Detecting Critical Fluctuations in Ternary Model Membrane Systems of DOPC, DPPC, and Cholesterol Using NMR Spectroscopy

by

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ABSTRACT

DETECTING CRITICAL FLUCTUATIONS IN TERNARY MODEL MEMBRANE SYSTEMS OF DOPC, DPPC, AND CHOLESTEROL USING NMR SPECTROSCOPY

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This study investigated the critical behaviour of ternary mixtures of DOPC and DPPC, with cholesterol. The properties of model membranes such as these are studied in order to provide insight into aspects of complex biological systems. Experiments were performed using the Jeener echo, a static solid-state NMR technique, however no information about the critical phenomena was obtained. Conversely, the sideband linewidths measured from $^2$H MAS NMR are sensitive to temperature and dependent upon the phase behaviour. By fitting the linewidth data to an equation from Suwelack et al. (J. Chem. Phys., 1980; 73(6):2559-2569), the critical temperature and the critical exponent for the correlation length of the system were calculated. The critical exponent values obtained from these samples ranged between $\nu_c = 0.65$ and $\nu_c = 1.2$, which encompasses the critical exponents for both the 2D and 3D Ising models within error. The universality class for these model membranes cannot be unambiguously assigned yet.
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<tr>
<td>α-d₄-DPPC</td>
<td>DPPC with deuterons at the first four chain positions</td>
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<td>CHOL</td>
<td>cholesterol</td>
</tr>
<tr>
<td>DLPC</td>
<td>1,2-dilauroyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPPC-d₆₂</td>
<td>chain perdeuterated DPPC</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>l_d</td>
<td>liquid disordered</td>
</tr>
<tr>
<td>l_o</td>
<td>liquid ordered</td>
</tr>
<tr>
<td>MAS</td>
<td>magic angle spinning</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PAS</td>
<td>principle axis system</td>
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<tr>
<td>r.f.</td>
<td>radio frequency</td>
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Chapter 1

Introduction

The structure and function of biological membranes have been intriguing researchers for a long time. By studying membranes one can gain insight into how they function and how outside factors can affect their behaviour. Since true biological membranes are very complex and contain many different lipids, cholesterol and proteins, researchers tend to study simpler model systems to mimic the behaviour of certain aspects of full biological membranes. Ternary mixtures of two phospholipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), with cholesterol (CHOL) form two coexisting fluid bilayer phases over a broad range of temperatures and compositions. The two fluid phases are known as the liquid disordered ($l_d$) phase, and the liquid ordered ($l_o$) phase. The phase separation in these ternary mixtures can be observed in nuclear magnetic resonance (NMR) spectra as well as fluorescence images. Phase diagrams for these ternary lipid/cholesterol mixtures have been presented by Davis et al., 2009 [1] and Veatch et al., 2007 [2]. The phase coexistence leads to the question of whether or not functional lipid rafts are present in biological membranes [3]. The work presented here is a study of DOPC, DPPC, and cholesterol model membranes and their phase behaviour near the critical points in the phase diagram.

The overall goal of this project was to look into critical compositional fluctuations in model membrane systems in more detail and determine which universality class these model membranes fit into based on the NMR results. Although lipid bilayers are very thin compared to their width, they are not perfectly two-dimensional, so it is of interest to see whether the critical behaviour in these systems can be described by the 2D Ising
An objective was to find a static NMR technique rather than a magic angle spinning (MAS) NMR technique to avoid introducing a temperature gradient across the sample as a result of the friction between the probe and rotor while spinning. It would also be advantageous to use $^1$H detection that does not require labelling of any of the lipids. The Jeener echo pulse sequence [5] which probes the relaxation of the dipolar order created by the local fields of the nuclei seemed like a good candidate for this. The sample composition 35:35:30 mol% (DOPC/DPPC/CHOL) was investigated using the Jeener echo and the results of this work are presented in Chapter 4. Despite the efforts to optimize a Jeener echo pulse sequence, no information about the critical behaviour in model membrane systems was obtained from this static NMR technique. It then seemed most useful to turn back to MAS NMR and investigate the $^2$H MAS line broadening for a series of samples with a constant molar concentration of cholesterol of 30%, using specifically deuterated DPPC ($\alpha$-d$_4$-DPPC) rather than the chain perdeuterated DPPC (DPPC-d$_{62}$) used previously [6]. By using specifically labelled DPPC, the contributions to the spectra are simplified and inhomogeneities in the line shapes caused by specific environments, such as the methyl groups, can be eliminated. $^2$H MAS NMR is a useful technique for studying critical phenomena since the linewidths are sensitive to the phase behaviour of the systems and changes in the sideband linewidths are relatively easy to measure. The $^2$H MAS study can be found in Chapter 5 of this thesis. In the following sections and chapters the theory and methodology used to investigate critical phenomena in model membrane systems of DOPC, DPPC, and cholesterol are set out and the current findings are presented.
1.1 Biological Membranes and Lipid Rafts

Biological membranes are complex systems containing many different types of lipids, and proteins along with cholesterol. Lipids are divided into classes; all eukaryotic plasma membranes contain glycerophospholipids, and most contain sphingolipids and sterols as well. Glycerophospholipids contain a head group that is attached to two acyl chains via a glycerol group. The two chains may be different lengths and one or both may be unsaturated. Sphingolipids are split into two sub-categories, sphingomyelin and glycosphingolipids, both types are made up of a polar head group, a ceramide backbone, and non-polar acyl chains. The head group for sphingomyelin is a phosphocholine group, while glycosphingolipids contain a carbohydrate head group [7, 8]. Examples of the structures of glycerophospholipids and sphingolipids are found in Figure 1.1.

![Diagram of membrane components](image)

Figure 1.1: Comparison of the components making up (a) DPPC, a glycerophospholipid, with the head group, glycerol, and fatty acid chains indicated, (b) Egg Sphingomyelin, a sphingolipid, with the head group, ceramide group, and fatty acid chains indicated.
Sterols are another common class of compounds found in biological membranes. Sterols are based on a complex of four rigid rings, three 6-carbon rings and one 5-carbon ring. The hydroxyl head group is attached on one side of this ring-structure, while a side chain (at C-3), the identifying feature for each different sterol, is attached on the other side of the ring-structure (at C-17) [8]. The most well-known sterol is cholesterol, which is shown in Figure 1.2.

![Figure 1.2: Structure of cholesterol with the four ring complex, hydroxyl head group and side chain indicated.](image)

Many of the lipids found in biological membranes have two acyl chains, this 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 [9]. 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 [7].

There has been a lot of discussion of the possible existence of cholesterol-rich microdomains called lipid rafts in biological membranes. It has been suggested that rafts may be important for a variety of biological processes including signalling pathways, and as a site of entry into a cell and for protein binding [3, 7]. Lipids are able to move laterally throughout the membrane via diffusion, and thus the proposed raft systems are thought to be dynamic [3]. Though the possible existence of lipid rafts has garnered attention, the biological requirement for these rafts has not been proven [1, 3, 10, 11]. The possibility of raft-like phenomena in biological membranes provides motivation for understanding
the structure and dynamics of simple, model systems. These techniques and insights gained through studying model systems can then be applied to more complex models and eventually full biological membranes. The work presented here looks to describe one of the aspects of the phase behaviour of model lipid/cholesterol membranes, namely critical fluctuations in composition near the critical points in the phase diagram.

1.2 Introduction to Phases, Phase Diagrams and Critical Points

The phase of a substance is based on its physical properties, and often substances can be classified as belonging to one of three general states of matter; solid, liquid, or gas. Factors such as temperature, pressure, and volume can affect the phase of a material. Water is used as an example to discuss briefly the concept of phase diagrams. Lines on a phase diagram represent the boundaries between different phases. On either side of the line, the specified phase(s) are distinguishable, but at the line (phase transition), the properties are such that the phases exist simultaneously. Special points may be present on the phase diagram; these include triple points, where three phases coexist, and critical points, which will be discussed in some more detail below. The classical pressure-temperature phase diagram for water can be found in Figure 1.3. Note that in this diagram the line between the gas and liquid phases has a finite length, and the point at which it ends is called a critical point. Here, the conversion between the liquid and gas phases is continuous and the two phases are indistinguishable.

Phase diagrams can also be presented as functions of an appropriate order parameter. The order parameter is something that differentiates the phases; in the example shown in Figure 1.4, the order parameter of choice is the difference in densities between the two phases. If a horizontal line corresponding to a constant pressure in the case of the Figure 1.4 would intersect the isotherm at more than one point, then one can draw a
straight line between these two points. This is known as the Maxwell construction and it is used to connect the two coexisting phases. These horizontal portions correspond to a two-phase coexistence region. The point at which the isotherm just changes concavity such that a horizontal line at each pressure only ever intersects with the isotherm once, is the critical point [12]. An example is shown in Figure 1.4.

Figure 1.3: Phase diagram of water.

Figure 1.4: Pressure as a function of density for a fluid with a liquid phase, gas phase, and a two phase region, isotherms are indicated for temperatures above, below and at the critical temperature [12].
Critical phenomena can be described mathematically by functions that diverge as the critical temperature is approached. These functions can be written in terms of a power-law dependence on the reduced temperature, \( t = \frac{T}{T_c} - 1 \), near the critical point. The following are some examples of thermodynamic quantities as functions of the reduced temperature and their corresponding critical exponents.

Zero-field magnetization:

\[
M \sim (-t)^\beta
\]  \hspace{1cm} (1.1)

Liquid-gas density difference:

\[
\rho_L - \rho_G \sim (-t)^\beta
\]  \hspace{1cm} (1.2)

Specific heat at a constant volume:

\[
C_V \sim (t)^\alpha
\]  \hspace{1cm} (1.3)

Correlation lengths:

\[
\xi \sim (t)^{-\nu}
\]  \hspace{1cm} (1.4)

Values of these critical exponents can be calculated (or estimated when full calculations are not possible) for various models. In statistical mechanics, theoretical models are used to describe physical observations such as phase transitions. Systems that exhibit similar overall physical behaviour, regardless of the mechanism, are said to belong to the same universality class. Universality classes include mean field theory, the 2D Ising model, and the 3D Ising model. Each universality class results in a specific set of critical exponents [4, 12]. Experimentalists probe physical properties, and then by fitting the data can in principle find the critical exponent and thus the universality class that describes a given system.

The Ginzburg criterion can be used to determine the region in which critical phenomena can be observed, that is where fluctuations dominate the thermodynamics and
mean order parameter value of the system. At temperatures far from the critical point, the ratio between the correlation function and the equilibrium order parameter for the system should be much less than 1. As a result, the reduced temperature, $t = \frac{T}{T_c} - 1$ follows the relationship [13]

$$|t|^{\frac{4-d}{2}} > \frac{k_B}{4\Delta C \xi(1)^d} \equiv t_G^{4-d}$$

(1.5)

where $|t|$ is the reduced temperature, $d$ is the dimension of the system, $k_B$ is the Boltzmann constant, $\Delta C$ is the change in the heat capacity at the transition, $\xi(1)$ is the linear dimension, and $t_G$ is the Ginzburg reduced temperature. Equation 1.5 is known as the Ginzburg criterion, where it breaks down indicates the onset of the critical regime. The upper limit of the critical region can be determined from $t_G$. The critical region therefore satisfies $|t| < t_G$. $t_G$ can be estimated by noting that $\Delta C \xi(1)^d$ will be approximately $k_B$ per particle (so $\frac{\Delta C}{k_B} \sim O(1)$) [13]. Equation 1.5 will be simplified as shown below for $t_G$ to get an order of magnitude estimate for the critical region.

$$\frac{4-d}{t_G^2} \approx \frac{1}{\xi(1)^d}$$

(1.6)

In the case of the lipid bilayers, $\xi(1)$ is on the order of 1 nm or 10 Å (the size of a single lipid molecule). In two dimensions this means that

$$\frac{4-2}{t_G^2} \approx \frac{1}{(O(1))^2}$$

$$(1.7)$$

$$t_G \approx 0.01$$

(1.8)

which results in $T \approx 295$ K for $T_c = 292$ K. This means that the critical region in two dimensions is within $\sim 3$ K of the critical temperature. In the three dimensional case, the critical region will be smaller, here

$$\frac{4-3}{t_G^2} \approx \frac{1}{(O(1))^3}$$

(1.9)

$$\frac{1}{t_G^2} \approx 0.001$$

(1.10)
which results in \( T \approx 292.3 \text{ K} \) for \( T_c = 292 \text{ K} \). This means that the critical region in three dimensions is within \( \sim 0.3 \text{ K} \) of the critical temperature. These are just rough approximations but show that it should be possible to probe the critical region using NMR. Phases, phase diagrams and critical phenomena in the context of model lipid membrane systems are discussed below.

### 1.3 Model Membrane Systems

Model membranes are often comprised of mixtures of long chain saturated and unsaturated phospholipids along with cholesterol. The behaviour of ternary mixtures of two phospholipids and cholesterol is of interest in the current work. These mixtures contain an unsaturated lipid with a low melting temperature (DOPC), a saturated lipid with a high melting temperature (DPPC), and cholesterol. Cholesterol is a sterol which is known to stiffen bilayers and is an essential component in biological membranes \( [8, 14] \). Cholesterol favours interacting with saturated lipids over unsaturated lipids. The interactions between the lipids and cholesterol result in hydrocarbon chain lengthening in the surrounding lipids, which increases the thickness of the membrane \( [15] \). DOPC/DPPC/CHOL mixtures are useful as model membranes because they exhibit two phase coexistence over a broad range of temperatures and compositions \( [1, 2] \). The overall phase behaviour of these model membrane systems is discussed in the following section.

#### 1.3.1 Model Membrane Phases, Phase Diagrams and Critical Points

The phase diagram for binary systems of DPPC and cholesterol \( [14] \), and DOPC and DPPC \( [16] \), and for ternary systems of DOPC/DPPC/CHOL \( [1, 2] \) have been determined using NMR spectroscopy and fluorescence microscopy (in the case of the latter). The lipid bilayers formed by these mixtures can exist in two distinct membrane phase classes:
fluid and gel. Bilayers in the gel phase result in broad static $^2$H NMR spectra due to the slow molecular motions and high degree of molecular order. The gel phase is the most rigid of the phase-types, but is not of interest to the work presented here. Ternary mixtures of DOPC/DPPC/CHOL exhibit two coexisting fluid phases over a broad range of temperatures and compositions [1, 2]. The two fluid phases, $l_d$, and $l_o$ are differentiated based on the degree of order of the lipid chains. Cholesterol is known to have an ordering effect on the lipid chains, and it has been found that cholesterol-rich regions of the membrane can form $l_o$-phase domains, while cholesterol-poor regions only form $l_d$-phase domains [1, 2, 14, 17, 18]. An illustration of the make-up of these phases is shown in Figure 1.5.

![Figure 1.5: Cholesterol-rich and cholesterol-poor regions of a membrane representing the liquid ordered and liquid disordered membrane phases respectively.](image)

In static $^2$H NMR, phases are identified by the size of their quadrupolar splittings. It is the C-$^2$H bond order that governs the width of the splitting, and the higher this order is, the larger the quadrupolar splitting. The $l_o$-phase has a higher degree of order than the $l_d$-phase so contributions to the static $^2$H NMR spectra from the $l_o$ phase are broader than those from the $l_d$ phase [1, 14, 19].

Davis et al. determined the phase diagram for a three-component lipid/cholesterol mixture [1]. An isotherm from their phase diagram for DOPC/DPPC-d$_{62}$/CHOL determined using static $^2$H NMR is shown in Figure 1.6. In this diagram the various single
phase, two phase and three phase coexistence regions can be seen as a function of the ternary mixture composition. The full phase diagram is built up of many isothermal planes like that in Figure 1.6, and a three dimensional figure with planes separating the various regions is produced. The star indicates an expected critical point where the $l_o$ and $l_d$ phases are indistinguishable. In the full phase diagram, there is a line of critical points [1].

1.3.2 Critical Phenomena in Model Membranes

In the two phase coexistence region of a phase diagram such as the isotherm shown for DOPC/DPPC-d$_{62}$/CHOL in Figure 1.6, if the spectra for these two samples are comprised of the same underlying spectra but in different proportions, then a tie-line can be drawn to connect the two compositions. Tie-lines are straight lines that link the two sides of the two phase region [1]. As mentioned above, critical points are places on the phase diagram where coexisting phases become identical and the transition between them becomes continuous. This occurs when the tie-lines become a single point [10, 12].
One of the key differences between the two fluid membrane phases, the $l_o$ phase and the $l_d$ phase, is the concentration of cholesterol. Thus, in the two phase region cholesterol is not distributed uniformly throughout the sample, instead, there are cholesterol-rich and cholesterol-poor areas [1, 2]. The behaviour near a critical point can be described in terms of fluctuations in the concentration of cholesterol [6, 10]. The composition (or local cholesterol concentration) is the relevant order parameter in these model membrane systems [4]. A discussion of previous work that indicates the presence of critical fluctuations in model membrane systems and provides motivation for the research presented here is given below.

**1.3.3 Previous Work on Critical Fluctuations**

A large body of work on ternary DOPC/DPPC/CHOL model membrane systems has been presented by Veatch and Keller et al. [2, 4, 10, 19, 20, 21]. The primary technique used for their work is fluorescence microscopy on giant unilamellar vesicles, but they also use deuterium NMR on multilamellar vesicles [2, 19, 21]. The following is a short motivation for why the Ising model may be useful for describing the fluid phase coexistence and critical behaviour in model membrane systems. As discussed by Honerkamp-Smith et al. [4], the $l_d$ and $l_o$ phases can be represented by a two dimensional lattice of Ising spins. The phases can also be modelled as thin sheets containing two states provided that the correlation length (characteristic size of the domains) of these states is greater than the thickness. An adaptation of a figure found in Honerkamp-Smith et al. [4] which depicts these representations can be seen in Figure 1.7. Near the critical point, the observed fluctuations in the phase domains are on the order of microns, which is much larger than the bilayer thickness which is on the order of nanometres [4, 21].

Compositional fluctuations occur at temperatures above and below the critical temperature. These fluctuations are limited to a characteristic correlation length, $\xi$, for the system. As the critical temperature is approached, the correlation length diverges ac-
Figure 1.7: (a) Lipid bilayer showing two phase coexistence, (b) these two phases represented by Ising spins where up represents disordered lipid chains, and down represents ordered lipid chains, and (c) the two phases modelled by two states, 1 and 2. Adapted from Honerkamp-Smith et al. [4].

According to the power law $\xi \approx |T - T_c|^{-\nu}$, where $\nu$ is the critical exponent. By measuring the line tension at temperatures below $T_c$, and the correlation length via the structure factors and membrane composition distribution above $T_c$, they determined the critical exponent for the correlation length to be $\nu = 1.2 \pm 0.2$, which corresponds to the 2D Ising model. The line tensions were measured by tracing the outlines of the $l_o$ domains in the vesicles as they fluctuate over time; the line tension goes to zero at the critical point. They expect the critical behaviour to follow the 2D Ising model because the lipid bilayer thickness ($\sim 1 \text{ nm}$) is much less than the radius of the vesicles ($>10 \mu \text{m}$) being studied, so the system is close to two dimensional [4, 21]. One must point out that there are however experimental limitations to fluorescence microscopy, including the spatial resolution, the length of time required to obtain an image through the scanning process, the ability to control the temperature with enough precision to get close to $T_c$, and the variations in the composition of each vesicle [20, 21]. Overall, Veatch and Keller et al. propose that critical fluctuations provide a mechanism for lipids and proteins to organize themselves at the submicron scale near a critical point, and that the critical fluctuations they observe adhere to the 2D Ising universality class [4].
McConnell et al., have proposed a mechanism for explaining the observed $^2$H NMR line broadening [22, 23, 24, 25, 26, 27, 28]. They present the idea that there is only one thermodynamic phase in the region where critical fluctuations are proposed, and that the observations of increased splittings in NMR spectra are due to the formation of complexes. They use condensed complexes of phospholipids and cholesterol at a stoichiometric ratio 2:1 (phospholipid/cholesterol) to calculate fluctuations observed in model membranes containing cholesterol. These condensed complexes are said to be more ordered than the free lipids, so the formation of complexes increases the order of the system. They assert that the processes of complex formation and dissociation contribute the most to the line broadening in static $^2$H spectra, and that concentration fluctuations due to the critical point are smaller effects on top [25, 26].

Previous work by Veatch et al. [2] and Davis et al. [1] on DOPC/DPPC-$d_{62}$/CHOL systems using static $^2$H NMR found line broadening of the spectra near the proposed critical points in the phase diagram. They discuss the possibility that this observed line broadening may be a result of increases in the transverse relaxation rate as a consequence of critical composition fluctuations [1, 2]. More recently, Davis et al., have investigated a series of compositions of DOPC/DPPC-$d_{62}$/CHOL mixtures both near and far from the expected critical points using $^2$H MAS NMR [6]. They found that there was significant line broadening, up to a 10 fold increase in the linewidths as a critical temperature was approached. This increase in the linewidth was much more prominent for samples that were closer to critical compositions. A theoretical model describing the relationship between the MAS peak linewidths and slow motions within the sample was developed by Suwelack et al. [29] and used to fit the linewidth and temperature data for the DOPC/DPPC-$d_{62}$/CHOL samples. This model is discussed in more detail later in this thesis since it was also used for the analysis of the MAS experiments on the specifically deuterated ternary mixtures presented here.
Chapter 2

NMR Theory

NMR spectroscopy is a versatile experimental physics technique that is used by physicists, chemists and biologists. NMR can be used to study solid, liquid, and gas phase samples, but the first two are most prevalent. In NMR spectroscopy, the response of the nuclear spins in a sample under specific conditions (controlled via the applied radio frequency (r.f.) pulses in the pulse sequence) is probed to learn about the environment of the nucleus [30]. NMR can provide valuable information about the structure of a molecule, and the phase behaviour, for example. Here the basics of how NMR works are discussed very briefly.

Nuclei possess spin which is a form of angular momentum that is not caused by a physical motion but rather is an intrinsic property of the atom. Nuclei can have integer or half-integer spin values, these values dictate the types and relative strengths of the interactions between nuclei. Some of the most commonly used NMR active nuclei are $^1\text{H}$ - spin $\frac{1}{2}$, $^2\text{H}$ - spin 1, $^{13}\text{C}$ - spin $\frac{1}{2}$, and $^{31}\text{P}$ - spin $\frac{1}{2}$. Samples are placed in an NMR spectrometer which produces a strong external magnetic field that acts on the sample. In equilibrium under the influence of the external magnetic field, the nuclei precess at a frequency given by

$$\omega_0 = -\gamma B_0$$  \hspace{1cm} (2.1)

where $\omega_0$ is called the Larmor frequency, $\gamma$ is the gyromagnetic ratio (which is specific to each isotope), and $B_0$ is the magnitude of the external magnetic field. It should be noted that $\omega$ refers to an angular frequency in units of rad/s, while $\nu$ will refer to a frequency in units of Hertz [30].
Information about the spin systems is gathered via their free induction decay (FID). The system starts in thermal equilibrium with the external field. A pulse sequence is then applied to affect the spins in a desired manner, and then the r.f. signal given off by the free precession of the nuclear spins is amplified and detected. It is this signal that is recorded as a function of time and is presented as the FID. A Fourier transform is usually performed to convert the time domain FID into a spectrum in the frequency domain which we analyze [30]. Figure 2.1 shows a simple quadrupolar echo pulse sequence which results in the FID given and the corresponding spectrum obtained via a Fourier transform of this FID. The sample depicted in Figure 2.1 contains two lipids, DOPC and DPPC-d$_{62}$ and the data was collected on a 500 MHz spectrometer which has a $^2$H frequency of 76.77 MHz. The following sections describe in more detail pulse sequences and techniques used specifically for the work presented in this thesis.

2.1 $^1$H Jeener Echo

The Jeener echo pulse sequence was designed to probe the direct relaxation of the nuclear magnetic dipolar spin energy to the lattice [31]. The principle is to convert some of the abundant Zeeman order (induced by the external field, $B_o$) into dipolar order and thus make the dipolar order detectable. Dipolar order is much weaker than the Zeeman order as it is dependent upon local fields (due to the magnetic moments of other nuclei) which tend to be on the order of $10^{-4}$ T while the external magnetic field is on the order of 10 T. Zeeman order occurs because the spins tend to orient with the external magnetic field, while dipolar order is a result of the alignment of the nuclear magnetic moments due to the presence of a field created by the neighbouring nuclear magnetic moments. The Jeener echo pulse sequence has the form $90_x-t-45_y-\tau-45_y-T$ [5]. The effect of this pulse sequence on the system can be described thermodynamically. Initially the system is in a state of thermal equilibrium, or Zeeman order which has an associated equilibrium
Figure 2.1: Static $^2$H NMR spectrum obtained via a Fourier transform of the FID from a quadrupolar echo sequence. This example shows a powder spectrum for a sample containing DOPC and DPPC-$d_{62}$. 
lattice temperature. The $90_x$-$t$-$45_y$ pulse pair acts to adiabatically transfer some of this Zeeman order into dipolar order. There is now a large amount of dipolar order, but since the entropy of the system is fixed, this requires the dipolar spin states to be at a much lower temperature than the original lattice temperature. The effect is maximized when a $45^\circ$ pulse is used [31, 32]. After this cooling has occurred, the dipolar levels are assumed to reach a common spin temperature at a rapid rate, and then relax back to the lattice temperature at a slower rate. After a variable delay, the second $45^\circ$ pulse is used to produce the signal by converting the remaining dipolar order back into Zeeman order which can be detected [31].

The pulse sequence can also be thought about in another way in order to explain the time constants and information that can be extracted from the Jeener echo experiments. The first $90^\circ$ pulse is the excitation pulse which creates the magnetization in the $\pm x$ or $\pm y$ direction depending on the phase of the pulse. The first delay, $t$, determines which couplings contribute to the dipolar order in the case of proton experiments, or to the quadrupolar order in the case of deuterium experiments. The dipolar (quadrupolar) order depends on the length of the delay, shorter delay times probe stronger couplings. The first $45^\circ$ pulse creates the dipolar order and is followed by a variable delay, $\tau$, that allows us to measure the decay of dipolar (quadrupolar) order, the time constant $T_{1D}$ ($T_{1Q}$), from a series of experiments. The second $45^\circ$ pulse converts the remaining dipolar order back to magnetization that is detected by the receiver. The Jeener echo and modified Jeener echo pulse sequences differ in the phasing of the two $45^\circ$ pulses. The Jeener echo and modified Jeener echo pulse sequences are shown in Figure 2.2.

As shown in *Deuterium Nuclear Magnetic Resonance Spectroscopy in Partially Ordered Systems* [33], these differences in the phase lead to different contributions from the quadrupolar order and double quantum coherence in the resulting signals after each pulse sequence. The contributions to the relaxation from the quadrupolar order ($T_{1Q}$) and the double quantum coherence ($T_{DQ}$) to the signal can be separated by either adding or sub-
tracting the Jeener echo and modified Jeener echo results from one another. Addition of
the Jeener echo and modified Jeener echo heights corresponds to the quadrupolar order,
while, subtraction of the modified Jeener echo height from that of the Jeener echo corre-
sponds to the double quantum order. Since the system of interest contains protons, not
deuterium, the significant order will be the dipolar order since there is no quadrupolar
coupling for nuclei with spins less than one. The discussion in the aforementioned paper
is for deuterium nuclei, but is valid here since protons are always coupled to one another
via dipolar interactions. A pair of interacting protons leads to two states, a triplet state
that behaves like deuterium and an isolated singlet state that does not affect NMR [33].
It should be noted that higher order, multiple quantum coherences are also created using
these pulse sequences but these are not discussed here.

2.2 \textsuperscript{2}H Magic Angle Spinning NMR

2.2.1 Brief Introduction to Deuterium NMR

Deuterium is a spin 1 nucleus that is often substituted for hydrogen (spin \( \frac{1}{2} \)) when study-
ing systems using NMR. Static deuterium NMR spectra are dominated by quadrupolar
interactions which are small enough that they can be treated using first order perturbation theory on the Zeeman interaction [33]. When an external magnetic field is applied to a nucleus, it breaks the energy level degeneracy via the Zeeman interaction. The number of energy levels for a given spin is equal to \(2I + 1\) where \(I\) is the spin of the nucleus. Spin states are defined according to their magnetic quantum number, \(m\). For deuterium, there are 3 energy levels, from \(-I\) to \(+I\) in integer steps, so the spin states are \(m = -1\), \(m = 0\), and \(m = +1\) [33]. These energies along with the perturbations due to the quadrupolar Hamiltonian are illustrated in Figure 2.3. The quadrupole energy shift is caused by 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 of the nucleus).

![Energy levels for a spin-1 nucleus](image)

**Figure 2.3:** Energy levels for a spin-1 nucleus arising from (a) the Zeeman Hamiltonian (where \(E_m = -m\hbar\omega_0 = m\hbar\gamma B_0\)) and (b) the shifts due to the quadrupolar interactions (as a first order perturbation to the Zeeman interaction) [33].

The Zeeman Hamiltonian has the form

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

(2.2)

where \(\mu\) is the magnetic moment, \(B_0\) is the external magnetic field, \(\gamma\) is the gyromagnetic ratio for the spin, \(\hbar\) 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

\[ E_m = -mh\omega_0 = mh\gamma B_0 \]  \hspace{1cm} (2.3)

In the presence of the first order quadrupolar Hamiltonian, the \(m = \pm 1\) levels are shifted up by

\[ \Delta = \frac{e^2 q Q}{8\hbar} \left[ (3\cos^2\beta - 1) + \eta\sin^2\beta \cos 2\alpha \right] \]  \hspace{1cm} (2.4)

while the \(m = 0\) level is shifted down by twice this amount. Notice that this energy shift is dependent upon the orientation of the electric field gradient tensor relative to the magnetic field. The angles governing this orientation dependence are the Euler angles \(\alpha\), and \(\beta\), the third Euler angle, \(\gamma\), is chosen to zero because the magnetic field is an axis of symmetry for the coordinate system. The Euler angles are defined using the convention of Rose [34] and are shown for the transformation from the (X,Y,Z) frame to the (x,y,z) frame in Figure 2.4.

![Figure 2.4: Definition of Euler angles [35].](image)

The principal value of the electric field gradient is \(eq\), and \(eQ\) is the electric quadrupole moment. The asymmetry parameter is called \(\eta\). For \(^2\)H in C-\(^2\)H bonds, \(\eta \leq 0.02\) and is often taken as zero. The quadrupolar splitting in the absence of molecular motions is

\[ \omega_Q = 6\Delta = \frac{3e^2 q Q}{4\hbar} \left[ (3\cos^2\beta - 1) + \eta\sin^2\beta \cos 2\alpha \right] \]  \hspace{1cm} (2.5)
which is dependent upon the orientation of the system with respect to the external magnetic field. In the case where \( \eta \) is set to zero, this equation becomes

\[
\omega_Q = 6\Delta = \frac{3e^2qQ}{4\hbar} \left[ 3\cos^2\beta - 1 \right]
\]  

(2.6)

In the presence of molecular motion which results in fluctuations of angle between the C-\(^2\)H bond vector and the bilayer normal, an average bond order parameter can be defined as \( \langle S_{CD} \rangle = \langle \frac{1}{2} (3\cos^2\beta - 1) \rangle \) where \( \beta \) is the angle between the C-\(^2\)H bond and the bilayer normal [33]. Equation 2.6 then takes the form

\[
\omega_Q = 6\Delta = \frac{3e^2qQ}{4\hbar} \langle S_{CD} \rangle
\]  

(2.7)

The samples studied here are partially ordered systems made up of multilamellar dispersions of the ternary phospholipid and cholesterol mixtures. 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 [33].

In previous work on ternary mixtures using static deuterium NMR, spectral broadening was observed near the proposed critical point [1, 2]. As the degree of order within a sample increases, so too does the width of the quadrupolar splitting for each nucleus, thus spectra from samples in fluid phases are much narrower than 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. \(^2\)H MAS NMR was used for the current experiments.
2.2.2 Magic Angle Spinning NMR

MAS is a widely used solid-state NMR technique. Experimentally, one spins the sample in a rotor oriented at an angle of 54.7° (the so-called magic angle) to the magnetic field of the spectrometer, $\vec{B}_0$ [36, 37]. This configuration is shown in Figure 2.5. MAS is highly popular because of its ability to simplify otherwise extremely complex spectra. The effect of MAS is to essentially average out the anisotropic contributions to the spectra. The ternary mixtures used for these experiments are partially deuterated. As a result, there will be very small $^2\text{H}^-\text{H}$ dipolar interactions, however, these are eliminated by MAS. The following is an overview of how MAS modulates the quadrupolar interactions that dominate static deuterium spectra.

The quadrupolar Hamiltonian in the principle axis system (PAS) of the electric field gradient tensor is

$$\mathcal{H}_Q = \frac{e^2 qQ}{4I(2I-1)} \left[ (3I_z^2 - I^2) + \eta \left( I_x^2 - I_y^2 \right) \right]$$

[38]. Here, $I$ is the spin of the nucleus, $e q$ is the principal value of the electric field gradient, $eQ$ is the electric quadrupole moment and $I_x, I_y, \text{and } I_z$ 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 shown in the aforementioned paper. 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. Figure 2.6 shows the axis systems for each of the steps in the transformation from the PAS of the
The electric field gradient tensor to the laboratory frame.

\[ F_{P,0}^2 = \sqrt{\frac{3}{2}} \epsilon q \]
\[ F_{P,\pm 1}^2 = 0 \]  \hspace{1cm} (2.9)
\[ F_{P,\pm 2}^2 = \frac{\eta}{2} \epsilon q \]  \hspace{1cm} (2.10)

Since the C-H bond is nearly axially symmetric, the asymmetry parameter, \( \eta \), is nearly zero, so the \( F_{P,\pm 2}^2 \) term is usually ignored. In addition, the second rank spherical tensors for the spin variables are given as

\[ T_{2,0} = \frac{1}{\sqrt{6}} \left[ 3I_z^2 - I(I + 1) \right] \]  \hspace{1cm} (2.12)
\[ T_{2,\pm 1} = \mp \frac{1}{2} [I_z I_{\pm} + I_{\pm} I_z] \]  \hspace{1cm} (2.13)
\[ T_{2,\pm 2} = \frac{1}{2} I_{\pm}^2 \]  \hspace{1cm} (2.14)

The quadrupolar Hamiltonian is the product of these electric field gradient tensor components, and the second rank spherical tensors as shown below

\[ \mathcal{H}_Q = \frac{eQ}{2} \sum_{m'''=-2}^{2} (-1)^{m'''} T_{2,m'''} F_{2,-m''' \rightarrow m''} \]  \hspace{1cm} (2.15)
The first transformation takes the electric field gradient tensor from its PAS to the appropriate axis of symmetry for the system. In this case, the bilayer normal is an axis of symmetry for the molecular motion. It should be noted that the electric field gradient tensor components can be transformed in the same way as spherical harmonics.

\[
F_{2,m'}^B = \sum_{m=-2}^{2} F_{2,m}^P D_{m,m'}^{(2)} (\alpha, \beta, \gamma) \tag{2.16}
\]

In the above equation, \(D_{m,m'}^{(2)}\) is the Wigner rotation matrix which is defined as

\[
D_{m,m'}^{(2)} = e^{-i\alpha m} d_{m,m'}^{(2)}(\beta) e^{-i\gamma m'} \tag{2.17}
\]

and \(\alpha, \beta,\) and \(\gamma\) are the Euler angles for the given transformation [39]. The only non-zero component of \(F_{2,m}^P\) is for \(m = 0\). In addition, if \(\beta\) is defined as the angle between the C-2H bond in a lipid and the bilayer normal, then both \(\alpha\) and \(\gamma\) can be taken to be zero. Now,

\[
F_{2,m'}^B = F_{2,0}^P D_{0,m'}^{(2)} (0, \beta, 0) \tag{2.18}
\]

is the electric field gradient tensor in the bilayer normal frame. The next transformation takes the electric field gradient from the bilayer normal frame to the rotor frame for MAS.

\[
F_{2,m''}^R = \sum_{m'=-2}^{2} F_{2,m'}^B D_{m',m''}^{(2)} (\alpha', \beta', \gamma') \tag{2.19}
\]

Since the bilayer normal is an axis of symmetry for the motion in the system and the whole sample is evenly distributed about the long axis of the rotor, the angle \(\alpha'\) can be chosen to equal zero.

\[
F_{2,m''}^R = \sum_{m'=-2}^{2} F_{2,m'}^B D_{m',m''}^{(2)} (0, \beta', \gamma') \tag{2.20}
\]

The final transformation is from the rotor frame to the laboratory frame.

\[
F_{2,m''}^L = \sum_{m'=m''=-2}^{2} F_{2,m''}^R D_{m'',m'''}^{(2)} (\alpha'', \beta'', \gamma'') \tag{2.21}
\]

Again, based on the axially symmetry of the frame, this time 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, so $\gamma''$ is set equal to zero. In addition, since the rotor frame is at a fixed angle to the laboratory frame, rename $\alpha'' = \phi_{\text{magic}}$, and $\beta'' = \theta_{\text{magic}}$.

$$F_{L,2,0}^{P} = 2 \sum_{m''=-2}^{2} F_{2,0}^{P} D_{0,m''}^{(2)} (0,0,0) D_{m',m''}^{(2)} (0,\beta',\gamma') D_{m'',m''}^{(2)} (\phi_{\text{magic}},\theta_{\text{magic}},0)$$

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

$$F_{L,2,0}^{P} = 2 \sum_{m''=-2}^{2} \sum_{m'=-2}^{2} F_{2,0}^{P} D_{0,m''}^{(2)} (0,0,0) D_{m',m''}^{(2)} (0,\beta',\gamma') D_{m'',0}^{(2)} (\phi_{\text{magic}},\theta_{\text{magic}},0)$$

In this situation, $\phi_{\text{magic}}$ is dependent upon the spin rate and time, and can be defined as $\phi_{\text{magic}} = -\text{const.} \cdot \omega_r t$ and $\theta_{\text{magic}} = 54.7^\circ$. Only one of the components of the second rank spherical tensors for the spin variables, $T_{2,0}$, commutes with the Zeeman Hamiltonian, so in first order only $F_{2,0}^{P}$ will contribute to the quadrupolar Hamiltonian [33].

$$F_{2,0}^{L} = \sum_{m''=-2}^{2} \sum_{m'=-2}^{2} F_{2,0}^{P} D_{0,m''}^{(2)} (0,0,0) D_{m',m''}^{(2)} (0,\beta',\gamma') D_{m'',0}^{(2)} (\phi_{\text{magic}},\theta_{\text{magic}},0)$$

Using the definition of the Wigner rotation matrices found in Equation 2.17, gives

$$F_{2,0}^{L} = \sum_{m''=-2}^{2} \sum_{m'=-2}^{2} F_{2,0}^{P} d_{0,m''}^{(2)} (\beta) d_{m',m''}^{(2)} (\beta') e^{-im''\gamma'} e^{im''\text{const.} \cdot \omega_r t} d_{m'',0}^{(2)} (\theta_{\text{magic}})$$

Terms that depend on $d_{0,0}^{(2)} (\theta_{\text{magic}})$ will be eliminated due to magic angle spinning since $d_{0,0}^{(2)} (\theta_{\text{magic}}) = \frac{1}{2} (3 \cos^2 \theta_{\text{magic}} - 1) = 0$ [39]. The Hamiltonian will also depend upon the Euler angles from each transformation. The time dependence comes in through $e^{im''\text{const.} \cdot \omega_r t}$.

Under MAS conditions, the signal following an initial 90° excitation pulse decays quickly, within a rotor period, as a result of the interference of different spin packets at different orientations specified by $\alpha'$ and $\beta'$. These spin packets however each go through a set pattern of changing Larmor frequencies which causes them to constructively interfere at a time corresponding to each full rotation of the sample about the magic angle. This periodic constructive interference results in the formation of a train of rotational spin echoes as can be seen in Figure 2.7.
Figure 2.7: Sample $^2$H MAS signal from a Hahn echo pulse sequence.
The Fourier transform of an FID such as the one shown is a series of peaks spaced at intervals of the spin rate about a central isotropic peak [40, 29]. Figure 2.8 shows simulated spectra displaying the effect of MAS at different spin rates from 1 kHz to 18 kHz compared to a static spectrum containing a single deuterium nucleus.

Figure 2.8: Simulated spectra depicting static and MAS $^2$H NMR for a single deuterium nucleus. Note that 100 Hz linebroadening was used in all cases.

The Hahn echo pulse sequence used in the present experiments is described in the next section.
2.2.3 The Hahn Echo

The Hahn echo is a simple two-pulse sequence consisting of a $90^\circ$ pulse and a $180^\circ$ pulse that may be $90^\circ$ out of phase from one another. This sequence is illustrated in Figure 2.9. The $90^\circ$ pulse creates the magnetization in the x-y plane, a delay, $\tau$, allows the magnetization to evolve, the $180^\circ$ pulse then inverts the magnetization and after another time, $\tau$, an echo is formed. The theory is discussed in detail in *Deuterium Nuclear Magnetic Resonance Spectroscopy in Partially Ordered Systems* [33]. When using the Hahn echo pulse sequence under MAS conditions, the length of the delay should be rotor-synchronized to avoid destructive interference in the formation of the rotary echoes [41]. A characteristic spectrum for a sample spinning at 3 kHz is shown in Figure 2.10.

In the case of static $^2$H NMR, a quadrupolar echo pulse sequence is used. The quadrupolar echo pulse sequence has the form, $90_x-\tau-90_y-\tau$. This sequence results in full echo formation after a time of $2\tau$ from the first pulse. For the $^2$H MAS experiments described here however, the Hahn echo pulse sequence with the form $90_x-\tau-180_y-\tau$ is used. A simple argument for why the Hahn echo is preferable over the quadrupolar echo is presented here. In both pulse sequences, the purpose of the first $90_x$ pulse is to transform the original magnetization, which is aligned with the external field, into magnetization in the y-direction. This magnetization then evolves for a time, $\tau$, before the second pulse is applied. In the case of MAS, the sample returns to its initial state (neglecting relaxation) after every full rotation of the rotor. This is to say, following each full rotor period there has been no evolution due to $\mathcal{H}_Q$. This results in the characteristic
Figure 2.10: Example spectrum from a $^2$H MAS sample rotating at 3 kHz.

Train of rotational echoes seen for MAS samples [40]. If the second pulse is applied at an interval of the rotor period from the first pulse, then the signal will be maximized, and a full echo can be formed. If a 90° pulse is used as the second pulse, as is the case for the quadrupolar echo, then a full echo is not formed since any of the magnetization that had spread out over time in the x-y plane, is now in the y-z plane, and there is nothing to refocus via a 90° pulse. On the other hand, if a 180° pulse is used, then the magnetization in the x-y plane is inverted and after another time, $\tau$, a full echo will be formed. This explains why the signal is reduced if a quadrupolar echo pulse sequence is used under MAS instead of a Hahn echo.
Chapter 3

Lipids/Cholesterol Used For These Studies

The following diagrams show the lipids used to make the ternary mixtures for the $^1$H Jeener Echo and $^2$H MAS NMR experiments.

Figure 3.1: Structure of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC).

Figure 3.2: Structure of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

Figure 3.3: Structure of $\alpha$-d$_4$-1,2-dipalmitoyl-sn-glycero-3-phosphocholine ($\alpha$-d$_4$-DPPC).

DOPC is an unsaturated lipid and has two 18-carbon acyl chain tails with one double bond at the 9-10 carbon position in each chain. DPPC is a saturated lipid with two
Figure 3.4: Structure of cholesterol.

16-carbon acyl chain tails. DOPC and DPPC were obtained from Avanti Polar Lipids (Alabaster, AL). α-d₄-DPPC was synthesized previously in the lab using the method developed by Gupta et al. [42], and cholesterol was obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of each lipid and cholesterol were made by weighing out the dry lipid or cholesterol and adding a proportionate amount of freshly distilled ethanol to achieve a concentration of 2.5 mg/mL for each solution. These stock solutions were then used to prepare the various sample compositions as discussed in the following chapters.
Chapter 4

Jeener Echo Experiments

4.1 Introduction/Motivation

One of the undertakings of this project was to find a solid-state static NMR technique that could effectively probe relatively slow motions such as critical fluctuations. A potential way to do this would be to use dipolar order, which is relaxed by fluctuations in the local fields of the nuclei (typically a few kHz in strength) and described by the associated time constant $T_{1D}$. NMR detection of dipolar order, which is ordinarily much smaller than the Zeeman order, is possible using the Jeener echo pulse sequence [5]. In ternary lipid/cholesterol systems the proposed critical fluctuations are slow motions and in turn change the rate of the relaxation of dipolar order. This technique should be sensitive to relatively slow motions (the motions in question are on the order of 0.3 ms) which makes it a good candidate for investigating the existence of fluctuations near a critical point. Critical fluctuations arising from variations of the local cholesterol concentration will result in changes to the local field. As the critical point is approached, substantial changes in the value of $T_{1D}$ are expected. A ternary mixture of composition 35:35:30 (DOPC/DPPC/CHOL) was investigated using the Jeener echo pulse sequence to see if there were any differences in the results of these experiments that were comparable with the line-broadening observed in the $^2$H NMR static and MAS spectra [1, 2, 6]. In this chapter the Jeener echo pulse sequence is introduced, the sample preparation and experimental setup are described, and the results of these experiments are presented.
4.2 Materials and Methods

4.2.1 Sample Preparation Method for $^1$H Jeener Echo Experiments

The sample preparation method described below is similar to the one found in Vist and Davis [14] and Davis et al. [1]. The samples were made at a molar ratio of 35:35:30 (DOPC/DPPC/CHOL). Appropriate amounts of each 2.5 mg/mL stock solution were measured and mixed in a 50 mL round-bottomed flask. Samples were made with 10 mg (dry weight) of DPPC and corresponding amounts of DOPC and CHOL. Once the three components were mixed, the solvent was removed using rotary evaporation. The flask was then placed under vacuum for several hours or overnight in order to ensure that any residual solvent was removed. The dry sample was then carefully scraped from the round-bottomed flask walls and transferred into a snap-cap vial. Samples were then hydrated with $^2$H 50 mM phosphate buffer, at a total dry lipid weight to buffer volume ratio of 4:3. The $^2$H 50 mM phosphate buffer was made by lyophilizing 10 mL of 50 mM phosphate buffer (obtained from Fisher Scientific, Whitby, ON), and then rehydrating the salts first using 6 mL of 99.99% D$_2$O, $^2$H 7.4, (obtained from Cambridge Isotope Laboratories Inc, Andover, MA), then freeze-drying again before finally rehydrating the buffer using 10 mL of the same D$_2$O. The sample was gently centrifuged and then mixed by hand using a small glass stir-rod. The centrifuging and mixing process was repeated several times until the sample appeared uniform in colour and texture. The end of the snap-cap vial was cut off and the sample was spun down into a glass Pasteur pipette with one end sealed. Samples were typically 2.5 to 3 cm long with a tapered diameter ranging from 1.8 to 2 mm. A rubber pipette bulb was placed on the end to prevent evaporation and then the sample was sealed using a small torch. The mass of the sample in the holder was recorded both before and after running experiments to ensure that no water was lost during the experimental process.
4.2.2 NMR Setup

Two variations of the three-pulse sequence developed by Jeener and Broekaert were used for these experiments [5]. The Jeener echo pulse sequence has the form $90_x - t - 45_y - \tau - 45_y - T$, and the modified Jeener echo pulse sequence has the form, $90_y - t - 45_x - \tau - 45_y - T$. The first $90^\circ$ pulse is the excitation pulse which puts the magnetization from the z-direction (aligned with the magnetic field of the spectrometer) into the x-y plane. The first delay, $t$, is set to maximize the dipolar order in this case. The first $45^\circ$ pulse creates the dipolar order and is followed by a variable delay, $\tau$, that allows for the measurement of the decay of dipolar order, the time constant $T_{1D}$, from a series of experiments. The second $45^\circ$ pulse converts the remaining dipolar order back to magnetization that is detected by the receiver. Phase cycling was used to eliminate artifacts in the data due to after-pulse effects and asymmetries in the receiver [43]. Significant artifacts were observed in the signal when phase cycling was not employed for these experiments. The phase cycling used for each the Jeener echo and modified Jeener echo pulse sequences are outlined in Table 4.1 and Table 4.2.

The experimental procedure was optimized by using different variations of the pulse sequence to get the appropriate information from the data and the sequence was tested by looking at $(2,2^{2H_2})$-1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) and comparing with known $^2H$ results for the Jeener echo pulse sequence found in Davis [33] to confirm that the pulse sequence behaved as expected. Example results are discussed briefly later in this chapter.

All NMR experiments were performed on a 500 MHz wide-bore Bruker Avance II spectrometer (Bruker BioSpin, Milton, ON) using a static probe with a homemade coil. The software used for collecting and visualizing the data at the spectrometer was Bruker TopSpin 2.1 (Bruker BioSpin, Karlsruhe, Germany). Pseudo 2D $^1H$ experiments were set up with a $90^\circ$ pulse length of 1.75 $\mu$s with $t = 12$ $\mu$s and the variable delay, $\tau$, ranging from 0.01 ms to 3000 ms. Typically 32 scans were run at each of the 30 variable delay
Table 4.1: Phase cycling for the Jeener echo pulse sequence

<table>
<thead>
<tr>
<th>Pulse 1 (90°)</th>
<th>Pulse 2 (45°)</th>
<th>Pulse 3 (45°)</th>
<th>Receiver</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>-Y</td>
<td>-Y</td>
<td>Y</td>
</tr>
<tr>
<td>X</td>
<td>-Y</td>
<td>Y</td>
<td>-Y</td>
</tr>
<tr>
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Table 4.2: Phase cycling for the modified Jeener echo pulse sequence

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times. The time-domain data for each slice of the pseudo-2D acquisition contained 8192 points. The echo heights were analyzed in the time-domain and the procedure for this is
described in the following section.

Experimental Considerations

The delay between the 90° pulse and the first 45° pulse, t, dictates which of the couplings the experiment is sensitive to. Relaxation is generally faster for spins that are strongly coupled to other spins. The spins in the more ordered states are more strongly coupled and shorter delay times are required to probe their order. By varying the length of t, different hydrogen atoms can be probed based on their coupling strength. A series of experiments in which t was set at values between 6 µs and 240 µs was run. A delay of 12 µs provided a good amount of signal intensity while maintaining a relatively short delay.

Echo Height Measurements

The pseudo 2D experimental data was split into 1D slices for each variable delay time using Bruker TopSpin 2.1 (Bruker BioSpin, Karlsruhe, Germany) and converted into a text file which could be read into Origin 8.5 (OriginLab, Northampton, MA) for analysis. An example is shown in Figure 4.1.

The maximum value was found using the Column Statistics function in Origin 8.5 (OriginLab, Northampton, MA) and ten values on either side of this point were averaged with the maximum to get a reasonable estimate of the height since there is some noise in the results. The standard deviation of these values was calculated and used as a measurement of the error in the echo height. Plots of Jeener echo amplitude as a function of variable delay time were produced at eight temperatures ranging from 316.8 K to 299.6 K. Note that experiments were always run from the highest temperature to the lowest temperature.
The ternary mixture of molar composition 35:35:30 (DOPC/DPPC/CHOL) was chosen as the starting point for these experiments because of the significant broadening effects observed in both the $^2\text{H}$ static, and $^2\text{H}$ MAS spectra [1, 6]. Two phase cycling schemes were used for the pulse sequence: the Jeener echo which has pulses $90_x$-$t$-$45_y$-$\tau$-$45_y$-$T$ and the modified Jeener echo which has pulses $90_y$-$t$-$45_x$-$\tau$-$45_y$-$T$. In the Jeener echo sequence, the two $45^\circ$ pulses have the same phase whereas in the modified Jeener echo sequence the two $45^\circ$ pulses are $90^\circ$ out of phase with one another. The Jeener echo and
modified Jeener echo sequences produced significantly different results in the (2,2-$^2$H$_2$)-DLPC experiments as shown in Figure 4.2.

Figure 4.2: Amplitude of the Jeener echo and modified Jeener echo as a function of variable delay time for $^2$H NMR on (2,2-$^2$H$_2$)-DLPC.

These results follow the expected trends for the signal after the Jeener echo and modified Jeener echo pulse sequences [33]. These results can be either added together or subtracted from one another to separate the contributions to the signal from the quadrupolar order ($T_{1Q}$) shown with open circles, and the double quantum coherence ($T_{DQ}$) shown with closed squares in Figure 4.3.
Figure 4.3: Added and subtracted results of the Jeener echo and modified Jeener echo sequences on $(2,2\text{-}^2\text{H}_2)$-DLPC, corresponding to the double quantum coherence and quadrupolar order respectively.

Both pulse sequences were employed again for the $^1\text{H}$ experiments on 35:35:30 (DOPC/DPPC/CHOL) and it was found that the difference between the Jeener echo and modified Jeener echo results were quite minimal. An example is shown in Figure 4.4 at 316.8 K.
Figure 4.4: Amplitudes of the Jeener echo and modified Jeener echo for 35:35:30 (DOPC/DPPC/CHOL) at 316.8 K, NS = 32.

Since the Jeener echo and modified Jeener echo pulse sequences gave nearly identical results, only a summary of the temperature dependence of the Jeener echo sequence is presented. Figure 4.5 shows the Jeener echo data for each variable delay time at 299.6 K.
Figure 4.5: Jeener echo data for varying delay times, $\tau$, at $T = 299.6$ K. (NS = 32).

Note that for delay times longer than about 50 ms, the initial, sharp component of the signal is barely (if at all) distinguishable from the broad peak. The points used to calculate the height of the sharp peak after 50 ms were all taken for the same acquisition time range. The graph in Figure 4.6 gives the Jeener echo height as a function of the variable delay time at temperatures from 316.8 K to 299.6 K.
There was no substantial change in the $T_{1D}$ observed across the range of temperatures from 316.8 K to 299.6 K. This range includes the critical point where substantial line broadening was observed in the $^2$H MAS experiments on the same molar composition of DOPC/DPPC-d$_{62}$/CHOL. Note that DPPC-d$_{62}$ has a chain melting temperature of 311 K [1] which is lower than that of pure DPPC which has a chain melting temperature of 314 K (Avanti Polar Lipids, Alabaster, AL). Thus, the two phase coexistence region of the mixture should be shifted up a little bit in temperature from where the most line broadening in the corresponding $^2$H MAS experiments was observed for samples with DPPC-d$_{62}$. Initially, the plan was to complete a series of $T_{1D}$ measurements for several
sample compositions both near and far from the line of critical points on the ternary
phase diagram [1]. Since nothing was observed in this sample which should be close to
the critical line, the Jeener echo experiments were discontinued. It is possible that since
the dipolar coupling between $^1$H atoms is strong, the whole system may relax back to the
lattice as one unit resulting in a single, average decay time for the dipolar order at all
temperatures. Only some of the protons (specifically those that are located on the chains
of DPPC) will be sensitive to the fluctuations in the cholesterol concentration, while most
of the other protons will be insensitive to these changes. As a result, the average $T_{1D}$
that is observed is not influenced by the cholesterol concentration fluctuations. One could
attempt to eliminate as many of these dipolar couplings as possible through deuteration,
but this would be very difficult, and probably not very effective.

Conclusions

The Jeener echo pulse sequence appeared to be a likely candidate for a static solid-
state NMR technique to observe relatively slow motions such as critical fluctuations
based on the expected effects on the local field and the time-scale of these interactions.
Experiments were conducted on a sample of molar ratio 35:35:30 (DOPC/DPPC/CHOL).
The phase behaviour of this composition had previously been investigated using static
and $^2$H MAS NMR where the DPPC was chain perdeuterated with 62 deuterons. These
experiments showed both static and MAS line broadening at the transition temperature
from the $l_d$ to the two-phase ($l_d$ and $l_o$) region. No effect due to critical fluctuations
was observed using the current experiment and this method was abandoned in favour of
using $^2$H MAS NMR to investigate the linewidths of specifically deuterated DPPC in the
ternary mixture samples.
Chapter 5

$^2$H MAS Experiments

5.1 Introduction/Motivation

Previously, Davis et al. studied a series of ternary DOPC/DPPC-d$_{62}$/CHOL samples with various compositions using $^2$H MAS NMR. All 62 deuterium atoms on the acyl chains of DPPC contribute to the spectral linewidths, including the two methyl groups which give rise to additional slightly shifted components for sidebands below $\sim$10 kHz. To eliminate the methyl contributions, the 15 kHz sideband at a spin rate of 3 kHz was chosen for these studies [6]. As an extension to this work, the experiments presented here were performed using specifically deuterated DPPC ($\alpha$-d$_4$-DPPC) in order to simplify the contributions to the spectra and to eliminate complications from the methyl groups. In this case, rather than having the spectra made of components coming from all 62 chain deuteron positions, including the two methyl groups which behave much differently than the methylene groups that make up the rest of the chain, there are only contributions from the four deuterons closest to the head group of the DPPC molecule. In addition, two of the four labelled positions are equivalent, so there are only three unique contributions to the spectra. An example of a static $^2$H spectrum and a corresponding $^2$H MAS spectrum for one of these ternary mixtures is shown in Figure 5.1.

Initially, a molar composition of 35:35:30 (DOPC/$\alpha$-d$_4$-DPPC/CHOL) was chosen based on the large broadening effect seen in the $^2$H MAS experiments on the chain perdeuterated DPPC sample of the same composition. Four other sample compositions were chosen with varying ratios of DOPC/$\alpha$-d$_4$-DPPC with 30 mol% CHOL in each
Figure 5.1: Comparison of $^2$H static and MAS spectra of 35:35:30 (DOPC/$\alpha$-d$_4$-DPPC/CHOL). (a) Static spectrum at 310.6 K, NS = 8192, (b) MAS spectrum at 311.2 K, $\nu_r = 3$ kHz, NS = 16384.

In the following sections, the sample preparation and experimental methods used to study these samples are described, and the results of this experiments are discussed.

## 5.2 Materials and Methods

### 5.2.1 Sample Preparation Method for $^2$H MAS Experiments

The same sample preparation method is discussed in Vist and Davis [14], and Davis et al. [1]. Ternary mixtures of DOPC, $\alpha$-d$_4$-DPPC, and cholesterol were prepared in the following manner for $^2$H MAS NMR experiments. The 2.5 mg/mL stock solutions of each lipid and cholesterol in ethanol were used to prepare the various sample compositions. Appropriate volumes of each stock solution were measured and mixed in a 50 mL round-
bottomed flask. Samples were made so that they either had \( \sim 25 \) mg (dry weight) of \( \alpha\text{-d-4-DPPC} \), or a total lipid and cholesterol dry weight of \( \sim 50\text{-}60 \) mg in order to ensure enough signal from the \( \alpha\text{-d-4-DPPC} \) and that the sample fit into a 4 mm rotor. Once the three components were mixed, the solvent was removed using rotary evaporation. The flask was then placed under vacuum for several hours or overnight in order to ensure that the sample was completely dry and any remnants of solvent were removed. The dry sample was then carefully scraped off the wall of the round-bottomed flask and transferred into a snap-cap vial. Samples were hydrated with 50 mM potassium phosphate buffer, pH 7.0 (obtained from Fisher Scientific, Whitby, ON), at a total dry lipid weight to buffer volume ratio of 4:3. Centrifugation and gentle mixing using a small glass stir-rod was repeated several times until the sample appeared uniform in colour and texture. Finally, the end of the snap-cap vial was cut, the sample was spun into the 4 mm rotor using the centrifuge, and the cap was then placed on the rotor to seal the sample. The mass of the sample in the rotor was recorded both before and after running experiments to ensure that no water was lost during the experimental process. Samples for this series of experiments had the following molar ratios of DOPC/\( \alpha\text{-d-4-DPPC}/\text{CHOL} \): (21:49:30), (28:42:30), (35:35:30), (42:28:30), and (49:21:30).

5.2.2 \( ^2\text{H} \) MAS Experiments

All NMR experiments were performed on a 500 MHz wide-bore Bruker Avance II spectrometer (Bruker BioSpin, Milton, ON) at a \( ^2\text{H} \) NMR frequency of 76.77 MHz. The pulse sequence used was the Hahn echo, which is comprised of two pulses with a set delay between them. First, a 90° pulse is applied to flip the magnetization into the \( x-y \) plane where it is then allowed to evolve for a time, \( \tau \). After this delay, a 180° pulse is applied to invert the magnetization. Since this is a MAS experiment, the length of \( \tau \) is such that the 180° pulse is synchronized with a rotor period in order to get a full echo and avoid distortions. The second delay time is just slightly shorter than \( \tau \) so that the
data collection starts before the top of the echo is reached. By collecting just before the echo maximum is reached, the echo maximum can easily be identified and there is no question as to whether the echo has been missed. It is then possible to discard the points before the top of the echo to ensure that the maximum signal intensity and a symmetric spectrum are obtained [43].

Pulse lengths were optimized and kept as short as possible to minimize spectral distortions due to the pulse length [43]. For the experiments here, the 90° pulse length was 2.4 µs. Depending on the sample size, either 8192 or 16384 scans were collected at each temperature. CYCLOPS phase cycling was used to get rid of artifacts that can arise due to asymmetries in the detection [43, 44]. Samples were equilibrated overnight by spinning at 3 kHz at a temperature of 312.3 K. Two to five degree set-point temperature steps were used on the first two days of experiments, and a full set of temperatures were run over the course of one to two days (continuously) for the third run [6]. Temperature scans were completed from high temperature to low temperature over a maximum range from 318 K to 283 K. The FID was Fourier transformed using Bruker TopSpin 2.1 (Bruker BioSpin, Karlsruhe, Germany) with 5 Hz of line-broadening added in the process. The spectral analysis used for these studies is described in the following section.

5.2.3 Experimental Considerations

The effect of spin rate on the results was studied by looking at the 18 kHz sideband of a sample of composition 37:37:26 (DOPC/DPPC-d$_{62}$/CHOL) at spinning frequencies of 2, 3, 4.5, 6, and 9 kHz. A summary of these results is presented in this thesis as well. For the other experiments, a spin rate of 3 kHz was chosen, this rate ensures stable spinning while reducing the effects of temperature gradients and heating across the sample due to friction. In previous MAS experiments on ternary mixtures with chain-perdeuterated DPPC, it was found that the linewidths were not consistent between runs until after the second time through the temperatures. It was also noted that when the sample was left
overnight at a high temperature (where the sample was in the disordered fluid phase) the linewidths decreased significantly over a period of about 8 to 12 hours. It is believed that these effects are due to the excess water in the sample requiring time to equilibrate in the spinning system [6].

5.2.4 Linewidth Measurements

The first spectrum for each sample was phased in TopSpin 2.1 (Bruker BioSpin, Karlsruhe, Germany) and the conditions for the zeroth order and first order phase corrections were recorded and used for all subsequent spectra. The exact zoom function was used to select the spectral range from 7.5 kHz to 19.5 kHz (half the distance between sidebands past the furthest peaks of interest). Both the real and imaginary parts of the spectrum were saved in a text file. The real and imaginary parts of the spectra were separated into columns when imported into Origin 8.5 (OriginLab, Northampton, MA) for graphing and fitting. The frequencies of each point were calculated based on the spectral resolution given in TopSpin 2.1 (Bruker BioSpin, Karlsruhe, Germany). In this case, the spectral resolution was 1.525879 Hz and 7865 points were used to make the graphs.

Sidebands were chosen for the fits rather than the central peak because they are close to Lorentzian in shape and do not have the added complications due to water that the central peak does. Sideband linewidths were measured by fitting both the real and the imaginary components of the data simultaneously to a Lorentzian line shape [38]. Four sidebands, at 9, 12, 15, and 18 kHz, were fit at one time to ensure that contributions to the line shape due to overlap, especially for broad peaks, were taken into account. The equations used to fit the real and imaginary components of the four peaks are shown below.
\[ y_r = \left(1 - \frac{\phi^2}{2}\right) \cdot [A_1 + B_1 \cdot x + \frac{\alpha_0}{(1 + (x - x_0)^2 \cdot d_0^2)} + \frac{\alpha_1}{(1 + (x - x_1)^2 \cdot d_1^2)} + \frac{\alpha_2}{(1 + (x - x_2)^2 \cdot d_2^2)} + \frac{\alpha_3}{(1 + (x - x_3)^2 \cdot d_3^2)}] \]

\[ y_i = \phi \cdot [A_1 + B_1 \cdot x + \frac{\alpha_0}{(1 + (x - x_0)^2 \cdot d_0^2)} + \frac{\alpha_1}{(1 + (x - x_1)^2 \cdot d_1^2)} + \frac{\alpha_2}{(1 + (x - x_2)^2 \cdot d_2^2)} + \frac{\alpha_3}{(1 + (x - x_3)^2 \cdot d_3^2)}] - \frac{\phi^2}{2} \cdot [A_2 + B_2 \cdot x + \alpha_0 \cdot (x - x_0) \cdot \frac{d_0}{(1 + (x - x_0)^2 \cdot d_0^2)} + \alpha_1 \cdot (x - x_1) \cdot \frac{d_1}{(1 + (x - x_1)^2 \cdot d_1^2)} + \alpha_2 \cdot (x - x_2) \cdot \frac{d_2}{(1 + (x - x_2)^2 \cdot d_2^2)} + \alpha_3 \cdot (x - x_3) \cdot \frac{d_3}{(1 + (x - x_3)^2 \cdot d_3^2)}] \]

The four parameters \( A_1, B_1, A_2, \) and \( B_2 \) define the baseline. There is a small term, \( \phi \), which is a phase correction which has a magnitude which was always less than \(| \phi | \leq 0.15 \) radians and most often less than \(| \phi | \leq 0.10 \). Instead of using full sine and cosine terms in the linewidth expressions, a small angle approximation was used. The small values of \( \phi \) prove the validity of using the small angle approximation here. The amplitudes of the peaks are defined by \( \alpha_0, \alpha_1, \alpha_2, \) and \( \alpha_3 \) and their linewidths are related to \( d_0, d_1, d_2, \) and \( d_3 \). All of the previously mentioned parameters were varied in turn for each spectrum. The frequencies of the sidebands were found by fitting \( x_0, x_1, x_2, \) and \( x_3 \) for the highest temperature spectrum for each composition. These frequencies were then held constant for the rest of the fits for that sample. This was done because the peaks are sharpest at high temperature and thus the frequencies are best defined at the highest temperature of the run. A weighting scheme was applied to the raw data such that the portions of the spectra from 10.5 kHz to 16.5 kHz were four times more heavily weighted than those
from 7.5 kHz to 10.5 kHz, and 16.5 kHz to 19.5 kHz. As a result, the peaks at 12 kHz and 15 kHz were forced to fit more accurately than the peaks at 9 kHz and 18 kHz. This weighting was especially important for ensuring that the baseline fit in the region of interest was as accurate as possible. A detailed description of the steps used in this fitting process can be found in Appendix A of this thesis.

Figure 5.2 and Figure 5.3 show examples of the real and imaginary parts of the spectrum for 28:42:30 (DOPC/α-d$_4$-DPPC/CHOL) at 306.3 K, and 296.3 K with the regression fits to the data. As the linewidths get broader, the noise becomes more significant and the linewidth fits become less accurate.

Figure 5.2: Real and imaginary spectral components for spinning sidebands from 9 kHz to 18 kHz for 28:42:30 (DOPC/α-d$_4$-DPPC/CHOL) at 306.3 K with fits, \( NS = 16384 \).
The linewidths for the 12 kHz and 15 kHz sidebands were calculated from the values of \( d_1 \) and \( d_2 \), respectively, with \( \Delta \nu_{12kHz} = \frac{2}{d_1} \) and \( \Delta \nu_{15kHz} = \frac{2}{d_2} \). When the other side of the spectrum was fit in the same manner, the resulting linewidths were the same within a couple of percent. The error in the linewidth measurements was estimated as \( \delta \nu = 2.5 \text{ Hz} + 0.025 \cdot \Delta \nu \). This was chosen because 5 Hz of line broadening was added in *TopSpin 2.1* (Bruker BioSpin, Karlsruhe, Germany), typically the sharpest peaks were on the order of 100 Hz in width, and the uncertainty in the linewidth becomes larger as the peaks get broader.
5.2.5 Theoretical Model for Finding the Critical Temperature

The portion of the linewidth versus temperature data that is well above the critical temperature was fit using an equation developed by Suwelack et al. [29]. The following provides the reasons for choosing this model, as well as a discussion on the methods of data analysis used for this study.

Model from Suwelack et al.

The spinning sidebands observed for ternary lipid/cholesterol mixtures have very close to Lorentzian line shapes, as a result the linewidth can be related to the transverse relaxation time, $T_2$, in the following manner

$$
\Delta \nu = \frac{\Delta \omega}{2\pi} = \left( \frac{1}{2\pi} \right) \left( \frac{2}{T_2} \right) = \frac{1}{\pi \cdot T_2}
$$

(5.3)

[38]. In addition, following Abragam’s discussion of motional narrowing in solids, the linewidth can be related to $\Delta M_2$ and the correlation time, $\tau_c$. $\Delta M_2$ describes the degree of fluctuations of the mean-squared strength of the orientation dependent interactions, which can include the quadrupolar interaction, chemical shift anisotropy, and dipole-dipole interactions [45]. This relationship is shown below

$$
\Delta \nu = \frac{\Delta \omega}{2\pi} = \frac{1}{\pi} \Delta M_2 \tau_c
$$

(5.4)

and gives the linewidth expected for a non-spinning sample ($\nu_s=0$) [38].

Suwelack et al. present a model for fitting the linewidths due to slow motions in their paper called *Slow molecular motion detected in the NMR spectra of rotating solids* [29]. The derivation is based on the chemical shift Hamiltonian, but will also hold true for other anisotropic interactions including the quadrupolar coupling. The equation is based upon solving the equation of motion describing the spin density matrix of the system and is used to describe the effect that slow motions have on the linewidths of MAS peaks.
The equation as given by Suwelack et al. for the chemical shift has the following form

\[ \frac{1}{T_2} = \frac{\omega_0^2 \delta^2}{15} \cdot \left( 1 + \frac{\eta^2}{3} \right) \cdot \left[ \left( \frac{1}{1 + 4\omega_r^2 \cdot \tau_0^2} \right) + \left( \frac{2}{1 + \omega_r^2 \cdot \tau_0^2} \right) \right] \] (5.5)

[29]. If the model is extended to other types of motion, critical fluctuations, in particular the correlation time in the presence of critical fluctuations can be defined as \[ \tau_c \approx \xi^2 D \] where \( D \) is the diffusion coefficient and \( \xi \) is the correlation length equal to \[ \xi = \xi_0 \left( \frac{T}{T_c} - 1 \right)^{-\nu_c} \].

Now, \[ \tau_c = \tau_0 \cdot \left( \frac{T}{T_c} - 1 \right)^{-p} \] where \( \tau_0 \approx \xi_0^2 D \) and \( p = 2\nu_c \). As discussed previously, for these systems \( \eta \) is taken to be zero. In addition, for quadrupolar relaxation the term \( (\omega_0 \delta)^2 \) from the chemical shift anisotropy case is replaced with \( (\frac{3}{4}e^2 qQ)^2 \). Critical fluctuations are considered slow motions because as one approaches a critical point, the correlation length, and consequently the correlation time diverges and as a result this model seems appropriate. The form for the equation used to fit the sideband linewidths as a function of temperature is

\[ \Delta \nu = K \cdot \tau_0 \cdot \left( \left( \frac{T}{T_c} - 1 \right)^{-p} \right) \cdot \left[ \left( \frac{1}{1 + 4\omega_r^2 \cdot \tau_0^2 \cdot \left( \frac{T}{T_c} - 1 \right)^{-2p}} \right) + \left( \frac{2}{1 + \omega_r^2 \cdot \tau_0^2 \cdot \left( \frac{T}{T_c} - 1 \right)^{-2p}} \right) \right] \] (5.6)

where \( K \) is a constant that depends on the type of interaction. In the case of the chemical shift \( K = \frac{1}{\pi} \frac{\omega_0^2 \delta^2}{15} \). In the quadrupolar case, \( \Delta M_2 = \frac{3}{15} (\frac{3}{4}e^2 qQ)^2 \), this gives \( K = \frac{\Delta M_2}{3\pi} \) in terms of \( \Delta M_2 \). The approximate magnitude of \( K \) can be estimated based on fluctuations in \( M_2 \). The size of \( M_2 \) is proportional to the square of the average Hamiltonian of the system, so \( M_2 \approx < H >^2 \). Fluctuations in this Hamiltonian give \( \Delta M_2 \approx < H^2 - < H >^2 > \), which is proportional to the linewidth of the spectrum. At certain temperatures and compositions of ternary DOPC/DPPC/CHOL mixtures, fluctuations in the local cholesterol concentration result in contributions to the spectrum with different quadrupolar splittings, which result in an overall broadening of the spectrum. In the two phase region, the quadrupolar splittings of the two distinct phases can be identified. From these splittings it is possible to obtain a good estimate of the value of \( K \). Figure 5.4 gives a static spectrum for the composition 35:35:30 (DOPC/DPPC-d_{62}/CHOL) in the two phase region.
The splitting characteristic of the \( l_d \) phase is \( \sim 36 \) kHz, and the splitting characteristic of the \( l_o \) phase is \( \sim 54 \) kHz. This gives \( K = \frac{\Delta M_2}{3\pi} = \frac{(54 \times 10^3 - 36 \times 10^3)^2}{3\pi} = \sim 3.4 \times 10^7 \).

Figure 5.4: Static \( ^2 \)H NMR spectrum for 35:35:30 (DOPC/DPPC-d\(_{62}\)/CHOL) at 292.5 K, NS = 4096 for determining an approximate value of \( \Delta M_2 \). Reproduced with permission from Davis et al. [1].

**Fitting to the Theoretical Model**

The linewidth as a function of temperature data was fit in *Origin 8.5* (OriginLab, Northampton, MA) using equation 5.6 in the following form for fitting the 12 kHz and 15 kHz sidebands.
\[ y_{12kHz} = A + \left( \frac{B}{3000} \right) + K_{12} \cdot \tau_0 \cdot \left[ \left( \frac{T}{T_c} - 1 \right)^{-p} + \Delta t \right] \]
\[ \frac{1}{1 + 1.42122303 \times 10^9 \cdot \tau_0^2 \cdot \left( \left( \frac{T}{T_c} - 1 \right)^{-2p} + \Delta t \right) + 2 \cdot \frac{1}{1 + 3.5530576 \times 10^8 \cdot \tau_0^2 \cdot \left( \left( \frac{T}{T_c} - 1 \right)^{-2p} + \Delta t \right)}] \] (5.7)

and

\[ y_{15kHz} = A + \left( \frac{B}{3000} \right) + K_{15} \cdot \tau_0 \cdot \left[ \left( \frac{T}{T_c} - 1 \right)^{-p} + \Delta t \right] \]
\[ \frac{1}{1 + 1.42122303 \times 10^9 \cdot \tau_0^2 \cdot \left( \left( \frac{T}{T_c} - 1 \right)^{-2p} + \Delta t \right) + 2 \cdot \frac{1}{1 + 3.5530576 \times 10^8 \cdot \tau_0^2 \cdot \left( \left( \frac{T}{T_c} - 1 \right)^{-2p} + \Delta t \right)}] \] (5.8)

The constant, \( A \), was assigned a value of 5 Hz since 5 Hz of line broadening were used during the spectral analysis. \( B \) was allowed to vary during the fitting process but is a term that depends on the spin rate which in this case is 3000 Hz. The initial correlation time is \( \tau_0 \approx \frac{\xi_0^2}{D} \). For the lipids used in these experiments, a good starting value for \( \tau_0 \) can be found using \( D \approx 4 \times 10^{-12} \) m\(^2\)/s [46] and \( \xi_0 \approx 1 \times 10^{-9} \) m which is on the order of the size of a single lipid molecule. \( T_c \) is the critical temperature and \( p \) is twice the value of the critical exponent for the system, \( \nu_c \). A small correction has been added to the reduced temperature in the form of \( \Delta t \). The value of \( \Delta t \) is 0.0034 (~1 K/294 K), and is used to prevent the reduced temperature term from blowing up at \( T_c \) since there is a temperature gradient across the sample. \( K_{12} \) and \( K_{15} \) are related to the second moment, \( \Delta M_2 \), and constant terms and are the only values that may differ between the two sets of data as they are fit simultaneously. During the fitting process, the values of \( A \), and \( \Delta t \) were kept constant. First \( B \), \( K_{12} \), \( K_{15} \), and \( \tau_0 \) were allowed to vary and were fit using the simplex grid search mode, then \( T_c \) and \( p \) were released as well and the simplex mode was used two to three times to get a good estimate of the fit. Iterations were then performed using the Levenberg-Marquardt method for non-linear least squares fitting until the convergence criteria were met.
5.3 Results and Discussion

The first sample in the set was 35:35:30 (DOPC/α-d₄-DPPC/CHOL), since in the chain perdeuterated DPPC samples this composition showed a significant increase in the side-band linewidths near the critical point [6]. The ternary mixtures of DOPC/α-d₄-DPPC/CHOL were chosen to have 30 mol% cholesterol and varying ratios of DOPC/α-d₄-DPPC. Four additional compositions, two on either side of the sample with equal parts of DOPC and DPPC, were chosen for this set of experiments.

The linewidths of the spinning sidebands for ²H MAS spectra of these ternary mixtures are sensitive to temperature and phase behaviour. The goal of this work was to quantify these changes and fit them to a theoretical model for critical fluctuations. An example of how the linewidths change qualitatively is given first, and then the quantitative results are presented. In Figure 5.5, the peaks at 12 kHz and 15 kHz are shown at temperatures ranging from 312.3 K to 294.1 K. From Figure 5.5 it can be seen that the peaks at 312.3 K are the sharpest of the entire series shown. As the temperature is decreased, the peaks broaden and around 301 K they are at their widest thus far. Below ~301 K, the peaks get narrower again for a few degrees, and then begin to broaden again. This second broadening is attributed to the formation of gel phase in the spectra and is not relevant to the critical phenomena being investigated.
Figure 5.5: 12 kHz and 15 kHz sidebands for 35:35:30 (DOPC/α-d₄-DPPC/CHOL) showing the changes in the linewidths as a function of temperature. (NS = 16384).

The spin rate dependence of the $^2$H MAS sideband linewidths was investigated using a sample with the composition 37:37:26 (DOPC/DPPC-d₆₂/CHOL). The 18 kHz sideband linewidth data for spin rates of 2, 3, 4.5, and 9 kHz were fit simultaneously using the Suwelack et al. equation [29]. These fits are depicted in Figure 5.6. The following results were obtained from this fit, $T_c = 294.5$ K, $p = 1.306$, and $K = 3.33 \times 10^7$. Since $p$ is 1.306, $\nu_c$ is about 0.653 which is very close to the value for the 3D Ising model of 0.63 [12]. The value of $K$ here is very close to the estimated value based on the quadrupolar splittings of a static spectrum in the two phase region, which provides a rationale for using equation 5.6 for modelling critical behaviour in model membrane...
systems under MAS.

Figure 5.6: 18 kHz linewidth data as a function of temperature for MAS spin rates of 2, 3, 4.5, and 9 kHz with fits to the equation 5.6. Only the data used for the fit is shown. All four curves were fit with \( A = 5 \text{ Hz} \), \( B = 100471 \), \( \tau_0 = 7.6 \times 10^{-8} \), \( T_c = 294.5 \text{ K} \), \( K = 3.33 \times 10^7 \), \( \Delta t = 0.0034 \), and \( p = 1.306 \).

The linewidths of the DOPC/\( \alpha \)-d4-DPPC/CHOL samples were fit using the method outlined above, and plots of the linewidth as a function of temperature were created for each of the five sample compositions. One of the biggest challenges in fitting this data was to fit the baseline accurately. It is possible to obtain reasonable results by fitting both the real and imaginary components of the spectra simultaneously. Care needed to be taken in the experimental setup to achieve as flat a baseline as possible so that it could be fit with a straight line to ensure that the fits were as accurate as possible. As
the peaks get broader, the noise and imperfections in the shape of the baseline introduce more uncertainty into the value of the linewidth. This has been accounted for in the error bars, which increase as the linewidth increases. In addition, since the 12 kHz sideband is sharper than the 15 kHz sideband, the lineshape fitting, and thus the linewidth values are more accurate. To account for this in the fits, the weights for the 15 kHz linewidths was doubled so that they would not be weighted as heavily when both sets of data were fit simultaneously to equations 5.7 and 5.8.

The 12 kHz and 15 kHz sideband linewidths as a function of temperature for each of the compositions are shown in the figures below. The corresponding fits to the equations 5.7 and 5.8 for the high temperature portion of the data are also shown on the same figures. The compositions are presented in order of increasing molar concentration of DOPC. Figure 5.7 shows the results for the 21:49:30 (DOPC/α-d_{4}-DPPC/CHOL) sample.

![Figure 5.7: 12 kHz (closed circles) and 15 kHz (open squares) linewidths as a function of temperature for the ternary DOPC/α-d_{4}-DPPC/CHOL mixture with composition 21:49:30 with fits to equation 5.6 for temperatures ranging from 318.0 K to 298.4 K.](image)
Figure 5.8 gives the 28:42:30 (DOPC/$\alpha$-d$_4$-DPPC/CHOL) linewidths and fits to equations 5.7 and 5.8.

Figure 5.8: 12 kHz (closed circles) and 15 kHz (open squares) linewidths as a function of temperature for the ternary DOPC/$\alpha$-d$_4$-DPPC/CHOL mixture with composition 28:42:30 with fits to equation 5.6 for temperatures ranging from 318.0 K to 300.4 K.

Notice in Figure 5.8 that below 297 K the 12 kHz and 15 kHz linewidths no longer follow the same trends. This may be a result of inaccuracies in the measurements due to low signal to noise in the spectra below these temperatures. Figure 5.9 gives the results for the 35:35:30 (DOPC/$\alpha$-d$_4$-DPPC/CHOL) sample.
Figure 5.9: 12 kHz (closed circles) and 15 kHz (open squares) linewidths as a function of temperature for the ternary DOPC/α-d4-DPPC/CHOL mixture with composition 35:35:30 with fits to equation 5.6 for temperatures ranging from 312.3 K to 301.1 K.
The results for 42:28:30 (DOPC/α-d₄-DPPC/CHOL) are shown in Figure 5.10.

Figure 5.10: 12 kHz (closed circles) and 15 kHz (open squares) linewidths as a function of temperature for the ternary DOPC/α-d₄-DPPC/CHOL mixture with composition 42:28:30 with fits to equation 5.6 for temperatures ranging from 318.0 K to 292.7 K.

In Figure 5.10, one main difference between the two sidebands at ~290.5 K was noticed, while the rest of the points track fairly closely. Finally, the 49:21:30 (DOPC/α-d₄-DPPC/CHOL) sample linewidths and fits are shown in Figure 5.11.
Figure 5.11: 12 kHz (closed circles) and 15 kHz (open squares) linewidths as a function of temperature for the ternary DOPC/α-d4-DPPC/CHOL mixture with composition 49:21:30 with fits to equation 5.6 for temperatures ranging from 318.0 K to 288.2 K.
In this sample, there was a relatively large difference between the 12 kHz and 15 kHz linewidths at 286.6 K. In addition, the peak in the linewidths was not well defined, as there is only a one point drop before the linewidth increases again. Whether or not this maximum was real or a result of error in the fit to very broad lines is unclear.

Overall, the trends in the linewidths of the two sidebands vary at lower temperatures when the linewidths are broader. This variation is quite significant in some cases. This is one reason why it is imperative to use a model that fits the high temperature part of the data as opposed to just the low temperature data. The results of the critical temperatures and critical exponents determined for each composition are summarized in the Table 5.1 below. Note that the estimates for the errors in the calculated values of the critical temperature and exponents were obtained from the standard error given with the fit results in *Origin 8.5* (OriginLab, Northampton, MA) and rounded to the first significant digit.

The results in the Table 5.1 show that all the critical exponent, $\nu_c$, values are between 0.65 and 1.2. Mean field theory predicts $\nu_c = 0.5$, overall, these results do not conform to this model. The 2D Ising model gives a result of $\nu_c = 1$, while the 3D Ising model gives a result of $\nu_c = 0.63$, and some of our results are close to each of these critical exponents [12]. From these results, one cannot say conclusively which universality class, 2D or 3D Ising model, these systems belong to since these results show critical exponents close to each of these models, and with the error, some results could fit into both at once. In most cases, the larger critical exponent values also had the largest uncertainties associated with both the critical temperature and the critical exponent calculated.
Table 5.1: Summary of the critical temperatures and critical exponents for the $^2$H MAS experiments

<table>
<thead>
<tr>
<th>Composition</th>
<th>Quality of Fit</th>
<th>Critical Temp.</th>
<th>Calculated Exp.</th>
<th>Critical Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$ Value</td>
<td>$T_c$ (K)</td>
<td>$p$</td>
<td>$\nu_c$</td>
</tr>
<tr>
<td>21:49:30</td>
<td>0.993</td>
<td>292 ± 3</td>
<td>2.4 ± 0.6</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>28:42:30</td>
<td>0.997</td>
<td>297.8 ± 0.5</td>
<td>1.5 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>35:35:30</td>
<td>0.996</td>
<td>299.8 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>42:28:30</td>
<td>0.998</td>
<td>282 ± 4</td>
<td>2.2 ± 0.7</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>49:21:30</td>
<td>0.992</td>
<td>282 ± 6</td>
<td>1.4 ± 0.8</td>
<td>0.7 ± 0.4</td>
</tr>
</tbody>
</table>

The same data sets were fit again, this time holding the critical exponent, $p$ constant at either the value for the 3D Ising model ($p = 1.34$) or the 2D Ising model ($p = 2$). The results for the critical temperatures and quality of each of these fits are shown in Table 5.2 for comparison between the two models.
Table 5.2: Critical temperatures for the $^2$H MAS experiments with fixed critical exponents

<table>
<thead>
<tr>
<th>Composition</th>
<th>Quality of Fit</th>
<th>Critical Temp. $T_c$ (K)</th>
<th>Calculated Exp. $p$</th>
<th>Critical Exp. $\nu_c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>21:49:30</td>
<td>0.977</td>
<td>296.6 ± 0.2</td>
<td>1.26</td>
<td>0.63</td>
</tr>
<tr>
<td>21:49:30</td>
<td>0.992</td>
<td>293.0 ± 0.6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>28:42:30</td>
<td>0.997</td>
<td>298.5 ± 0.1</td>
<td>1.26</td>
<td>0.63</td>
</tr>
<tr>
<td>28:42:30</td>
<td>0.998</td>
<td>296.3 ± 0.2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>35:35:30</td>
<td>0.992</td>
<td>299.3 ± 0.2</td>
<td>1.26</td>
<td>0.63</td>
</tr>
<tr>
<td>35:35:30</td>
<td>0.997</td>
<td>298.5 ± 0.2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>42:28:30</td>
<td>0.998</td>
<td>288.5 ± 0.2</td>
<td>1.26</td>
<td>0.63</td>
</tr>
<tr>
<td>42:28:30</td>
<td>0.998</td>
<td>283.5 ± 0.4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>49:21:30</td>
<td>0.993</td>
<td>284.2 ± 0.5</td>
<td>1.26</td>
<td>0.63</td>
</tr>
<tr>
<td>49:21:30</td>
<td>0.992</td>
<td>279.3 ± 0.9</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

In all cases, the quality of the fits are very good and are similar between the two models. The critical temperature is always a couple of degrees higher (closer to the cut off temperature of the data used for the fit) for the 3D Ising model. The challenges encountered when trying to measure accurate linewidths definitely contribute to the uncertainty in the results. Although model membrane systems are relatively two-dimensional, they are not completely two-dimensional and thus it is plausible that critical phenomena in these systems could fit a three-dimensional model. Further, careful experiments need to be performed in order to differentiate between the two models. It is also possible that neither of these two universality classes describes the critical behaviour of ternary model membrane systems precisely, so other models should be explored as well.
5.4 Conclusions

Previously, the critical behaviour of a series of ternary mixture samples with chain perdeuterated DPPC was investigated using $^2$H MAS NMR [6]. Due to the complexity of the contributions to the spectra, including the contributions from the methyl groups which result in inhomogeneous line shapes for sidebands below 12 kHz, the experiments presented here used samples with specifically deuterated DPPC ($\alpha$-d$_4$-DPPC) instead of DPPC-d$_{62}$. The high temperature linewidth data was fit using an equation based on the critical behaviour of the correlation length within the sample which is described by Suwelack et al. [29]. The critical exponents obtained from these samples ranged from $\nu_c = 0.65$ to $\nu_c = 1.2$. The 2D Ising model gives a value of $\nu_c = 1$, while the 3D Ising model predicts a value of $\nu_c = 0.63$ [12]. At this time, it is not possible to definitively assign a universality class to these model membrane systems.
Chapter 6

Final Summary and Future Directions

The overlying goal of this project was to study the critical fluctuations in ternary DOPC, DPPC, cholesterol model membrane systems using NMR spectroscopy and to fit the results to a theoretical model describing critical phenomena. One of the subsequent goals was to find a static solid state NMR technique that was able to probe these slow motions. The Jeener echo pulse sequence, which enhances the dipolar order caused by fluctuations in the local field of each nucleus to a level that can be measured, seemed like a good candidate. It seemed as though one should be able to measure changes in the relaxation time constant for the dipolar order as a function of the temperature using this pulse sequence. Unfortunately, no useful information about critical phenomena was obtained from the data collected for the 35:35:30 (DOPC/DPPC/CHOL) sample using this technique. $^2$H MAS NMR was then used to investigate the critical behaviour of these systems. Instead of using chain perdeuterated DPPC as was done previously [6], for the current study specifically labelled $\alpha$-d$_4$-DPPC was chosen. Using specifically labelled samples instead of the chain perdeuterated samples greatly simplifies the contributions to the spectra. Five compositions, all with 30 mol% cholesterol, were used for this work. The samples had the following molar ratios of 21:49:30, 28:42:30, 35:35:30, 42:28:30, and 49:21:30 (DOPC/$\alpha$-d$_4$-DPPC/CHOL). The sideband linewidth were analyzed as a function of temperature for two sideband frequencies, 12 kHz and 15 kHz, under MAS at a rate of 3 kHz. The high temperature linewidth data was fit to a function described
by Suwelack _et al._ [29] to determine the critical temperature and critical exponent for
the correlation length of the system for each composition. The critical exponent values
obtained from these samples ranged between $\nu_c = 0.65$ and $\nu_c = 1.2$, which encompasses
the critical exponents for both the 2-D and 3-D Ising models within experimental error.
As a result, the question of which universality class these model membranes belong to
cannot be definitively answered yet.

One of the biggest challenges with MAS NMR is that it creates a temperature gradient
across the sample as a result of friction from the spinning. This temperature gradient
is especially significant since observing critical phenomena is highly dependent upon the
temperature of the sample. If the temperature is not uniform, it can skew the results
since some portions of the sample may be at the critical temperature while others are
not. The impact of the temperature gradient will depend on the size of the gradient, but
a temperature gradient can affect the accurate determination of $T_c$ and $\nu_c$, specifically,
the value of $\nu_c$ will be underestimated. The fact that there is a temperature gradient has
been taken into account when fitting to the Suwelack _et al._ equation [29] via the $\Delta t$ term
which prevents the equation from going to infinity at the critical point. Nevertheless,
the exact details of this temperature gradient are unknown so the estimate for the size
of this gradient may not be correct. In the fits presented here, a correction of 1 K was
used. The effects of the temperature gradient are limited by spinning relatively slowly.
A measurement of the temperature gradient across the sample should be looked into in
order to accurately quantify the uncertainty in the results. Ideally, one would like to use a
static NMR technique where the temperature can be controlled more precisely, but as of
yet an ideal method of probing critical phenomena using static NMR has not been found.
Moving forward, a more complete set of sample compositions across the phase diagram
should be studied to get a better picture of how the critical exponent and temperature
behave as a function of composition. It would be interesting to try different labelling
schemes for the DPPC, since perhaps the $\alpha$-d$_4$-DPPC is not the ideal labelling scheme
to use to detect critical fluctuations. There may be other positions on the chain that are more sensitive to the compositional fluctuations which would lead to better defined, or more consistent linewidth versus temperature trends for each sideband.
Appendix A

$^{2}$H MAS Line Shape Fitting

The FID was converted from digital to analog data in *Bruker TopSpin 2.1* (Bruker BioSpin, Karlsruhe, Germany). The points before the echo were eliminated to give symmetric spectra with a flat baseline. The phase was adjusted based on the highest temperature spectrum and these conditions were used on all the other spectra for that composition. Four sidebands at 9, 12, 15, and 18 kHz on the right hand side of the spectrum were normally analyzed so the exact zoom function was used to select the region from -7500 to -19500 Hz (in the Origin files these were indexed as positive numbers). The text file was modified such that the real and imaginary parts could be separated into columns when imported into Origin for graphing and fitting. Both the real and imaginary components of the spectra were fit at the same time using the following equations

$$y_r = \left(1 - \frac{\phi^2}{2}\right) \cdot (A_1 + B_1 \cdot x + \frac{\alpha_0}{(1 + (x - x_0)^2 \cdot d_0^2)}) + \frac{\alpha_1}{(1 + (x - x_1)^2 \cdot d_1^2)} + \frac{\alpha_2}{(1 + (x - x_2)^2 \cdot d_2^2)} + \frac{\alpha_3}{(1 + (x - x_3)^2 \cdot d_3^2)}$$

and

$$y_i = \phi \cdot (A_2 + B_2 \cdot x + \frac{\alpha_0}{(1 + (x - x_0)^2 \cdot d_0^2)}) - \frac{\phi^2}{2} \cdot (A_2 + B_2 \cdot x + \frac{\alpha_0}{(1 + (x - x_0)^2 \cdot d_0^2)})$$

$$- \frac{\alpha_1}{(1 + (x - x_1)^2 \cdot d_1^2)} - \frac{\alpha_2}{(1 + (x - x_2)^2 \cdot d_2^2)} - \frac{\alpha_3}{(1 + (x - x_3)^2 \cdot d_3^2)}$$
\[ +\alpha_1 \cdot (x - x_1) \cdot \frac{d_1}{1 + (x - x_1)^2 \cdot d_{1}^2} + \alpha_2 \cdot (x - x_2) \cdot \frac{d_2}{1 + (x - x_2)^2 \cdot d_{2}^2} + \alpha_3 \cdot (x - x_3) \cdot \frac{d_3}{1 + (x - x_3)^2 \cdot d_{3}^2} \]  \hspace{1cm} (A.2)

Note that the terms \( \phi \) and \( 1 - \frac{\phi^2}{2} \) are the small angle approximations for sine and cosine respectively. These phase terms correspond to the real and imaginary contributions to the spectra.

## A.1 Fitting Procedure for the Highest Temperature Spectrum

The initial parameters were obtained by looking at the data for the real part of the spectrum. The maximum amplitude value near 9, 12, 15, and 18 kHz frequencies were used to define \( \alpha_0, \alpha_1, \alpha_2, \) and \( \alpha_3, \) respectively. The corresponding frequency values were assigned to \( x_0, x_1, x_2, \) and \( x_3. \) The initial value for the values defining the linewidths \( (d_0, d_1, d_2, \) and \( d_3) \) was 0.016, which corresponds to a linewidth of 125 Hz. \( A1, B1, A2, \) and \( B2 \) were used to define the baseline and were initially set to 0. The phase adjustment, \( \phi, \) was also initially set to 0.

### A.1.1 Steps Used to Fit Data

1. Fix all parameters.

2. Allow \( A1, B1, A2, \) and \( B2 \) to vary, perform 2 iterations.

3. Fix \( A1, B1, A2, \) and \( B2, \) allow \( \alpha_0, \alpha_1, \alpha_2, \) and \( \alpha_3 \) to vary, perform 2 iterations.

4. Fix \( \alpha_0, \alpha_1, \alpha_2, \) and \( \alpha_3, \) allow \( x_0, x_1, x_2, \) and \( x_3 \) to vary, perform 2 iterations.

5. Fix \( x_0, x_1, x_2, \) and \( x_3, \) allow \( d_0, d_1, d_2, \) and \( d_3 \) to vary, perform 2 iterations.

6. Now vary \( d_0, d_1, d_2, \) and \( d_3, \) and \( \alpha_0, \alpha_1, \alpha_2, \) and \( \alpha_3, \) perform 2 iterations.
7. Now vary $d_0$, $d_1$, $d_2$, and $d_3$, $\alpha_0$, $\alpha_1$, $\alpha_2$, and $\alpha_3$, and $A_1$, $B_1$, $A_2$, and $B_2$ to vary, perform 2 iterations.

8. Allow everything except for $\phi$ to vary, perform 2 iterations.

9. Allow everything to vary, including the phase, perform 2 iterations.

10. Fix all parameters and save the values.

11. Unfix all parameters and fit until the convergence criterion is met.

A.2 Fitting Procedure for All Subsequent Spectra for the Same Sample

Lineshape fitting was done systematically from highest temperature to lowest temperature and the values from the fit of the previous spectrum were used at the starting point for the current fit. Note that the initial value for the phase factor, $\phi$, was always set as zero. The series of steps in the fitting algorithm described above was the same except that the frequency values for the peaks were kept constant throughout.
Bibliography


