NMR Studies of Liquid Disordered and Liquid Ordered Phase Coexistence in Model Membranes

by

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ABSTRACT

NMR STUDIES OF LIQUID DISORDERED AND LIQUID ORDERED PHASE COEXISTENCE IN MODEL MEMBRANES

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University of Guelph, 2016  Professor James H. Davis

Biological membranes separate the cell from the environment. A fundamental understanding of multi-component model membrane systems may provide insight into the phase behaviour and the relationship between the lipids, proteins, water and temperature in biological membranes containing phase separated domains or ‘rafts’. Model membranes composed of saturated and unsaturated phospholipids with cholesterol can exhibit coexisting liquid disordered ($\ell_d$) and liquid ordered ($\ell_o$) phases. Peptides can modify the phase behaviour of these model membranes and may also partition preferentially into either fluid phase. Several model membranes were investigated using $^2$H NMR to determine the phase behaviour of lipid bilayers with and without peptides.

Critical fluctuations have been observed in systems exhibiting $\ell_d$-$\ell_o$ coexistence. Multilamellar dispersions of DOPC/DPPC/cholesterol exhibit critical phase behaviour that agrees with systems belonging to the 3D Ising universality class. Coexisting $\ell_d$-$\ell_o$ phases were observed in magnetically aligned DMPC/cholesterol/DCPC bicelles with choles-
terol mole fractions ≥0.13. DPOPC/DMPC/cholesterol/DCPC or DOPC/DPPC/cholesterol/DHPC bicelles also showed two fluid phase coexistence. 2D exchange experiments were used to study the exchange of lipids between the \( \ell_d \) and \( \ell_o \) phase domains. DPOPC/DMPC-\( \text{d}_{54} \)/cholesterol multilamellar dispersions showed broadening due to lipids exchanging between the two phases. Corresponding bicelles however did not show exchange. The presence of the short chain lipid may move the sample away from a critical composition eliminating the exchange phenomena.

Multilamellar dispersions of DOPC/DPPC/cholesterol with gramicidin A resulted in very broad and weak \(^2\text{H} \) signals and did not provide evidence of preferential partitioning into either of the two fluid phases. Two short peptides, Conolysin-Mt1 and ALGA, were studied in aligned lipid bilayer samples. The lipid chain order in DMPC/DCPC bicelles was affected slightly by Conolysin-Mt1. DPOPC/DMPC/cholesterol with ALGA on glass slides showed that some of this peptide is undergoing rapid axial reorientation while the rest is in a powder environment. Preferential partitioning of ALGA into either the \( \ell_d \) or \( \ell_o \) phase was not observed in these experiments.
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<tr>
<td>$\ell_d$</td>
<td>liquid disordered</td>
</tr>
<tr>
<td>$\ell_o$</td>
<td>liquid ordered</td>
</tr>
<tr>
<td>CP</td>
<td>cross-polarization</td>
</tr>
<tr>
<td>DCPC</td>
<td>1,2-dicaproyl-$sn$-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DHPC</td>
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<td>chain methyl-deuterated DPPC</td>
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<td>G-protein coupled receptor</td>
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<tr>
<td>GUV</td>
<td>giant unilamellar vesicle</td>
</tr>
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<td>magic angle spinning</td>
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<td>SM</td>
<td>sphingomyelin</td>
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<tr>
<td>TFE</td>
<td>trifluoroethanol</td>
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Chapter 1

Introduction

1.1 Biological Membranes

The cell membrane is vital to cell function and protection. It separates the cell from the outside world and functions as a semi-permeable barrier allowing required ions and molecules into the cell, and keeping unwanted molecules out of the cell. The cell membrane also plays an important role in signalling pathways and the interactions of cells with one another. Biological membranes are complex mixtures of lipids, sterols, and proteins. The current picture of the membrane is based on the fluid mosaic model which was presented by Singer and Nicolson in 1972 [1]. Figure 1.1 shows an illustration of a cell membrane with various types of proteins.

Membranes are complex and dynamic systems, in which motions occur transversely across the membrane as well as laterally in each leaflet allowing for interactions among proteins and between lipids and proteins. The two leaflets of the bilayer do not necessarily have the same composition and in nature the leaflets of the plasma membrane are not identical. Certain proteins, lipids, and carbohydrates are required on the one side rather than the other for cell processes. For example, in the case of the plasma membrane, most of the sphingolipids are in the outer leaflet [3].
Membrane proteins play critical roles in biological processes acting as channels, receptors, enzymes and anchors. Proteins can be associated with the membrane in several different ways. Integral transmembrane proteins span the two leaflets of the bilayer, and peripheral proteins can be associated with one leaflet of the bilayer. There are also some proteins that have fatty acid chains and link to the membrane via these chains. Since the membrane isolates the inside of the cell from the rest of the environment, interactions with the cell begin at the cell membrane. Sensory systems depend on receptor proteins which may be activated by different types of stimuli such as chemicals, light, and forces. Some membrane proteins function as channels which regulate the flow of ions and molecules such as water and amino acids into and out of the cell as required. In excitable cells, voltage-gated ion channels are responsible for the propagation of action potentials.
Lipids also have essential roles in cellular functions and can act on the proteins in two ways. First, there can be specific protein-lipid interactions which depend on the specific chemical and structural properties of the lipids including the head group, backbone, chain length, degree of unsaturation, chirality, and chelating properties. Second, general membrane properties can affect the proteins and membrane functions through the fluidity of the membrane, bilayer thickness, shape, surface charge, and packing properties [4].

1.2 Lipids, Cholesterol, and Proteins

Lipids are diverse and can be divided into classes based on their composition. Lipids are amphiphilic molecules that are made up of three parts: a head group, a backbone, and hydrocarbon chain(s). Common classes of lipids in eukaryotic plasma membranes are glycerophospholipids, sphingolipids, and sterols. In glycerophospholipids, the head group is attached to the two hydrocarbon chains via the backbone which is a glycerol 3-phosphate group. There are several possible head groups such as choline, serine, and ethanolamine. The variety of lipids is further increased due to the numerous chain lengths and the possibility of both saturated and unsaturated hydrocarbon chains. Lipids may have one hydrocarbon chain, two matching hydrocarbon chains, or two different hydrocarbon chains. Sphingolipids are made up of a sphingosine which has a long hydrocarbon chain, and a second hydrocarbon chain which is connected to the sphingosine via an amide bond. Sphingomyelins are sphingolipids with a phosphatidylcholine head group. Figure 1.2 shows the general structure of glycerolipids, sphingolipids and cholesterol.

Many of the lipids found in biological membranes have two hydrocarbon chains, which
Figure 1.2: Structure of glycerolipids, sphingolipids and cholesterol. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, Sprong et al. [5], copyright (2001)
Table 1.1: Lipid Composition (in mol %) of Major Lipids in Selected Biomembranes [7]

<table>
<thead>
<tr>
<th>Membrane</th>
<th>PC</th>
<th>PS + PE</th>
<th>SM</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human erythrocyte plasma membrane</td>
<td>21</td>
<td>29</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>Human neuron myelin membrane</td>
<td>16</td>
<td>37</td>
<td>13</td>
<td>34</td>
</tr>
<tr>
<td>E. coli plasma membrane</td>
<td>0</td>
<td>85</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rat inner mitochondrial membrane</td>
<td>45</td>
<td>45</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Rat outer mitochondrial membrane</td>
<td>34</td>
<td>46</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

makes them generally cylindrical in shape. As a result, bilayers are readily formed with the hydrophilic head groups exposed to the surroundings while the hydrophobic tails of each leaflet are contained within the bilayer [6]. The composition of each leaflet of a bilayer need not be the same; in fact in plasma membranes almost all of the sphingolipids are restricted to the outer leaflet [3, 7, 4]. Table 1.1 gives the compositions of several biomembranes in mol % of the major lipid components of biomembranes [7, 4]. The lipids that are included in this data are phosphatidylcholine (PC), phosphatidylethanolamine and phosphatidylserine (PE + PS), sphingomyelin (SM), and cholesterol. PC and SM are primarily found on the exoplasmic side of the membrane, PE and PS are primarily found on the cytosolic side of the membrane, and cholesterol is found in both leaflets.

Sterols have a unique structure that is based on a fused four-ring core. Cholesterol is a common component of eukaryotic cell membranes and regulates the fluidity of the membrane through interactions with the hydrocarbon chains of the other lipids. Sterols are also often used as hormones or signaling molecules in biology [8]. Cholesterol has
a similar basic structure to other sterols with three 6-carbon rings and one 5-carbon ring. The head group is a hydroxyl and is attached on one side of this ring-structure and the hydrocarbon chain tail group is attached on the other side of the ring-structure [8].

About one third of genes code for membrane proteins [9], but there are challenges associated with determining the structures of these proteins. They are made up of amino acid building blocks and their primary structure is the linear amino acid sequence that is unique to the protein. Amino acids bind to one another through peptide bonds. A peptide bond occurs between the carboxyl terminal of one amino acid and the amino terminal of the next. These amino acid sequences fold into various configurations or secondary structural elements, of which the most common are the $\alpha$ helix and $\beta$ sheets. There are also random coils and other types of helices. The overall conformation of the protein is the tertiary structure in which the helices, beta sheets and/or loops are stabilized by interactions between the amino acid side chains with one another, the backbone and surrounding lipids in the case of membrane proteins. Some proteins function as oligomers which are made up of individual proteins (monomers). The amino acid sequence determines the structure of the protein and the structure determines the function. Any mistakes in folding a protein can result in the protein not performing as expected. Protein misfolding has been implicated in several human diseases including type 2 diabetes, Parkinson’s disease and Alzheimer’s disease [10].

Integral membrane proteins have two basic motifs, helical bundles and beta barrels. G-protein coupled receptors (GPCRs) are a large family of proteins that play many key roles in cell function including regulating ion channels and signal transduction. These proteins have a common seven transmembrane helical bundle structure, however, their amino acid
sequences vary greatly [7, 11]. Visual rhodopsins in multicellular organisms are GPCRs whereas rhodopsins in bacteria often function as proton pumps. Rhodopsins have seven transmembrane $\alpha$-helices which are connected by short interhelical loop regions. Retinal is found inside the bundle and photoisomerizes upon the absorption of a photon causing changes in the protein’s conformation.

Porins are beta barrel proteins which form channels in the membrane which are large enough to allow for passive diffusion of specific molecules to occur. They are trimers which are made up of three identical subunits that each contain 16 $\beta$ strands. These proteins have a hydrophobic exterior which interacts with the lipid bilayer and a hydrophilic interior (channel) which allows for the passage of small hydrophilic molecules such as disaccharides. Porins are found in the outer membrane of many bacteria (especially Gram-negative bacteria), and the membranes of mitochondria and chloroplasts [7].

Peripheral proteins do not interact with the hydrophobic core of the membrane directly, instead they interact with other integral membrane proteins or the lipid head groups. G-proteins (guanine nucleotide-binding proteins) are examples of peripheral proteins. There are small monomeric G-proteins which interact with the membrane and can have lipid modifications. Heterotrimeric G-proteins have $G\alpha$, $G\beta$, and $G\gamma$ subunits and are activated by changes in the receptor protein to which they associate.

Since structure often determines the function of a protein, in order to understand a protein it is important to know the structure. Due to the fact that membrane proteins are associated with the membrane and depend on their lipid environment, the most popular method of protein structure determination, X-ray crystallography, is often not possible for these proteins because they do not form crystals or may not be in their native/functional
form in crystals. Nuclear magnetic resonance (NMR) and cryo-electron microscopy can also be used to determine protein structures. Other biophysical techniques such as small angle X-ray scattering, neutron scattering, circular dichroism, and Fourier transform infrared spectroscopy can provide some information about the structure of proteins, including the orientation in the membrane and secondary structure elements. It can also be advantageous to combine results from different techniques when determining protein structures, this is often done with crystallographic and NMR results. In many cases, the conditions under which the structure of a protein is determined are not the native conditions of that protein. This can lead to differences in the determined structure from the active structure especially in the case of membrane bound proteins whose structures are sometimes determined in the absence of a native-like lipid environment. Solid state NMR provides a technique which allows for membrane proteins to be studied in a native-like environment. In most cases simplified model membranes are used, however some work is being done on proteins in cell membranes [12]. Another important consideration for studying proteins in membranes is temperature which affects the properties of the lipid bilayer. The effect of temperature on model membrane systems is a focus of the current work.

1.3 Model Membranes

Model membranes can have a very simple composition, with a single type of lipid, or be more complex containing multiple types of lipids, cholesterol, and peptides or proteins. There are many different ways to prepare model membrane samples for NMR studies. These include multilamellar dispersions, magnetically oriented lipid bilayers (bicelles),
and mechanically oriented samples (on glass plates for example). Multilamellar dispersions produce powder pattern NMR spectra and are often referred to as powder samples despite the fact that they are fully hydrated bilayer samples. Powder in this sense refers to the fact that all orientations are equally likely. Static NMR spectra are significantly simpler for oriented samples than for powder samples because they contain a single orientation rather than a distribution of all possible orientations. The shapes of some typical NMR spectra are presented in Chapter 2.

Magnetically aligned lipid bilayers or bicelles align spontaneously when placed in an external magnetic field. Bicelles can be made by mixing a long chain phospholipid with a short chain phospholipid in a molar ratio known as q (mol long chain lipids/mol short chain lipids) [13, 14]. The morphology of the lipid particles found in these orientable phases has been the subject of much discussion and depends on the temperature and variables of sample composition including the ratio q, and buffer concentration/water content in the sample [15, 16, 17, 18]. At low q values, q ≤ 2.5, fast tumbling bicelles can be formed. Magnetically aligned bilayers occur for moderate values, ~ 2.5 < q <~ 7.5. These bicelles can exhibit different morphologies depending on the water content and q value including the commonly pictured disk-shaped bicelles, chiral nematic ribbons, multilamellar vesicles, and perforated lamellae [19]. The disk shaped bicelles are thought to have the long chain lipid making up the flat part of the disk while the short chain lipids are found on the edges where higher curvature is required. Similarly, in the perforated lamellae the holes are lined with the short chain lipids due to the curvature at these points as shown in Figure 1.3 [20]. At high q values, q ≥ 7.5, there is relatively little short chain lipid and multilamellar vesicles are formed [19]. A very commonly used combination of long and short chain
lipids for bicelle preparations is 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC or di(14:0)PC) with 1,2-dicaproyl-sn-glycero-3-phosphocholine (DCPC or di(6:0)PC) (often referred to in the literature as DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine)). Bicelles are discussed in more detail in Chapter 7.

![Common bicelle morphologies, disks and perforated lamellae. Reprinted with permission from Prosser et al. [20]. Copyright (2006) American Chemical Society.](image)

### 1.4 Phase Separation in Lipid Mixtures

The amphiphilic nature of lipids results in their self-assembly into a wide variety of different structures in water depending on their overall shape and the water concentration. It is possible to predict the types of structures that are most likely going to be formed by a given lipid by calculating the lipid’s packing parameter: $V/a_o l_c$ where $V$ is the volume of the lipid, $a_o$ is the optimal surface area of the headgroup, and $l_c$ is the chain length [6]. Lipids with a large headgroup or lipids with a single hydrocarbon chain (lysolipids) have
an overall cone shape (packing parameter $< \sim 1/3$) and tend to form micelles and the hexagonal H$_f$ phase. Lipids with a small head group such as phosphatidylethanolamines, phosphatidic acids and phosphotidylserines, have an inverted cone shape (packing parameter $> 1$) and will form various phases including inverted micelles, the hexagonal H$_{II}$ phase and cubic phases. Lipids with similar head group and hydrocarbon chain cross sectional areas have a cylindrical shape (packing parameter $\sim 1$) and form bilayer (lamellar) phases which include different fluid/liquid crystalline phases and gel phases. Mixtures of lipids of different shapes can also form bilayer phases as seen in the formation of cell membranes. Cone and inverted-cone shaped lipids are considered non-bilayer forming lipids and when incorporated into a bilayer will induce stress into the system depending on the curvature.

A phase transition occurs at the chain melting temperature for pure phospholipid systems. This transition temperature, $T_m$, depends on the hydrocarbon chain length and the degree of unsaturation. As the chain length increases, the transition temperature increases. As the degree of unsaturation (number of double bonds) increases, the transition temperature decreases. If multiple lipid species are mixed, different phases and coexisting phases can occur due to the different natures of the lipids involved. Gel phases occur below $T_m$, while liquid crystalline (fluid) phases occur above $T_m$. Many of the different morphologies and phases that lipids can form are shown in Figure 1.4.

The fluid phases and fluid phase coexistence are of interest in the present work. The liquid crystalline (L$_{a}$) or liquid disordered ($L_d$) phase is characterized by highly flexible hydrocarbon chains and is the most common fluid phase. Cholesterol affects the fluidity of the membrane, acting as a bidirectional regulator of membrane fluidity. At high temperatures, cholesterol stabilizes the membrane favouring a less fluid, more ordered structure.
Figure 1.4: Examples of lipid phases. I. Lamellar phases: (A) subgel, Lc, (B) gel, Lβ, (C) interdigitated gel, Lβint, (D) gel, tilted chains, Lβ′, (E) rippled gel, Pβ′, and (F) liquid crystalline, Lα. II. Micellar aggregates: (G) spherical micelles, M1, (H) cylindrical micelles (tubules), (J) disks, (K) inverted micelles, MII, and (L) liposomes. III. Non-lamellar liquid-crystalline phases: (M) hexagonal phase H1, (N) inverted hexagonal phase HII, (O) inverted micellar cubic phase QII[MI], and (P, Q, and R) bilayer cubic phases. Reprinted from Koynova and Tenchov [21] CC BY http://creativecommons.org/licenses/by/4.0/. 
than the liquid crystalline phase. At low temperatures, the presence of cholesterol between the chains of the other lipids prevents them from clustering together to form the gel phase. In some cases, when phospholipids are mixed with cholesterol, a more ordered fluid phase known as the liquid ordered ($\ell_o$) phase can be observed at certain temperatures and compositions. Cholesterol preferentially interacts with saturated lipids so it is possible to get domains which are cholesterol and saturated lipid-rich ($\ell_o$), and regions which are cholesterol-poor and are rich in unsaturated lipids ($\ell_d$) [22, 23, 24, 25]. The $\ell_d$ and $\ell_o$ phases are depicted in Figure 1.5.

Figure 1.5: The $\ell_d$ and $\ell_o$ fluid phases of lipid membranes. Note that the cholesterol-rich $\ell_o$ phase with the stiffened hydrocarbon chains is thicker than the cholesterol-poor $\ell_d$ phase.

$^2$H NMR is often chosen for studies of lipid phase behaviour since changes in the molecular motion and orientational order of the phospholipid chains can be observed via the deuterium quadrupolar splittings [26]. The phase of the membrane can often be easily determined from the shape and width of the spectrum. Quantitative analysis such as the calculations of the moments of the spectrum or orientational order parameters ($S_{CD}$) can
be performed as well. The quadrupolar splitting is related to the amount of molecular motion, splittings are smaller for fluid phases that are highly mobile and broader for gel phases where motions are significantly slower [27]. Figure 1.6 shows three spectra of a model membrane system composed of 1,2-di-(d$_{31}$)palmitoyl-sn-glycero-3-phosphocholine (DPPC-d$_{62}$) and cholesterol in a molar ratio of 75:25. Part (a) shows a typical powder pattern for the liquid disordered ($\ell_{id}$) fluid phase. Part (b) shows the liquid ordered ($\ell_{oi}$) phase; note the inequivalence of the methyl groups (around ±5 kHz) in this phase. Part (c) shows the spectrum characteristic of the gel phase membrane and is broad with few distinct peaks which is characteristic of the slow motions and limited chain mobility in the gel phase [25].

$^2$H NMR has been used to investigate the phase behaviour of many different lipid mixtures. Phase diagrams for binary lipid or lipid/sterol mixtures that have been established using $^2$H NMR alone or in combination with another technique include that of DP-PC/cholesterol [24], DMPC/1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) [28], N-(lignoceroyl)galactosylceramide/1-stearoyl,2-oleoyl-PC (SOPC) [29], 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE)/cholesterol [30], and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)/DPPC [31]. Phase diagrams for ternary lipid and cholesterol mixtures have also been determined. Examples of ternary systems for which phase diagrams have been established using $^2$H NMR results include 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine (POPC)/sphingomyelin (SM)/cholesterol [32, 33, 34], DOPC/DPPC/cholesterol [35, 25], palmitoyl,2-elaidoyl-PC (PEPC)/SM/cholesterol [36], and 1-palmitoyl,2-dodecahexaenoyl-PC (PDPC)/SM/cholesterol [36]. Other chemical and physical techniques such as differential scanning calorimetry, fluorescence depolarization, and
electron spin resonance can be used to study lipid systems. A complete survey of lipid properties including phase transition temperatures and phase diagrams established prior to 2013 can be found in Marsh’s *Handbook of Lipid Bilayers, Second Edition* [37].

Phase separation in ternary lipid/cholesterol mixtures is readily observed using $^2$H NMR spectroscopy. These coexisting fluid phases can also be seen using fluorescence

\[\text{Figure 1.6: Static }^2\text{H NMR spectra of multilamellar dispersions of DPPC-d}_{62}\text{ and cholesterol (75:25) in (a) the } \ell_d \text{ phase, (b) the } \ell_o \text{ phase, and (c) the gel phase. Reprinted from Davis et al. [25] with permission from Elsevier.}\]
microscopy. Figure 1.7 [38] shows the onset of the $\ell_d$-$\ell_o$ fluid phase coexistence in giant unilamellar vesicles (GUVs) of 32:48:20 mol % DOPC/DPPC/cholesterol as the temperature is lower from 36.5 °C in (a), to 35.7 °C in (b), to 35.5 °C in (c). At the highest temperature, the fluorescence intensity is uniform and the vesicles are in the $\ell_d$ phase region. The fluorescent probe used preferentially partitions into the $\ell_d$ phase. As the temperature is lowered, fluctuations in the fluorescence intensity are observed and some of these are indicated by the arrows in part (b). As the temperature is lowered further, these dark regions coalesce into larger domains. In part (c) the two coexisting fluid phases can be seen. The light regions are in the $\ell_d$ phase while the dark regions are in the $\ell_o$ phase.

Davis et al. and Veatch et al. have presented phase diagrams for the ternary mixtures of DOPC, DPPC and cholesterol using deuterium NMR spectroscopy [35, 25] and fluorescence microscopy [35]. The phase diagrams for DOPC/DPPC/cholesterol contain a region of fluid-fluid phase coexistence. The coexisting phases are the liquid disordered and liquid ordered phases. An isothermal plane from the DOPC/DPPC/cholesterol phase diagram by Davis et al. [25] is shown in Figure 1.8. The $\ell_d$-$\ell_o$ phase coexistence region is indicated in blue and the critical point is indicated by a magenta star in this diagram. The two sides of the two phase region of the phase diagram are linked by straight lines called ’tie-lines’. The $^2$H NMR spectra in the $\ell_d$-$\ell_o$ coexistence region can be decomposed into the same two end-point spectra via pair-wise subtractions [39]. If two samples with different compositions lie on the same tie-line, then at temperatures where these samples are in the $\ell_d$-$\ell_o$ coexistence region the compositions of the end-points can be determined. This technique has been used to determine the phase diagrams for DOPC/DPPC and DOPC/DPPC/cholesterol model membranes [25, 31]. Critical points occur where the coexisting phases become
Figure 1.7: Confocal microscopy images showing the onset of the $\ell_d$-$\ell_o$ fluid phase coexistence in GUVs of 32:48:20 mol % DOPC/DPPC/cholesterol as the temperature is lower from (a) 36.5 °C, to (b) 35.7 °C, and finally to (c) 35.5 °C. The arrows in (b) indicate the fluctuating fluorescence intensity, the $\ell_o$ domains are dark in (b) and (c) because the fluorescent probe preferentially partitions into the $\ell_d$ phase. Reprinted from Juhasz et al. [38] with permission from Elsevier.
identical and the transition between the phases is thus continuous and the tie-line disappears. There is a line of critical points in the phase diagrams for ternary lipid/cholesterol mixtures, specifically mixtures of DOPC/DPPC/cholesterol, where the $\ell_d$ and $\ell_o$ phases become indistinguishable [25]. Critical phenomena are discussed in more detail in the next section.

The phase behaviour of model membrane systems, especially liquid disordered - liquid ordered fluid phase coexistence in these systems is of interest because of the question of whether or not functional lipid rafts are present in biological membranes. In 1997 Simons and Ikonen proposed lipid rafts to describe membrane subcompartmentalization for various cellular functions including endocytosis and signalling [40]. The current definition of membrane rafts is that they are sterol and sphingolipid-rich domains which
are small (10-200 nm), heterogeneous and dynamic. These rafts may be stabilized to form larger systems through specific lipid-lipid, lipid-protein, and protein-protein interactions [3, 41, 42, 43, 44, 45].

## 1.5 Introduction to Critical Phenomena

The properties of systems close to the critical point of a continuous phase transition are independent of the microscopic details of the material undergoing the transition. Rather, the phase behaviour can be classified by features such as the symmetry of the Hamiltonian, and the dimensionality of the system. Systems which have critical phenomena that behave in the same way belong to the same universality class [46]. For example, multilamellar dispersions of ternary lipid and cholesterol mixtures appear to belong to the 3D Ising universality class as do uniaxial ferromagnets and binary liquids. For all of these systems, as a critical point is approached, the specific heat, susceptibility, order parameter, and correlation length for the phase transition all vary with temperature according to a power law of the form

\[ \left( \frac{T - T_c}{T_c} \right)^n \]  

(1.1)

where \( n \) is the relevant critical exponent for the quantity. Models that can be used to describe critical behaviour include mean field theory, the 2D Ising model, and the 3D Ising model. Each universality class results in a specific set of values for the critical exponents [47, 48] which are the same for all systems belonging to that class. Of particular interest in the investigation of the critical behaviour of ternary lipid/cholesterol mixtures are the 2D and 3D Ising models. The critical exponent, \( n \), is \(-\alpha_c\) for the specific heat,
$-\gamma_c$ for the susceptibility, and $\beta_c$ for the order parameter for the phase transition. An order parameter is a physical quantity that has a value of zero above the phase transition temperature, and a non-zero value below the transition. The order parameter allows changes in the properties of the system that occur as it passes through the phase transition to be described quantitatively. In both the 2D and 3D cases, these exponents follow the relationship $\alpha_c + 2\beta_c + \gamma_c = 2$, but the values of these critical exponents differ in the two cases [49]. For the 2D Ising model, $\alpha_{2D} = 0$ (the specific heat diverges logarithmically), $\beta_{2D} = 0.125$, and $\gamma_{2D} = 1.75$. In the case of the 3D Ising model, $\alpha_{3D} = 0.11$, $\beta_{3D} = 0.325$, and $\gamma_{3D} = 1.24$. The correlation length ($\xi$) describes how far the fluctuations extend around the average value of the physical observable and gives a characteristic length scale. The correlation length varies according to the critical exponent $\nu_c$ which is related to the critical exponent for the specific heat and the dimensionality, $d$, of the system as $2 - \alpha_c = \nu_c \times d$. For the 2D Ising model $\nu_{2D} = 1$ and for the 3D Ising model $\nu_{3D} = 0.63$ [49, 50].

The divergence of the length scale, $\xi$, describes fluctuations in the order parameter as the critical point is approached. When $T \gg T_c$ then the order parameter is zero, that is for example there is no correlation between the orientations of neighbouring Ising spins. On the other hand, as $T \to T_c$, although the long range order is still zero, but now there are local correlations between neighbouring spins. It is possible to measure the order parameter directly below $T_c$, while above $T_c$ one can study local fluctuations using physical techniques including NMR.

Previous work on critical phenomena in ternary DOPC/DPPC/cholesterol model membrane systems has been presented by Veatch and Keller et al. [51, 52, 35, 53, 54, 48] and Davis et al [55, 56]. The primary technique used by Veatch and Keller et al. is fluores-
cence microscopy on GUVs, but they also used deuterium NMR on multilamellar vesicles. GUVs are formed by a single bilayer which has a thickness of 4-6 nm, and as a result one might expect these systems to approximate a 2D system. Indeed, this is what is claimed by Veatch and Keller et al. in their studies of GUVs; near T$_c$ they obtained results for the critical exponents that agree with those of the 2D Ising universality class.

The work by Davis et al. was performed using static and magic angle spinning (MAS) deuterium NMR spectroscopy on multilamellar dispersions. These samples contain multiple bilayers that may result in critical behaviour that gives critical exponents which match the expected values for 3D systems rather than 2D systems due to interactions between the bilayers. Indeed the work reported on these systems indicates the best fit for the critical phenomena in these systems is for the 3D Ising model [55, 56]. The 3D Ising model for ternary lipid mixture phase behaviour was explored further in the current work using static $^2$H NMR experiments and is presented in Chapter 5.

1.6 Peptides in Model Membrane Systems

Membrane proteins are found in a dynamic environment where there are many different motions which occur on different time scales. These motions depend on the membrane composition, temperature and corresponding phase behaviour and the characteristics of the protein. Model membrane systems can be made to mimic various physical properties of real membranes in a simplified and more controlled manner. Peptides can be added to the model lipid membranes to provide more complexity. There can be interactions within peptides, between peptides, and between peptides and the surrounding lipids.
The lipid membrane is flexible and the thickness of the membrane depends on its composition, but it is able to expand or compress in order to match the hydrophobic length of a protein or peptide. In addition, transmembrane peptides are often found with their long axis tilted at some angle with respect to the bilayer normal [57]. The angle of the tilt can change and will depend on the properties of the bilayer. Simple transmembrane peptides often undergo fast axial diffusion about their long axis with a correlation time on the order of $10^{-7}$ s, and slower off-axis reorientation on the order of $10^{-5}$ to $10^{-6}$ s [58, 59, 60]. They may also undergo further rotational averaging about the bilayer normal [61].

The motions and interactions of peptides with one another and with other components of the membrane are influenced by the lipid environment. The particular properties of this membrane environment depend on factors such as the types of lipids present and the phase behaviour of the lipids. Motions of lipids in the fluid phases are generally much more rapid than those in the gel phases [27]. When cholesterol is present in the membrane, there is the possibility of two fluid ($\ell_d$-$\ell_o$) phase coexistence or fluctuations in the local membrane composition if the system is close to a critical point [25, 35, 56]. The $\ell_d$ and $\ell_o$ phases have similar rates of axial reorientation and lateral diffusion within the membrane; however, the chains of the lipids in the $\ell_o$ phase are significantly more ordered than those of the lipids in the $\ell_d$ phase making the $\ell_o$ phase thicker than the $\ell_d$ phase in the bilayer.

1.7 Thesis Objectives and Motivation

Membranes are complex systems which are essential to many cell functions. Model membranes are used in order to mimic some of the attributes of biological membranes in a
simplified and controlled setting. The aim of the current work is to gain more insight into the phase behaviour, molecular order, and molecular dynamics of model membranes with or without peptides.

The phase behaviour of model lipid membranes, specifically the coexistence of two fluid phases and critical phenomena in multilamellar and bicelle systems, is studied in more detail in this work. Three different aspects of lipid model membrane phase behaviour are presented. First, it has been previously established that ternary DOPC/DPPC/cholesterol mixtures exhibit critical behaviour as the $\ell_d$ and $\ell_o$ phases become indistinguishable. The nature of this critical behaviour and the universality class of multilamellar dispersions of ternary lipid/cholesterol mixtures are investigated using static NMR experiments. Next, the phase behaviour including the formation of coexisting $\ell_d$ and $\ell_o$ phases in aligned bicelle mixtures containing unsaturated lipids and cholesterol is characterized. Finally, 2D $^2$H exchange experiments are used to determine whether there is exchange between the domains of the $\ell_d$ and $\ell_o$ phases in multilamellar dispersions and magnetically oriented bicelle samples at temperatures close to the onset of the two fluid phase coexistence region.

The addition of peptides into model membranes will affect the system and it is of interest to determine both how the presence of a peptide in a model membrane system affects the phase behaviour of the bilayer and whether the helical peptides partition preferentially into one of the two coexisting fluid phases in these model membranes. In this work, several different model membranes with peptides are investigated. Samples include gramicidin in lipid/cholesterol multilamellar dispersions, Conolysin-Mt1 in DMPC/DCPC bicelles, and ALGA in both magnetically (bicelle) and mechanically (on glass slides) aligned lipid and cholesterol mixtures.
The purpose of this work is to further the fundamental understanding of model membrane systems containing saturated and unsaturated phospholipids with cholesterol and peptides. This may help us understand the phase behaviour and relationship between the lipids, proteins, water and temperature in biological membranes which may contain raft-like or phase separated domains.
Chapter 2

NMR Theory

NMR spectroscopy is a widely used technique with diverse applications in physics, biology and chemistry. Valuable information about a sample such as the molecular structure of a newly synthesized chemical or protein, or the phase behaviour of a lipid sample can be obtained from NMR spectroscopy. NMR takes advantage of the fact that nuclei have an intrinsic angular momentum property known as 'spin' which can be influenced by the application of radio frequency (r.f.) pulses while inside a strong magnetic field such as that of an NMR spectrometer. Frequently used NMR-active nuclei in biophysics include $^1$H, $^2$H, $^{13}$C, $^{15}$N and $^{31}$P. Table 2.1 summarizes the commonly used and biologically relevant nuclei with their spin and natural abundance.

Nuclei with non-zero integer or half-integer spin values are NMR-active. The value of the nuclear spin determines the relative strengths of the possible interactions between nuclei. These interactions can be expressed mathematically in terms of a Hamiltonian.

$$\mathcal{H} = \mathcal{H}_{\text{external}} + \mathcal{H}_{\text{internal}}$$ (2.1)

$\mathcal{H}_{\text{external}}$ arises from the Zeeman interaction of the nuclei with the static external magnetic field ($\mathcal{H}_Z$) and the applied r.f. pulses ($\mathcal{H}_{\text{rf}}$).

$$\mathcal{H}_{\text{external}} = \mathcal{H}_Z + \mathcal{H}_{\text{rf}}$$ (2.2)
Table 2.1: NMR Active Nuclei

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Spin</th>
<th>Natural Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1\text{H}$</td>
<td>$\frac{1}{2}$</td>
<td>$\sim 100%$</td>
</tr>
<tr>
<td>$^2\text{H}$</td>
<td>1</td>
<td>0.015%</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>$\frac{1}{2}$</td>
<td>1.1%</td>
</tr>
<tr>
<td>$^{14}\text{N}$</td>
<td>1</td>
<td>99.6%</td>
</tr>
<tr>
<td>$^{15}\text{N}$</td>
<td>$\frac{1}{2}$</td>
<td>0.37%</td>
</tr>
<tr>
<td>$^{31}\text{P}$</td>
<td>$\frac{1}{2}$</td>
<td>$\sim 100%$</td>
</tr>
</tbody>
</table>

When magnetic nuclei are in equilibrium in an external magnetic field, their magnetic moments undergo precession at a frequency called the Larmor frequency, $\omega_0$, which is related to the magnitude of the magnetic field, $B_0$ by a constant of proportionality, $\gamma_I$, the gyromagnetic ratio specific to each type of isotope.

$$\omega_0 = -\gamma_I B_0$$  \hfill (2.3)

The Zeeman interaction of the nuclei with the external magnetic field is usually the largest interaction for all nuclei since the external fields that are applied are normally larger than any of the internal interactions.

$\mathcal{H}_{\text{internal}}$ is made up of the various internal interactions that can occur within a sample including the direct dipole-dipole interaction ($D$), the chemical shift ($CS$), the J-coupling ($J$), and the quadrupolar interaction ($Q$).

$$\mathcal{H}_{\text{internal}} = \mathcal{H}_D + \mathcal{H}_{CS} + \mathcal{H}_J + \mathcal{H}_Q$$  \hfill (2.4)
Under the high field approximation, these internal interactions appear as perturbations to the large Zeeman interaction.

For spin-$\frac{1}{2}$ nuclei: dipolar coupling > chemical shift > J-coupling, for quadrupolar nuclei with spin $\geq 1$: quadrupolar interaction $\gg$ dipolar coupling > chemical shift > J-coupling [62]. The relative importance of these interactions also depends on the conditions under which it is being investigated, namely solution state NMR, static solid state NMR, or magic angle spinning (MAS) NMR.

$\mathcal{H}_D$ is the direct dipole-dipole interaction between both like and unlike nuclei. The dipolar interaction is a through-space interaction which is stronger for nuclei that are close together ($1/r^3$ distance dependence) and thus is often most important in $^1$H NMR since protons are abundant and are often found in close proximity to one another (on the order of 1 Å). $\mathcal{H}_{CS}$ arises from the chemical shift anisotropy due to orientation dependent interactions of the nucleus with the local electron environment. $\mathcal{H}_J$ is a relatively small dipole-dipole interaction that occurs indirectly via bonding electrons. Finally, $\mathcal{H}_Q$ is the quadrupolar interaction which is an interaction between the nuclear quadrupole moment and the electric field gradient at the nucleus and only occurs for nuclei with spins greater than $\frac{1}{2}$ [62, 63]. The quadrupolar coupling can be very large and leads to extremely broad spectra. Typical magnitudes of the various interactions are given in Table 2.2.

In the following sections the quadrupolar interaction for deuterium and the chemical shift for phosphorus will be introduced in more detail.
Table 2.2: Typical Sizes of Hamiltonian Contributions for NMR [62]

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Interaction</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_Z$</td>
<td>Zeeman: from static external field</td>
<td>$10^6 - 10^9 \text{ Hz}$</td>
</tr>
<tr>
<td>$H_{rf}$</td>
<td>Zeeman: from applied r.f. field (pulses)</td>
<td>up to $10^5 \text{ Hz}$</td>
</tr>
<tr>
<td>$H_D$</td>
<td>Direct dipole-dipole: between like and unlike nuclei</td>
<td>up to $10^5 \text{ Hz}$</td>
</tr>
<tr>
<td>$H_{CS}$</td>
<td>Chemical shift: shielding due to electronic environment</td>
<td>$10^3 - 10^4 \text{ Hz}$</td>
</tr>
<tr>
<td>$H_J$</td>
<td>J-coupling: indirect dipole-dipole coupling</td>
<td>up to $10^3 \text{ Hz}$</td>
</tr>
<tr>
<td>$H_Q$</td>
<td>Quadrupolar Interaction: for spin $\geq 1$</td>
<td>$10^3 - 10^7 \text{ Hz}$</td>
</tr>
</tbody>
</table>

2.1 $^2\text{H NMR}$

Deuterium is an isotope of hydrogen which has an extra neutron giving it spin-$1$ rather than spin-$\frac{1}{2}$. As a result, deuterium is a useful nucleus which can be substituted for hydrogen in order to investigate the properties of a sample via quadrupolar interactions as is the case in this work, or to eliminate protons in order to enhance the resolution of spectra when studying proteins for example. Since the quadrupolar interaction, though large compared to other internal interactions in static deuterium NMR, is small compared to the interaction between the nucleus and the external field, it can be treated as a perturbation to the Zeeman interaction using first order perturbation theory. A nucleus of spin $I$ has $2I + 1$ degenerate energy levels. In the presence of an external magnetic field, the Zeeman interaction with the nucleus breaks down the energy level degeneracy. Deuterium has a spin $I = 1$ and thus has three energy levels from $-I$ to $+I$ at integer intervals, therefore the spin states for deuterium are $m = -1$, $m = 0$, and $m = +1$. The interaction
between the electric field gradient at the nucleus (from the electron distribution) and the
electric quadrupole moment of the nucleus (from the charge distribution within the nu-
cleus) causes the quadrupolar energy shift. The splitting of the ground state into three
non-degenerate levels due to the Zeeman interaction between the $I = 1$ nucleus and the
external magnetic field, and the perturbations to this interaction due to the quadrupole
interaction are depicted in Figure 2.1 [64].

![Figure 2.1: Energy levels for a spin-1 nucleus arising from the Zeeman Hamiltonian
(where $E_m = -m\hbar\gamma B_0$) and the shifts due to the quadrupolar interactions as
a first order perturbation to the Zeeman interaction [64].](image)

Below is a brief mathematical description of the Zeeman and quadrupolar interactions.
The Zeeman Hamiltonian has the form

$$
\mathcal{H}_Z = -\vec{\mu} \cdot \vec{B}_0 = \hbar \omega_0 I_z
$$

(2.5)

where $\mu$ is the magnetic moment, $\vec{B}_0$ defines the axis of quantization of nuclear angular
momentum which is chosen to be along the $z$-axis, $\hbar$ is the reduced Planck constant ($\hbar/2\pi$),
\( \omega_0 \) is the Larmor frequency of the nucleus, and \( I_z \) is the \( z \)-component of the spin angular momentum. The associated energies for the \( m^{th} \) spin state are

\[
E_m = -m\hbar\gamma_1 B_0
\]  

In the presence of the first order quadrupolar Hamiltonian, the \( m = \pm 1 \) levels are shifted up by an amount \( \Delta \) while the \( m = 0 \) level is shifted down by \( 2\Delta \). The energy shift, \( \Delta \), depends on the orientation of the electric field gradient tensor relative to the magnetic field and can be calculated using the following equation

\[
\Delta = \frac{e^2 q Q}{8\hbar} \left[ (3 \cos^2 \beta - 1) + \eta \sin^2 \beta \cos 2\alpha \right]
\]

The principal value of the electric field gradient is \( e_\text{q} \), and \( eQ \) is the nuclear electric quadrupole moment. The asymmetry parameter is called \( \eta \). For \( ^2\text{H} \) in C-\( ^2\text{H} \) bonds, \( \eta \leq 0.02 \) and is often taken as zero. The angles used to describe this orientation dependence are the Euler angles \( \alpha \), \( \beta \), and \( \gamma \). The Euler angles are defined using the convention of Rose [65] and the transformation from the (X,Y,Z) frame (purple) to the (x,y,z) frame (red) is shown in Figure 2.2. In order to go from one frame to another, up to three rotations are required as follows: \( \alpha \): rotation about \( Z \) to bring \( Y \) to the line of nodes, \( \beta \): rotation about the line of nodes to bring \( Z \) to \( z \), and \( \gamma \): rotation about \( z \) to bring \( Y \) to \( y \) [65, 66, 67].

The quadrupolar splitting in the absence of molecular motions is

\[
\omega_Q = 6\Delta = \frac{3e^2 q Q}{4\hbar} \left[ (3 \cos^2 \beta - 1) + \eta \sin^2 \beta \cos 2\alpha \right]
\]

which is dependent upon the orientation of the system with respect to the external magnetic field. Here only two of the Euler angles are required for the rotation, \( \alpha \), and \( \beta \), the third
Euler angle, $\gamma$, is chosen to zero because the magnetic field is an axis of symmetry for the laboratory coordinate system. In the case where $\eta$ is zero, this equation becomes

$$\omega_Q = 6\Delta = \frac{3e^2qQ}{4\hbar} \left[ 3\cos^2 \beta - 1 \right]$$  \hspace{1cm} (2.9)$$

In the presence of molecular motion which results in fluctuations of the angle between the C-$^2$H bond vector and the bilayer normal, an average bond order parameter can be defined as $< S_{CD} > = \frac{1}{2} < (3\cos^2 \beta' - 1) >$ where $\beta'$ is the angle between the C-$^2$H bond and the bilayer normal [64]. Equation 2.9 then takes the form

$$\omega_Q = 6\Delta = \frac{3e^2qQ}{4\hbar} \left[ 3\cos^2 \beta - 1 \right] < S_{CD} >$$  \hspace{1cm} (2.10)$$

The samples studied here are partially ordered systems of phospholipids, cholesterol, and peptides. The motions in these systems are anisotropic meaning that they do not average to
zero so there are contributions to the spectra from the quadrupolar interactions, the nuclear
dipole-dipole interactions and the anisotropic chemical shift. The quadrupolar splitting for
deuterium can be as large as 250 kHz. For deuterium, the chemical shift anisotropy and
dipole-dipole interactions are relatively small (at most about 10 kHz) compared to the
quadrupolar interaction, so they do not contribute much to the observed spectra and will
not be considered further [64].

As the degree of order within a sample increases, so too does the size of the quadrupolar
splitting for each nucleus, thus the quadrupolar splittings of spectra from samples in
fluid phases are much smaller than those of spectra from samples in gel phases. Since the
liquid ordered phase is more ordered than the liquid disordered phase, these phases are
distinguishable in static $^2$H NMR spectra due to their differences in quadrupolar splitting.

2.1.1 $\mathcal{H}_Q$: Mathematical Formalism

The quadrupolar Hamiltonian in the principal axis system (PAS) of the electric field gra-
dient tensor is

$$\mathcal{H}_Q = \frac{e^2 q Q}{4I(2I-1)} \left[ (3I_Z^2 - I^2) + \eta(I_X^2 - I_Y^2) \right]$$

[69]. Here, $I$ is the spin of the nucleus, $e q$ is the principal value of the electric field
gradient, $e Q$ is the electric quadrupole moment and $I_X$, $I_Y$, and $I_Z$ are the components of
the spin angular momentum operators in the PAS of the electric field gradient tensor.

The transformation from the PAS to the laboratory frame for the static case is treated
explicitly here. Figure 2.3 shows the axis systems for each of the steps in the transfor-
mation from the PAS of the electric field gradient tensor to the laboratory frame. In the
situation where the sample is spinning at the magic angle, an extra transformation is used to account for the set angle of the rotor but this will not be discussed further in this work.

![Figure 2.3: Axis systems for transformation from the PAS to the laboratory frame. Note that P represents the PAS of the electric field gradient tensor, B represents the bilayer frame, and L represents the laboratory frame.](image)

The development used here is analogous to that shown in Davis [64]. The quadrupolar Hamiltonian can be written as the product of the electric field gradient tensor components, and the second rank spherical tensors as shown below

\[ \mathcal{H}_Q = \frac{eQ}{2} \sum_{m'=-2}^{2} (-1)^{m''} T_{2,m''} F_{2,-m''}^L \]

(2.12)

In the PAS, the electric field gradient tensor components are given by the following set of equations.

\[ F_{2,0}^P = \frac{\sqrt{3}}{2} e q \]  \hspace{1cm} (2.13)

\[ F_{2,\pm1}^P = 0 \]  \hspace{1cm} (2.14)

\[ F_{2,\pm2}^P = \frac{1}{2} \eta e q \]  \hspace{1cm} (2.15)
The asymmetry parameter, $\eta$, for the nearly axially symmetric C-$^2$H bond is almost zero; thus, the $F^p_{2,\pm 2}$ term is usually ignored.

The second rank spherical tensors for the spin variables are given as

$$T_{2,0} = \frac{1}{\sqrt{6}} \left[ 3I_z^2 - I(I + 1) \right]$$

(2.16)

$$T_{2,\pm 1} = \mp \frac{1}{2} \left[ I_z I_\pm + I_\pm I_z \right]$$

(2.17)

$$T_{2,\pm 2} = \frac{1}{2} I_\pm^2$$

(2.18)

**Transformation From PAS Directly to Laboratory Frame**

Beginning with the general equation for the quadrupolar Hamiltonian, equation 2.12, a single transformation takes the electric field gradient tensor from its PAS to the laboratory frame. The transformation of the electric field gradient tensor components is done in the same way that spherical harmonics are transformed.

$$F^L_{2, -m''} = \sum_{m'=-2}^{2} F^p_{2,m'} D^{(2)}_{m', -m''} (\alpha, \beta, \gamma)$$

(2.19)

where $D^{(2)}_{m,m'}$ is the Wigner rotation matrix which is defined as

$$D^{(2)}_{m,m'} = e^{+i\alpha m} d^{(2)}_{m,m'} (\beta) e^{+i\gamma m'}$$

(2.20)

and $\alpha$, $\beta$, and $\gamma$ are the Euler angles for the given transformation [66, 67]. Now the quadrupolar Hamiltonian can be rewritten in terms of the electric field gradient tensor in its principal axis system.

$$\mathcal{H}_Q = \frac{eQ}{2} \sum_{m''=-2}^{2} (-1)^{m''} T_{2,m''} \sum_{m'=-2}^{2} F^p_{2,m'} D^{(2)}_{m', -m''} (\alpha, \beta, \gamma)$$

(2.21)
Due to the axial symmetry of the laboratory frame ($B_0$ is along the $z$-axis) we may take $\gamma_{PL} = 0$. The only part of the spin tensor that commutes with the Zeeman Hamiltonian is $T_{2,0}$, so in first order perturbation theory we may take $m'' = 0$. In addition, if there is symmetry about the C-$^2$H bond, $\eta \approx 0$ which allows $\alpha_{PL}$ to be taken as zero, and $m' = 0$. The equation for the quadrupolar Hamiltonian becomes

$$H_Q = \frac{eQ}{2} T_{2,0} F_{2,0}^P D_{0,0}^{(2)} (0, \beta_{PL}, 0)$$  \hspace{1cm} (2.22)$$

Using the definition of the Wigner rotation matrices found in Equation 2.20, gives

$$H_Q = \frac{eQ}{2} T_{2,0} F_{2,0}^P d_{0,0}^{(2)} (0, \beta_{PL}, 0)$$  \hspace{1cm} (2.23)$$

Substituting in the expression for $d_{0,0}^{(2)} (0, \beta_{PL}, 0) = \frac{1}{2} \left( 3 \cos^2 \beta_{PL} - 1 \right)$ gives

$$H_Q = \frac{eQ}{2} T_{2,0} F_{2,0}^P \left( \frac{1}{2} \left( 3 \cos^2 \beta_{PL} - 1 \right) \right)$$  \hspace{1cm} (2.24)$$

### Transformation From PAS to Bilayer Normal to Laboratory Frame

Here, two transformation steps will be used: the first is from the PAS to the bilayer normal frame ($P \rightarrow B$), the second is from the bilayer normal frame to the laboratory frame ($B \rightarrow L$). The bilayer normal serves as an axis of symmetry for the motion of the system. As above, if $\eta = 0$, the only non-zero

$$\beta_{PB}$$

is defined as the angle between the C-$^2$H bond in a lipid and the bilayer normal and is an axis of symmetry for the motion of the system. As above, if $\eta = 0$, the only non-zero
component of $F_{2,m'}^P$ is for $m' = 0$, and thus $\alpha_{PB}$ can be taken to be zero. The equation for the electric field gradient tensor in the bilayer normal frame can now be rewritten as

$$F_{2,m}^B = \sum_{m'=-2}^{2} F_{2,m'}^P D_{m',-m}^{(2)} (0, \beta_{PB}, \gamma_{PB}) \tag{2.26}$$

The next transformation takes the electric field gradient from the bilayer normal frame to the laboratory frame

$$F_{2,m''}^L = \sum_{m=-2}^{2} F_{2,m}^B D_{m,-m''}^{(2)} (\alpha_{BL}, \beta_{BL}, \gamma_{BL}) \tag{2.27}$$

The axial symmetry of the frame coming from the alignment of the z-axis of the laboratory frame with the magnetic field of the spectrometer, one of the Euler angles can be eliminated. Here $\gamma_{BL}$ is set equal to zero and $m=0$.

$$F_{2,0}^L = \sum_{m=-2}^{2} F_{2,m}^B D_{m,0}^{(2)} (\alpha_{BL}, \beta_{BL}, 0) \tag{2.28}$$

Now the transformations back from the laboratory frame to the bilayer normal frame to the PAS can be substituted into $F_{2,0}^L$ to get an equation in terms of the PAS electric field gradient tensor and the rotation matrices.

$$F_{2,0}^L = \sum_{m=-2}^{2} F_{2,0}^P D_{0,m}^{(2)} (0, \beta_{PB}, \gamma_{PB}) D_{m,0}^{(2)} (\alpha_{BL}, \beta_{BL}, 0) \tag{2.29}$$

Rewriting the Wigner matrices in equation 2.20 gives

$$F_{2,0}^L = \sum_{m=-2}^{2} F_{2,0}^P e^{i(\gamma_{PB})m} e^{i(\gamma_{PB}+\alpha_{BL})m} d_{m,0}^{(2)} (\beta_{BL}) \tag{2.30}$$

Motional averaging causes $e^{i(\gamma_{PB}+\alpha_{BL})m} \rightarrow 1$ and now $m = 0$. The equation for $F_{2,0}^L$ is now

$$F_{2,0}^L = F_{2,0}^P d_{0,0}^{(2)} (\beta_{PB}) d_{0,0}^{(2)} (\beta_{BL}) \tag{2.31}$$
Only one of the components of the second rank spherical tensors for the spin variables, $T_{2,0}$, commutes with the Zeeman Hamiltonian. The quadrupolar Hamiltonian can then be written as

$$\mathcal{H}_Q = \frac{eQ}{2} T_{2,0} F_{2,0} d_{b,0}^{(2)}(\beta_{PB}) d_{b,0}^{(2)}(\beta_{BL})$$

(2.32)

Substituting in the expressions for $d_{b,0}^{(2)}(\beta)$ to get $\mathcal{H}_Q$ in terms of $P_2(\cos \theta)$ functions.

$$\mathcal{H}_Q = \frac{eQ}{2} T_{2,0} F_{2,0} \left( \frac{1}{2} \left( 3 \cos^2 \beta_{PB} - 1 \right) \right) \left( \frac{1}{2} \left( 3 \cos^2 \beta_{BL} - 1 \right) \right)$$

(2.33)

### 2.1.2 Quadrupolar Echo Pulse Sequence and Typical $^2$H Spectral Shapes

The quadrupolar echo pulse sequence is made up of two 90° pulses which are separated by a time delay, $\tau$. These two pulses are 90° out of phase from one another, and there is a second delay before the signal can be acquired which allows for the formation of the echo at a time of approximately $2\tau$ from the first pulse. Thus, the quadrupolar echo pulse sequence has the form, 90°-$\tau$-90°-$\tau$. A schematic diagram of the quadrupolar echo pulse sequence is shown in Figure 2.4. For a detailed mathematical description of the quadrupolar echo pulse sequence and the transformations, see Davis, 1991 [64].

The typical quadrupolar powder pattern for an axially symmetric system such as phospholipids in a model membrane is shown in Figure 2.5. Here $\eta = 0$ and the quadrupolar splitting is chosen to be 25 kHz. For a single deuterium label in a fluid lipid bilayer oriented with its bilayer normal perpendicular to the magnetic field (90° orientation) an oriented sample spectrum would have two peaks that are separated by the quadrupolar splitting.
Oriented sample spectra for $^2$H NMR result in a doublet for each deuteron. Two sample spectra from well-oriented ternary lipid/cholesterol mixtures in the $\ell_d$ phase are shown in Figure 2.6. These samples are aligned on glass slides and placed in the magnetic field such that the bilayer normal is perpendicular with the field ($90^\circ$ orientation) in (A) and parallel with the field ($0^\circ$ orientation) in (B). In Figure 2.6(A), the sample contains DPPC(7,7,d$_4$) which has two deuterons on each chain of the lipid. In the $\ell_d$ phase these deuterons are equivalent and give rise to the doublet at $\pm 20$ kHz. The doublet in the centre of the spectrum is from the D$_2$O used to monitor the hydration of the system and it is ordered in this case. In Figure 2.6(B), the sample contains chain perdeuterated DMPC-d$_{54}$ . There are 54 deuterons on the lipid which give rise to the many doublets seen in this spectrum. The small, sharp peak at 0 kHz is due to the water in this sample.
Figure 2.5: Simulated line shape for a quadrupolar powder pattern with $\Delta \nu = 25$ kHz and $\eta = 0$. 
Figure 2.6: Oriented $^2$H spectra in the $\ell_d$ phase. (A) Spectrum of 30:45:25 molar composition (DOPC/DPPC(7,7,d$_4$)/cholesterol) oriented on glass slides with the bilayer normal perpendicular to the magnetic field. The outer peaks ($\pm$20 kHz) are from the labelled lipid and the central peaks are the ordered D$_2$O in this sample which was used to monitor the hydration. Spectrum courtesy of J. Clair, University of Guelph. (B) Spectrum of 32:48:20 molar composition (DPoPC/DMPC-d$_{54}$/cholesterol) oriented on glass slides with the bilayer normal parallel to the magnetic field. The fine structure arises from the 54 deuterons on the DMPC lipid chains. The small, sharp peak in the center comes from the water.
2.2 $^{31}$P NMR

The local electronic environment partially shields the nucleus from the external magnetic field giving rise to the chemical shift. The chemical shift describes the interaction between the local field due to the electrons and the nucleus and is important for spin $\frac{1}{2}$ nuclei such as $^{31}$P. The external magnetic field causes the electron cloud which surrounds the nucleus to shift slightly resulting in a small change in the Larmor frequency of the nucleus that is seen as a shift in the spectrum. Except in cases where nuclei are in a position with very high symmetry, the chemical shift is dependent on orientation (anisotropic). The chemical shift anisotropy (CSA) is proportional to $\gamma B_0$, and is often discussed in terms of a chemical shielding, $\sigma$.

The chemical shift Hamiltonian has the form

$$H_{CS} = -\gamma I \cdot \vec{\sigma} \cdot \vec{B}_0$$

(2.34)

As in the quadrupolar case discussed previously, a set of transformations from the principal axis system to the laboratory frame defined by the Euler angles (see Section 2.1.2, Figures 2.2 and 2.3) is required to find the magnitude of the chemical shift.

2.2.1 $H_{CS}$: Mathematical Formalism

In the laboratory frame the Hamiltonian for the chemical shift interaction can be written in terms of second rank spherical tensors as follows [70]

$$H_{CS} = \sum_{k=0}^{2} \sum_{m=-k}^{k} (-1)^m T_{k,m} F_{k,-m}^I$$

(2.35)
In the PAS, the spatially dependent components of the chemical shift spherical tensors are given by the following set of equations.

\[ F_{0,0}^p = -\frac{1}{\sqrt{3}} \gamma I r[\vec{\sigma}] = -\sqrt{3} \gamma \delta_0 \]  
\[ (2.36) \]

\[ F_{1,0}^p = i \sqrt{2} \gamma I \sigma_{12} \]  
\[ (2.37) \]

\[ F_{1,\pm 1}^p = \gamma I (\sigma_{13} \pm \sigma_{23}) \]  
\[ (2.38) \]

\[ F_{2,0}^p = \frac{\sqrt{3}}{2} \gamma I \left( \sigma_{33} - \frac{1}{3} r[\sigma] \right) = \sqrt{\frac{3}{2}} \gamma \delta \]  
\[ (2.39) \]

\[ F_{2,\pm 1}^p = 0 \]  
\[ (2.40) \]

\[ F_{2,\pm 2}^p = \frac{1}{2} \gamma I (\sigma_{11} - \sigma_{22}) = \frac{1}{2} \gamma \eta \delta \]  
\[ (2.41) \]

The isotropic part of the chemical shift comes from the \( F_{0,0} \) component which is scalar and independent of the orientation. The first rank tensor components, \( F_{1,m} \), are negligible and will not be looked at further here [71]. The chemical shift powder pattern arises due to the second rank tensor components, \( F_{2,m} \). These are orientation dependent and are formed from the principal values of the Cartesian chemical shift tensor \( \vec{\sigma} \) (\( \sigma_{11} \), \( \sigma_{22} \), and \( \sigma_{33} \)). It is conventional to assign the principal values such that \( |\sigma_{33} - \delta_0| \geq |\sigma_{11} - \delta_0| \geq |\sigma_{22} - \delta_0| \).

The spin dependent components of the second rank spherical tensors are given as

\[ T_{0,0} = -\frac{1}{\sqrt{3}} \vec{I} \cdot \vec{B}_0 \]  
\[ (2.42) \]

\[ T_{1,0} = 0 \]  
\[ (2.43) \]

\[ T_{1,\pm 1} = -\frac{1}{2} \vec{I}_\pm \cdot \vec{B}_0 \]  
\[ (2.44) \]
\[ T_{2,0} = \sqrt{\frac{2}{3}} \vec{i}_z \cdot \vec{B}_0 \]  \hspace{1cm} (2.45) \\
\[ T_{2,\pm 1} = \pm \frac{1}{2} \vec{i}_z \cdot \vec{B}_0 \] \hspace{1cm} (2.46) \\
\[ T_{2,\pm 2} = 0 \] \hspace{1cm} (2.47)

As before, transformations between the different frames (PAS, bilayer normal, and laboratory) depend on Euler angles. The second rank tensor component \( F^B_{2,m} \) in the bilayer frame can be written in terms of the PAS as follows

\[ F^B_{2,m} = \sum_{m''=-2}^{2} F^P_{2,m''} D^{(2)}_{m''m'}(\alpha_{PB}, \beta_{PB}, \gamma_{PB}) \] \hspace{1cm} (2.48)

Since the bilayer normal is an axis of symmetry for the motion in lipid samples, not all of the Euler angles are required for the rotation between frames. Here \( \gamma_{PB} \) is set to zero.

\[ F^B_{2,m} = \sum_{m''=-2}^{2} F^P_{2,m''} D^{(2)}_{m''m'}(\alpha_{PB}, \beta_{PB}, 0) \] \hspace{1cm} (2.49)

The next transformation required is from the bilayer normal frame to the laboratory frame. Only one angle, \( \beta_{BL} \) is required because of the axial symmetry of both the bilayer normal and laboratory frames.

\[ F^L_{2,m} = \sum_{m''=-2}^{B} F^P_{2,m''} D^{(2)}_{m'',m}(0, \beta_{BL}, 0) \]  \hspace{1cm} (2.50)

In the secular approximation, only the \( F^L_{2,0} \) component is required in order to calculate the orientation dependent resonance frequency since only \( T_{2,0} \) commutes with the Zeeman Hamiltonian.

\[ F^L_{2,0} = \frac{1}{4} \gamma I \delta \sqrt{\frac{3}{2}} \left[ (\eta \sin^2 \beta_{PB} \cos(2\alpha_{PB}) + (3 \cos^2 \beta_{PB} - 1)) \right] \left( 3 \cos^2 \beta_{BL} - 1 \right) \] \hspace{1cm} (2.51)
The resonance frequency in terms of the angles used to transform from $P \rightarrow B \rightarrow L$ is

$$\omega_{CS} = \gamma B_0 (1 - \delta_0) + \frac{1}{4} (3 \cos^2 \beta_{BL} - 1) [(3 \cos^2 \beta_{PB} - 1) + \eta \sin^2 \beta_{PB} \cos(2\alpha_{PB})] \delta \gamma B_0 \tag{2.52}$$

### 2.2.2 Typical $^{31}$P Spectral Shapes

Powder samples or samples in which all orientations can occur with equal probability such as multilamellar dispersions of lipids result in spectra that are quite broad because the chemical shift of each orientation is different and they all contribute to the overall powder pattern. The powder pattern can be thought of as a sum of the spectra for all of the individual orientations and can take on two general shapes depending on the symmetry of the system. Figure 2.7 shows powder patterns for an arbitrary chemical shift tensor where $\sigma_{11} < \sigma_{22} < \sigma_{33}$, and one for an axially symmetric chemical shift tensor where $\sigma_{11} = \sigma_{22} < \sigma_{33}$. The values of the principal shielding components ($\sigma_{11}$, $\sigma_{22}$, and $\sigma_{33}$) can be determined directly from the spectrum. These values are affected by changes in the electronic environment [63, 62].

Oriented samples simplify the shapes of the resulting spectra. Two types of oriented samples are used in the current work: magnetically aligned bilayers (bicelles), and mechanically aligned bilayers on glass slides. The $^{31}$P line shapes observed for these types of spectra are shown in Figure 2.8. Well-oriented samples on glass slides result in a single peak as shown in Figure 2.8 (A). Magnetically oriented bicelle samples give rise to two or three peaks as shown in Figure 2.8 (B). The largest peak corresponds to the long chain
Figure 2.7: Theoretical powder line shapes for the chemical shift interaction for (A) an arbitrary chemical shift tensor, and (B) an axially symmetric chemical shift tensor.

lipid or the flat parts of the bicelle, the middle peak corresponds to the edge regions, and the isotropic peak corresponds to the phosphate buffer in this case (this peak will not be present if the buffer does not contain $^{31}$P, unless there is an isotropic phase present for the lipids themselves). $^{31}$P NMR is one approach used in this work to look at the behaviour and orientation of lipid bilayers as a function of temperature.

2.3 Analysis Methods

2.3.1 Moment Analysis

Molecular motions give rise to the observed quadrupolar splittings in $^2$H spectra. These motions tend to be anisotropic and result in incomplete averaging of the static electric-quadrupole interactions (the same is true for dipolar interactions). Since the dominant
Figure 2.8: $^{31}$P spectra for (A) 37.5:37.5:25 (DOPC/DPPC-d$_{62}$/cholesterol) lipid bilayers oriented on glass slides at 0° (red) and 90° (black) (collected at 202.46 MHz, 300.8 K, 128 scans), and (B) magnetically aligned DMPC-d$_{54}$/DCPC bicelles with the bilayer normal of the long chain lipids perpendicular to the magnetic field (90°) (collected at 202.46 MHz, 329.2 K, 64 scans).
interaction for deuterium spectra is the quadrupolar interaction, the spectra are dependent on this interaction and the quadrupolar splittings can be used to determine the molecular order of the sample. Moment analysis is a quantitative way to compare the molecular order of the phospholipids giving rise to deuterium spectra [72, 73].

We define the n’th moment of a spectrum as

$$M_n = \frac{1}{A} \int_{-\infty}^{\infty} |\omega'|^n f (\omega') d\omega'$$

(2.53)

where $\omega' = \omega - \omega_0$, $\omega_0$ is the Larmor frequency and $f (\omega')$ is the function describing the line shape of the spectrum. The area of the spectrum, $A$, is

$$A = \int_{-\infty}^{\infty} f (\omega') d\omega'$$

(2.54)

Practically, these calculations are done in two pieces for symmetric deuterium spectra. The moments for each half of the spectrum are calculated separately and the average is taken. On the right side, the limits of integration range from the central frequency $\omega_0$ to the positive limit, and on the left side, the limits of integration range from the central frequency $\omega_0$ to the negative limit. The positive and negative limits are equidistant from the central frequency and are sufficiently far that all of the spectral intensity is contained between them.

The first moment is of particular interest here because for axially symmetric line shapes it is directly proportional to the average carbon-deuterium bond order parameter, $S_{CD}$. For a powder sample in which every orientation occurs with equal likelihood, the bond order parameter is related to the first moment as

$$M_1 = \frac{4\pi}{3 \sqrt{3}} \left( \frac{3e^2qQ}{4\hbar} \right) \langle |S_{CD}| \rangle$$

(2.55)
where $\frac{3e^2 q Q}{4h}$ is the electric quadrupolar coupling constant (126 kHz for C-D bonds) and the coefficient $\frac{4\pi}{3\sqrt{3}}$ is used for samples with all possible orientations equally likely.

For a sample oriented with its bilayer normal perpendicular to the magnetic field, the bond order parameter is related to the first moment as

$$M_1 = 2\pi \frac{3e^2 q Q}{4h} \langle |S_{C,D}| \rangle$$

(2.56)

Note that here the only remaining coefficient is $2\pi$ which is required to convert to the correct frequency units. The first moments as a function of temperature can be used to compare the molecular order and behaviour of lipid samples with varying concentrations of cholesterol, unsaturated lipids or peptides.

### 2.3.2 $^2$H Line Shape Fitting

Line shape fitting can be used to determine the parameters defining the component spectra making up a more complex $^2$H NMR spectrum. Powder patterns can be characterized by their area, quadrupolar splitting and line broadening.

Line shape fitting was used in the investigation of the critical behaviour of DOPC, methyl deuterated DPPC (DPPC-d$_6$) and cholesterol mixtures by static $^2$H NMR. At temperatures above the critical temperature ($\ell_o$ phase region) a single Gaussian or Lorentzian broadening powder pattern can be used to fit the spectra. For ternary mixtures containing DOPC, DPPC-d$_6$, and cholesterol in the $\ell_d$-$\ell_o$ coexistence region, the $^2$H NMR spectra are made up of a superposition of three Gaussian or Lorentzian broadened quadrupolar doublet powder patterns. Two of these powder patterns correspond to the inequivalent methyl groups characteristic of the $\ell_o$ phase domains (high cholesterol) and the other corresponds
to the methyl groups in the $\ell_d$ phase domains.

For multilamellar dispersions, the superposition of splittings was weighted by a factor of $\sin \theta$ where $\theta$ is the angle between the bilayer normal and the external magnetic field. This weighting factor is what would be expected for spherical liposomes. It was found, however, that these fits resulted in too much intensity at the $0^\circ$ shoulders of the spectrum as compared to the experimental results. This result indicates that there is a slight distortion of the liposomes by the magnetic field that results in slightly prolate ellipsoidal liposomes [74]. The weighting of the doublets for these prolate ellipsoidal liposomes is

$$P(\theta) = \frac{\sin(\theta)}{(\sin^2 \theta + \rho^2 \cos^2 \theta)^2}$$

(2.57)

where $\rho$ is the ratio of the semimajor to semiminor axis of the prolate ellipsoid [75].

A comparison of the fits using the Gaussian and Lorentzian broadened powder patterns for a $^2$H NMR spectrum of 37.5:37.5:25 (DOPC/DPPC-d$_6$/cholesterol) at 310.39 K is shown in Figure 2.9. This spectrum was taken well above the critical temperature in the $\ell_d$ phase region for the sample. The best fit to a Gaussian broadened powder pattern is shown in red and uses a value of $\rho = 1.07$ (semimajor/semiminor axis). The best fit to a Lorentzian broadened powder pattern is shown in blue and uses a value of $\rho = 1.27$ (semimajor/semiminor axis). In both cases, the quadrupolar splitting is estimated well, however there is too much intensity in the shoulders of the fitted spectrum. The Gaussian broadened powder pattern fits the wings of the experimental spectrum better than the Lorentzian broadened powder pattern but the experimental line shape is not perfectly reproduced by this model. It is possible to fit using a combination of Lorentzian and Gaussian line shape characteristics; however, in this work the Gaussian broadened powder pattern with ellipsoidal distortion fit was deemed sufficient to represent the spectra [76].
Figure 2.9: Fits of a $^2$H NMR spectrum for the 37.5:37.5:25 (DOPC/DPPC-d$_6$/cholesterol) sample at 310.39 K. The experimental spectrum is shown in black, the small, sharp peak near the centre is due to deuterium in the buffer. The fit shown in red uses Gaussian broadened powder patterns, and the fit shown in blue uses Lorentzian broadened powder patterns. Note that both fits overestimate the size of the 0° shoulders of the experimental powder pattern.

2.3.3 Relaxation Measurements

Spin-lattice relaxation or longitudinal relaxation is characterized by the time constant $T_1$. $T_1$ is the time it takes to re-establish the normal Boltzmann distribution of spins in a sample. Spin-spin relaxation or transverse relaxation is characterized by the time constant $T_2$. $T_2$ is the loss of coherence between spins in a sample. For spin $\frac{1}{2}$ nuclei, the rel-
ative importance of the relaxation mechanisms are as follows: dipole-dipole > CSA >
spin-rotation. For spins 1 or greater, the relative importance of the relaxation mechanisms
are: quadrupolar ≫ dipole-dipole > CSA > spin-rotation [62]. The electric quadrupole
relaxation dominates the spin relaxation for quadrupolar nuclei, just as the quadrupolar
interactions dominate the spectra for these nuclei [62].

The line width of an NMR signal is governed by $T_2$, while the maximum repetition rate
for the acquisition NMR signal is determined by $T_1$. A shorter $T_2$ results in broader lines
according to the relationship for the line width at half the maximum height, $\Delta \nu_2 = \frac{1}{\pi T_2}$. The line width depends on the decay rate of the FID signal. A shorter $T_1$, however, is
advantageous because it means that the signal can be acquired faster (more scans can be
collected in the same amount of experiment time) [69, 62].

$T_2$ can be determined by measuring the intensity of the signal from a series of spin
echo experiments with different delays. The effective quadrupolar echo relaxation time,
$T_{2e}$, was used to determine the contribution of relaxation to the static powder pattern line
widths for the investigation of critical phenomena in DOPC/DPPC/cholesterol systems in
Chapter 5.
Chapter 3

Lipids and Peptides

3.1 Lipids

Several phospholipids and cholesterol were used in the samples investigated here. Both saturated and unsaturated long chain phospholipids were required. Schematics of the long chain phospholipids are shown in Figure 3.1: the saturated lipids are (a) 1,2-dimyristoyl-\textit{sn}-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-\textit{sn}-glycero-3-phosphocholine (DPPC), the unsaturated lipids are (c) 1,2-dipalmitoleoyl-\textit{sn}-glycero-3-phosphocholine (DPoPC) and (d) 1,2-dioleoyl-\textit{sn}-glycero-3-phosphocholine (DOPC).

For $^2$H NMR experiments, it is advantageous to use deuterium labelled lipids. Here chain perdeuterated DMPC and DPPC were used as probes to investigate the phase behaviour of the lipid mixtures. Chain perdeuterated 1,2-dimyristoyl-d$_{54}$-\textit{sn}-glycero-3-phosphocholine (DMPC-d$_{54}$) and chain perdeuterated 1,2-di-(d$_{31}$)palmitoyl-\textit{sn}-glycero-3-phosphocholine (DPPC-d$_{62}$) are shown in Figure 3.2 (a), (b) respectively. In addition, for the critical fluctuations work, methyl-deuterated DPPC-d$_6$ was used. DPPC-d$_6$ is shown in Figure 3.2(c).

Two saturated short chain phospholipids were used, 1,2-dicaproyl-\textit{sn}-glycero-3-phosphocholine (DCPC) which has two chains with 6 carbons each, and 1,2-diheptanoyl-
Figure 3.1: (a) DMPC, (b) DPPC, (c) DPoPC, and (d) DOPC.

Figure 3.2: (a) DMPC-d$_{54}$, (b) DPPC-d$_{62}$, and (c) chain methyl-deuterated DPPC-d$_6$. 
sn-glycero-3-phosphocholine (DHPC) which has two chains with 7 carbons each. Note that DCPC is often referred to in the literature as DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine) but in this work DHPC will refer to 1,2-diheptanoyl-sn-glycero-3-phosphocholine. A schematic diagram of DCPC is shown in Figure 3.3(a) and DHPC is shown in Figure 3.3(b).

![Diagram of DCPC and DHPC](image)

Figure 3.3: (a) DCPC, and (b) DHPC.

Lipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) in powder form and used without further purification. Because of their hygroscopic nature, the short chain lipids, DCPC and DHPC were dissolved in ethanol to give a stock solution with a concentration of 2.5 mg/mL for use in sample preparation. Cholesterol was purchased from Sigma Aldrich (St. Louis, MO).

### 3.2 Peptides

Gramicidin is a 15 amino acid long peptide that dimerizes to form pores in the membrane. Gramicidin A has an alternating L- and D- amino acid sequence formyl-L-Val-Gly-L-Ala-
D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Y-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. In the case of gramicidin A, Y is Trp. In gramicidin B the tryptophan is replaced by phenylalanine, while in gramicidin C the tryptophan is replaced by tyrosine. Exchange labelling of gramicidin can be achieved by dissolving the peptide in deuterated methanol. This results in labelling of the amide protons on all 15 residues. In addition the indole groups on the four tryptophans have exchangeable hydrogens [64]. Labelled gramicidin A was synthesized by Prosser et al. [77] and gramicidin D from *Bacillus brevis* was obtained from Sigma Aldrich (St. Louis, MO). Figure 3.4 shows the structure of a gramicidin dimer which forms a transmembrane channel.

![Diagram of the crystal structure of a gramicidin dimer](image)

Figure 3.4: Diagram of the crystal structure of a gramicidin dimer (a) end view, and (b) side view.

Conolysin-Mt1 is a 23-residue peptide with sequence: FHPSLWVLIPQYIQLIRK-ILKSG. Conolysin-Mt1 has a hydrophobic face and a hydrophilic face and appears to interact with the surface of the bilayer. Synthesis of Conolysin-Mt1 used in the current work was done by Erik H. Nielsen, inSPIN and iNANO, Aarhus University, Denmark. Fig-
Figure 3.5 shows the lowest energy structure of Conolysin-Mt1 in bicelles as calculated by molecular dynamics simulations from NMR data. Structure calculations were performed by Jakob T. Nielsen, inSPIN and iNANO, Aarhus University, Denmark [78].

ALGA has the amino acid sequence Ac-KKPV ALIFALGALA\(^\text{\textsuperscript{\textdegree}}\)FIL A VGKK-NH\(_2\). This peptide is hydrophobic and has a helical structure in trifluoroethanol (TFE). In addition to a non-labelled version of ALGA, a specifically \(^2\text{H}\) labelled version was synthesized with the label on the fourth alanine (indicated by the \(^\text{\textdegree}\)). Both versions of ALGA used in this work were synthesized by Biomatik (Cambridge, ON). Figure 3.6 gives a visualization for a helical representation of the ALGA peptide.
Figure 3.6: Helical representation of the structure of ALGA (a) end view, and (b) side view.

3.3 Buffers

Powder samples and most bicelle samples were hydrated using 50 mM phosphate buffer, pH 7.0. In some cases, bicelle samples containing peptides were hydrated using a higher concentration phosphate buffer (75 mM). Lanthanide salts were used to change the orientation of some bicelle samples; YbCl$_3$ and EuCl$_3$ were purchased from Sigma Aldrich (St. Louis, MO). These lanthanide salts were added to the 50 mM phosphate buffer and this mixture was used to hydrate the lipids to form flipped bicelles.
Chapter 4

Sample Preparation and Experimental Setup

4.1 Sample Preparation

4.1.1 Powder Samples

Multilamellar dispersions of lipids are referred to here as ‘powder samples’ because they give rise to the typical deuterium NMR powder pattern since there is an equal probability for every orientation of the lipids. These multilamellar dispersions were prepared by either mixing the appropriate quantities of the dry, powdered lipids and cholesterol in a round-bottomed flask and adding ethanol in order to completely dissolve all components, or by mixing together appropriate volumes of stock solutions for each component (2.5 mg/mL lipid or cholesterol in ethanol). The solvent was removed by freezing the mixture using liquid nitrogen and lyophilizing overnight. The dry sample was then carefully scraped from the flask and weighed. Phosphate buffer (50 mM, pH 7.0) was added at a ratio of 4:3 (lipid weight to buffer volume) and the mixture was stirred multiple times alternately by hand using a glass rod and gentle centrifugation until the mixture was homogeneous. This hydration and mixing was done at ambient temperature. The sample was transferred into a
small glass sample tube which was sealed using silicone or a rubber stopper to prevent any water loss during the experiments. This sample preparation technique is also discussed elsewhere [25].

**Powder Samples with Peptides**

Samples with peptides in multilamellar dispersions were prepared in much the same manner as the samples without peptide. For these samples the lipids and peptide were codissolved in an appropriate solvent (for gramicidin TFE is used in order to have the correct configuration of the peptide). The mixture was then lyophilized to remove the solvent and then scraped and hydrated with buffer at a ratio of 4:3 (lipid weight to buffer volume).

**4.1.2 Bicelle Samples**

Bicelle samples were made in a manner very similar to the powder samples. Important differences are that these samples include the short chain lipid, DCPC, such that the desired ratio (q) between the long-chain lipid and short-chain lipid is obtained, and the amount of buffer in the sample is significantly higher for bicelle samples than typically used for the multilamellar dispersions. DMPC-d$_{54}$, DPOPC, and cholesterol were weighed out as dry powders, while the DCPC was added as an appropriate volume of a 2.5 mg/mL (DCPC/ethanol) stock solution due to its highly hygroscopic nature. The lipids were dissolved in ethanol, the solvent was removed by lyophilizing overnight, then the dry mixture was scraped from the round-bottomed flask. Buffer was added such that the final ratio of buffer/total sample (w/w) was 0.6 (unless otherwise noted). 50 mM phosphate buffer was used except in cases where the bicelles were to be flipped to have their normals ori-
ented parallel to the magnetic field with the use of lanthanide ions. In these cases, the buffer was mixed with lanthanide salts giving the final dry sample/lanthanide molar ratio of 10:1 when the weight ratio of buffer/total sample is maintained as 0.6. All samples were transferred into small glass tubes which were sealed with silicone to ensure that the water content was constant throughout the experiments.

Typically bicelle preparation methods include steps of freezing and thawing the hydrated samples in order to create uniformly sized particles. These freeze-thaw cycles are omitted from the current sample preparation as the essential feature of the samples used here is that the bilayers are well-oriented within the magnetic field of the spectrometer and that the particles are large enough to support \( \ell_d-\ell_o \) phase coexistence meaning that they should be \( \sim 200 \) nm or larger. It is generally thought that bicelle samples with \( q \sim 3 \) and water concentrations on the order of 60\%, corresponding to the bicelle compositions used in this work, are made up of perforated bilayers with the possibility of some disk shaped particles as well. This morphology should allow for the coexistence of the \( \ell_d \) and \( \ell_o \) phases within the same particle.

**Bicelles with Peptides**

For bicelle samples containing both lipids and peptide, the sample preparation technique was much the same as above; however, in some cases the buffer used was changed from the 50 mM phosphate buffer to a 75 mM phosphate buffer. In addition, depending on the characteristics of the peptide, another solvent such as methanol was required to dissolve all components of the sample. For samples with 4 mol \% Conolysin-Mt1, 75 mM phosphate buffer was used in order to achieve a long-lasting sample. For samples with ALGA, a
mixture of ethanol and methanol was used to dissolve the lipid and peptide components.

4.1.3 Samples Oriented on Glass Slides

Mechanically oriented samples were aligned on glass slides. Appropriate amounts of the lipids, cholesterol and peptide were combined and dissolved in a small volume of ethanol (or ethanol/methanol). This solution was pipetted onto glass slides and allowed to dry, this was repeated until all of the sample was deposited on the slides. The dry slides with sample on them were then stacked and placed in a plastic channel to maintain their alignment and provide stability. The sample was then put through a series of hydration/dehydration cycles at \( \sim 50^\circ \text{C} \) ending with hydration. A couple of small pieces of wetted filter paper was placed on top of a clean glass slide in order to provide extra water for the lipids. In order to monitor the hydration of the sample and ensure that there was available water in the sample holder, a drop of D\(_2\)O was added to the H\(_2\)O used for hydrating the samples with deuterated lipids. Pure H\(_2\)O was used for samples with labelled peptide. Typically samples with deuterated lipids had 10 mg of DMPC-d\(_{54}\) whereas samples with protonated lipids had 12 mg of DMPC and both contained up to 6 mol % peptide.

4.2 Experimental Setup

NMR experiments were performed on either a 500 MHz, 600 MHz or 800 MHz Bruker BioSpin (Milton, ON) spectrometer. Home-made coils were used and the 90° pulses were optimized and kept as short as possible in order to minimize any artefacts [79]. Typically, the 90° pulse lengths used were 2.25 \( \mu \text{s} \) at 76.77 MHz, 1.70 \( \mu \text{s} \) at 92.15 MHz, and 2.75 \( \mu \text{s} \) at
122.84 MHz. The echo delay was 40 µs at 76.77 and 92.15 MHz, and 25 µs at 122.84 MHz.

The temperature for each probe used was calibrated using Pb(NO₃)₂ [80, 81], and the temperatures presented here are the corrected temperatures. The melting points of DPPC and DMPC were used as references for the calibrations.

The majority of the ²H spectra were collected using a quadrupolar echo pulse sequence. The second delay in the pulse sequence is set such that some points before the top of the echo are recorded. The FID signal is manually phase corrected and the points before the top of the echo in the time domain were removed. The points are shifted so that the first point sits exactly at the top of the echo. This is an important process which results in symmetric spectra with a flat baseline [72]. In some cases a modified quadrupolar echo sequence (for water suppression for example) is used and details of these sequences are specified with the resulting data.

Two dimensional ²H exchange experiments were performed on powder and bicelle samples exhibiting ℓ₋ℓᵤ coexistence. The pulse sequences have the form 90° y - t₁ - 54.7° - τₘ (variable mixing time) - 54.7° - Δ - 90° x - Δ - t₂. Two datasets are recorded for ²H exchange experiments with different phases for the 54.7° storage and reconversion pulses. For dataset 1, the storage pulse is 54.7° y, and the reconversion pulse is 54.7° y. For dataset 2, the storage pulse is 54.7° x, and the reconversion pulse is 54.7° x. [82]. These experiments are discussed in detail in Chapter 9.

The ³¹P NMR experiments were performed using a simple 90° pulse and detection. The probe used could only be set to a single channel in the 600 MHz spectrometer so no decoupling could be applied. The ³¹P spectra were used to determine whether some samples were oriented in the magnetic field.
Chapter 5

Critical Fluctuations

The results presented in this chapter are published in Biophysical Journal [76].

5.1 Introduction

Phase diagrams for ternary model membranes composed of DOPC, DPPC, and cholesterol have been determined using NMR spectroscopy and fluorescence microscopy [51, 35, 25]. In addition, phase diagrams for the binary mixtures of DPPC and cholesterol [24], and DOPC and DPPC [31] have also been presented. Ternary mixtures composed of DOPC/DPPC/cholesterol can exhibit two coexisting fluid phases over a broad range of temperatures and compositions [35, 25] and the phase diagrams for these mixtures contain a line of critical points at which the \( \ell_d \) and \( \ell_o \) phases become indistinguishable. Cholesterol is known to have an ordering effect on phospholipid chains and interacts preferentially with saturated hydrocarbon chains. The more ordered \( \ell_o \) phase consists of cholesterol-rich domains of the membrane, while cholesterol-poor domains will only form the less ordered \( \ell_d \) fluid phase [23, 22, 24, 25, 35]. The degree of lipid chain order provides a means for differentiating the \( \ell_o \) and \( \ell_d \) fluid phases. At some compositions and temperatures, fluctuations in the local concentration of cholesterol have been
observed [53, 48, 76]. Critical phenomena in ternary DOPC/DPPC/cholesterol model membrane systems have been explored previously using experimental data from fluorescence microscopy on giant unilamellar vesicles and $^2$H NMR on multilamellar vesicles or multilamellar dispersions [51, 35, 54, 55, 56, 76], and have been interpreted in terms of an Ising model [54, 48, 55, 76] or of condensed complexes of cholesterol and phospholipids [83, 84, 85, 86, 87, 88, 89].

Our previous work on critical fluctuations in ternary DOPC, DPPC, cholesterol model membrane systems fit the results from magic angle spinning (MAS) NMR spectroscopy to a theoretical model describing critical phenomena [55, 56]. Critical fluctuations in ternary lipid/cholesterol systems can be detected by MAS NMR as extra broadening in the MAS sideband line widths, which provides a useful method for studying critical fluctuations near $T_c$ [35, 87, 90, 91, 92]. The function used to fit the sideband line widths as a function of temperature was described by Suwelack et al. [93] in the context of line broadening due to slow motions and was used to determine the critical temperature and critical exponent for the correlation length of the DOPC/DPPC/cholesterol bilayer samples. Specifically labelled $\alpha$-$d_4$-DPPC was used here for some experiments in order to simplify the MAS NMR spectra. The four deuterium labels on the $\alpha$-$d_4$-DPPC are found at the top of the acyl chains (closest to the headgroup). Several samples were studied with molar compositions 21:49:30, 28:42:30, 35:35:30, 42:28:30, and 49:21:30 (DOPC/$\alpha$-$d_4$-DPPC/cholesterol). The exponent values obtained from the DOPC/$\alpha$-$d_4$-DPPC/cholesterol samples ranged between $\nu_c = 0.65$ and $\nu_c = 1.2$ [55]. Not all of these compositions are close to a critical composition, and unless the sample is near a critical composition, it is not possible to determine the critical exponents or universality class. However, critical fluctuations in
composition, specifically cholesterol concentration, in DOPC/DPPC-d$_{62}$/cholesterol samples with compositions which were chosen to be close to the critical compositions were studied using $^2$H MAS NMR and resulted in an experimental value for the critical index for the correlation length, $\nu_c = 0.628$ which is consistent with the 3D Ising model [56].

Since critical phenomena are highly dependent on the temperature of the sample, one of the biggest drawbacks of using MAS NMR is that there is a temperature gradient across the sample due to the friction from the spinning and the size of this gradient depends strongly on the spinning rate. It is therefore advantageous to perform static NMR experiments where there is no temperature gradient due to spinning the sample. We investigated the critical behaviour of DOPC/DPPC/cholesterol mixtures by fitting the lineshapes of static $^2$H NMR spectra. The results presented here are from static solid state NMR experiments on DOPC/DPPC/cholesterol multilamellar dispersions. The first moments and quadrupolar splittings of the $^2$H NMR spectra were analysed as a function of temperature [76].

### 5.2 Experimental Details

The compositions for the samples used to investigate critical phenomena were verified by solution state $^1$H NMR using a 600 MHz cryoprobe (Bruker Biospin, Milton, ON).

It is important to have a consistent temperature across the sample when studying critical phenomena. This work was done using a home built probe which has a copper oven for even heating and a reduced temperature gradient across the sample as compared to conventional air flow heated probes. The temperature is measured using a Pt-100 resistance
thermometer and a calibration was performed using Pb(NO\(_3\))\(_2\) [80, 81]. The temperature gradient was estimated to be ±0.05 K or \(T_{\text{grad}} < 0.1\) K.

Moment analysis was performed as described in Chapter 2.3.1. Uncertainties for the samples containing DPPC-d\(_{62}\) were estimated to be ~2 % for the area, 0.5 % for \(M_1\), and 1 % for \(M_2\). Note that the uncertainties for the first and second moments are smaller than the uncertainty for the area. This is because the intensity of the spectra depends on variations in probe tuning which will affect the area but not the overall shape of the spectra.

Quadrupolar splittings for the methyl-deuterated sample spectra were measured by fitting the spectra to Gaussian broadened powder pattern(s) as described in Chapter 2.3.2, Appendix A and in more detail below.

## 5.3 Results

### 5.3.1 Chain Perdeuterated versus Methyl-Deuterated DPPC

Figure 5.1 shows static \(^2\)H NMR spectra for multilamellar dispersion samples with molar compositions 37.5:37.5:25 (DOPC/DPPC/cholesterol) with two different labelling schemes. In part (A) chain perdeuterated DPPC-d\(_{62}\) was used, while in part (B) methyl-deuterated DPPC-d\(_6\) was used. These spectra show the onset of the \(\ell_d-\ell_o\) two phase coexistence region for these samples. Notice the different frequency scales of the two parts of the figure since the methyl splittings are much smaller than those of the methylenes. At the highest temperatures, both samples are in the \(\ell_d\) region of the phase diagram. As the temperature is lowered and approaches the critical point, the spectra begin to broaden. Below the critical temperature, \(T_c\), two coexisting fluid phases can be distinguished in the spectra.
(below 301.09 K for the sample containing DPPC-d$_{62}$ and below 303.38 K for the sample containing DPPC-d$_{6}$ sample). Note that the critical temperature is slightly higher for the methyl-deuterated (DPPC-d$_{6}$) sample than for the chain perdeuterated (DPPC-d$_{62}$) sample which is expected due to the different numbers of deuterons on the acyl chains of DPPC in the two samples.

At temperatures below $T_c$ for the 37.5:37.5:25 (DOPC/DPPC-d$_{6}$/cholesterol) sample, three quadrupolar splittings can be seen in Figure 5.1 (B). The smallest quadrupolar splitting corresponds to the $\ell_d$ phase which is the most disordered. The two larger quadrupolar splittings arise from the inequivalent methyl groups in the $\ell_o$ phase. There is a small, sharp peak in the centre of these spectra which is due to the natural abundance HDO in the buffer.

### 5.3.2 Critical Behaviour in DOPC/DPPC-d$_{6}$/Cholesterol Mixtures

The $^2$H NMR spectra from the 37.5:37.5:25 (DOPC/DPPC-d$_{6}$/cholesterol) sample were fit with a single Gaussian broadened doublet at temperatures above $T_c$ and a superposition of three Gaussian broadened doublet powder patterns at temperatures below $T_c$ as described in Chapter 2.3.2. The fits were characterized by an area, a quadrupolar splitting, and a Gaussian broadening factor, $\sigma$ for each component. The parameter, $\rho$, which is the ratio between the semimajor and semiminor axes of the prolate ellipsoidal liposomes ranged between 1.05 and 1.08 for temperatures above $T_c$. When the temperature was below $T_c$ and three powder pattern contributions were included, all three contributions used the same value for $\rho$ which varies between 1.0 and 1.08. In addition, the areas of the two $\ell_o$ phase components were constrained to remain within 10% of one another. The fitting functions used to fit the experimental spectra both above and below $T_c$ can be found in Appendix A.
Figure 5.1: Static $^2$H NMR spectra of multilamellar dispersions of (A) 37.5:37.5:25 (DOPC/DPPC-d$_{62}$/cholesterol), and (B) 37.5:37.5:25 (DOPC/DPPC-d$_{6}$/cholesterol) at various temperatures showing the onset of $^d$/$^o$ fluid phase coexistence. Note that the critical temperature is slightly higher for the methyl-deuterated (DPPC-d$_6$) sample than for the chain perdeuterated (DPPC-d$_{62}$) sample. Collected at 76.77 MHz, (A) 4096 scans, (B) 2048 scans. [76]

The temperature dependence of the quadrupolar splittings for the $\ell_d$ and $\ell_o$ contributions to the $^2$H NMR spectra are shown in Figure 5.2. Splittings due to the $\ell_d$ phase are shown by solid circles above $T_c$ and open circles below $T_c$ ($v_1$ component). Splittings arising from the $\ell_o$ contributions to the spectra below $T_c$ are given by open triangles ($v_2$
component) and solid squares (ν₃ component). Note that the small discontinuity that can be seen near 297 K in Figure 5.2 is a result of the necessity for a slight change in the air flow rate in order to reach lower temperature.

![Figure 5.2: Quadrupolar splittings for the d and o phase components of ²H NMR spectra of 37.5:37.5:25 (DOPC/DPPC-d₆/cholesterol) as a function of temperature. The d phase components are shown by the solid circles above T_c and the open circles below T_c (ν₁). The o phase components are represented by the open triangles and solid squares (ν₂ and ν₃). [76] ](image)

As shown in Figure 5.2, at temperatures above T_c, there is a single quadrupolar splitting. The ²H powder pattern for 37.5:37.5:25 (DOPC/DPPC-d₆/cholesterol) splits into three identifiable components at the critical temperature. The uncertainty of the quadrupo-
lar splitting values increases near the critical point since the three quadrupolar splittings rapidly approach the same value as the critical temperature is approached from below. There are two factors that contribute to the observed changes in the quadrupolar splittings of this sample as a function of temperature. First, in the absence of phase changes, as the temperature is lowered, the splittings generally increase. Second, there are continuous changes in the composition of the domains (\(\ell_d\) and \(\ell_o\)) compared to the average sample composition as the temperature is lowered. For example, in the \(\ell_d\) phase domains the cholesterol concentration is lower than the nominal sample composition of 25 mol \%, whereas in the \(\ell_o\) phase domains the cholesterol concentration is higher than the nominal sample composition.

The Gaussian broadening factor, \(\sigma\), used to characterize the powder patterns is shown as a function of temperature in Figure 5.3. The value of \(\sigma\) changes dramatically as the critical temperature is approached from above and below. The Gaussian factors for the \(\ell_d\) phase are shown by solid circles for temperatures above \(T_c\) and open circles for temperatures below \(T_c\) (\(\sigma_1\)). The Gaussian factors for the two components corresponding to the two inequivalent \(\ell_o\) contributions to the spectra below \(T_c\) are given by open triangles (\(\sigma_2\) component) and open squares (\(\sigma_3\) component). The \(\sigma_2\) and \(\sigma_3\) values which are related to the \(\ell_o\) domains differ slightly from one another and do not show the strong temperature dependence observed for \(\sigma_1\) which corresponds to the \(\ell_d\) domains.

Static deuterium powder pattern line widths for spectra obtained from lipid bilayer samples are dependent on a number of factors including slow fluctuations of the quadrupolar interaction, temperature gradients across the sample and the compositional homogeneity of the sample. The methyl group quadrupolar splitting depends on cholesterol con-
Figure 5.3: Gaussian line widths as a function of temperature for $^2$H NMR spectra of 37.5:37.5:25 (DOPC/DPPC-d$_6$/cholesterol). The $\ell_d$ phase components are shown by the solid circles above $T_c$ and the open circles below $T_c$ ($\sigma_1$). The $\ell_o$ phase components are represented by the open triangles ($\sigma_2$) and open squares ($\sigma_3$). [76]

centration, and can vary from about 4 kHz without cholesterol to 12 kHz with 30 mol % cholesterol or more depending on the temperature of the sample. For a sample with a local cholesterol concentrations that varies by no more than 1 mol % across the sample, there can be a contribution to the line width of up to 200-300 Hz. At temperatures slightly below $T_c$, a temperature gradient of 0.1 K across the sample may contribute up to 200 Hz to the
Gaussian line width for the $\ell_d$ phase, $\sigma_1$. Note in Figure 5.3 that the $\ell_o$ line widths, $\sigma_2$ and $\sigma_3$, stay quite small and do not change dramatically; however, the quadrupolar splittings, $\nu_2$ and $\nu_3$, as seen in Figure 5.2 increase quickly as the temperature is decreased. This indicates that the temperature gradient across the sample in the probe used here is less than $\sim 0.1$ K near $T_c$.

Orientational fluctuations in the quadrupolar Hamiltonian are important to the spectral line width of deuterium NMR spectra. Fluctuations in the local cholesterol concentration due to critical behaviour result in a modulation of the deuterium quadrupolar Hamiltonian. The modulation of the quadrupolar Hamiltonian occurs as a result of the strong ordering effect that cholesterol has on the acyl chains of the phospholipids [94, 24, 95] and provides an effective relaxation/line broadening mechanism [93]. Previous work on critical phenomena in DOPC/DPPC/cholesterol systems was completed using $^2$H MAS NMR, where critical fluctuations can be directly detected through extra broadening in the sideband line widths [56].

Quadrupolar echo relaxation measurements were performed at 308.8 K where the sample is in the $\ell_d$ phase region, and 292.6 K where the sample is in the $\ell_d$-$\ell_o$ coexistence region. Echo delays were varied from 50 to 800 $\mu$s. These measurements allow the echo decay time, $T_{2e}$, to be determined. The relaxation of the deuterium spectral line widths across the powder pattern were fit to an exponential function using the following relationship

$$2\sqrt{2\log 2} \times \sigma_{2e} = \frac{1}{\pi \times T_{2e}}$$

(5.1)

where $\sigma_{2e}$ is the Gaussian width, and $T_{2e}$ is the echo decay time. This relationship connects the full width at half-maximum to the Gaussian width. At 308.8 K, $T_{2e} = 711 \pm 1$ $\mu$s and
the calculated value of the Gaussian width is $\sigma_{2e} = 190$ Hz. This is much smaller than the static powder pattern linewidth which was measured as $\sigma = 502.4 \pm 1.4$ Hz. Similarly, at 292.6 K, the component relaxation times are $T_{2e}^{(1)} = 893 \pm 7$ $\mu$s, $T_{2e}^{(2)} = 843 \pm 6$ $\mu$s, and $T_{2e}^{(3)} = 656 \pm 5$ $\mu$s. These $T_{2e}$ values correspond to Gaussian line widths of $\sigma_{2e}^{(1)} = 151$ Hz, $\sigma_{2e}^{(2)} = 160$ Hz, and $\sigma_{2e}^{(3)} = 205.4$ Hz. Again, these values for the Gaussian widths are lower than the measured values at 292.6 K which are $\sigma^{(1)} = 303.5 \pm 3$ Hz, $\sigma^{(2)} = 336 \pm 3$ Hz, and $\sigma^{(3)} = 368.4 \pm 4$ Hz. These results indicate that relaxation accounts for only about half of the observed line widths of the static $^2$H NMR spectra of the 37.5:37.5:25 (DOPC/DPPC-d$_6$/cholesterol) sample. As a result, it is not possible to use the same fitting models that were used for the MAS sideband analysis to analyse the critical behaviour of this system under static $^2$H NMR conditions. The method of analysis used to determine the critical exponent and best model for the critical behaviour of DOPC/DPPC/cholesterol ternary mixtures from static $^2$H NMR is developed below.

The fraction, $g_{\ell_o}$, describes the amount of DPPC which is found in the $\ell_o$ phase domains of the sample. For the DOPC/DPPC-d$_6$/cholesterol sample, $g_{\ell_o}$ can be found by determining the ratio of the areas of the spectra coming from the two inequivalent methyls in the $\ell_o$ phase (the $A_2$ and $A_3$ components) to the total spectral area as follows

$$g_{\ell_o} = \frac{A_2 + A_3}{A_1 + A_2 + A_3}$$

(5.2)

Note that $A_1$ is the area of the $\ell_d$ component of the spectrum, and $A_2$ and $A_3$ are the areas of the two $\ell_o$ components.

The fraction of DPPC-d$_6$ that is in the $\ell_o$ phase is plotted as a function of temperature in Figure 5.4. The data was fit to an expression in terms of $T_{c} - T$ which allows values of
The parameters which gave the best fit for this function were $a = 0.1516 \pm 0.014$, $b =$ 

Figure 5.4: Temperature dependence of $g_{\ell_o}$, the fraction of the DPPC-$d_6$ in the $\ell_o$ phase. $g_{\ell_o}$ was determined from the ratio of the areas of the spectral components from the $\ell_o$ phase to the total spectral area for the 37.5:37.5:35 (DOPC/DPPC-$d_6$/cholesterol) sample. The data was fit to an arbitrary function of $(T_c - T)$ in order to allow for a comparison with the first moment data from the 37.5:37.5:35 (DOPC/DPPC-$d_{62}$/cholesterol) sample. [76]
0.0102 ± 0.0008, \( c = 0.553 \pm 0.013 \), and \( d = 1.59 \pm 0.07 \). This expression was used to determine the appropriate value for \( g_{e_o} \) based on the critical temperature for the sample which was required for the analysis of the first moment data from the DPPC-d_{62} sample.

The quadrupolar splittings of the \( \ell_d \) and \( \ell_o \) phase components of the \(^2\)H NMR spectra of the DPPC-d_{6} sample can be used to directly evaluate the average change in the splitting, \( \langle \Delta (\delta v) \rangle \) below \( T_c \).

\[
\langle \Delta (\delta v) \rangle = [(\nu_2 + \nu_3) / 2] - \nu_1
\]  

This expression describes the difference between the average methyl group quadrupolar splitting of the \( \ell_o \) phase components and the methyl group quadrupolar splitting of the \( \ell_d \) phase.

Here the assumption is that \( \Delta (\delta v) \) is proportional to the order parameter (local cholesterol concentration) for the phase transition. Figure 5.5 shows \( \Delta (\delta v) \) as a function of temperature for the 37.5:37.5:25 (DOPC/DPPC-d_{6}/cholesterol) sample. Note that the error bars represent the standard errors from the fitting function for the splittings. The data was fit to an expression in terms of \( T_c - T \) to determine the critical exponent for the system.

\[
\Delta (\delta v) = \Delta (\delta v)_0 [(T_c - T) / T_c]^{\beta_c}
\]  

The solid line in Figure 5.5 represents the best fit of the data and was for \( \Delta (\delta v)_0 = 17.37 \pm 0.5 \) kHz and a critical exponent of \( \beta_c = 0.338 \pm 0.009 \). This value for \( \beta_c \) is close to the value expected for the 3D Ising model, \( \beta_{3D} = 0.325 \). On the other hand, the critical exponent for the 2D Ising model is \( \beta_{2D} = 0.125 \). The resulting curve for the 2D Ising model is shown by the dashed line in Figure 5.5 and does not fit the data.

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Figure 5.5: Difference between the average methyl group quadrupolar splitting of the \( \ell_o \) phase components and the methyl group quadrupolar splitting of the \( \ell_d \) phase as a function of temperature below the critical temperature, \( T_c \), for the 37.5:37.5:25 (DOPC/DPPC-d\(_6\)/cholesterol) sample. \( T_c \) is indicated by the dotted line at 303.74 \( \pm \) 0.07 K. The best fit for the critical exponent is for \( \beta_c = 0.338 \pm 0.009 \). [76].

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5.3.3 Critical Behaviour in DOPC/DPPC-\textit{d}_{62}/Cholesterol Mixtures

As discussed previously, moment analysis is a method for quantifying the chain order of the lipids in the samples as a function of temperature. The first moments for the 37.5:37.5:25 (DOPC/DPPC-\textit{d}_{62}/cholesterol) sample are shown in Figure 5.6.

![Figure 5.6: $M_1$ as a function of temperature for the 37.5:37.5:25 mol ratio (DOPC/DPPC-\textit{d}_{62}/cholesterol) sample. The dotted line indicates the baseline temperature dependence. The experimental data deviates from the baseline value as the temperature is lowered beginning at the critical temperature, $T_c$. The solid curve for temperatures below $T_c$ is the best fit to a power law with $\beta_c = 0.391 \pm 0.02$. The dashed curve for temperatures below $T_c$ is the 2D Ising model prediction which has $\beta_{2D} = 0.125$. [76]]
In the $\ell_d$ single phase region which occurs at temperatures above the critical temperature, $M_1$ increases approximately linearly as the sample temperature is decreased. This baseline temperature dependence is indicated by a dotted line in the figure. In the two fluid phase coexistence region below $T_c$, the experimental data deviates from the baseline value and there is a significant change in the slope of the $M_1$ versus temperature graph. Cholesterol has an ordering effect on the chains of the saturated lipid and thus the quadrupolar splitting for lipids in the $\ell_o$ phase is larger than for lipids in the $\ell_d$ phase. The $\ell_o$ phase is rich in cholesterol and the saturated lipid (DPPC), while the $\ell_d$ phase is rich in the unsaturated lipid (DOPC), and the different quadrupolar splittings for the two phases are readily observed in the $^2$H NMR spectra. For the methyl-deuterated lipid, $\langle \Delta (\delta \nu) \rangle$ corresponds to $\Delta M_1$ for the chain perdeuterated DPPC. The first moment for a $^2$H NMR spectrum collected in the two fluid phase coexistence region of the DOPC/DPPC/cholesterol system can be written as a sum of two parts

$$M_1^{\text{exp}} = g_{\ell_o} \times M_1^{\ell_o} + (1 - g_{\ell_o}) \times M_1^{\ell_d}$$

$$= g_{\ell_o} \times (M_1^{\ell_o} - M_1^{\ell_d}) + M_1^{\ell_d}$$

$$= g_{\ell_o} \times \Delta M_1 + M_1^{\ell_d} \tag{5.6}$$

where $M_1^{\ell_d}$ and $M_1^{\ell_o}$ are the first moments which arise from each of the phases present in the spectrum, and $g_{\ell_o}$ is the fraction of DPPC which is in the $\ell_o$ phase. $M_1$ is proportional to the average quadrupolar splitting of the spectrum [73, 72], meaning $M_1^{\ell_d} < M_1^{\ell_o}$ and in equation 5.6, $\Delta M_1 = (M_1^{\ell_o} - M_1^{\ell_d}) > 0$. The value of $\Delta M_1 = \frac{4\pi}{(3\sqrt{3})} \Delta \langle (\delta \nu Q) \rangle$ is dependent upon the difference in the cholesterol concentrations in the $\ell_d$ and $\ell_o$ phases. Note that the relative amounts of the saturated and unsaturated lipids also differ between the $\ell_d$ and $\ell_o$ phase domains. $M_1$ was found to be proportional to the cholesterol concentration.
for cholesterol concentrations ranging from 0 to 30 mol% in samples with equal molar proportions of the two lipids DOPC and DPPC-d_{62}. In these cases $M_1(z_{\text{chol}}) = 8.75 \times 10^4 \times z_{\text{chol}} + 5.17 \times 10^4$ with an adjusted $R^2$ value of 0.9986.

The behaviour of $M^{exp}_1$ (Figure 5.6) below $T_c$ is dependent on $\Delta M_1$ and the fraction of DPPC-d_{62} in the $\ell_o$ phase, $g_{\ell_o}$. The parameters for the fit in Figure 5.4 for the DPPC-d_6 sample can be used to calculate the fraction of $\ell_o$ phase in the DPPC-d_{62} sample by substituting the appropriate value for the critical temperature into the expression for $g_{\ell_o}$. In the case of the DPPC-d_6 sample, the critical temperature was $T_c = 303.74$ K, while in the case of the DPPC-d_{62} sample, the critical temperature was $T_c = 301.51$ K. It is now possible to fit the expression for $M^{exp}_1$ to the data shown in Figure 5.6. Here $\Delta M_1 = \Delta M_1^0 \times [(T_c - T)/T_c]^{\beta_c}$ and the fit is indicated by the solid line on the graph. The parameters used to fit the data in Figure 5.6 are $\Delta M_1^0 = (7.1 \pm 0.7) \times 10^4$ s$^{-1}$ and $\beta_c = 0.391 \pm 0.02$. The value for the critical exponent, $\beta_c$ obtained is close to the value expected for the 3D Ising model, $\beta_{3D} = 0.325$. The best fit of the data using the critical exponent value for the 2D Ising model, $\beta_{2D} = 0.125$, is also shown on the graph for comparison but clearly does not fit the data.

## 5.4 Conclusions

Fluid-fluid, $\ell_d-\ell_o$, phase coexistence occurs for many different compositions of DOPC/DPPC/cholesterol mixtures. For high cholesterol concentrations ($> 20$ mol %), this two phase coexistence occurs over a broad range of temperatures. In this work, two samples were used, both with the same molar composition of 37.5:37.5:25 (DOPC/DPPC/cholesterol-d_{62}).
terol). In one sample chain perdeuterated DPPC-d_{62} was used, while in the other sample methyl-deuterated DPPC-d_{6} was used. In the two phase region, the $\ell_d$ and $\ell_o$ phases have different molar compositions. There is a higher concentration of cholesterol and saturated phospholipids in the $\ell_o$ phase domains. Near the critical point for these mixtures, compositional fluctuations become important and the correlation length diverges.

The critical behaviour of the two samples used in this work was analysed using the $^2$H NMR spectra. In the case of DPPC-d_{62}, the first moments were used and a fit to $\Delta M_1$ below the critical temperature was used to find the critical exponent. In the case of DPPC-d_{6}, the quadrupolar splittings arising from the parts of the spectra representing the two phases were measured and the difference between the average quadrupolar splitting in the $\ell_o$ phase and the quadrupolar splitting in the $\ell_d$ phase ($\Delta(\delta\nu)$) was used to find the critical exponent. Both $\Delta M_1$ and $\Delta(\delta\nu)$ are proportional to the order parameter, $\eta$. As the critical point is approached, the order parameter varies according to the power law $\eta = \left[\frac{T-T_c}{T_c}\right]^{\beta_c}$ where $\beta_c$ is the critical exponent. The value for the critical exponent for the DPPC-d_{6} sample was found to be $\beta_c = 0.338 \pm 0.009$ using the quadrupolar splittings of the $\ell_d$ and $\ell_o$ components of the spectra. The value for the critical exponent for the DPPC-d_{62} sample was found using a fit to $M_1^{exp}$ and was $\beta_c = 0.391 \pm 0.02$. Both of these values for the critical exponent are in close agreement with the 3D Ising model value of $\beta_{3D} = 0.325$. These results, along with our previously obtained value of $\nu_c = 0.628 \pm 0.01$ ($\nu_{3D} = 0.63$) for the critical exponent of the correlation length from $^2$H MAS NMR [56], indicate that multilamellar dispersions of phospholipids with cholesterol belong to the 3D Ising universality class. On the other hand, Veatch and Keller et al. [51, 52, 35, 53, 54, 48] have studied GUVs using fluorescence microscopy and reported critical exponents for
these systems that agree with those of the 2D Ising universality class.

Fluid phase coexistence of the $\ell_d$ and $\ell_o$ phases in lipid mixtures with cholesterol have been observed in systems since the 1980s [96]. Membrane rafts in biochemistry are a topic that has received much attention and debate since its introduction by Simons and Ikonen in 1997 [40]. Critical fluctuations are observed in model membrane systems with cholesterol concentrations that are comparable to those found in many cytoplasmic membranes. Critical behaviour, specifically fluctuations in the local membrane lipid composition may have a role to play in membrane biochemistry.
Chapter 6

Peptides in Non-oriented Model Membranes

6.1 Introduction

Biological membranes are dynamic and heterogeneous systems composed of multiple lipid species, sterols, and proteins. The phase behaviour of model lipid membranes has been characterized for many different lipid compositions [37]. Ternary mixtures of a saturated long chain phospholipid, an unsaturated long chain phospholipid, and cholesterol can exhibit two fluid phase coexistence of the $\ell_d$ and $\ell_o$ phases. Since the phase behaviour of DOPC/DPPC/cholesterol mixtures has been studied and these mixtures show $\ell_d$-$\ell_o$ phase coexistence over a broad range of temperatures and compositions [35, 25, 76, 97], they provide an interesting system in which to study peptide behaviour. The discussion of lipid rafts of cholesterol rich $\ell_o$ domains provides motivation for investigating the partitioning of peptides into the two coexisting fluid phases.

Gramicidin is a pentadecapeptide which is made by the soil bacterium Bacillus brevis and is often used as an antibiotic. There are variations of the sequence of gramicidin and these are called gramicidin A, B, C, and D. Gramicidin D is a mixture of the others and
contains about 85% gramicidin A. Gramicidin forms a dimer which spans the membrane and each subunit has a left handed \( \beta_{L,D}^{6,3} \) helical structure [98]. This channel structure allows small monovalent ions to pass through the membrane. Here gramicidin is used as a peptide component of a model membrane. Gramicidin spans the membrane in a multilamellar dispersion sample and \(^2\text{H}\) NMR is used to probe whether the peptide partitions preferentially into the \( \ell_d \) or \( \ell_o \) fluid phase, and to determine what effect the presence of the peptide has on the phase behaviour of the lipid membrane.

### 6.2 Experimental Details

A sample with a composition 35:35:30 (DOPC/DPPC/cholesterol) with 6 mol % synthetic 3,5-d\(_2\)-alanine gramicidin A (relative to the lipids: i.e. 94 mol % lipid and 6 mol % peptide) was used for this study. The DPPC component was made up of a mixture of unlabelled DPPC and methyl deuterated DPPC-d\(_6\) such that there was the same amount of the DPPC-d\(_6\) as gramicidin in the sample. The sample was prepared using the method for powder samples and was hydrated with a 4:3 (lipid weight/buffer volume) ratio of 50 mM phosphate buffer (deuterated buffer was used for the exchange labelled sample). It is important to codissolve the components of the sample in an appropriate solvent such as TFE in order to ensure the correct structure for gramicidin [99, 100].

Data was collected at 76.77 MHz using an inversion recovery pulse sequence, which is the quadrupolar echo pulse sequence with an alternating inversion pulse prior to the two 90° pulses. The delay between the inversion pulse and the quadrupolar echo sequence is 0.15 ms. This pulse sequence was used in order to suppress the water signal.
6.3 Results

Figure 6.1 shows an example of the full $^2$H spectrum for the sample composed of a mixture of DOPC/DPPC/cholesterol at a molar ratio of 35:35:30 with 6 mol % 3,5-d$_2$-alanine gramicidin A at a single temperature of 297.4 K. Since there are labels on both the lipid and the peptide, the behaviour of both can be monitored as a function of temperature. Note that the signal from the methyl groups of DPPC-d$_6$ is much sharper (with peaks near ±5 kHz) and easier to see than the peptide peaks (around ±50 kHz).

Figure 6.1: $^2$H spectrum for the 35:35:30 (DOPC/DPPC/cholesterol) sample with 6 mol % gramicidin A at 297.4 K. Note that the strong lipid methyl peaks are around ±5 kHz and the weaker peptide peaks are around ±50 kHz. The sharp peak at 0 kHz is due to deuterium in the buffer. Spectrum was collected with 160 000 scans at 76.77 MHz.
Figure 6.2: $^2$H spectra as a function of temperature for the 35:35:30 (DOPC/DPPC/cholesterol) sample with 6 mol % gramicidin A focussing on the DPPC-d$_6$ methyl peaks. Spectra were collected with 160 000 scans at 76.77 MHz.

In Figures 6.2 and 6.3, the same series of spectra are shown highlighting the different parts of the spectra. In Figure 6.2, the focus is on the methyl peaks of the labelled DPPC-d$_6$ as a function of temperature. As the temperature is decreased, the methyl splittings increase and become inequivalent which is indicative of the presence of the $\ell_o$ phase. This sample may have a smooth transition between the $\ell_d$ and $\ell_o$ phase without the two fluid phase coexistence.

In Figure 6.3, the focus is on the peptide peaks which have quadrupolar splittings on the order of 100 kHz. Again the splittings increase as the temperature decreases indicating
Figure 6.3: $^2$H spectra as a function of temperature for the 35:35:30 (DOPC/DPPC/cholesterol) sample with 6 mol % gramicidin A focusing on the gramicidin peaks. Spectra were collected with 160 000 scans at 76.77 MHz.

Increasing order as expected. Overall, these spectra show a high proportion of the liquid ordered phase across a broad range of temperatures. This may mean that gramicidin A promotes the liquid ordered phase in this DOPC/DPPC/cholesterol model membrane. Other samples were made using exchange labelled gramicidin D (a mixture of gramicidin A and other isoforms of the peptide) and different molar proportions of DOPC, DPPC, and cholesterol. Even though there are at least 20 exchangeable sites on this peptide [58], these samples also had very low signal to noise ratios and did not lead to conclusive results about preferential partitioning of gramicidin D into the $\ell_d$ and $\ell_o$ phases.
6.4 Conclusions

From this data, it appears that there is a large proportion of $\ell_\alpha$ phase over a broad range of temperatures which may be a result of ordering promoted by the presence of the peptide. However, the peptide signals in the powder spectra for these gramicidin in DOPC/DPPC/-cholesterol multilamellar dispersions are very broad and weak. Oriented samples would be advantageous for investigating peptides in model membranes since spectra from oriented samples are much simpler than those from powder samples. There are different ways to obtain oriented samples for NMR, these include mechanically orienting the sample on glass slides and magnetically orienting the samples by adding a short chain lipid to form bicelles. Magnetically oriented samples are preferable in terms of sample volume to signal ratio since no substrate is required for the alignment of the sample. The phase behaviour of bicelles which include cholesterol is described in Chapter 7 and investigations of peptides in bicelles are presented in Chapter 8, results for samples oriented on glass plates are presented in Chapter 10.
Chapter 7

Bicelle Investigations

Many of the results presented in this chapter are published in *Biochimica et Biophysica Acta (BBA) - Biomembranes* [97].

7.1 Introduction

Bicelles are useful as a membrane mimetic for NMR since they can give more signal for the same sample volume because there is no substrate needed to align them. Bicelles are hydrated mixtures of long and short chain lipids which spontaneously orient in a magnetic field [13, 14]. Long chain DMPC and short-chain DCPC readily form bicelles when mixed with water or buffer. Bicelle mixtures can be made from various types of long-chain lipids and short-chain lipids/detergents but DMPC/DCPC bicelles are most widely used [101, 102, 103, 16, 20, 104, 17]. Bicelles provide an oriented membrane mimetic that can be used for investigations of membrane-associated peptides and proteins.

The level of saturation of the long chain lipids as well as the addition of another component such as cholesterol will have an effect on the phase behaviour of the membrane in general. The effect of 1-palmitoyl-2-linoleoyl-*sn*- glycerol-3-phosphatidylcholine (PLiPC) which is a polyunsaturated lipid, and cholesterol on DMPC/DCPC bicelles has been in-
vestigated by Minto et al. [105]. In their work, they found that cholesterol increases the minimum alignment temperature of the bicelles, whereas the PLiPC decreases the minimum alignment temperature of the bicelles. Phase separation into the $\ell_d$ and $\ell_o$ phases of a bicelle system with multiple long chain lipids and cholesterol was investigated by Cho et al. [106] using $^1$H magic angle spinning NMR. They used lateral diffusion measurements to study POPC/DMPC/cholesterol/DCPC bicelles and reported micrometre-scale domains in these bicelles. Two fluid phase coexistence within aligned lipid samples is of interest for the investigation of peptides and proteins in these more complex and biologically relevant model lipid membrane systems.

In this chapter, the phase behaviour of bicelles with varying concentrations of cholesterol and unsaturated lipids is discussed and the coexistence of the $\ell_d$ and $\ell_o$ fluid phases is directly observed using static, solid-state $^2$H NMR. In addition, bicelles containing peptides were investigated. Many of the results from the DMPC/DCPC/cholesterol bicelles, DPoPC/DMPC/cholesterol/DCPC bicelles and the effects of lanthanide ions on these bicelles are presented by Schmidt and Davis [97].

### 7.2 Results

#### 7.2.1 DMPC/DCPC/Cholesterol Bicelles

A series of DMPC/DCPC/cholesterol bicelle samples with a constant ratio of DMPC-$d_{54}$/DCPC (q=3.2) and different amounts of cholesterol was made to investigate the effect of cholesterol on the phase behaviour of DMPC/DCPC bicelles. All samples were hydrated with buffer at a ratio of 0.6 (buffer/total hydrated sample (w/w)). The mole fraction
of cholesterol, $x_c$, is defined for these samples as $x_c = \frac{\text{mol cholesterol}}{\text{mol DMPC}+\text{mol DCPC}+\text{mol cholesterol}}$ and will be used to identify the different sample compositions.

The $^2$H NMR spectra of DMPC-$d_{54}$/DCPC ($q=3.2$) bicelles with no cholesterol are shown in Figure 7.1 over the temperature range from 329.2 K to 281.1 K. The $^2$H NMR spectra of DMPC-$d_{54}$/DCPC ($q=3.2$) bicelles with $x_c=0.037$ are shown in Figure 7.2 from 329.2 K to 292.5 K. Well-oriented bicelles are obtained for both of these samples over a broad range of temperatures. At high temperatures (above those shown in these figures) and at lower temperatures, below 286.7 K in the absence of cholesterol and below 292.5 K with $x_c=0.037$, an isotropic phase is observed which gives rise to a peak at 0 kHz. Several isotropic phases where molecules undergo isotropic reorientation on the NMR time scale exist such as micellar and cubic phases. For the sample with $x_c=0.037$, at temperatures below 290 K, this isotropic phase dominates the spectra, and a similar result is expected for DMPC/DCPC bicelles though a low enough temperature was not reached in this set of experiments.

Figures 7.3, 7.4, 7.5, and 7.6 show $^2$H spectra of DMPC-$d_{54}$/DCPC ($q=3.2$) bicelles with $x_c=0.071$, $x_c=0.087$, $x_c=0.10$, and $x_c=0.12$ respectively. Again, note the isotropic phase that begins to appear as the temperature is lowered. The results for $x_c=0.087, 0.10$ and 0.12 all give very similar spectra with the onset of the isotropic phase (as the sample temperature is lowered) occurring at a slightly increasing temperature as the cholesterol concentration increases. There is no evidence of two fluid phase coexistence for these values of $x_c$. A smooth transition from the $\ell_d$ to the gel phase (or gel + isotropic phases) is observed for these bicelle samples. The $^2$H spectra collected at temperatures about one degree apart are shown from 304.5 to 289.0 K in Figure 7.7 and illustrate this transition.
Figure 7.1: $^2$H spectra of DMPC-d$_{54}$/DCPC bicelles, $q=3.2$ at various temperatures. For the spectra at 286.7 K and 281.1 K, the vertical scale was increased by a factor of 4. Collected at 76.77 MHz, 512 scans.
Figure 7.2: $^2$H spectra of DMPC-\textsubscript{d54}/DCPC bicelles, q=3.2 with x\textsubscript{c}=0.037 at various temperatures. For the spectrum at 292.5 K, the vertical scale was increased by a factor of 2. Collected at 92.15 MHz, 512 scans.
from \( \ell_d \) to gel for the bicelle sample with \( x_c = 0.087 \).

Figures 7.8 and 7.9 show DMPC-d$_{54}$/DCPC (\( q = 3.2 \)) bicelles with \( x_c = 0.13 \) and \( x_c = 0.16 \). The major differences between these samples and all the other samples which had less cholesterol are the presence of a large isotropic peak throughout the entire temperature range investigated, and the appearance of the \( \ell_o \) fluid phase. The regions of the sample that are not in the isotropic phase are well oriented and give rise to relatively sharp peaks, many of which can be resolved from 304.5 K to 292.5 K in the \( x_c = 0.13 \) sample and from 316.8 K to 292.5 K for the \( x_c = 0.16 \) sample. At these levels of cholesterol, the quadrupolar splittings are much larger than those observed in the other bicelle samples, indicating that the fluid phase is the more ordered \( \ell_o \) phase.

A phase diagram for the DMPC-d$_{54}$/DCPC bicelle mixtures (\( q = 3.2 \)) with varying concentrations of cholesterol is summarized in Figure 7.10. The different single and coexisting phases are represented by each of the colours. The isotropic phase is magenta, the gel phase is green, the \( \ell_d \) phase is violet, the gel + isotropic coexisting phases are shown in yellow, the \( \ell_o \) + isotropic coexisting phases are shown in red, the \( \ell_d \) + isotropic coexisting phases are shown in cyan, and the \( \ell_d \) + \( \ell_o \) + isotropic coexisting phases are shown in orange. For the \( x_c = 0.087 \) mol fraction cholesterol bicelles at the highest temperatures investigated here, the bicelles are magnetically aligned and in the \( \ell_d \) phase down to about 298 K. Below that there is a rather gradual transition into a gel phase which can be seen in the spectra shown in Figure 7.7. The gel phase is more ordered than the liquid disordered phase and is characterized by larger quadrupolar splittings and broader lines as a result of a shorter \( T_2 \) but the sample is still oriented as seen in the spectra. As the temperature is lowered further the gel (+ isotropic) phase is reached. Eventually, at low enough tempera-
Figure 7.3: $^2$H spectra of DMPC-$d_{54}$/DCPC bicelles, q=3.2 with $x_c=0.071$ at various temperatures. For the spectrum at 292.5 K, the vertical scale was increased by a factor of 2. Collected at 92.15 MHz, 512 scans.
Figure 7.4: $^2$H spectra of DMPC-d$_{54}$/DCPC bicelles, q=3.2 with $x_c=0.087$ at various temperatures. Collected at 92.15 MHz, 512 scans.
Figure 7.5: $^2$H spectra of DMPC-d$_{54}$/DCPC bicelles, q=3.2 with $x_c=0.10$ at various temperatures. Collected at 76.77 MHz, 512 scans.
Figure 7.6: $^2$H spectra of DMPC-d$_{54}$/DCPC bicelles, q=3.2 with $x_c=0.12$ at various temperatures. Collected at 92.15 MHz, 512 scans.
Figure 7.7: $^2$H spectra demonstrating the continuous change from the $\ell_d$ to the gel (gel + isotropic) phase for DMPC-d$_{54}$/DCPC bicelles, q=3.2 with $x_c$=0.087. Collected at 92.15 MHz, 512 scans.
Figure 7.8: $^2$H spectra of DMPC-$d_{54}$/DCPC bicelles, $q=3.2$ with $x_c=0.13$ at various temperatures. Collected at 92.15 MHz, 512 scans.
Figure 7.9: $^2$H spectra of DMPC-d$_{54}$/DCPC bicelles, q=3.2 with $x_c=0.16$ at various temperatures. Collected at 92.15 MHz, 512 scans.
tures, the whole sample goes into an isotropic phase. The dashed line is used to represent the transition between the oriented $\ell_d$ phase and the onset of an isotropic peak and gel phases at lower temperatures. At higher temperatures and low cholesterol concentrations, an isotropic phase is observed in addition to the $\ell_d$ phase. An isotropic phase contribution to the spectra can also be seen in the $x_c=0.13$ and $x_c=0.16$ bicelle samples throughout the full range of temperatures. Data represents the full range of temperatures sampled in the experiments, from 341.5 K to 281.1 K but not all temperatures were sampled for each cholesterol concentration. The upper limit for the oriented $\ell_d$ phase was not reached for the $x_c=0.071$ to $x_c=0.12$ cholesterol/DMPC bicelle samples.

The phase behaviour of bicelle samples is sensitive to the temperature history of the sample. For example, spectra for a bicelle sample which has been exposed to a sufficiently high temperature environment where a significant isotropic phase is present in addition to the fluid phase will contain the isotropic peak down to a lower temperature than if the temperature of the sample had not been raised so high. This is illustrated in Figure 7.11 for two DMPC-$d_{54}$/DCPC $q=3.2$ bicelle samples. The isotropic peak in the spectra is present down to 318.0 K in Figure 7.11(A) which had a maximum temperature of 343.9 K and to 330.5 K in (B) which was only taken up to a maximum of 331.7 K. The temperature history for these types of samples is important and can affect the values of the calculated moments and the phase behaviour of the samples. Nevertheless, a broad range of temperatures and compositions, for which oriented bicelles containing DMPC, DCPC and cholesterol can be investigated, has been found in this investigation.

The first moment, $M_1$, for the $^2$H spectra of the DMPC-$d_{54}$/DCPC ($q=3.2$) bicelles with $x_c = 0$ to 0.16 were calculated as described in Chapter 2.3.1. The first moment of a $^2$H
Figure 7.10: Summary of phases observed in DMPC-d_{54}/DCPC bicelle mixtures ($q=3.2$) with varying concentrations of cholesterol. Each colour represents a different phase or coexisting phases. Magenta: isotropic, green: gel, violet: $\ell_d$, yellow: gel + isotropic, red: $\ell_o$ + isotropic, cyan: $\ell_d$ + isotropic, and orange: $\ell_d$ + $\ell_o$ + isotropic. The dashed line indicates the transition between the oriented $\ell_d$ phase and the onset of an isotropic peak and gel phases at lower temperatures. In the 0.071 to 0.12 mol fraction cholesterol bicelle samples the upper limit for the oriented $\ell_d$ phase was not reached.

The spectrum provides a quantitative method for comparing dependence of the molecular order and phase behaviour of the bicelles on both cholesterol concentration and temperature. The calculations of $M_1$ include the entire spectrum for $x_c=0$ to 0.12. For the $x_c=0.13$ and $x_c=0.16$ samples which have a large isotropic peak in the spectra throughout the entire temperature range investigated, the isotropic peak was not included in the calculation of
Figure 7.11: $^2$H NMR spectra from two DMPC-d$_{54}$/DCPC q=3.2 bicelle samples. (A) Sample was taken to a maximum temperature of 343.9 K. (B) Sample was taken to a maximum temperature of 331.7 K. All spectra were collected at 92.15 MHz with 512 scans.
the moments. The isotropic peak in these spectra does not contribute significantly to the
description of the chain order of the lipids in the lamellar phase of this system but it does
have a large impact on the area because of its relative size (approximately 5 to 40 % of
the spectral area). As a result, including the isotropic peak in these cases would skew
the calculated moments to a value that was much smaller than they should be based on
their quadrupolar splittings, \( \delta v_Q \). In these cases, the isotropic peak was excluded from the
calculation of the moments by cropping it out at the level at its base when the integration
region for the moment calculation was selected. To do this, the centre of the spectrum was
selected, and then the lower limit for the integration was chosen to begin just before the
methyl peaks.

A comparison of the first moment over a temperature range from 330 K to 285 K for
the entire series from 0 to \( x_c = 0.16 \) in DMPC-d\(_{54}\)/DCPC (q=3.2) bicelles is shown in Fig-
ure 7.12. The \( M_1 \) values are shown for all cholesterol concentrations as a function of tem-
perature. Generally, as the cholesterol concentration is raised, the first moment increases at
high temperature. The presence of the \( \ell_0 \) phase at high cholesterol concentrations results
in the large jump in the value of first moment of the NMR spectra between cholesterol
concentrations of \( x_c = 0.12 \) and \( x_c = 0.13 \) below \( \sim 308 \) K. As the temperature is lowered, the
first moment rises which indicates an increase in the amount of molecular order, or a de-
crease in the molecular mobility. The sharp drop in the first moment is observed for the
\( x_c = 0.087, 0.10, \) and 0.12 samples near 300 K when the spectra become dominated by the
isotropic phase.
Figure 7.12: Comparison of $M_1$ for DMPC-d$_{54}$/DCPC bicelles, $q=3.2$ with varying concentrations of cholesterol over a range of temperatures from 285 K to 330 K. Black squares: no cholesterol, red circles: $x_c=0.037$, green triangles: $x_c=0.071$, blue down triangles: $x_c=0.087$, cyan diamonds: $x_c=0.10$, magenta left triangles: $x_c=0.12$, olive right triangles: $x_c=0.13$, and purple hexagons: $x_c=0.16$.

7.2.2 Bicelles with DPPC

A large body of work on the phase behaviour of model lipid membranes has been done using the saturated long chain lipid DPPC [24, 25, 52, 35, 107, 31, 56, 76]. Previous work on ternary mixtures which exhibit two fluid phase coexistence has focussed on mixtures of DOPC, DPPC, and cholesterol. Phase diagrams for these DOPC/DPPC/cholesterol mix-
tures have been established using systems of multilamellar dispersions or giant unilamellar vesicles [25, 52, 35, 107]. In the present work, in order to investigate peptides in model membranes which exhibit $\ell_d$-$\ell_o$ fluid phase coexistence, it is desirable to have oriented samples, especially magnetically aligned samples for improved signal/sample volume. For the DMPC bicelles, DCPC (chain length: 6 carbons) is used as the short chain lipid, but since DPPC is longer than DMPC, DHPC (chain length: 7 carbons) is used here [108]. The formation of oriented samples depends on the ratio $q$ of the long chain to the short chain lipids as well as the amount of buffer or water in the sample. Figure 7.13 shows $^2$H spectra for DPPC-d$_{62}$/DHPC bicelles ($q=3$) with a buffer ratio (buffer/total sample (w/w)) of 0.6 at several temperatures.

In order to investigate the phase behaviour of more complex oriented model membranes, a second long chain lipid, DOPC, was added to the DPPC-d$_{62}$/DHPC bicelle mixtures. Figure 7.14 shows $^2$H NMR spectra for DPPC-d$_{62}$/DOPC (1:1) + DHPC bicelles, $q=3$ with a buffer ratio of 0.6.

Figure 7.15 shows a comparison of three different $q$ values, where $q=($mol DOPC + mol DPPC-d$_{62}$)/mol DHPC for 40:40:20 (DOPC/DPPC-d$_{62}$/cholesterol) + DHPC bicelles and a buffer ratio of 0.5 at (A) 329.2 K and (B) 316.8 K. At both of these temperatures, $q=2.3$ produced the best oriented samples. The quality of the orientation of the samples was judged based on sharpness of the peaks and how close to the baseline level the gap between the methyl and methylene peaks was.

Coexisting $\ell_d$ and $\ell_o$ fluid phases are readily observed in DOPC/DPPC/cholesterol mixtures as multilamellar dispersions and vesicles. This phase coexistence was also observed in bicelles made with DOPC, DPPC, cholesterol and the short chain lipid DHPC as seen
Figure 7.13: $^2$H spectra for DPPC-d$_{62}$/DHPC bicelles, $q=3$, buffer ratio 0.6 at various temperatures. Collected at 92.15 MHz, 1024 scans.
Figure 7.14: $^2$H spectra for DPPC-d$_{62}$/DOPC (1:1) + DHPC bicelles, q=3, buffer ratio 0.6 at various temperatures. Collected at 92.15 MHz, 1024 scans.
Figure 7.15: Comparison of $^2$H spectra for 40:40:20 (DOPC/DPPC-d$_{62}$/cholesterol) + DHPC bicelles, with three different q values, buffer ratio: 0.5. (A) at 329.2 K, and (B) at 316.8 K. Collected at 76.77 MHz, 512 scans.
in Figure 7.16. This sample has a composition of 40:40:20 (DOPC/DPPC-d_{62}/cholesterol) with q=2.3. The sample was hydrated at a ratio of 0.5 with 50 mM phosphate buffer.

Though it was possible to make bicelles using DOPC, DPPC-d_{62}, cholesterol and DHPC, the DPoPC/DMPC-d_{54}/cholesterol/DCPC bicelles were favoured because the sample preparation and results were more repeatable. In addition, the DPoPC/DMPC-d_{54}/cholesterol/DCPC bicelles were consistently better aligned with less of the isotropic phase in the temperature region of interest. Results from the DPoPC/DMPC/cholesterol/DCPC bicelles are presented in the next section.

### 7.2.3 DPoPC/DMPC/Cholesterol/DCPC Bicelles

Although it is possible to make bicelles with longer chained phospholipids including DPPC, as demonstrated in the previous section, DMPC is used most frequently and easily produces well-oriented bicelles. Here a ternary mixture analogous to DOPC, DPPC, cholesterol was used. DMPC has a chain length of 14 carbons whereas DPPC has a chain length of 16 carbons, consequently DPoPC (chain length: 16 carbons) was used instead of DOPC (chain length: 18 carbons) for the unsaturated lipid. Cholesterol was used in all cases. The bicelle samples used in this work were prepared without the freeze-thaw cycles that are typical of many bicelle preparations. This was done to preserve the largest possible structures in these oriented samples in order to have a large matrix for the two fluid phase coexistence.

First, the phase behaviour, specifically the two phase coexistence of the $\ell_d$ and $\ell_o$ phases, for DPoPC/DMPC-d_{54}/cholesterol multilamellar dispersions was established. Figure 7.17 (A) shows the onset of the two phase $\ell_d$–$\ell_o$ region in the powder sample of
Figure 7.16: Onset of the $\ell_d$-$\ell_o$ phase coexistence region in 40:40:20 (DOPC/DPPC-$d_{62}$/cholesterol) + DHPC bicelles, $q=2.3$, buffer ratio: 0.5. Collected at 76.77 MHz, 512 scans.
32:48:20 (DPoPC/DMPC-d54/cholesterol). This behaviour follows what is expected from the work completed on DOPC/DPPC/cholesterol mixtures. Similarly, Figure 7.17 (B) shows the onset of the \( \ell_d - \ell_o \) phase coexistence in 32:48:20 (DPoPC/DMPC-d54/cholesterol) bicelles with \( q = 3.5 \) ((DPoPC+DMPC)/DCPC). Various values of \( q \) were investigated ranging from 2.6 to 3.5, and it was found that the isotropic peak was smaller for the higher \( q \) values used here with the 0.6 (buffer:total sample w/w) hydration ratio.

The onset of the two phase region in these DPoPC/DMPC/cholesterol mixtures follows the same trend as DOPC/DPPC/cholesterol mixtures, as the concentration of the saturated lipid, DMPC, is increased the two phase region occurs at a higher temperature. In Figure 7.18 the amount of \( \ell_o \) phase increases as the relative concentration of DMPC-d54 or cholesterol increases. The three compositions, 32:48:20, 30:55:15, and 20:60:20 (DPoPC/DMPC-d54 /cholesterol) with \( q = 3.5 \) ((DPoPC+DMPC)/DCPC), all have different relative proportions of the \( \ell_o \) phase at 299.5 K.

For the bicelle samples there is a small fraction of the \(^2\text{H}\) spectra that exhibits the \( \ell_o \) phase at high temperatures where only the \( \ell_d \) phase is observed in the powder samples. It is proposed that this arises because the samples are prepared and hydrated at ambient temperatures where these mixtures are in the two phase region. This means that it is possible that some of the bicelles can be formed with a higher concentration of cholesterol than the sample average. If these bicelles are not able to exchange molecules with the bulk of the sample, there may be some isolated regions of the sample which, even at high temperatures, will exhibit the \( \ell_o \) phase due to the high cholesterol content of those bicelles. Figure 7.19 is a cartoon representation of the different morphologies of bicelles, perforated bilayers and discs, with \( \ell_d - \ell_o \) coexistence.
Figure 7.17: The onset of the two phase $\ell_d - \ell_o$ region in powder and bicelle samples of DPOPC, DMPC-$d_{54}$ and cholesterol. (A) $^2$H spectra of powder 32:48:20 (DPOPC/DMPC-$d_{54}$/cholesterol) at various temperatures. Collected at 92.15 MHz, 1024 scans. (B) $^2$H spectra of 32:48:20 (DPOPC/DMPC-$d_{54}$/cholesterol) + DCPC bicelles, q=3.5 ((DPOPC+DMPC)/DCPC) at various temperatures. Collected at 92.15 MHz, 1024 scans.
7.2.4 Bicelles and Lanthanide Ions

Lanthanide ions can be used to flip the orientation of bicelles in a magnetic field [109, 110]. This flipped orientation with the bicelle bilayer normals parallel to the external magnetic field may be advantageous for samples containing proteins or peptides. Two different lanthanide salts, EuCl$_3$ and YbCl$_3$, were added to the stock 50 mM phosphate buffer in order to determine which performed better for the DPOPC/DMPC-d$_{54}$/cholesterol + DCPC bicelles. The weight ratio of buffer/total sample in these samples was always 0.6, and the
Figure 7.19: Cartoon representation of bicelles as perforated lamellar and disc shapes with two phases. Light blue represents the $\ell_d$ phase and dark blue represents the $\ell_o$ phase.

The final dry sample/lanthanide molar ratio was 10:1. Figure 7.20 demonstrates the effect of adding the two different lanthanide salts to the bicelle mixtures and compares these spectra to a sample made without any lanthanide salts. The dashed vertical line indicates the outer peak of the bicelles with the plain buffer and serves as a visual guide for comparing the three spectra.

The addition of Eu$^{3+}$ to the buffer resulted in samples that were no longer oriented in either direction when cholesterol was in the bicelle mixtures. On the other hand, the addition of Yb$^{3+}$ to the sample resulted in a doubling of the quadrupolar splittings indicative of flipped bicelles with their bilayer normals now parallel to the external magnetic field. This occurred for both DMPC-based and DPPC-based bicelles containing cholesterol.

Two phase coexistence between the $\ell_d$ and $\ell_o$ phases is observed in the flipped bicelles and spectra showing the onset of this two phase region in 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles, $q=3.5$ are shown in Figure 7.21(A). Since the quadrupo-
Figure 7.20: The effect of adding two different lanthanides to 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) with DCPC, q = 3.5 bicelles (dry sample/lanthanide molar ratio 10:1). (a) plain 50 mM phosphate buffer, (b) 50 mM phosphate buffer with EuCl$_3$, and (c) 50 mM phosphate buffer with YbCl$_3$. Spectrometer $^2$H frequency: 92.15 MHz, 1024 scans.

Larger splittings of the bicelles with their normal oriented parallel to the magnetic field (flipped by Yb$^{3+}$) should be twice those of the bicelles oriented perpendicular to the magnetic field, the first moment for the flipped bicelles should be twice that of the ordinary bicelles. Indeed, this can be seen in Figure 7.21(B) which shows both sets of moments (plain buffer: squares, buffer with Yb$^{3+}$: triangles) and also 2×$M_1$ (circles) for the bicelles made with plain buffer. At high temperature and again at low temperature, the values of $M_1$ for bicelles with YbCl$_3$ and 2×$M_1$ for bicelles with plain buffer are in good agreement. However,
the shape of the curves of the first moment as a function of temperature differs between the sample with and without Yb$^{3+}$. This indicates that presence of the lanthanide ions has an effect on the phase behaviour of the bicelles.

### 7.3 Conclusions

For DMPC-d$_{54}$/DCPC (q=3.2) bicelles with varying quantities of cholesterol from $x_c=0$ to 0.16, $M_1$ of the $^2$H spectra the first moment increases at high temperatures as the relative amount of cholesterol increases. This result is expected since cholesterol is known to have a stiffening effect on the membrane and promotes the formation of a more ordered fluid phase ($\ell_o$). There is a significant difference in the first moment at the highest temperatures between the bicelles with $x_c=0.13$ and 0.16 compared to lower cholesterol concentrations due to the appearance of the $\ell_o$ phase, see Figure 7.12. As the temperature is lowered, the first moment rises which indicates an increase in the amount of molecular order. At the lowest temperatures, there is a conversion to an isotropic phase, and the isotropic phase is also persistent in samples with high cholesterol concentrations (0.13 and 0.16 cholesterol/DMPC). There is a relatively wide range of temperatures and compositions of the DMPC/cholesterol/DCPC bicelles which align in the magnetic field and can provide an environment for investigating membrane integrated peptides or proteins.

The coexistence of two fluid phases is of interest and bicelles which exhibit $\ell_d-\ell_o$ phase coexistence could provide a medium in which to study membrane proteins and peptides under different phase conditions by varying temperature. Similar to the often used DOPC/DPPC/cholesterol system, mixtures of DPoPC, DMPC and cholesterol exhibit $\ell_d$-
Figure 7.21: (A) Onset of the two phase region in flipped 32:48:20 (DPOPC/DMPC-d\textsubscript{54}/cholesterol) + DCPC bicelles, q=3.5 with YbCl\textsubscript{3} in the 50 mM phosphate buffer at various temperatures. (B) Comparison of the first moment of the 32:48:20 (DPOPC/DMPC-d\textsubscript{54}/cholesterol) + DCPC q=3.5 bicelle spectra with plain buffer (M\textsubscript{1}: squares and 2× M\textsubscript{1}: circles) and buffer with YbCl\textsubscript{3} (triangles).
Fluid phase coexistence. Bicelles can be made by combining DOPC/DPPC/cholesterol or DPoPC/DMPC/cholesterol mixtures with a short chain lipid, DHPC or DCPC respectively. These multi-component bicelles exhibit two fluid phase coexistence and are of interest for studying peptides and proteins in oriented environments with both the \( \ell_d \) and \( \ell_o \) phases without a supporting material such as glass plates. In these samples, a small amount of \( \ell_o \) phase is observed in the \(^2\text{H}\) spectra even at high temperature. This feature is more pronounced in the bicelles with DMPC than it is in the bicelles with DPPC. This contribution to the spectra is attributed to a small fraction of the bicelles that are physically isolated (not able to exchange molecules with the rest of the sample) which contain a higher concentration of cholesterol. As a result, these isolated bicelles can display different phase behaviour than the bulk sample, posing a challenge for studying the partitioning of peptides or proteins into the different fluid phases.

It is well established that lanthanide ions can be used to flip bicelles. Typical bicelles which are made with DMPC and DCPC align such that their bilayer normal is perpendicular to the external magnetic field. In the presence of lanthanide ions, these bicelles align such that their bilayer normal is parallel with the external magnetic field. Here, bicelles containing DPoPC/DMPC/cholesterol and DCPC were found to exhibit \( \ell_d-\ell_o \) phase coexistence in the parallel orientation when \( \text{YbCl}_3 \) was added to the buffer. It was found that the addition of \( \text{EuCl}_3 \) did not flip the bicelles when cholesterol was present and it interfered with the alignment/structure of the bicelles. This ‘flipped’ orientation can be advantageous when peptides/proteins that preferentially align with the magnetic field are present in order to minimize stress between the orientation of the lipids and the protein [111, 112].
Chapter 8

Peptides in Bicelles

DMPC/DCPC bicelles are often used as an orienting liquid crystalline medium which can be used to align water soluble molecules including proteins solution-state NMR experiments. Membrane proteins can be embedded into DMPC/DCPC bicelles for solid-state NMR experiments [103, 20, 113, 19, 17]. The types of samples containing proteins and bicelles are illustrated in Figure 8.1. As shown in Chapter 7, bicelles made with DPoPC, DMPC, cholesterol and DCPC can have coexisting $\ell_d$ and $\ell_\alpha$ phases. In this chapter, two peptides Conolysin-Mt1 and ALGA are investigated in bicelle mixtures.

![Figure 8.1](image)

Figure 8.1: Illustration of four ways in which bicelles can be used to study proteins: (A) aligned bicelles containing integral membrane proteins, (B) isotropically tumbling bicelles containing integral membrane proteins, (C) bicelles aligning soluble proteins for residual dipolar coupling measurements, and (D) isotropic and aligned bicelles for soluble protein-membrane interactions. Reprinted from Dürr et al. [19] with permission from Elsevier.
8.1 Conolysin-Mt1

8.1.1 Introduction

Conolysin is a short peptide which is found in the venom of the predatory sea snail *Conus mustelinus*. This venom contains many different components including two variants of the cytolytic peptide Conolysin-Mt. Conolysin-Mt1 has the amino acid sequence: FHPSLWVLIPQYIQLIRKILKS, while Conolysin-Mt2 has the amino acid sequence: FHPSLWVLIPQYIQLIRKILKS-NH$_2$. Of the two peptides, Conolysin-Mt1 is the more active and potent species, while Conolysin-Mt2 is the more abundant species. Conolysin-Mt1 elicits neurotoxic responses when injected into mice and its purpose in the venom may be to work in combination with the other components to affect the neurological pathways and cause paralysis of the prey [114]. Since Conolysin has the ability to disrupt the membrane, sample preparation conditions are important in order to lengthen the period of stability for the samples. Previous work by Hansen [78] indicated that for a sample with 2 mol % Conolysin-Mt1 the samples were significantly changed after a relatively short time (18 hours) which makes these samples impossible to use for longer NMR experiments using nuclei such as $^{15}$N which have inherently low signal to noise ratios.

The work presented here was completed in collaboration with Sara Krogh Hansen and Prof. Thomas Vosegaard (Aarhus University, Denmark). In this work, the phase behaviour of DMPC/DCPC bicelles containing Conolysin-Mt1 was investigated using $^2$H NMR.
8.1.2 Experimental Details

Bicelle samples were prepared with DMPC-d54/DCPC, q = 3.2 and different concentrations of Conolysin. The method used is described in Chapter 4.1.2. Samples with 2 mol% Conolysin were hydrated with the usual 50 mM phosphate buffer. For samples containing more than 2 mol% Conolysin, 75 mM phosphate buffer was used in order to increase the lifetime and stability of the bicelles. In all cases the samples were hydrated such that the ratio between the dry lipid/peptide weight and the total sample weight was 0.6.

$^2$H NMR experiments were performed on a wide bore 600 MHz spectrometer at a $^2$H frequency of 92.15 MHz. A quadrupolar echo pulse sequence was used and the 90° pulses were 1.70 µs at 600 W power.

8.1.3 Results

In this work, we investigated the effect of the cytolytic peptide Conolysin-Mt1 on the phase behaviour and stability of DMPC/DCPC bicelles. A helical wheel projection of the Conolysin-Mt1 peptide is shown in Figure 8.2. The hydrophobicity and charge of the amino acid residues are indicated by the shape and colour of the residues. Conolysin-Mt1 contains hydrophobic, hydrophilic, and positively-charged residues, as well as a proline (purple). From the helical wheel, Conolysin-Mt1 appears to be amphipathic with a hydrophobic side and a hydrophilic side.

To better understand how cytolytic peptides affect bicellar assemblies, we investigated the interaction of Conolysin-Mt1 with regular, zwitterionic DMPC-d54/DHPC bicelles as well as the effect of this peptide on the phase properties of the bicellar systems. DMPC-d54/DCPC bicelle (q=3.2) samples containing various molar ratios of Conolysin-Mt1 with
Figure 8.2: Helical wheel projection of Conolysin-Mt1. The hydrophobicity and charge of the amino acid residues are indicated by the shape and colours of the residues. Hydrophobic residues are indicated by blue squares, hydrophilic residues are indicated by red diamonds, positively charged residues are indicated by black octagons, and prolines are shown in purple. Image was created using http://www.bioinformatics.nl/cgi-bin/emboss/pepwheel

respect to DMPC-d$_{54}$ were prepared for these NMR studies. $^2$H NMR was performed on the bicelle samples as a function of temperature in order to investigate the effect that the peptide has on the general order and phase behaviour of the DMPC-d$_{54}$/DCPC bicelles.

$^2$H spectra were collected as a function of temperature for a series of samples with different concentrations of Conolysin-Mt1. Examples of these spectra as a function of temperature are shown in Figures 8.3, 8.4, and 8.5 for 0 mol %, 2 mol % and 4 mol % (with respect to DMPC-d$_{54}$) respectively. In all cases, the samples are well oriented in the magnetic field. At high temperatures, there is an isotropic peak in the spectra for the sample without Conolysin-Mt1, but this is relatively small and disappears as the temperature
Figure 8.3: $^2$H spectra for DMPC-$d_{44}$/DCPC bicelles $q=3.2$ without Conolysin-Mt1 at various temperatures. Spectra were collected at 92.15 MHz with 512 scans.

is lowered. There is no evidence of an isotropic phase at high temperatures in the spectra for the samples containing Conolysin-Mt1. The whole system becomes isotropic with and without Conolysin-Mt1 at low temperatures (spectra not shown).

Figure 8.6 shows a comparison between the $^2$H spectra for the bicelle samples containing no Conolysin, 2 mol % Conolysin and 4 mol % Conolysin at three temperatures. The presence of Conolysin changes the shape of the spectra relative to the bicelles without the
Figure 8.4: $^2$H spectra for DMPC-d$_{54}$/DCPC bicelles q=3.2 with 2 mol% Conolysin-Mt1 (with respect to DMPC-d$_{54}$) at various temperatures. Spectra were collected at 92.15 MHz with 512 scans.
Figure 8.5: $^2$H spectra for DMPC-d$_{54}$/DCPC bicelles q=3.2 with 4 mol\% Conolysin-Mt1 (with respect to DMPC-d$_{54}$) at various temperatures. Spectra were collected at 92.15 MHz with 512 scans.
peptide at all temperatures; however, all spectra indicate that the samples are well aligned throughout the temperature range investigated.

The moments for the $^2$H spectra were calculated for all temperature steps for these samples. Figure 8.7 shows $M_1$ for DMPC-$d_{54}$/DCPC bicelles $q=3.2$ with 0, 2, and 4 mol% Conolysin-Mt1 (with respect to DMPC-$d_{54}$) as a function of temperature. The zoomed in area shows the region where the moments for the bicelles with and without Conolysin-Mt1 cross. The presence of Conolysin-Mt1 in bicelles affects the temperature dependence of the lipid chain order of these bicelles relative to the pure DMPC-$d_{54}$/DCPC bicelles. These results indicate that Conolysin-Mt1 increases the order of the lipid chains at high temperatures (345-320 K) whereas it decreases the order of the lipid chains at lower temperatures (320-298 K) relative to the bicelles with no peptide. As the temperature is lowered fur-
ther, the gel phase and then the isotropic phase dominate the spectra, these phases occur at different temperatures depending on the concentration of Conolysin-Mt1. These results indicate that Conolysin-Mt1 has a stabilizing effect on the bicelles at these concentrations and temperatures.

Figure 8.7: $M_1$ for DMPC-d$_{54}$/DCPC bicelles $q=3.2$ with 0, 2, and 4 mol% Conolysin-Mt1 (with respect to DMPC-d$_{54}$) as a function of temperature. The zoomed in area shows the region where the moments cross for the bicelles with and without Conolysin-Mt1.
8.1.4 Conclusions

This work shows that it is possible to form stable, long-lived bicelle samples that contain Conolysin-Mt1 with care to increase the buffer concentration for higher concentrations of the peptide. These samples are well aligned over a broad range of temperatures. Moment analysis of these samples indicates that the presence of Conolysin-Mt1 in the bicelles increases the order of the lipid chains relative to the bicelles without peptide at high temperatures (345-320 K), and decreases the order of the lipid chains relative to the bicelles without peptide at lower temperatures (320-298 K). At temperatures below 298 K the spectra become much broader and there is an onset of the gel phase which was not of interest in this work. The orientation and phase behaviour of these samples was performed using $^2$H NMR and then the samples were used for other studies using MAS NMR since bicellar samples provide better resolution for $^{13}$C and $^{15}$N experiments. Further investigations of Conolysin-Mt1 using other methods are presented by Sara Krogh Hansen [78].

8.2 ALGA

8.2.1 Introduction

ALGA is a 22-residue peptide with the sequence Ac-KKPVLIFALGALAFILAVGKK-NH$_2$. ALGA was designed to have a well resolved proton NMR spectrum. ALGA has a helical structure, is hydrophobic and is long enough to span the hydrophobic thickness of the bilayer. The lysine residues at either end are charged and serve to interact with the headgroup regions as an anchor. The positive charges are also intended to prevent
aggregation of the peptides. In this work, ALGA is used as a model peptide to study the phase behaviour of bicelles with and without cholesterol in the presence of a helical peptide.

### 8.2.2 Experimental Details

Bicelle samples were prepared as described previously in Chapter 4.1.2. The ratio of the long chain to short chain lipids was calculated as \( q = \frac{\text{mol DMPC}}{\text{mol DCPC}} \) for DMPC/DCPC bicelles, or \( q = \frac{\text{mol DPoPC} + \text{mol DMPC}}{\text{mol DCPC}} \) for the DPoPC/DMPC/cholesterol/DCPC bicelles. ALGA was added to the lipids at different concentrations relative to the long chain lipids (DMPC or DPoPC + DMPC). In all cases the bicelles were hydrated such that the buffer weight/total sample weight is 0.6. The \(^2\text{H}\) NMR experiments were performed on a 500 or 600 MHz spectrometer at a \(^2\text{H}\) frequency of 76.77 or 92.15 MHz respectively. Results presented here were collected using the usual quadrupolar echo pulse sequence.

### 8.2.3 Results

Previously, labelled gramicidin was added to non-oriented model membrane systems which exhibit \( \ell_d-\ell_o \) fluid phase coexistence. The results described in Chapter 6 indicated that it would be advantageous to use oriented systems to investigate peptides in model membranes. The feasibility of magnetically aligned bicelles with ALGA was investigated using unlabelled ALGA and chain perdeuterated DMPC-\(d_{54}\) to determine the lipid phase behaviour and the quality of the alignment of the sample. A series of DMPC-\(d_{54}/\text{DCPC}\) bicelle samples containing different molar proportions of ALGA were made to determine
how much ALGA could be added to the bicelle system without disrupting the alignment of the bicelles. Then ALGA was added to the more complex DPOPC/DMPC-d$_{54}$/cholesterol/DCPC bicelles which were previously shown to exhibit two fluid phase coexistence.

Figures 8.8 and 8.9 show $^2$H NMR spectra of the DMPC-d$_{54}$/DCPC (q = 3.2) bicelles with 1 and 2 mol % ALGA as a function of temperature respectively. These spectra show that the samples are very well aligned over a range of temperatures from 316.8 K to 298.4 K. For both the 1 and 2 mol % samples, the transition to the isotropic phase occurs at a higher temperature than for the bicelles which do not contain any peptide (see Chapter 7).

The $^2$H NMR spectra as a function of temperature are shown for samples with 4, 5 and 6 mol % ALGA in DMPC-d$_{54}$/DCPC bicelles in Figure 8.10, 8.11, and 8.12. Several samples were made with different q values and the best aligned sample spectra obtained for each composition are shown in these figures. Figure 8.10 shows spectra from the sample with 4 mol % ALGA and q = 2.8. This sample is fairly well aligned and has little to no isotropic phase throughout the temperature range presented. The best spectra from a sample with 5 mol % ALGA and q = 3.2 are shown Figure 8.11. Figure 8.12 shows the spectra as a function of temperature for the sample containing 6 mol % ALGA and q = 3.2. This sample is not very well aligned but does still follow the general phase behaviour seen for the other DMPC/DCPC bicelles with q = 3.2.

A comparison between three different q ratios (2.8, 3.2, and 3.5) for the DMPC-d$_{54}$/DCPC bicelles with 4 mol % ALGA is shown in Figure 8.13. In this case q = 2.8 results in the best aligned bicelles. For bicelles containing 5 mol % ALGA, q = 3 resulted in very poorly aligned samples (data not shown) whereas q = 3.2, and 3.5 gave better
Figure 8.8: $^2$H NMR spectra for DMPC-d$_{54}$/DCPC bicelles ($q = 3.2$) with 1 mol % ALGA (relative to DMPC) at various temperatures. Spectra were collected at 91.15 MHz with 512 scans.
Figure 8.9: $^2$H NMR spectra for DMPC-d$_{54}$/DCPC bicelles ($q = 3.2$) with 2 mol % ALGA (relative to DMPC) at various temperatures. Spectra were collected at 91.15 MHz with 512 scans.
Figure 8.10: $^2$H NMR spectra for DMPC-d$_{54}$/DCPC bicelles ($q = 2.8$) with 4 mol % ALGA (relative to DMPC) at various temperatures. Spectra were collected at 76.77 MHz with 512 scans.
Figure 8.11: $^2$H NMR spectra for DMPC-d$_5$4/DCPC bicelles ($q = 3.2$) with 5 mol% ALGA (relative to DMPC) at various temperatures. Spectra were collected at 76.77 MHz with 512 scans.
Figure 8.12: $^2$H NMR spectra for DMPC-$d_{54}$/DCPC bicelles ($q = 3.2$) with 6 mol % ALGA (relative to DMPC) at various temperatures. Spectra were collected at 91.15 MHz with 512 scans.
Figure 8.13: $^2$H NMR spectra at 304.5 K for DMPC-d$_{54}$/DCPC bicelles with 4 mol % ALGA (relative to DMPC) and various q values. Spectra were collected at 91.15 MHz with 512 scans.

Aligned samples. The sample with q = 3.5 resulted in spectra that were quite similar to those from the sample with q = 3.2. None of the samples with 4 mol % ALGA or higher were aligned as well as the bicelles with 0 to 2 mol % ALGA.

Next ALGA was added to DPoPC/DMPC/cholesterol/DCPC bicelle systems that have been shown previously to exhibit two fluid phase coexistence. Figure 8.14 shows $^2$H NMR spectra for the sample with 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles (q = 3.2) and 2 mol % ALGA (relative to the long chain lipids) as a function of temperature. This sample is not well aligned, however it does show two fluid phase coexistence. As
was seen previously for DPoPC/DMPC/cholesterol/DCPC bicelles, there is evidence of the \( \ell_o \) phase even at high temperatures but the bulk of the sample enters the two phase coexistence region around 298.4 K. Figure 8.15 shows \( ^2\text{H} \) NMR spectra for the sample with 32:48:20 (DPoPC/DMPC-\( d_{54} \)/cholesterol) + DCPC bicelles (\( q = 3.2 \)) and 4 mol % ALGA as a function of temperature. This sample is not aligned in the magnetic field and contains a large, persistent isotropic peak throughout the temperature range. There is still evidence of \( \ell_d-\ell_o \) phase coexistence in this sample.

![Figure 8.14: \( ^2\text{H} \) NMR spectra from 32:48:20 (DPoPC/DMPC-\( d_{54} \)/cholesterol) + DCPC bicelles (\( q = 3.2 \)) and 2 mol % ALGA (relative to the long chain lipids) at various temperatures. Spectra were collected at 76.77 MHz with 512 scans.](image)
Figure 8.15: $^2$H NMR spectra from 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.2$) and 4 mol % ALGA (relative to the long chain lipids) at various temperatures. Spectra were collected at 76.77 MHz with 512 scans.
The lanthanide salt YbCl$_3$ was added to samples containing 2 and 4 mol % ALGA to determine whether flipping the bicelles would improve the alignment by reducing the competition between the preferred parallel orientation of the helical peptide and the perpendicular orientation of the bilayer normal in the magnetic field. The results for the ALGA bicelle samples containing YbCl$_3$ were inconsistent; in some cases, the samples did flip but were not well aligned, in other cases the samples did not flip and were also poorly aligned.

Overall, this work indicates that bicelles may not be an ideal candidate for a model system in which to determine the effect of ALGA on the phase behaviour of DPOPC/DMPC/cholesterol mixtures or the partitioning of the peptide into the $\ell_o$ and $\ell_d$ phases. It is difficult to obtain well-aligned bicelle samples with ALGA and there is an $\ell_o$ phase component of the sample even at high temperatures.

### 8.2.4 Conclusions

Though bicelles are desirable samples to use for aligning peptides in a magnetic field because of their ability to spontaneously align without the need for a mechanical support, making bicelle samples with multiple components can be challenging. There are many variables for the sample preparation including the long chain lipid composition, the long chain to short chain ratio $q$, the peptide concentration and the water content of the sample. Changing any of these variables can upset the balance and disrupt ability for the sample to be magnetically aligned.

In the present work, the limit for obtaining aligned DMPC/DCPC bicelle samples with ALGA was 4 mol %. In addition, there were challenges with the reproducibility of results
for samples with the same composition and which were prepared in the same manner. As was seen previously, bicelles are sensitive to their temperature history and this makes it challenging to determine quantitative information about the phase behaviour.

None of the samples made with ALGA and DPoPC/DMPC/cholesterol/DCPC were sufficiently well aligned to be used for investigations with the deuterium labelled ALGA in the two fluid phase coexistence region. Samples aligned on glass slides are more likely to have consistent alignment due to the supportive glass plates and will be used to further investigate ALGA in DPoPC, DMPC, cholesterol mixtures. These results are presented in Chapter 10.
Chapter 9

Exchange Experiments

9.1 Introduction

Motions with correlation times on the order of $10^{-5}$ to $10^2$ s can be studied using exchange experiments. The time scale depends on the value of $T_1$ for the nucleus. For example, the upper limit for the correlation time that can be investigated by exchange experiments on $^2$H is about 0.1 s. Two-dimensional exchange experiments correlate the resonance frequency of a molecule at the beginning and end of a delay. These experiments are sensitive to processes such as a change in conformation or a change in phase in which a molecule may switch from precessing at a resonant frequency of $\omega_A$ in one state to $\omega_B$ in the other state or visa versa. The general form of the pulse sequence used for exchange experiments for spin-$\frac{1}{2}$ and spin-1 nuclei are shown in Figure 9.1 (A) and (B) respectively [115, 68, 82]. Initially, the transverse magnetization is excited by a 90° pulse or cross-polarization (CP). There is then a period of evolution, $t_1$, during which time the transverse magnetization will evolve at its characteristic frequency, $\omega_A$. Following $t_1$, a pulse is applied to flip the magnetization up into the z-direction and it is stored for a mixing time, $\tau_m$. This storage pulse is a 90° pulse for spin-$\frac{1}{2}$ nuclei, and a 54.7° pulse for spin-1 nuclei. During $\tau_m$, molecular reorientation which changes the precession frequency for $\omega_A$ and $\omega_B$ can occur.
In a series of experiments different values for $\tau_m$ may be used. Following $\tau_m$ another pulse is applied to flip the magnetization back into the transverse plane where it can be detected. This flip back pulse is a 90° pulse for spin-$\frac{1}{2}$ nuclei, and a 54.7° pulse for spin-1 nuclei. The magnetization now evolves with a characteristic frequency $\omega_B$ which is recorded in the FID. In the case of the $^2$H experiment, there is an additional echo sequence ($\Delta$-90°-$\Delta$) in order to record the powder line shapes without distortion.

Data processing results in a 2D spectrum which correlates the observed spin’s offset frequencies ($\omega_A$ during $t_1$ and $\omega_B$ during $t_2$). Since molecular orientation can be directly correlated with offset frequency, the spectrum describes the molecular reorientation during $\tau_m$. Two datasets are recorded for $^2$H exchange experiments so that pure absorption line shapes can be obtained in the final 2D spectrum. For dataset 1, the storage pulse is 54.7°, and the reconversion pulse is 54.7°, whereas for dataset 2, the storage pulse is 54.7°, and the reconversion pulse is 54.7°. These two data sets can be summed together to obtain the pure absorption line shape. Scaling may be required for this addition because the relaxation processes occurring during $\tau_m$ are not the same for the two datasets. For dataset 1, the cosine spectrum, the system is subject to spin-lattice relaxation which is described by $T_{1Z}$. For dataset 2, the sine spectrum, the system is subject to quadrupolar order relaxation which is described by $T_{1Q}$. $T_{1Z}$ and $T_{1Q}$ are usually different and can lead to different amplitudes in the spectra for the two datasets [115, 68, 82]. Figure 9.2 shows an example of 2D $^2$H exchange NMR spectra using the two different storage/reconversion pulse phasing schemes ((a) and (b)) as well as the final pure absorption spectrum in (c) [115]. Note that the ridges seen in the base plane of the pure absorption powder spectrum (Figure 9.2(c)) arise as a result of molecular reorientation.
In systems that do not exhibit any slow dynamics, the frequencies $\omega_A$ and $\omega_B$ are equal, that is $\omega_A$ does not change during the mixing time and thus the signal is confined to a line.

Figure 9.1: (A) The general pulse program for exchange experiments using spin-$\frac{1}{2}$ nuclei. This pulse program has the form: excitation - $t_1$ - 90° storage pulse - $\tau_m$ (mixing time) - 90° reconversion pulse - $t_2$. The signal is detected during $t_2$. (B) The general pulse program for exchange experiments using spin-1 nuclei. This pulse program has the form: 90° excitation pulse - $t_1$ - 54.7° storage pulse - $\tau_m$ (mixing time) - 54.7° reconversion pulse - $\Delta$ - 90° pulse - $\Delta$ - $t_2$. Note that $\Delta$ - 90° pulse - $\Delta$ is an echo sequence which allows $^2$H powder signal to be recorded without distortion. Two datasets are recorded for $^2$H exchange experiments. For dataset 1, the storage pulse is 54.7°$_x$, and the reconversion pulse is 54.7°. For dataset 2, the storage pulse is 54.7°$_y$, and the reconversion pulse is 54.7°$_x$. [82]
Figure 9.2: Deuterium 2D exchange spectra of dimethyl sulfone at 310 K. (a) cosine spectrum (dataset 1), (b) sine spectrum (dataset 2), and (c) absorption mode spectrum (sum of (a) and (b)). Adapted from Schmidt et al. [115]

where $\omega_A = \omega_B$, i.e. the diagonal. Frequency changes due to reorientations of the molecule during $\tau_m$, result in spectral intensity off the diagonal [68].

Deuterium 2D exchange experiments can be used to study motions with correlation times on the order of $10^{-5}$ to 0.1 s. In this chapter, deuterium 2D exchange experiments are used to investigate the behaviour of lipids at temperatures where ternary lipid/cholesterol mixtures exhibit $\ell_d\ell_o$ phase coexistence in both powder samples and bicelle samples.
9.2 Experimental Details

Experiments were performed on an 800 MHz spectrometer at a $^2$H frequency of 122.84 MHz. Pulse programs including phase cycling used are given in Appendix B and are of the form shown in Figure 9.1 (B).

Data processing follows the procedures outlined by Schmidt et al. [115] for the two different datasets. For the first dataset, the Fourier transform in F2 was performed, then the imaginary part of the spectrum was zeroed, and finally the Fourier transformation in F1 was performed to get the resulting symmetric 2D spectrum. For the second dataset, the Fourier transform in F2 was performed with the phase such that it is anti-symmetric, then the imaginary part of the spectrum was zeroed, the Fourier transformation in F1 was performed and a phase correction of $90^\circ$ added in order to get the resulting anti-symmetric 2D spectrum. Once both data sets were phased appropriately, they were added together in order to get the final 2D spectrum. Note that because of the different mechanisms that affect the relaxation in the two experiments, a small scaling factor may be required for the addition in some cases since the spectra may not have equal amplitudes.

Figures 9.3 and 9.4 show two different representations of two collected data sets (A) and (B) as well as the resulting sum (C) = (A) + 1.3(B) for the experiments at 292.21 K with a mixing time of 0.25 ms.
Figure 9.3: $^2$H exchange spectra for 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at 292.2 K with $\tau_m = 0.25$ ms. (A) Symmetric spectrum, (B) antisymmetric spectrum and (C) sum of (A) and 1.3×(B). Collected at 122.84 MHz.
Figure 9.4: Stacked plot view of the $^2$H exchange spectra for 32:48:20 (DPoPC/DMPC-$d_{54}$/cholesterol) multilamellar dispersions at 292.2 K with $\tau_m = 0.25$ ms. (A) Symmetric spectrum, (B) antisymmetric spectrum and (C) sum of (A) and 1.3×(B). Collected at 122.84 MHz.
9.3 Results

9.3.1 Multilamellar Dispersions

The phase behaviour of DPoPC/DMPC/cholesterol mixtures is similar to that of the well-studied DOPC/DPPC/cholesterol mixtures. Two fluid phase coexistence can be observed for a range of temperatures and compositions. A powder sample with a composition of 32:48:20 (DPoPC/DMPC-d54/cholesterol) was prepared for deuterium 2D exchange experiments at a temperature just above the onset of the \( \ell_d - \ell_o \) coexistence region. The 1D \(^2\)H NMR spectra for this sample across the transition from the \( \ell_d \) phase to the \( \ell_d - \ell_o \) phase coexistence region are shown in Figure 9.5. Broadening due to fluctuations which occur as the \( \ell_d - \ell_o \) phase region begins is observed starting at 292.2 K, and at temperatures below this there are two distinct fluid phases. The \(^2\)H 2D exchange experiments were performed at 292.2 K, 291.3 K, and 289.6 K for this sample.

Figures 9.6 and 9.7 show the results of the 2D exchange experiments with mixing times of 0.25, 5, 10, 20, and 40 ms at 292.2 K. These spectra have been scaled to have the same maximum amplitude for the methyl peaks. In addition, the same contour levels were used in order to compare the exchange effects for the different mixing times. The exchange spectra shown in Figure 9.6 display some broadening indicative of exchange of lipids between the two phases for \( \tau_m = 5 \) and 10 ms. The projections from the spectra are shown in Figure 9.7. The decrease in the signal as the mixing time is increased can be seen in these projections. This loss of intensity occurs due to \( T_1 \) and/or \( T_{1Q} \).

Figures 9.8 and 9.9 show the results for 2D exchange experiments with a single mixing time of 5 ms at 292.2, 291.3, and 289.6 K. Again, these spectra have been scaled to the
same maximum amplitude for the methyl peaks and the same contour levels. The most evidence of exchange of the lipids between the two phases is seen at 292.2 K. The onset of the two phase region is near 292.2 K, and by 291.3 K, the two phases are distinct and have established domains as seen in the projections of the spectra shown in Figure 9.9.

Figure 9.5: $^2$H NMR spectra for 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at various temperatures used to determine phase behaviour of the sample for 2D exchange experiments in the $\ell_d$-$\ell_o$ phase coexistence region. Collected at 122.84 MHz, 256 scans.
Figure 9.6: $^2$H 2D exchange NMR spectra for 32:48:20 (DPoPC/DMPC-d_{54}/cholesterol) multilamellar dispersions at 292.2 K. These spectra were obtained from sums of the two datasets collected with each $\tau_m$ value. Spectra were collected at 122.84 MHz.
Figure 9.7: Projections from $^2$H exchange NMR spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at 292.2 K with various mixing times. Note that the signal strength decreases as the mixing time increases due to $T_1/T_{1Q}$ relaxation.
Figure 9.8: $^2$H 2D exchange NMR spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at various temperatures with $\tau_m = 5$ ms. Collected at 122.84 MHz.
Figure 9.9: Projections from $^2$H exchange NMR spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at various temperatures with a mixing time of 5 ms.
9.3.2 Bicelles

As discussed in Chapter 7, 32:48:20 (DPoPC/DMPC-d54/cholesterol) bicelles with q=3.5 ((DPoPC+DMPC)/DCPC) also exhibit $\ell_d$-$\ell_o$ phase coexistence. Figure 9.10 shows $^2$H NMR spectra for this bicelle sample as a function of temperature. Note that in these samples some $\ell_o$ phase is observed even at high temperatures; however, the onset of the bulk of the sample moving from the $\ell_d$ phase to the $\ell_d + \ell_o$ phases occurs around 297.5 K. The 2D $^2$H exchange experiments were performed at 297.5 and 296.6 K for this sample.

Bicelles are oriented samples which result in much sharper NMR peaks than the multilamellar dispersion samples. This leads to imperfect subtractions for the two datasets as seen by presence of intensity on the anti-diagonal in the spectra. The additions of the symmetric and antisymmetric datasets was performed 1:1 for the bicelle sample. Figure 9.11 shows a stacked plot for the 32:48:20 (DPoPC/DMPC-d54/cholesterol) + DCPC bicelles with q = 3.5 at 297.5 K, and $\tau_m = 5$ ms. Note that the intensities of the diagonal peaks (positive) are much higher than the negative peaks that occur due to imperfect subtractions for the sharp lines.

Figures 9.12 and 9.13 show the results of the 2D exchange experiments with mixing times of 0.25, 5, and 20 ms at 297.5 K. As for the powder samples, the bicelle sample spectra have been scaled to have the same maximum amplitude for the methyl peaks and the same contour levels. Diffusion leads to a spreading out of the intensity away from the diagonal of the spectrum. On the other hand, molecular reorientation via jumps between different orientations leads to ridges which are seen in the off-diagonal of the spectrum. There is no evidence of exchange in the 2D spectra shown in Figure 9.12. The corresponding projections of the summed spectra are shown in Figure 9.13.
Figure 9.10: $^2$H NMR spectra for 32:48:20 (DPOPC/DMPC-$d_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$ ((DPOPC+DMPC)/DCPC)) at various temperatures used to determine phase behaviour of the sample for 2D exchange experiments in the $l_d$-$l_o$ phase coexistence region. Collected at 122.84 MHz, 64 scans.
Figure 9.11: Stacked plot of the $^2$H 2D exchange experiment for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$ ((DPoPC+DMPC)/DCPC)) at 297.5 K, and $\tau_m = 5$ ms showing the intensities of the diagonal and off diagonal peaks. Collected at 122.84 MHz.
Figure 9.12: $^2$H 2D exchange NMR spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$) at 297.5 K with various mixing times. Collected at 122.84 MHz.
Figure 9.13: Projections of the $^2$H 2D exchange NMR spectra for 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$) at 297.5 K with various mixing times. Collected at 122.84 MHz.
Figure 9.14: $^3$H 2D exchange NMR spectra for 32:48:20 (DPoPC/DMPC-$d_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$) at 296.6 K with various mixing times. Collected at 122.84 MHz.

Figures 9.14 and 9.15 show the results of the 2D exchange experiments with mixing times of 5, 10, and 20 ms at 296.6 K. These spectra have also been scaled to have the same maximum amplitude for the methyl peaks and have the same contour levels. At this temperature, the $\ell_d$ and $\ell_o$ phases are well defined and the 2D spectra in Figure 9.14 do not show significant exchange between domains of the two fluid phases for these mixing times. The projections shown in Figure 9.15 decrease in intensity as $\tau_m$ increases which is the same trend seen for the multilamellar dispersion samples due to $T_1/T_{1Q}$.
Figure 9.15: Projections of the $^2$H 2D exchange NMR spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$) at 296.6 K with various mixing times. Collected at 122.84 MHz.
Figure 9.16: Overlay of the $^2$H 2D exchange spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$) at 296.6 K (blue) and 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at 292.21 K (red). Collected at 122.84 MHz.

Figure 9.16 shows an overlay of the $^2$H 2D exchange spectra for 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + DCPC bicelles ($q = 3.5$) at 296.6 K shown in blue and 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersions at 292.2 K shown in red. The spectra were scaled such that the methyl peak intensities are equal and the contour levels used were the same for both datasets. There is evidence of exchange in the powder sample, whereas the peaks from the bicelle sample are very sharp and there is no evidence of exchange between the $\ell_d$ and $\ell_o$ phases for the lipids in bicelles.
9.4 Conclusions

Deuterium exchange experiments were performed on a ternary mixture of DPOPC/DMPC/cholesterol as a multilamellar dispersion and as bicelles formed by the addition of the short chain lipid DCPC to the ternary mixture. These samples both exhibit two fluid phase coexistence of the $\ell_d$ and $\ell_o$ phases. The purpose of the exchange experiments was to investigate if there is exchange of the lipids between the two phases.

There is evidence of exchange of lipids between the $\ell_d$ and $\ell_o$ phases in the 32:48:20 (DPOPC/DMPC-d_{54}/cholesterol) multilamellar dispersion sample. This is seen as a broadening in the $^2$H 2D exchange spectra. The most dramatic effect is seen at 292.21 K where the static $^2$H spectrum begins to show broadening due to the fluctuations near the critical point for these mixtures. Mixing times of 5 ms and longer exhibit the broadening effect.

There is no evidence of exchange of lipids between fluid phase domains in the bicelle sample. This sample is composed of the same molar ratio of DPOPC, DMPC-d_{54}, and cholesterol as the multilamellar dispersion sample, but in order to form bicelles the short chain lipid DCPC was added. The final ratio of the long chain to short chain lipids for this bicelle sample was $q = 3.5$ ((DPOPC+DMPC)/DCPC). These mixtures exhibit $\ell_d$-$\ell_o$ coexistence, however due to the addition of the short chain lipid, this composition may not be near a critical point for the system. The largest exchange effect for the multilamellar dispersions was observed where the static $^2$H spectra for this sample show broadening near the critical temperature. If the sample is not near a critical point, then exchange of lipids between the two phases due to fluctuations may not be observed in these 2D $^2$H exchange experiments.
Chapter 10

Peptide Samples Oriented on Glass Slides

10.1 Introduction

As discussed in Chapter 8, bicelle samples containing higher concentrations of the short peptide ALGA did not provide a reproducibly alignable medium in which to study the phase behaviour of the lipids and peptide. Since oriented samples are desirable for investigating the peptide in the $\ell_d-\ell_o$ phase coexistence region for the lipid mixtures, the use of mechanically aligned samples on glass slides was revisited. Though the signal per unit volume of sample will be weaker for the samples on glass slides, the alignment of the samples should be much more reliable.

In this chapter, results for DPoPC/DMPC/cholesterol samples aligned on glass slides with varying concentrations of ALGA are presented. These samples were studied as a function of temperature to investigate the behaviour of the lipids and the peptide for these mechanically aligned samples.
10.2 Experimental Details

Sample compositions are indicated by the molar composition of DPoPC/DMPC/cholesterol, and the amount of ALGA is given as a mole percentage relative to the total lipids and cholesterol ((mol ALGA/(mol DPoPC + mol DMPC + mol cholesterol)) × 100%)

Samples aligned on glass slides are hydrated by placing them in a sealed jar which has water in the bottom and a stage above the water on which the sample sits. The whole system is placed in an oven at ~50°C. For samples with deuterated lipid, a drop of D₂O was added to the water to allow the hydration to be monitored during the experiments. For samples with deuterated ALGA, pure H₂O was used. ²H NMR experiments were performed at either 76.77 MHz or 92.15 MHz. For deuterated peptide samples, four spectra were collected each with 250 000 scans and added to obtain better signal.

10.3 Results

In order to establish the phase behaviour of the oriented lipid environment that the peptide will be exposed to, a sample of DPoPC/DMPC-d₅₄/cholesterol with a composition of 32:48:20 was prepared on glass slides. The static ²H spectra are presented in Figure 10.1. The onset of the \( \ell_d-\ell_o \) coexistence region occurs at 293.7 K for this sample. The phase behaviour of the 32:48:20 (DPoPC/DMPC-d₅₄/cholesterol) composition is preserved when going from a powder sample to a sample with the same composition aligned on glass slides.

Different concentrations of unlabelled ALGA were then added to the 32:48:20 (DPoPC/DMPC-d₅₄/cholesterol) mixture and oriented on glass slides to determine where the fluid
Figure 10.1: $^2$H spectra of 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) on glass plates at various temperatures. Note that the small, sharp peak near 0 kHz is due to the water in the sample. Spectra were collected at 76.77 MHz, 4096 scans.
phase coexistence region begins for samples containing the peptide. Figure 10.2 shows the
$^2$H spectra as a function of temperature for the sample containing 2 mol % ALGA aligned
on glass plates. The sample is mainly aligned; however, the 90° edges of the non-aligned
portion of the sample are indicated by the arrows in the figure. Another feature for a fully
aligned sample $^2$H spectrum is that the methyl peaks are resolved down to the baseline
level. A phosphorus NMR spectrum taken after several days of experiments had two com-
ponents corresponding to the 0° oriented and 90° powder components. For this 2 mol %
ALGA sample the two phase coexistence region begins around 294.8 K. The $^2$H spectra as
a function of temperature for the sample with 4 mol % ALGA are shown in Figure 10.3.
This sample is not as well aligned as the samples with less peptide and the onset of the
two phase region has been shifted to a higher temperature, now beginning at 298.4 K.

The highest concentration of ALGA investigated here was 6 mol %. The resulting $^2$H
spectra as a function of temperature are shown in Figure 10.4. Again, the higher concen-
tration of ALGA perturbs the alignment of the lipid bilayers on the glass slides. At high
temperatures, the quadrupolar splittings for the DMPC-d$_{54}$ peaks are already larger than
for the samples with 2 and 4 mol % ALGA. In addition, there appears to be a smooth
transition into the $\ell_o$ phase. There may still be some $\ell_d-\ell_o$ phase coexistence at lower
temperatures; however, the phase behaviour for this sample with 6 mol % ALGA is very
different from the phase behaviour of the pure lipid mixture. $^{31}$P NMR was also performed
on this 6 mol % ALGA sample and a sample spectrum at 298.4 K is shown in Figure 10.5.
There are three contributions to the spectrum, a peak from the 0° orientation, a powder
pattern from the non-oriented lipids, and an isotropic peak. This phosphorus spectrum
does not match the expected result for a well-oriented sample, nor is it similar to the spec-
Figure 10.2: $^2$H spectra of 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) with 2 mol % ALGA on glass plates at various temperatures. The 90° edges in the spectra are indicated by the arrows. Note that the small, sharp peak near 0 kHz is due to the water in the sample. Spectra were collected at 92.15 MHz, 8192 scans.
Figure 10.3: $^2$H spectra of 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) with 4 mol % ALGA on glass plates at various temperatures. Note that the small, sharp peak near 0 kHz is due to the water in the sample. Spectra were collected at 92.15 MHz, 8192 scans.
tra observed for the samples with lower concentrations of ALGA. From these results, it appears that 6 mol % ALGA is above the concentration limit for obtaining well aligned samples in which to study the possible partitioning of the peptide into the \( \ell_d \) and \( \ell_o \) phases of DPOPC, DMPC, cholesterol bilayers.

Finally, deuterated ALGA was added to 32:48:20 (DPOPC/DMPC/cholesterol) at concentrations of 2 and 4 mol %. A single alanine residue is methyl-deuterated, the position of the labelled alanine is indicated by an asterisk: Ac-KKPVALIFALGALAFILA\(^\ast\)VGKK-NH\(_2\). The alignment of these samples was tested using \( ^{31} \)P NMR. If the sample on glass slides is perfectly aligned, then a single peak is expected at the 0° position (\(-30 \) ppm). The non-oriented portion of the sample will result in a peak at the 90° position (\(-15 \) ppm), and any isotropic contribution will be at 0 ppm. A series of \(^2\)H NMR experiments were performed at temperatures in the \( \ell_d \) and \( \ell_d-\ell_o \) phase regions as determined for the lipids. Note that the temperatures for the onset of the two phase region will be higher for these samples because the melting point of DMPC is 24°C whereas for DMPC-d\(_{54}\) it is 19°C. The quadrupolar splitting for the CD\(_3\) group on the alanine was predicted from the moment of inertia frame coordinates of an alpha helical structure of ALGA as \(-20 \) kHz for the peptide undergoing rapid reorientation about the moment of inertia principle axis.

Figure 10.6 shows the \( ^{31} \)P spectrum for 32:48:20 (DPOPC/DMPC/cholesterol) with 2 mol % \(^2\)H ALGA on glass plates at 302.0 K. There are two peaks indicating that the sample is not fully aligned (approximately 12 % in the non-oriented part). The \(^2\)H NMR spectra of the same sample are shown as a function of temperature in Figure 10.7. As the temperature is lowered, the intensity of the peaks with the 19 kHz splittings decreases. The peaks with the 36 kHz quadrupolar splitting are not affected by temperature across
Figure 10.4: $^2$H spectra of 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) with 6 mol % ALGA on glass plates at various temperatures. Collected at 76.77 MHz using a modified quadrupolar echo to refocus the chemical shift, 8192 scans.
Figure 10.5: $^{31}$P spectrum for a sample with 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) + 6 mol % ALGA on glass plates at 298.4 K. Collected at 202.46 MHz with 1024 scans and a repeat rate of 5 s.
this range and remain constant throughout. There is no evidence from these spectra of partitioning of the ALGA between two different fluid phase domains. Two orientations, $0^\circ$ and $\sim 44 \pm 5^\circ$, were used at 298.4 K in order to determine the orientation dependence of the different spectral components. The resulting spectra are shown in Figure 10.8. The peaks with the 19 kHz splitting are affected by the orientation change, while the peaks with the 36 kHz quadrupolar splitting remain constant. Thus, the peaks with the 19 kHz quadrupolar splitting come from peptides which are undergoing axial diffusion, while the peaks with the 36 kHz quadrupolar splitting come from peptides which are a static powder within the sample.

![Figure 10.6: $^{31}$P spectra of 32:48:20 (DPoPC/DMPC/cholesterol) with 2 mol % $^3$H ALGA on glass plates at 302.0 K. Collected at 243 MHz with 1024 scans and a repeat rate of 5 s.](image)

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Figure 10.7: $^2$H spectra of 32:48:20 (DPoPC/DMPC/cholesterol) with 2 mol % $^2$H ALGA on glass plates at various temperatures. Collected at 92.15 MHz with 1 000 000 scans.

Figure 10.9 shows the $^{31}$P spectrum for 32:48:20 (DPoPC/DMPC/cholesterol) with 4 mol % $^2$H ALGA on glass plates at 298.4 K. Again, two peaks are present indicating that the sample is not fully aligned (approximately 20 % in the non-oriented part). The alignment of the 2 mol % ALGA sample is better than the alignment of 4 mol % ALGA
Figure 10.8: $^2$H spectra of 32:48:20 (DPoPC/DMPC/cholesterol) with 2 mol % $^2$H ALGA on glass plates at 298.4 K. The red spectrum is the 0° orientation and the black spectrum is at an orientation of ~ 44 ± 5°. Collected at 92.15 MHz with 1 000 000 scans.

The higher concentration of the peptide perturbs the lipid bilayer organization to a higher degree. The $^2$H NMR spectra of the 4 mol % sample are shown as a function of temperature in Figure 10.10. As with the 2 mol % ALGA sample, the intensity of the peaks with the 19 kHz splittings decreases as the temperature is lowered. The peaks with the 36 kHz quadrupolar splitting begin to increase as the temperature is decreased in this
case, indicating that more of the peptide is becoming immobilized perhaps as a result of aggregation. Up to \( \sim 28\% \) of the peptide is in the solid phase for the sample with 2 mol \% ALGA, and \( \sim 50\% \) of the peptide is in this phase for the sample with 4 mol \% ALGA. Only one mobile, fluid phase contribution to the peptide spectrum is observed and it is not possible to determine how the peptide partitions between the two phases of the membrane from these spectra.

Figure 10.9: \(^{31}\)P spectra of 32:48:20 (DPoPC/DMPC/cholesterol) with 4 mol \% \(^2\)H ALGA on glass plates at 298.4 K. Collected at 243 MHz with 1024 scans and a repeat rate of 5 s.
10.4 Conclusions

Samples containing ALGA were not as well aligned as samples of the pure lipids aligned on glass slides as seen in both the $^2$H and $^{31}$P NMR spectra. This decrease in alignment is more significant for higher concentrations of the peptide. The disruption of alignment of...
lipid samples by peptides is not unique to ALGA. Similar results have been observed by Ouellet et al. [116] for a 14-mer model amphipathic peptide in 1,2-dilauroyl-sn-glycero-3-phosphocholine, DMPC, and DPPC bilayers using \(^{31}\text{P}\) NMR. This particular 14-mer peptide is known to exhibit cytolytic activity in some membranes. The greatest disruption of alignment of the bilayer by the 14-mer peptide was for DPPC which has the longest acyl chains of the lipids investigated. For the ALGA samples, the alignment and phase behaviour of the systems were still acceptable for further studies using labelled peptide for concentrations of 2 and 4 mol % ALGA. However with 6 mol % ALGA the phase behaviour changed dramatically with a loss of the distinctive two fluid phase coexistence and poorer alignment of the sample including a large isotropic contribution in the \(^{31}\text{P}\) spectra.

The \(^2\text{H}\) spectra for the samples containing labelled peptide have peptide peaks with splittings of \(~19\) kHz indicative of peptide undergoing rapid axial reorientation, and a second contribution to the spectrum around \(36\) kHz. Some of the ALGA may be aggregating within the membrane resulting in domains that are static and giving rise to the larger splitting at \(~36\) kHz, very close to \(~40\) kHz which is characteristic of a static peptide in the sample (the rotation of the \(\text{CD}_3\) group still occurs). Sharpe et al. [117, 118] have seen similar \(^2\text{H}\) NMR results for a transmembrane peptide ErbB-2 (a class I receptor tyrosine kinase) labelled with deuterated alanine. Their peptide in POPC liposome samples gave rise to peaks with a quadrupolar splitting of \(38\) kHz which they have concluded are due to the self-association of this peptide within the membrane.

Samples aligned on glass plates tend to provide a more reliable level of alignment than bicelle samples with ALGA and exhibit very similar phase behaviour to the powder
samples. Experiments on samples with deuterated peptide take a significant amount of time to perform since a large number of scans is required in order to get reasonable signal to noise. In order to get better signal in the same experiment time, higher concentrations of peptide or larger samples are required. Currently, ALGA has proved to be a challenging peptide to work with, disrupting the membrane alignment significantly for concentrations greater than 4 mol%.
Chapter 11

Summary and Conclusions

11.1 Summary

Comprehensive $^2$H NMR spectroscopic studies of various model membrane systems with and without peptides were presented in this dissertation and are summarized below.

Model membranes that exhibit $\ell_d$-$\ell_o$ phase coexistence can be made by combining long chain saturated lipids, long chain unsaturated lipids and cholesterol. The $\ell_d$ phase is cholesterol-poor and less ordered than the $\ell_o$ phase which is cholesterol-rich and consequently more ordered. Lipid rafts which are cholesterol-rich, $\ell_o$ domains within the membrane have been a topic of much debate for the past two decades. Systems that exhibit two phase coexistence may also exhibit critical behaviour. Indeed critical fluctuations have been observed in model membranes with cholesterol concentrations similar to that of cytoplasmic membranes. Therefore, it seems possible that critical fluctuations play a role in membrane biochemistry.

Critical fluctuations have been observed in ternary mixtures of DOPC, DPPC, and cholesterol as both multilamellar and giant unilamellar vesicle samples at certain compositions and temperatures [53, 48, 56, 76]. Since critical phenomena are highly dependent on the temperature of the sample, it is important to control the temperature precisely and
minimize the temperature gradient across the sample. Static $^2$H NMR line shapes and first moments for samples with a composition of 37.5:37.5:25 (DOPC/DPPC/cholesterol) were analysed as a function of temperature in order to determine the universality class that describes the critical behaviour of these ternary mixtures. As the critical point is approached, the order parameter for these systems varies according to the power law $\eta = \left(\frac{T-T_c}{T_c}\right)^{\beta_c}$ where $\beta_c$ is the critical exponent. The expected value for $\beta_c$ is 0.325 for the 3D Ising model. The value obtained from the quadrapolar splittings of the $\ell_d$ and $\ell_o$ phases of the sample with DPPC-d$_6$ was $\beta_c = 0.338 \pm 0.009$. The value of the critical exponent for the sample with DPPC-d$_{62}$ was determined using the first moments and was found to be $\beta_c = 0.391 \pm 0.02$. These results suggest that multilamellar dispersions of lipids and cholesterol belong to the 3D Ising universality class and agree with previous results for these systems determined using $^2$H MAS NMR [56].

One of the objectives of this work was to investigate the effect of peptides on the model membrane phase behaviour and to determine whether helical peptides partition preferentially into one of the two fluid phases. Peptides and proteins are often $^{13}$C labelled for NMR experiments. Previously, $^{13}$C NMR experiments were performed on lipid mixtures with a labelled helical peptide with amino acid sequence K$_2$GL$_{16}$K$_2$A-amide (peptide-16) [60]. It was determined that $^{13}$C NMR was not a suitable method to explore the phase coexistence in these model membranes. $^2$H NMR is sensitive to the phase behaviour of lipid mixtures and has been used to determine the phase diagrams for model lipid membranes. Multilamellar dispersions in which every orientation is equally likely result in powder patterns which have quadrapolar splittings that depend on the amount of order within the sample. Gramicidin is a short peptide (15 amino acids) which forms dimers that span
the membrane, the phase behaviour of powder model membrane samples with gramicidin A and gramicidin D was explored using $^2$H NMR. Samples of DOPC/DPPC/cholesterol with specifically deuterated gramicidin A or exchange $^2$H labelled gramicidin D resulted in peptide signals that were very broad and weak and did not show evidence of partitioning of the peptide. These broad spectra motivated the use of either magnetically (bicelles) or mechanically (on glass slides) oriented samples for continuing the peptide investigations.

Mixing a short chain lipid with a long chain lipid results in the formation of bicelles which self-align in an external magnetic field. The classical bicelle mixture is DMPC + DCPC and the morphology of the bicelles can differ depending on the sample temperature, water content and ratio q (mol DMPC/mol DCPC) [15, 16, 17, 18, 19]. The effect of cholesterol on the phase behaviour of DMPC/DCPC bicelles was characterized for cholesterol ratios ($x_c = (\text{mol cholesterol})/(\text{mol DMPC}+\text{mol DCPC}+\text{mol cholesterol})$) of 0 to 0.16. A phase diagram has been presented which indicates the range of temperatures and compositions where DMPC/cholesterol/DCPC (q = 3.2) bicelles align in the magnetic field. Coexisting $\ell_d$ and $\ell_o$ fluid phases can be observed in bicelle mixtures of DPoPC/DMPC/cholesterol or DOPC/DPPC/cholesterol with either DCPC or DHPC as the short chain lipid component. In the DPoPC/DMPC/cholesterol bicelle samples and to a lesser extent in the DOPC/DPPC/cholesterol bicelle samples, a small fraction of the sample is in the $\ell_o$ phase at higher temperatures where only an $\ell_d$ phase was expected by comparison to multilamellar dispersions at similar compositions. There are likely physically isolated bicelles that have a higher than average concentration of cholesterol and which may not exchange lipids with the bulk giving rise to this observed $\ell_o$ phase at high temperatures. These experiments on bicelle mixtures show that there is a fairly large range of temperatures and
compositions where bicelles may provide a suitable aligned model membrane environment for investigating membrane integrated peptides or proteins.

Two different peptides were investigated in bicelle mixtures, Conolysin-Mt1 and ALGA. Though Conolysin-Mt1 is a potent component of the venom of a predatory sea snail, and exhibits cytolytic activity, it is possible to form bicelle samples suitable for long NMR experiments. In order to protect the lipids at higher peptide concentrations (above 2 mol %), the salt concentration of the buffer was increased slightly. The first moments as a function of temperature were calculated from $^2$H NMR spectra, and indicated that relative to bicelles without peptide, in the presence of Conolysin-Mt1 the order of the lipid chains is increased at high temperatures (345-320 K) and the order of the lipid chains is decreased at lower temperatures (320-298 K). In all cases, the bicelles containing Conolysin-Mt1 were well-aligned in the magnetic field.

ALGA is a short, helical synthetic peptide which spans the membrane. ALGA was added at different concentrations to DPOPC/DMPC/cholesterol/DCPC bicelles which had previously been found to be aligned in the magnetic field and exhibit $l_d$-$l_o$ coexistence. The limit for achieving reasonably aligned bicelles with ALGA was found to be about 4 mol % ALGA under the current sample preparation conditions. The challenges with the reproducibility and temperature sensitivity of these bicelles motivated the use of glass plates to investigate ALGA in DPOPC/DMPC/cholesterol further. It is possible that other q values and buffer concentrations or even different ratios for the DPOPC/DMPC/cholesterol components would result in better aligned phases with this peptide.

One question that this work raised was whether the lipids exchange between the two different fluid phase environments in our model membrane samples, especially in bicelles.
Two dimensional $^2$H exchange experiments can be used to study motions with correlation times on the order of $10^{-5}$ to 0.1 s. Here 2D $^2$H exchange experiments were used to investigate the exchange between the $\ell_d$ and $\ell_o$ phase lipids in DPoPC/DMPC-d$_{54}$/cholesterol mixtures that exhibit $\ell_d$-$\ell_o$ phase coexistence. The 32:48:20 (DPoPC/DMPC-d$_{54}$/cholesterol) multilamellar dispersion sample showed evidence of exchange of lipids between the $\ell_d$ and $\ell_o$ phases which is seen as a broadening in the $^2$H 2D exchange spectra. On the other hand, 2D exchange experiments on a bicelle sample with the same 32:48:30 (DPoPC/DMPC-d$_{54}$/cholesterol) and DCPC ($q = 3.5$) did not show the expected exchange phenomenon. Peaks in the bicelle spectra are very sharp and this may contribute to the imperfect addition of the two data collection schemes for elimination of the anti-diagonal. The bicelle mixtures exhibit two fluid phase coexistence but the addition of the short chain lipid may move the sample composition away from a critical composition. If this is the case and the sample is not near a critical point, then broadening and exchange of the lipids between the $\ell_d$ and $\ell_o$ phases as a results of compositional fluctuations may not be observable in these $^2$H 2D exchange NMR experiments. In addition, the broadening typically seen in the one dimensional $^2$H NMR spectra as the $\ell_d$-$\ell_o$ region is approached was not observed for bicelles.

The final series of experiments presented in this thesis were on ALGA in DPoPC/DMPC/cholesterol ternary mixtures aligned on glass slides. A comparison of the phase behaviour of 32:48:30 (DPoPC/DMPC-d$_{54}$/cholesterol) on glass slides to a powder sample shows that the same phase behaviour including the line broadening was present for the oriented sample unlike the bicelle case. Comparing aligned samples with and without ALGA indicates that samples containing the peptide are not as well aligned as samples of the
pure lipids and that the disruption of the alignment is greater for higher concentrations of ALGA. Samples with 2 and 4 mol % ALGA in 32:48:20 (DPOPC/DMPC-d$_{54}$/cholesterol) exhibit phase behaviour that is very similar to the phase behaviour of the multilamellar dispersions with the same composition. The peaks in the $^2$H NMR spectra broaden as the phase transition from the $\ell_d$ phase to the $\ell_d$-$\ell_o$ coexisting phases is approached. However, for 6 mol % ALGA the phase behaviour of the lipids changed dramatically. The distinctive two fluid phase coexistence typically observed in the $^2$H spectra was lost and the sample was more poorly aligned. This indicates that the presence of the peptide can disrupt the usual phase behaviour of the lipid model and may result in a loss of the two fluid phase coexistence altogether.

$^2$H NMR spectra from samples containing ALGA with a methyl-deuterated alanine have two distinct peptide peak quadrupolar splittings, one at $\sim$19 kHz indicative of peptide undergoing rapid axial reorientation, and a second at $\sim$36 kHz which is characteristic of a powder peptide. The quadrupolar splitting of the pure powder form of this $^2$H labelled ALGA was measured to be $\sim$37 kHz. A significant portion of the peptide appears to be in a solid, powder phase, likely as aggregates within the sample. The deuterium labelled methyl group allows for the peptide peaks to be seen since they are relatively sharp, however the signal to noise is still very poor. It is also possible that the methyl label position is not very sensitive to the difference between the $\ell_d$ and $\ell_o$ phase environments of the peptide. Unfortunately, it was not possible to determine anything about the partitioning of this peptide into either of the two fluid phases from the current data. Note that other experiments on samples containing ALGA that were run over the course of a couple of months showed evidence of lipid degradation. The stability of these lipid/peptide samples
is a concern when running experiments at physiological temperatures over a longer period of time. Since the peptide concentration is usually quite low, long experiments are often required in order to get a reasonable amount of signal.

### 11.2 Conclusions and Future Directions

Membranes separate the cell from the outside world and are integral to cell functions. These complex and dynamic systems of lipids and proteins are involved in signalling, controlling what gets into and out of the cell, and maintaining concentration gradients. Model membranes can serve as interesting systems to study various membrane properties in a simplified and controlled manner. In this work, the goal was to gain more insight into the phase behaviour, molecular order, and molecular dynamics of model membrane systems.

Ternary lipid and cholesterol samples can exhibit two fluid phase coexistence and critical fluctuations at certain compositions and temperatures which can be probed and monitored using 1D $^2$H NMR as well as 2D exchange experiments. The inclusion of a peptide in the lipid membrane with coexisting phases can change the phase behaviour of the membrane and the peptide may partition preferentially into one of the two fluid phases. In the current work, there was no evidence of preferential partitioning of the short, synthetic peptide ALGA or gramicidin A. It was found however, that the presence of a peptide in the lipid mixture did affect the overall phase behaviour of the model membrane.

Oriented samples can be advantageous for NMR experiments since the spectra from oriented samples are simpler and better resolved than those from powder samples. Bicelles
orient spontaneously in the magnetic field and give more signal for the same sample volume since they do not require a supporting substrate such as glass. The many components required to produce bicelle samples that can exhibit two fluid phase coexistence however, means that these are more complex model membranes and changing any of the variables can have a dramatic effect on the phase behaviour, morphology, and ability for the sample to align within a magnetic field. Adding a peptide or short chain lipid component to a lipid mixture which exhibits two phase coexistence may result in loss of two phase coexistence, or move the composition away from a critical point so that fluctuations are no longer observable. Characterizing the phase behaviour of bicelle mixtures, especially those with lipid mixtures that are expected to exhibit $\ell_d-\ell_c$ phase coexistence would provide a basis for future studies of peptides and proteins in these more complex membrane mimetics. A question that was raised from the exchange experiments on the bicelle systems investigated here is whether there are compositions for bicelle samples which exhibit $^2$H line broadening characteristic of critical fluctuations seen for certain compositions of DPOPC/DMPC/cholesterol and DOPC/DPPC/cholesterol powder samples. Different ratios of DPOPC/DMPC/cholesterol with DCPC should be studied to determine where the critical points for these bicelle mixtures are and to investigate what happens when peptides are exposed to this environment.

Critical behaviour in oriented systems could be investigated using both bicelles (when a critical composition is found) and bilayers aligned on glass slides. It would also be interesting to study single bilayers on glass beads using both NMR and fluorescence microscopy. The results for the critical exponents obtained for these different sample types could provide insight into the universality class or classes that the various systems belong
to and show how much the stacking of bilayers affects the nature of the critical fluctuations.

Biological membranes can contain many different species of lipids, sterols, and proteins and phase separated domains may be important for protein functions. By having fundamental understanding of these model membrane systems, we hope to gain insight into the important aspects of membrane phase behaviour and the relationship between the various components of biological membranes and their surrounding environments.
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Appendix A

Static $^2$H NMR Lineshape Fitting

A.1 Program for Fitting a Single Gaussian Broadened Powder Pattern

This program was used to fit $^2$H NMR spectra with a single Gaussian broadened powder pattern, i.e. for temperatures above $T_c$.

```c
#include <origin.h>
void _nlsfgauss_pp_3cf(
    // Fit Parameter(s):
    double A1, double nu_Q1, double sig_1, double d0, double rho,
    // Independent Variable(s)
    double nu,
    // Dependent Variable(s):
    double y)
{
    int count;
    double theti, d_theti, c_theti, s_theti, gx1, total1, N_fac_1;
    double twosig12, mu2, mu2_1p, mu2_1m, ecc, as_ecc;
```
12 ecc=sqrt(1-(1/rho)^2);
13 as_ecc=asin(ecc);
14 d_theti=3.14159265/(2.0*n);
15 N_fac_1=1.0/(sqrt(2.0*3.14159265)*sig_1);
16 twosig12=2.0*sig_1^2;
17 count=0;
18 total1=0.0;
19 do
20 { theti=(count – 1)*d_theti;
21 c_theti=cos(theti);
22 mu2=0.5*(3.0*c_theti^2-1);
23 mu2_lp=(x+nu_Q1*mu2+d0)^2/twosig12;
24 mu2_lm=(x-nu_Q1*mu2+d0)^2/twosig12;
25 s_theti=sin(theti);
26 gx1=exp(-mu2_lp)+exp(-mu2_lm);
27 total1=total1+s_theti*gx1/((s_theti^2+rho^2*c_theti^2)^2);
28 count ++;}
29 while(count < n + 1);
30 y=A1*exp(-14.7957704*tau*sig_1)*N_fac_1*total1*rho
^2/((1.0+(rho/ecc)*as_ecc)/2.0);}
A.2 Program for the Superposition of Three Gaussian Broadened Powder Patterns

This program was used to fit $^2$H NMR spectra with a superposition of three Gaussian broadened powder patterns, i.e. for temperatures below $T_c$.

```c
#include <origin.h>

void _nlsfgauss_pp_3cf(
    // Fit Parameter(s):
    double A1, double A2, double A3, double nu_Q1, double nu_Q2,
    double nu_Q3, double sig_1, double sig_2, double sig_3,
    double d0, double rho,
    // Independent Variable(s):
    double nu,
    // Dependent Variable(s):
    double y)
{
    const double tau=5e-005
    const double n=50
    #pragma numlittype (push; TRUE)
    int count;
    double theti, d_theti, c_theti, s_theti, gx1, gx2, gx3, total1, total2, total3, N_fac_1, N_fac_2, N_fac_3;
    double twosig12, twosig22, twosig32, mu2, mu2_1p, mu2_1m, mu2_2p, mu2_2m, mu2_3p, mu2_3m;
```
double ecc, as_ecc;

ecc=sqrt(1-(1/rho)^2);

as_ecc=asin(ecc);

d_theti = 3.14159265/(2.0*n);

N_fac_1 = 1.0/(sqrt(2.0*3.14159265)*sig_1);

N_fac_2 = 1.0/(sqrt(2.0*3.14159265)*sig_2);

N_fac_3 = 1.0/(sqrt(2.0*3.14159265)*sig_3);

twosig12 = 2.0*sig_1^2;

twosig22 = 2.0*sig_2^2;

twosig32 = 2.0*sig_3^2;

count = 0;
total1 = 0.0;
total2 = 0.0;
total3 = 0.0;

do

{ theti = (count - 1)*d_theti;

ctheti = cos(theti);

mu2 = 0.5*(3.0*c_theti^2 - 1);

mu2_1p = (nu+nu_Q1*mu2+d0)^2/twosig12;

mu2_1m = (nu-nu_Q1*mu2+d0)^2/twosig12;

mu2_2p = (nu+nu_Q2*mu2+d0)^2/twosig22;

mu2_2m = (nu-nu_Q2*mu2+d0)^2/twosig22;

mu2_3p = (nu+nu_Q3*mu2+d0)^2/twosig32;


38 \[ \text{mu}2.3\text{m} = (\text{nu} - \text{nu}_Q3 \times \text{mu}2 + \text{d}0)^2 / \text{twosig32}; \]
39 \[ s\_\text{theti} = \sin(\text{theti}); \]
40 \[ g_x1 = \exp(-\text{mu}2.1p) + \exp(-\text{mu}2.1m); \]
41 \[ \text{total1} = \text{total1} + s\_\text{theti} \times g_x1 / ((s\_\text{theti}^2 + \text{rho}^2 \times c\_\text{theti}^2)^2); \]
42 \[ g_x2 = \exp(-\text{mu}2.2p) + \exp(-\text{mu}2.2m); \]
43 \[ \text{total2} = \text{total2} + s\_\text{theti} \times g_x2 / ((s\_\text{theti}^2 + \text{rho}^2 \times c\_\text{theti}^2)^2); \]
44 \[ g_x3 = \exp(-\text{mu}2.3p) + \exp(-\text{mu}2.3m); \]
45 \[ \text{total3} = \text{total3} + s\_\text{theti} \times g_x3 / ((s\_\text{theti}^2 + \text{rho}^2 \times c\_\text{theti}^2)^2); \]
46 \[ \text{count} \; \text{++}; \]
47 \[ \text{while} (\text{count} < n + 1); \]
48 \[ y = \text{rho}^2 \times (A1 \times \exp(-14.7957704 \times \text{tau} \times \text{sig}_1) \times N\_\text{fac}_1 \times \text{total1} + A2 \times \exp(-14.7957704 \times \text{tau} \times \text{sig}_2) \times N\_\text{fac}_2 \times \text{total2} + A3 \times \exp(-14.7957704 \times \text{tau} \times \text{sig}_3) \times N\_\text{fac}_3 \times \text{total3}) / ((1.0 + (\text{rho} / \text{ecc}) \times \text{as\_ecc}) / 2.0); \]
Appendix B

Pulse Programs

B.1 Quadrupolar Echo

; written by HF 9.9.98
; 90–90 solid echo sequence for wide line observation
; written for DDS phase shifts (set edscon PHASPR to 2–3 usec)
; set d6 to 5–100 usec depending on dead time
; set d7 shorter than d6 by 3 usec plus 10 dw to make
; sure the evolution of the echo is observed, then left shift
; FID prior to ft to get smallest possible 1st order phase correction
; set receiver phase either with phcor0 or with 4–PM receiver phase
; to get maximum signal in ADC channel A and almost no signal in channel B
; for minimum 0 order phase correction (no signal in
channel B is only
; possible if O1 exactly in center and spectrum perfectly
symmetric).
; phase cycle will cancel dead time partially, but not
perfectly

"acqt0=-p1*2/3.1416"

1ze ; overwrite RCU data
2dl pl1:f1 ; relaxation delay, set power level
  (default)
  (pl ph1):f1 ; excitation pulse
d6 ; first echo delay according to probe dead
time
  (pl ph2):f1 ; refocussing pulse
d7 ; set to observe echo ascending
1u:f1 ph0:r ; reset DDS to reference phase, set
  phcor 0 for
go=2 ph31 ; all signal in one channel
wr #0
exit
ph0=0
ph1= 0 3 2 1 0 3 2 1 ; phase cycle after Ronemus and Vold
B.2 Exchange Experiments

;2dexch2H_qechA.jd
;avance−version (09/04/17)
;2D exchange with added quadrupolar echo
;phase sensitive
; phase cycling and sequence from Schmidt, Blumich and
  Spiess (1988)

;$CLASS=HighRes
;$DIM=2D
;$TYPE=
;$SUBTYPE=
;$COMMENT=

#include <Avance.incl>
"in0=inf1"
"p2=p1/1.645"
"d0=0.1u"
"acqt0=-p1*2/3.1416"
1 ze
2 d1
3 p1 ph1
d0
p2 ph2
d8
p2 ph3
d7
p1 ph4
d28
go=2 ph31
d1 mc #0 to 2 FlPH(calph(ph1, +90), caldel(d0, +in0))
exit

ph1=1 1 1 1 3 3 3 2 2 2 2 0 0 0 0
ph2=3 1 3 1 1 3 1 3 0 2 0 2 2 0 2 0
ph3=1 1 1 1 3 3 3 2 2 2 2 0 0 0 0
ph4=0 0 2 2 2 2 0 0 1 1 3 3 3 3 1 1
ph31=0 2 0 2 2 0 2 0 1 3 1 3 3 1 3 1

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; pl1 : f1 channel - power level for pulse (default)
; p1 : f1 channel - 90 degree high power pulse
; p2 : f1 channel - 54.7 degree high power pulse
; d0 : incremented delay (2D)
; d1 : relaxation delay; 1-5 * T1
; d8 : mixing time
; inf1: 1/SW = 2 * DW
; in0: 1/(1 * SW) = 2 * DW
; nd0: 1
; NS: 8 * n
; DS: 16
; tdl: number of experiments
; FnMODE: States-TPPI, TPPI, States or QSEQ

; Processing
; PHC0(F1): 90
; PHC1(F1): -180
; FCOR(F1): 1

; $Id: noesyph,v 1.8 2009/07/02 16:40:46 ber Exp$
;2dexch2H_qechB.jd
;avance-version (09/04/17)
;2D exchange with added quadrupolar echo
;phase sensitive
; phase cycling and sequence from Schmidt, Blumich and
; Spiess (1988)

;CLASS=HighRes
;DIM=2D
;TYPE=
;SUBTYPE=
;COMMENT=

#include <Avance.incl>

"in0=inf1"
"p2=p1/1.645"
"d0=0.1u"
"acqt0=-p1*2/3.1416"

1 ze
2 d1
3 p1 ph1
d0
p2 ph2
d8
p2 ph3
d7
p1 ph4
d28
go=2 ph31
d1 mc #0 to 2 F1PH(calph(ph1, +90), caldel(d0, +in0))
exit

ph1=1 1 1 1 3 3 2 2 2 0 0 0 0
ph2=2 0 2 0 0 2 0 2 3 1 3 1 3 1 3
ph3=0 0 0 0 2 2 2 2 1 1 1 3 3 3 3
ph4=0 0 2 2 2 2 0 0 1 1 3 3 3 3 1 1
ph31=0 2 0 2 2 0 2 0 1 3 1 3 3 3 3 3

; p11 : f1 channel – power level for pulse (default)
; p1 : f1 channel – 90 degree high power pulse
; p2 : f1 channel – 54.7 degree high power pulse
; d0 : incremented delay (2D)
; d1 : relaxation delay; 1−5 * T1
; d8 : mixing time

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; inf1: 1/SW = 2 * DW
; in0: 1/(1 * SW) = 2 * DW
; nd0: 1
; NS: 8 * n
; DS: 16
; tdl: number of experiments
; FnMODE: States – TPPI, TPPI, States or QSEQ

; Processing

; PHC0(F1): 90
; PHC1(F1): -180
; FCOR(F1): 1