the $^{31}$P NMR chemical shift line shapes. Experiments performed with $T>T_m$ provide information concerning the order of the PC head group in the $L_\alpha$ phase and can be compared to results obtained in Chapter 5 for mixed POPC/BMP MLVs.

Figure 6-11 shows a comparison between the $^{31}$P chemical shift line shapes of single component DPPC and DDPC/BMP (5 mol%) MLVs at temperatures above (43°C) and below (35°C) the main phase transition temperature of fully hydrated DPPC MLVs. The $^{31}$P spectra obtained from MLVs containing DPPC/BMP are composed of at least two line shapes but are not
simple superpositions of the individual components; as was also seen in the spectra obtained for POPC/BMP mixed MLVs in Chapter 5.

Plots A and B of Figure 6-11 and values listed in Table 6-8 show that both lamellar CSA patterns for DPPC/BMP (5 mol%) MLVs at either low (5 mM Na\(^+\)), or near biological (105 mM Na\(^+\)) ionic strength are narrowed with respect to pure DPPC MLVs at 43 °C. This result is consistent with \(^{31}\)P chemical shift measurements reported earlier for POPC/BMP MLVs in L\(_\alpha\) type assemblies, indicating the choline head group is tilted more toward the bilayer plane (larger angle from the bilayer normal when compared to single component DPPC) in the presence of negative charged amphiphiles. This result also provides more evidence that BMP\(_{18:1}\), even at concentrations below those found in late endosomes, changes the packing parameters of the head group region above the gel to liquid lamellar phase transition temperature of pure DPPC.

However, for chemical shift line shapes obtained at T< T\(_m\) (Figure 6-10 C and D) the head group region of mixed DPPC/BMP (5 mol%) MLVs is only slightly more disordered in low ionic strength buffer but is significantly more disordered in near biological strength buffer. These results are more easily visualized by comparing the plots in Figure 6-12 and values listed in Table 6-8. The increased concentration of positive counter ions at the buffer bilayer interface in near biological ionic strength buffers may in fact increase the choline head group tilt induced by incorporation of BMP into zwitterionic model bilayers.

Figure 6-12 A reports the span of single component DPPC as a function of temperature in low and near biological ionic strength buffers; the reported span values are in agreement within ±2 ppm of literature values for DPPC MLVs hydrated with water. Griffin and coworkers report values of -54 ppm (T = 37°C) and -48 ppm (T = 45°C)(85), while Seelig reports values of -54 ppm (T = 38°C) and -49 ppm (T = 44°C). (73) The values of the CSA span obtained from DPPC
MLVs as a function of ionic strength do not change; hence, these data indicate that there is no significant change in head group orientation in DPPC for the two ionic strength buffers used in our investigation.

A comparison of the span as a function of temperature for DPPC MLVs and DPPC doped with BMP (5 mol%) MLVs in low and at near biological ionic strength buffers are shown in Figures 6-12 B and C, respectively. It is evident that BMP induces a change in the PC head group orientation from that in single component DPPC for both buffer conditions and at all temperatures investigated. Moreover for DPPC/BMP (5 mol%) mixed MLVs in buffer near biological ionic strength the PC head group orientation is more affected than for DPPC/BMP (5 mol%) mixed MLVs at low ionic strength. This conclusion is drawn from the smaller CSA span values in Figure 6-11 D for the MLVs in near biological ionic strength buffer at all temperatures investigated. Further inspection Figure 6-11 D also shows evidence of a phase transition for DPPC/BMP mixed MLVs beginning at 37°C. It is clear that the chemical shift span and thus the head group order is decreasing in a linear fashion for mixed MLVs in both low and near biological strength buffer (the span decreases ~ 1 ppm per °C over the temperature range 37 to 41°C. The onset of this transition is consistent with that obtained from the total order parameter of the terminal methyl group as a function of temperature for mixed MLVs under similar conditions seen in Figure 6-3 B. However, more data points above 43°C are needed to fully characterize this thermotropic phase transition.

It is also interesting to note that as the temperature approaches T_m the values of the CSA span for the PC head group in DPPC/BMP (5 mol%) MLVs appear to rapidly approach -44 ppm. Recall the linear relationship between POPC span and mole fraction BMP obtained previously in Chapter 5 for POPC/BMP mixed MLVs; the predicted span for the POPC CSA span for χ_{BMP} of
5 mol% is -45 ppm. However, additional data points at temperatures above 43 °C are needed to confirm this limiting value. Further investigations at temperatures above the main phase transition and with mole fractions of BMP up to 0.3 would also substantiate the linear relationship between the $^{31}$P chemical shift span and mol fraction of negative amphiphile obtained for POPC/BMP mixed MLVs.

Figure 6-1. $^2$H NMR spectra of A) DPPC and B) DPPC/BMP (5 mol%) mixed MLVs showing the temperature dependence of the phase transition. Lipid samples (275 mM total lipid) were hydrated in a 5 mM HEPES, 0.1 mM EDTA, at pH 7.4.

Figure 6-2. $^2$H NMR spectra of A) DPPC MLVs and B) DPPC/BMP (5 mol%) mixed MLVs showing the temperature dependence of the phase transition. Lipid samples (275 mM total lipid) were hydrated in a 5 mM HEPES, 100 mM NaCl, at pH 7.4.
Figure 6-3. Total order parameter and residual quadrupolar splittings of terminal methyl groups as a function of temperature: A) and C) pure DPPC MLVs (▲) and DPPC/BMP mixed MLVs (△) in 5 mM HEPES, 0.1 mM EDTA, pH 7.4; B) and D) pure DPPC MLVs (●) and DPPC/BMP mixed MLVs (○) in 5 mM NaHEPES, 0.1 mM EDTA, 100 mM NaCl at pH 7.4. The error bars are estimated as approximately the FWHM (± 300 Hz) of the terminal methyl horn.
Figure 6-4. cw-EPR spectra of DPPC MLVs with 16-DOXYL spin probe (1 mol%) as function of temperature: 100mM DPPC, 5mM HEPES, 0.1 mM EDTA, at pH 7.4.

Figure 6-5. cw-EPR spectra of DPPC MLVs with 16-DOXYL spin probe (1 mol%) as function of temperature: 100mM DPPC, 5mM HEPES, 100 mM NaCl, 0.1 mM EDTA, at pH 7.4.
Figure 6-6. Various spectral parameters of 16-DOXYL labeled lipid incorporated into DPPC MLVs as a function of temperature: A) peak-to-peak width; B) second moment; C) order parameter; D) first derivative of sigmoidal fit to $\Delta H_{pp}(T)$ (low ionic strength); and E) $\Delta H_{pp}(T)$ with sigmoidal fit (dashed line) to $\Delta H_{pp}(T)$ (low ionic strength) MLVs in 5mM HEPES, 0.1 mM EDTA, at pH 7.4 (▲); MLVs in 5mM HEPES, 0.1 mM EDTA, 100 mM NaCl at pH 7.4 (●).
Figure 6-7. DPPC/BMP MLVs with 16-DOXYL spin probe as function of temperature at low ionic strength: sample contains 95mM DPPC, 5mM BMP<sub>18:1</sub> 5mM HEPES, 0.1 mM EDTA pH 7.4.
Figure 6-8. DPPC/BMP MLVs with 16-DOXYL spin probe as function of temperature (near biological ionic strength) 95mM DPPC, 5mM BMP_{18:1} 5mM HEPES, 0.1 mM EDTA pH 7.4.
Figure 6-9. Various spectral parameters of 16-DOXYL labeled lipid incorporated into DPPC/BMP (5 mol%) mixed MLVs as a function of temperature: A) peak-to-peak width; B) second moment; C) order parameter; D) first derivative of sigmoidal fit to $\Delta H_{pp}(T)$ (low ionic strength); and E) $\Delta H_{pp}(T)$ with sigmoidal fit (dashed line) to $\Delta H_{pp}(T)$ (low ionic strength) MLVs in 5mM HEPES, 0.1 mM EDTA, at pH 7.4 (Δ); MLVs in 5mM HEPES, 0.1 mM EDTA, 100 mM NaCl at pH 7.4 (○).
Figure 6-10. Various spectral parameters of 16-DOXYL labeled lipid incorporated into DPPC MLVs and DPPC/BMP (5 mol%) mixed MLVs as a function of temperature: A) peak-to-peak width of DPPC MLVs (▲) and DPPC/BMP MLVs (Δ) in 5mM HEPES, 0.1 mM EDTA, at pH 7.4; B) order parameter of DPPC MLVs (▲) and DPPC/BMP MLVs (Δ) in 5mM HEPES, 0.1 mM EDTA, at pH 7.4; C) peak-to-peak width of DPPC MLVs (●) and DPPC/BMP MLVs (○) in 5mM HEPES, 0.1 mM EDTA, ,100 mM NaCl at pH 7.4; D) order parameter of DPPC MLVs (●) and DPPC/BMP MLVs (○) in 5mM HEPES, 0.1 mM EDTA, ,100 mM NaCl at pH 7.4.
Figure 6-11. 31P NMR chemical shift of single component DPPC MLVs (solid line), DPPC/BMP (5 mol%) MLVs (dashed line): A) and C) 5 mM HEPES, 0.1 mM EDTA, at pH 7.4, 5; B) and D) 5 mM HEPES, 0.1 mM EDTA, 100 mM NaCl, at pH 7.4.
Figure 6-12. 31P CSA span of DPPC MLVs and DPPC/BMP (5 mol%) mixed MLVs as a function of temperature: A) DPPC low (▲) and high (Δ) ionic strength; B) DPPC (▲) and mixed DPPC/BMP (5 mol%) (●) both in low ionic strength buffer; C) DPPC (Δ) and mixed DPPC/BMP (5 mol%) (○) both in high ionic strength buffer; D) Mixed DPPC/BMP (5 mol%)low (●) and high (○) ionic strength buffer Error in span = ± 1 ppm.
Table 6-1  Total order parameter values for terminal methyls of d62-DPPC in DPPC MLVs.

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Table 6-2  Total order parameter values for terminal methyls of d62-DPPC in DPPC/BMP (5-mol%) mixed MLVs.

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Table 6-3  Parameters defining order of the 16-DOXYL nitroxide spin-probe in DPPC MLVs in 5 mM Na\(^+\) buffer.

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Table 6-4  Parameters defining order of the 16-DOXYL nitroxide spin-probe in DPPC MLVs in 105 mM Na\(^+\) buffer.

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<td>143</td>
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<td>143</td>
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<td>142</td>
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<td>1.6</td>
<td>142</td>
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<td>144</td>
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<td>1.6</td>
<td>142</td>
<td>0.10</td>
</tr>
<tr>
<td>50</td>
<td>1.6</td>
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Table 6-5  Pre-transition temperatures obtained from 16-DOXYL labeled lipid in various MLV lamellar structures.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta H_{pp}$</th>
<th>$2^{nd}$ Moment</th>
<th>$S_1(\theta)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC (5 mM Na$^+$)</td>
<td>34 °C</td>
<td>36 °C</td>
<td>35 °C</td>
</tr>
<tr>
<td>DPPC (105 mM Na$^+$)</td>
<td>35 °C</td>
<td>36 °C</td>
<td>35 °C</td>
</tr>
<tr>
<td>DPPC/BMP (5 mol%) (5 mM Na$^+$)</td>
<td>35 °C</td>
<td>35 °C</td>
<td>35 °C</td>
</tr>
<tr>
<td>DPPC/BMP (5 mol%) (105 mM Na$^+$)</td>
<td>34 °C</td>
<td>33 °C</td>
<td>29 °C</td>
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Table 6-6  Parameters defining order of the 16-DOXYL nitroxide spin-probe in DPPC/BMP (5 mol%) mixed MLVs in 5 mM Na⁺ buffer.

<table>
<thead>
<tr>
<th>T °C</th>
<th>ΔH&lt;sub&gt;pp&lt;/sub&gt;</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; Moment</th>
<th>S₁(θ)</th>
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</tr>
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<td>35</td>
<td>2.5</td>
<td>168</td>
<td>0.21</td>
</tr>
<tr>
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<td>2.2</td>
<td>158</td>
<td>0.14</td>
</tr>
<tr>
<td>37</td>
<td>1.9</td>
<td>142</td>
<td>0.11</td>
</tr>
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<td>1.8</td>
<td>138</td>
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<td>1.8</td>
<td>139</td>
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<td>138</td>
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Table 6-7 Parameters defining order of the 16-DOXYL nitroxide spin-probe in DPPC/BMP (5 mol%) mixed MLVs in 105 mM Na\(^+\) buffer.

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<th>2(^{nd}) Moment</th>
<th>(S_i(\theta))</th>
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<tbody>
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<tr>
<td>T °C</td>
<td>Δσ (ppm)</td>
<td>Δσ (ppm)</td>
<td>Δσ (ppm)</td>
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</tr>
<tr>
<td></td>
<td>DPPC 5 mM Na⁺</td>
<td>DPPC 105 mM Na⁺</td>
<td>DPPC/BMP 5 mM Na⁺</td>
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CHAPTER 7
SUMMARY AND CONCLUSIONS

The question posed in the beginning of this dissertation was “What effect does BMP have on model membrane morphology?” In order to answer this question membrane solubilization was first investigated using SDS detergent, since our original hypothesis was that BMP might have detergent properties, such as micelle formation and bilayer solubilization. However, we have presented data that contradicts our original hypothesis and further corroborates experimental results obtained by others that BMP self-assembles into a lamellar aggregate structure in an aqueous environment. Furthermore, our solubilization results indicate that the acyl chain packing motifs and the order parameters of BMP_{18:1} are similar to those found in POPC MLVs in that BMP is solubilized by SDS in a similar fashion. Also, the line shapes obtained from the 5-DOXYL labeled lipid are almost identical in either single component POPC or BMP LUVs indicating that the acyl chains pack in a similar manner.

Initial characterization of the interaction of the BMP lipid incorporated into model PC membrane structures indicated that negatively charged BMP causes choline head groups to tilt away from the bilayer normal as a function of BMP concentration as seen in other negatively charged amphiphile/PC membrane mixtures. This is a direct indication that BMP modulates the surface charge and molecular interactions in the vicinity of the polar region of these mixed model membrane systems.

Considering the results presented in this body of work there are some key experiments that should be revisited in order to further our understanding of lipid membrane structures and give us greater detail regarding the current observations.

$^2$H NMR experiments should be repeated for both DPPC and DPPC/BMP MLVs as a function of temperature with a larger deuterated lipid component. Increasing the number of
deuterium-enriched lipid molecules will increase the signal to noise ratio, allowing the spectra to be dePaked so the full order parameter profiles for each system can be reported. Also, spectra should be collected at a temperature well above \( T_m \) for DPPC, e.g. \( 55^\circ C \), to ensure that a single \( L_C \) component is present. This is necessary for proper dePaking and peak assignment.

Data points in the temperature region between 25 and 35\(^\circ\)C should be acquired for all of the EPR experiments involving the 16-DOXYL spin-labeled lipids. These data points will be useful in obtaining a better fit of the sigmoidal line shape and thus a better estimate of the thermotropic phase transition temperatures. Also, selected data points should be recollected to establish the error in the measurements using the 16-DOXYL probe.

Each EPR solubilization experiment should be repeated initially using only the 5-DOXYL labeled lipid. The preliminary results of these experiments seem to be very significant and the data should be obtained in triplicate and extended to both smaller and larger SDS/Lipid ratios.

Other useful experiments would include systematic studies of size distributions between POPC and POPC/BMP mixed vesicles and MLVs using light scattering techniques, confirmation of mesophase by cryogenic electron microscopy, and a comparison of these and results obtained previously with those obtained from mixed MLVs and LUVs containing negatively charged phosphatidylinositol.
LIST OF REFERENCES


44. K. Gawrisch. Buffer preparations in D2O (Private Communication 2007).


BIOGRAPHICAL SKETCH

Chad Mair was born on December 29, 1975 in Evansville, Indiana. He spent his childhood years in Owensville, Indiana where he attended Owensville Elementary. After completing secondary education, at Gibson Southern High School, he began his undergraduate education in Evansville at the University of Southern Indiana. Chad received a Bachelor of Science degree in chemistry from USI in 2001. His graduate education started at the University of Florida in the area of physical chemistry under the supervision of Dr. Valeria Kleiman. He spent four years working in the field of ultrafast laser spectroscopy on hyperconjugated polymers. Chad then began working on magnetic resonance spectroscopy of model biological membranes under the supervision of Dr. Gail E. Fanucci.