SOLID STATE DEUTERIUM NUCLEAR MAGNETIC RESONANCE
INVESTIGATION OF THE INTERACTION OF
POSITIVELY-CHARGED POLYELECTROLYTES WITH
NEGATIVELY-CHARGED LIPID BILAYER
MEMBRANE VESICLES

by

Kevin James Crowell

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Chemistry
University of Toronto

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SOLID STATE DEUTERIUM NUCLEAR MAGNETIC RESONANCE INVESTIGATION OF THE INTERACTION OF POSITIVELY-CHARGED POLYELECTROLYTES WITH NEGATIVELY-CHARGED LIPID BILAYER MEMBRANE VESICLES

Degree of Master of Science, 1997
by Kevin James Crowell
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Deuterium nuclear magnetic resonance (\(^2\)H NMR) is an important tool for studying many characteristics of lipid bilayers, in particular membrane electrostatics. The use of multilamellar vesicles (MLVs) containing specifically choline-deuterated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) produce \(^2\)H NMR spectra with quadrupole splittings (\(\Delta v_Q\)’s) that reflect the overall membrane surface charge. This molecule has been termed a “molecular voltmeter” because of this characteristic response to membrane surface charge. When MLVs containing choline-deuterated POPC and the anionic lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) are exposed to cationic polyelectrolytes \(\Delta v_Q\) changes in a specific manner characteristic of the polyelectrolyte. A survey of the effects of several different polyelectrolytes was carried out and one response in particular was explored in detail, that being the response to poly(vinylbenzyltrimethylammonium chloride) (PVTVA). This molecule was observed to induce the formation of two distinct domains. Through the combined use of spectral simulations and \(^2\)H NMR spectra, it was possible to accurately determine the composition of the two domains.
I must begin by expressing my gratitude to my supervisor Peter Macdonald, without whose guidance, support and encouragement this work would not have been possible. Equally important are my co-workers Carla Franzin, Peter Mitrakos and Darlene Semchyschyn. Their support, advice and assistance in all aspects of this research, especially the synthesis of deuterated lipids, were essential to its successful completion. I also wish to thank all of my fellow graduate students for making this a memorable experience, there being too many to name.

In addition, I have had the pleasure of knowing and working with many fine students and technicians in the Erindale Chemistry department. This would not have been the same without them.

Finally, I dedicate this thesis to my son Andrew, who was born just before I began my studies at Erindale. He has never been far from my heart and mind these last two years and I would not be what I am today without him.
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SYMBOLS AND ABBREVIATIONS

PC  phosphatidylcholine
POPA  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate
POPC  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPC-α-d₂  POPC deuterated in the alpha position
POPC-β-d₂  POPC deuterated in the beta position
POPG  1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol

PLYS  poly(lysine)
PMGU  poly(methylene-co-guanidine)
PEI  poly(ethyleneimine)
SD72  starburst dendrimer of d = 72Å
PVTA  poly(vinylbenzyltrimethylammonium chloride)

HEPES  4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid
TPS  2,3,5-triisopropylbenzenesulfonyl chloride
TPB  tetraphenylboron
TLC  thin layer chromatography

MLV  multilamellar vesicle
NMR  nuclear magnetic resonance
¹H NMR  proton nuclear magnetic resonance
²H NMR  deuterium nuclear magnetic resonance
³¹P NMR  phosphorus nuclear magnetic resonance
ΔνQ  deuterium NMR quadrupolar splitting
1. INTRODUCTION

1.1 Overview

The key to understanding many of the fundamental biological processes lies in acquiring a wide array of information on the manner in which molecules and supermolecular structures interact with one another. Since all living things are composed of cells with lipid-based cell membranes and since literally thousands of molecules either pass through this membrane or somehow send a signal through the membrane, interactions of molecules with lipid membranes assume a particular importance. Prior to being able to understand specific real-world examples one must become familiar with some of the more general classes of interactions that are possible, followed by an application of these principles to a particular problem. This research has been carried out with the aim of providing some of this necessary information on the interaction of positively-charged polyelectrolytes with negatively-charged lipid membranes.

Many methods exist which are capable of providing useful information on such interactions, and the past two decades have seen a tremendous growth in the applications of solid state nuclear magnetic resonance (NMR) to such questions, with deuterium (\(^{2}H\)) NMR being a particularly interesting technique for a number of reasons. In particular, \(^{2}H\) NMR has become a prominent means by which many characteristics of lipid membranes can be studied and important structural and motional information was obtained when \(^{2}H\) NMR was in its early stages (Seelig, 1977; Seelig & Seelig, 1980; Jacobs and Oldfield, 1981; Davis, 1983; Devaux, 1983; Bloom & Smith, 1985). \(^{2}H\) NMR has been used for studying lipid relaxation times (Brown, 1982; Bloom & Smith, 1985; Bloom & Sternin, 1987) and for probing lipid order.
When studying phospholipids one generally looks at either the acyl chains or the lipid headgroups since these two environments are distinctly different. An interesting and useful characteristic of the zwitterionic phosphatidylcholine (PC) headgroup is that, when selectively deuterated at one of the choline positions, it has a quadrupolar splitting whose value is changes as the membrane charge density changes. This surface potential sensitivity has been well documented (Brown & Seelig, 1976; Akutsu & Seelig, 1981; Scherer & Seelig, 1989), which has precipitated the labeling of the selectively deuterated PC headgroup as a “molecular voltmeter,” leading to its extensive use in a wide variety of studies (Roux et al., 1989; Macdonald et al., 1991; Marassi & Macdonald, 1991; Rydall & Macdonald, 1992, Franzin & Macdonald, 1996; Mitrakos & Macdonald, 1996; Crowell & Macdonald, 1997). These changes in quadrupolar splitting are thought to be the result of a conformational change of the PC headgroup relative to the plane of the membrane. This alleged conformational change is well approximated by the “choline tilt” model (Scherer & Seelig, 1986), though concrete proof supporting this model is still outstanding.

It has been shown that the interaction of charged electrolytes, such as Ca$^{2+}$, with negatively charged lipid membranes can be sensed by the molecular voltmeter (Macdonald & Seelig, 1987a; Macdonald & Seelig, 1987b). Therefore, it is plausible that the interaction of charged polyelectrolytes with lipid membranes could also be sensed by the molecular voltmeter, either directly or indirectly. In fact, several attempts at detecting such interactions, via $^2$H NMR, have been made. Roux, et al. (1988) were unable to show any appreciable effect of the binding of pentalysine to membranes composed of PC and phosphatidylserine (PS). However, other
& Macdonald, 1996; Mitrakos & Macdonald, 1996; Crowell & Macdonald, 1997). Ideally, one could observe the coexistence of regions where the polyelectrolyte was bound and regions where there was no polyelectrolyte bound.

This thesis is focused on the interaction of cationic polyelectrolytes with membranes that have a net negative charge, typically due to the presence of phosphatidylglycerol (PG). Initially, a survey of several different polyelectrolytes was made to determine what, if any, effect could be observed upon their addition to multilamellar vesicles (MLVs). MLVs were the vesicle type of choice because of their large size and ease of preparation.

There were two general classes of observations made, the first being essentially a classic molecular voltmeter response. The second type is of particular interest because it indicated the presence of two domains; a polyelectrolyte-bound and a polyelectrolyte-free domain. The ability to detect two domains was found to be dependent upon both the relative PG content and the ratio of polyelectrolyte to PG. These dependencies will be explored and an attempt at modeling them mathematically will be made.

1.2 Lipid Bilayer Structure and Characteristics

The role of the lipid bilayer in the function of cells is so significant that it can often be taken for granted. It is the lipid bilayer that provides the basis of the cell membrane of animal cells, the barriers through which ions, molecules, waste and nutrients must pass to enter or leave the cell. There are only a handful of constituent phospholipids available to create the lipid bilayer, which forms the framework in which membrane-bound and membrane-associated
which are essential for cellular life. Included among these are the regulation of the passage of ions, a complex process that controls many aspects of cell function. This includes the provision of binding sites for a large range of molecules (hormones, neurotransmitters, etc.), as well as the formation of ion-selective pores that pass through the membrane. That these proteins are so intricately associated with a lipid bilayer membrane implies a high degree of importance on their mutual interaction.

What exactly is a lipid bilayer? In simple terms, it is an arrangement of lipid molecules in which the polar headgroups are at the outer surfaces, facing the aqueous environment, while the non-polar portions are in the interior, isolated from the aqueous environment. A sketch of this arrangement is shown in figure 1.

The main component of lipid bilayers are phospholipid molecules, with the structures of three typical ones shown in figure 2. That these molecules are able to form lipid bilayers is largely due to the fact that they are amphipathic, with the polar headgroup being the hydrophilic portion and the two hydrocarbon tails being the hydrophobic portion. The tails of the phospholipid are often fatty acids that vary in length, usually from 14-24 carbon atoms. Generally, as illustrated in figure 2, one of the fatty acid tails is bent due to the presence of one (or more) cis-double bonds, which introduces a kink in the chain. In this research project the lipids used were composed of phospholipids containing the C_{18} palmitic acid and the 18-carbon oleic acid, designated 18:1cΔ9 because of the presence of a cis-double bond at carbon 9. The most prevalent phospholipids are those that contain the phosphatidylcholine (PC) headgroup, which has a positive charge, counteracting the negative phosphate charge. The result is a
Figure 1: Schematic representation of a typical lipid bilayer illustrating the orientation of the phospholipid molecules. This orientation results from the amphipathic nature of the lipid molecules, with the hydrophilic head groups facing the aqueous environment and the hydrophobic acyl chains being buried in the membrane interior.
Figure 2:  Examples of some typical phospholipid molecules. On the left is the anion POPA; in the middle is the anion POPG and on the right is the zwitterion POPC.
phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). PG, PI and PS are all negatively charged at physiological pH, while PE is neutral. In addition to phospholipids it is also common to have other membrane lipids present, such as cholesterol (a sterol) and sphingolipids.

Generally, there are two methods available for studying lipid bilayers: the use of cells or membranes extracted from cells, or the use synthetic liposomes with either natural or synthetic lipids. The former presents a host of problems due to the enormous complexity of the cell. The latter option, and the one used here, is far simpler because variables external to those which are under observation can be rigidly controlled, as can the exact composition of the bilayer. There are several types of liposomes that can be manufactured and they can be classified as either multilamellar or unilamellar. The latter group are classified by size and usually require more effort in their preparation. However, they more closely resemble the membranes of cells. The former group (multilamellar vesicles: MLVs) is much easier to prepare and has the further advantage of having a large number of available surfaces for binding due to the large number of lamellae, arranged much like the layers of skin on an onion.

1.3  **Bilayer Surface Electrostatics**

It has already been mentioned that natural membrane lipids can be either neutral or negatively charged. Furthermore, it is possible to synthesize lipids that have a net positive charge, though these are not common naturally. These charges are present at the headgroup of the lipids and the presence of these point charges at an otherwise neutral surface is responsible
introduction to the key points. Further detail can be found in reviews by McLaughlin (1989) and Cevc (1990).

To begin to calculate the surface potential, \( \psi(0) \), one has to first go back to Coulomb and his determination that the electrostatic potential a distance \( r \) from a fixed charge \( q \) in an infinite medium of dielectric constant \( \varepsilon_a \) is:

\[
\psi(r) = \frac{q}{4\pi\varepsilon_0\varepsilon_a r}
\]

where \( \varepsilon_0 \) is the permittivity of free space. Working from this basic premise and making several assumptions, this basic equation was modified by several researchers, including Debye and Huckel, until a general expression for \( \psi(0) \) was determined to be:

\[
\psi(0) = \frac{\sigma}{4\pi\varepsilon_0\varepsilon_a \kappa}
\]

where \( \sigma \) is the surface charge density and \( 1/\kappa \) is the Debye length, a measure of the thickness of the electrical double layer formed from the charged membrane surface and the counter-ions attracted to it. This expression was the culmination of the theory put forth by Gouy (1910) and Chapman (1913), whose work was done independently. This expression is remarkably simple due to the assumptions made in its determination. First, they assumed that the fixed charges on the surface were not discrete, but were instead spread uniformly over the surface. Second, they assumed that the aqueous environment was of uniform dielectric constant and that solvated ions in this phase were point charges. Therefore, the potential at a distance \( x \) from the membrane is given by
What many have found surprising about this theory is that it often works, despite its apparent simplicity. For a small surface potential this theory holds very well in cases that consider monovalent ions as the counterion in the aqueous phase. The obvious limitation here is that many ions present in biological solutions are not monovalent, and many are also polyelectrolytes of some sort. Fortunately, work on extending the standard Gouy-Chapman theory to account for larger polyelectrolytes has been done with some success and a detailed discussion of this extension has been given by Stankowski (1991).

The relevance of bilayer surface electrostatics lies in how it influences many biological processes. In the absence of an electrostatic potential many biologically active molecules would have no means by which to find the correct location on the membrane for binding or association. For example, it has been shown that acidic (ie: negatively-charged) phospholipid domains are important in the binding of extrinsic proteins (Lentz, 1995). Furthermore, it is known that the electrostatic potential serves to create so-called Gouy-Chapman double layer, an arrangement whereby charged lipid molecules attract bulk counter ions, forming a distinct layer on top of the outer surface of the membrane. Such interactions can be important in influencing the degree of protonation of certain molecules, a property that often determines their activity.

1.4 Characteristics of Polyelectrolytes

The value of polyelectrolytes cannot be overstated. Polyelectrolytes are present in numerous forms, including nucleic acids, proteins and a wide variety of synthetic polymers that
more than one charged site. The charges can be either positive (as with the amino acid lysine) or negative (as with DNA), can be evenly spread out (as with DNA) or they can be located at very specific locations (as with the vast majority of peptides and proteins). Synthetic polyelectrolytes have the advantage that the number and location of charged sites can be rigorously controlled.

There is no strict size requirement for a molecule to be considered a polyelectrolyte, and they come in sizes as varied as a dipeptide to large polymers containing thousands of monomeric units. And what about the possible shapes? They are almost limitless. The simplest case is that of a straight chain polymer with only one repeating (charged) unit. It is also possible for the polymer to be branched, to have more than one repeating unit, or to have both positive and negative charges present. A comprehensive introduction to polymers has been prepared by Young (1983). It is also possible to make polyelectrolytes with less conventional shapes, such as flat star-shaped polymers, or more interestingly, large, spherical cascade polymers, also referred to as starburst dendrimers. These polyelectrolytes are generally made by starting with a simple initiator core, such as NH$_3$$_2$, and successively adding a particular building block unit (usually -CH$_2$CH$_2$CONHCH$_2$CH$_2$NH$_2$). By controlling the number of generations that are added, the diameter of these dendrimers can be carefully regulated. They are characterized by the presence of terminal amino groups, and these can be protonated to give the outer surface a net positive charge. A detailed review of these molecules provides much of the history and theory underlying these compounds (Tomalia et al., 1990).

In discussing the shapes of polyelectrolytes it is essential to make the discussion relevant to the environment under consideration. The main environment of interest here is an aqueous
important of such a situation on the shape of flexible polyelectrolytes cannot be over-
emphasized. For chain polyelectrolytes one could expect either a long, rod-shaped molecule, or
a highly coiled and folded molecule, or something intermediate. Obviously, this assumes that
the polyelectrolyte has the flexibility necessary for such changes. Molecules such as DNA and
starburst dendrimers are not able to adopt such conformations.

What determines a molecule’s exact shape or morphology is the necessity of a
polyelectrolyte to maintain electrical neutrality. In considering this there are two classes of
interactions that come into play. First, there are interactions between the molecule and the
solvent (as well as the counter-ions present in the solvent). These interactions serve to counter
the polyelectrolyte charge, allowing the polyelectrolyte to “relax” and adopt a conformation
resembling a random coil. Neighbouring charges on the polyelectrolyte become screened from
each other. Second are the intramolecular interactions which, for a polyelectrolyte with only
positive or negative charges, repel one another, tending to lengthen and stiffen the
polyelectrolyte. The exact shape adopted is a balance of these two interactions. In the absence
of counter-ions there is little or no screening of adjoining charges on the polyelectrolyte,
producing a more elongated shape, while with increasing salt concentration there is
progressively more screening, allowing for a more relaxed shape. These sorts of considerations
must be kept in mind when considering the binding/adsorption of polyelectrolyte to lipid
bilayers. The details of these interactions are centered on the calculations of the electrostatic
free energy, and they will not be presented here. Such considerations for long rod-shaped ions
have been dealt with by Hill (1955), while for flexible linear polyelectrolytes initial calculations
1.5 Interactions of Polyelectrolytes With Lipid Bilayers - Domain Formation

A wide variety of ions and molecules are known to interact with either lipid membranes or with specific molecules somehow associated with lipid membranes. The molecular details of these interactions with lipid membranes are important in many aspects of cellular life, yet detailed information on how the interactions take place and how they are controlled and influenced is lacking in many areas. Recently there has been an increased interest in studying the interaction of polyelectrolytes with membranes, including peptides and proteins (Yang & Glaser, 1995; Seelig et al., 1995; Lentz, 1995; Buser et al., 1995; Carbone & Macdonald, 1996; and Seelig, 1997) as well as viruses (Luan & Glaser, 1994; Luan et al., 1995). As previously mentioned there has also been work done on the interaction of DNA with membranes (Mitrakos & Macdonald, 1996).

There are several methods available to attempt to study the interaction of polyelectrolytes and each has its own particular advantage. Vibrational spectroscopy has been used to study deuterated phospholipid membranes in the form of Raman spectroscopy (Mendelsohn & Tarashi, 1978; Mendelsohn & Koch, 1980; and Kouaouci et al., 1985) and Fourier transform infrared spectroscopy (FTIR) (Mendelsohn, et al., 1984). Another technique capable of quantitatively characterizing lipid-peptide interactions is that of high sensitivity titration calorimetry. This method involves measuring enthalpies of binding when either lipid is added to a peptide solution, or when a peptide is added to lipid vesicles. From these measurements it is possible to determine a binding constant, K (Beschiaschvili & Seelig, 1992; Seelig et al., 1993;
These two methods have the advantage of being able to provide quantitative binding information. However, they are unable to distinguish and characterize different domains present at the same time. In fact, they can't visualize domains at all. This shortfall has been partly overcome by the use of fluorescence digital imaging microscopy. This technique looks at single lipid vesicles and makes use of fluorescently labeled lipids and polyelectrolytes, enabling the visualization of distinct regions on the vesicle surface where there is polyelectrolyte bound (Luan & Glaser, 1994; Luan et al., 1995; and Yang & Glaser, 1995). The drawback of this technique is that it is purely qualitative in terms of determining the compositions of the different domains present. Quantitative determination of domain composition is not possible with this technique.

1.6 Theory of Solid State $^2$H NMR

NMR spectroscopy has become one of the most powerful analytical techniques available to the modern chemist. Initially used to probe molecular structure through the generation of $^1$H spectra, NMR expanded to be able to observe a wide variety of nuclei. In the early years the technique was limited mainly to those nuclei of high natural abundance and high magnetic moment, such as $^1$H and $^{19}$F. Otherwise, the signal-to-noise suffered prohibitively. A main reason for this was that early spectrometers operated as continuous wave instruments, meaning that a spectrum had to be obtained with one scan. It was possible to signal-average several spectra, but this was a very time-intensive process. With the advent of pulsed spectrometers making use of Fourier Transformation (FT) algorithms, spectra could be collected more rapidly.
up the ability to observe practically any NMR-active nucleus. In addition, the dimensionality of the spectra that could be acquired increased steadily, with numerous different pulse sequences designed to accomplish a specific goal and collect increasingly more information about the nuclear spins under observation. However, this was essentially limited to molecules in solution, where fast isotropic motions average out interactions that would otherwise broaden the signal.

The situation when discussing solid or semi-solid samples is quite different. There is no rapid isotropic tumbling of the molecules and consequently no isotropic averaging of those interactions that tend to broaden the NMR signal. Consequently, many anisotropic interactions are manifest, leading to extensive broadening of the NMR signal. The remainder of this section will introduce solid state NMR, discuss the nuclear spin Hamiltonian and outline the theory underlying \(^2\)H NMR spectroscopy. Solid state NMR theory has been covered extensively in the literature. What will be presented here is a summary based on Abragam (1961), Seelig (1977), Griffin (1981) and Davis (1983).

In order to understand the interaction of a nucleus with an external magnetic field \(B_0\), it is best to begin by introducing the nuclear spin Hamiltonian and its constituent terms. It can be written as:

\[
\mathcal{H} = \mathcal{H}_Z + \mathcal{H}_{RF} + \mathcal{H}_\alpha + \mathcal{H}_J + \mathcal{H}_{DD} + \mathcal{H}_Q
\]  

(4)

there the total Hamiltonian is the sum of six individual Hamiltonians that describe possible interactions of the nucleus with its environment. These hold for diamagnetic systems. In the
they are degenerate. $\mathcal{H}_z$ is the Zeeman Hamiltonian, which describes the interaction of the magnetic moment, $\mu$, with the external magnetic field. It is this interaction which is responsible for removing the degeneracy of the nuclear spin states, creating $2I + 1$ energy levels, where $I$ is the nuclear spin quantum number. This is the dominant interaction in NMR spectroscopy and it can be described as:

$$\mathcal{H}_z = -\mu \cdot B_o = -\gamma I \cdot B_o$$

(5)

where $\gamma$ is the magnetogyric ratio and $I$ is quantized along $B_o$. Bold-type indicates that the quantity is a vector quantity. Since the applied field is always defined to be along the z-axis in the laboratory frame of reference one need only consider the $z$-component of $I$, allowing expression of the Zeeman interaction as:

$$\hbar^{-1}\mathcal{H}_z = (-\gamma/2\pi)I_z B_o$$

(6)

$\mathcal{H}_{RF}$ is the Hamiltonian describing the interaction of the nucleus with an applied radio frequency (RF) field, $B_{RF}$. It is this RF field that is responsible for inducing transitions between the Zeeman energy levels. The RF field is applied perpendicular to $B_o$ and the energy absorbed by a nucleus corresponds exactly to the energy difference between Zeeman energy levels. This condition is referred to as resonance and the resonance frequency, termed the Larmor frequency, can be expressed as:

$$\nu_o = \gamma I B_o/2\pi$$

(7)
interactions, $\mathcal{H}_\sigma$ (chemical shift), $\mathcal{H}_j$ (scalar spin coupling), $\mathcal{H}_{DD}$ (dipolar) and $\mathcal{H}_Q$ (quadrupolar) are all what are termed internal Hamiltonians because they are not under the control of the operator. Instead, they are intrinsic to the particular environment of the nuclei in the sample. What must be determined is which interactions are significant enough to have an appreciable effect on the spectrum. Ideally some will be so small that they can effectively be ignored, and this is the case for $^2\text{H}$ spectra. Here the Zeeman Hamiltonian dominates over all other interactions by two or more orders of magnitude. All remaining interactions can be treated as first-order perturbations of the Zeeman interaction, the largest remaining being the quadrupolar interaction which arises because deuterium is a quadrupolar nucleus with $I = 1$. This allows one to rewrite the $^2\text{H}$ nuclear spin Hamiltonian as:

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

(8)

As the research in this thesis is based on the use of the information gathered from measured quadrupolar splittings, the remainder of this section will focus on the nature of the quadrupolar interaction and how it influences the appearance of $^2\text{H}$ NMR spectra.

The quadrupolar Hamiltonian arises as a result of an interaction between the nuclear quadrupolar moment, $eQ$, with the electric field gradient (EFG), $V$, at the nucleus. This interaction arises because quadrupolar nuclei possess a non-spherical charge distribution. The quadrupolar interaction is often expressed as:
\[
\hbar^{-1} H_Q = \frac{eQ}{4I(2I - 1)} (\mathbf{I} \cdot \mathbf{V} \cdot \mathbf{I})
\]

where \(e\) is the elementary charge, \(Q\) is the scalar quadrupolar moment, \(\mathbf{I}\) is the spin angular momentum vector and \(\mathbf{V}\) is the EFG tensor. Thus, the quadrupolar Hamiltonian is a scalar product of two tensors, both of which are second rank. The nuclear spin angular momentum tensor can be replaced by \(I_z\), as it is only the \(z\)-component of this tensor that interacts with the EFG tensor. The EFG tensor is a traceless second-rank tensor having five independent components, \(V_{ij}\), in an arbitrary reference frame. However, this tensor is better expressed in terms of a molecule-referenced principal axis system (PAS), and when this is done it becomes diagonal and takes the form:

\[
\begin{pmatrix}
V_{11} & 0 & 0 \\
0 & V_{22} & 0 \\
0 & 0 & V_{33}
\end{pmatrix}
\]

The nuclear spin angular momentum tensor is expressed in terms of a laboratory reference frame and in order for the EFG tensor to be compatible with the nuclear spin angular momentum tensor, they both must have the same frame of reference. The problem then becomes one of expressing \(V_{33}\) in terms of the laboratory-frame elements \(V_{xx}, V_{yy}\) and \(V_{zz}\). If \(V_{ij}\) are expressed in Cartesian coordinates then it is possible to accomplish the transformation through use of either a rotation matrix (Rose, 1957) or direction cosines. Instead, if \(V_{ij}\) are expressed in spherical coordinates, Wigner rotation matrices can be used to accomplish the
Regardless of which way the transformation is performed the result can generally be expressed as:

\[
\begin{pmatrix}
V_{xx} & V_{xy} & V_{xz} \\
V_{yx} & V_{yy} & V_{yz} \\
V_{zx} & V_{zy} & V_{zz}
\end{pmatrix}
\] (11)

When expressed in a laboratory-referenced manner there are two parameters by which \(V_{ij}\) can be characterized. First is the largest principal component of the tensor, \(V_{zz} = e_{zz}\) and the second is the asymmetry parameter, \(\eta = (V_{xx} - V_{yy})/V_{zz}\). This follows the convention that \(|V_{zz}| \geq |V_{yy}| \geq |V_{xx}|\), as well as the Laplacian condition that \(V_{xx} + V_{yy} + V_{zz} = 0\). In order to determine which component(s) are observable in NMR it must be determined which one(s) commute with the Zeeman Hamiltonian. That is, which one(s) are simultaneous eigenfunctions of both \(\mathcal{H}_z\) and \(\mathcal{H}_Q\). It turns out that \(V_{zz}\) is the only observable component. This allows the quadrupolar Hamiltonian to be rewritten as:

\[
\hbar^{-1} \mathcal{H}_Q = \frac{eQ}{4I(2I - 1)} \left[(3I_z^2 - I^2 + \eta(3I_x^2 - 3I_y^2)) V_{zz}\right] (12)
\]

There is a particularly useful simplification that can be taken advantage of to simplify the treating of quantum mechanical problems of this sort and it is based on the relative sizes of the Zeeman and quadrupolar interactions. When in a suitably high magnetic field the Zeeman
approximation makes it possible to disregard the nonsecular term, $\hbar(I_x^2 - I_y^2)$, from the above equation. This allows the quadrupolar Hamiltonian to be rewritten as:

$$\hbar^{-1} \mathcal{H}_Q = \frac{eQ}{4I(2I - 1)} (3I_Z^2 - I^2) V_{zz}$$

(13)

In trying to make the appropriate substitutions to be able to solve for the energies of the different spin states, an expression for $I_z$ is required. By defining the angle $\theta$ as that between the C-$^2$H bond axis and the EFG tensor ($V$), $I_z$ can be expressed as:

$$I_z = I_z \cos \theta + I_x \sin \theta$$

(14)

By substituting into equation (13) the truncated Hamiltonian becomes:

$$\hbar^{-1} \mathcal{H}_Q = \frac{e^2 qQ}{8I(2I - 1)} [(3\cos^2 \theta - 1) + \eta \sin^2 \theta \cos 2\varphi][3I_Z^2 - I^2]$$

(15)

where $V_{zz} = eq$. This can then be incorporated into the total Hamiltonian with the Zeeman Hamiltonian, giving:

$$\mathcal{H} = -\gamma \hbar \mathbf{I} \cdot \mathbf{B}_o + \frac{e^2 qQ}{8I(2I - 1)} [(3\cos^2 \theta - 1) + \eta \sin^2 \theta \cos 2\varphi][3I_Z^2 - I^2]$$

(16)

It is now possible to solve for the energies of the relevant spin states. It is obvious that in the
states. They are degenerate. For deuterium $I = 1$, allowing three possible energy levels, corresponding to $m$ values of -1, 0 and 1. They have the following expressions:

$$E_{+1} = -\gamma B_o + \frac{e^2qQ}{8} [(3\cos^2\theta - 1) + \eta\sin^2\theta \cos2\varphi]$$ (17) 

$$E_o = \frac{e^2qQ}{8} [(3\cos^2\theta - 1) + \eta\sin^2\theta \cos2\varphi]$$ (18) 

$$E_{-1} = +\gamma B_o + \frac{e^2qQ}{8} [(3\cos^2\theta - 1) + \eta\sin^2\theta \cos2\varphi]$$ (19) 

Knowing the expressions for the energies it is now possible to solve for the resonance energies, following the selection rule $\Delta m = \pm 1$. They are as follows:

$$h \nu^+ = (E_{-1} - E_o) = \gamma B_o + \frac{3e^2qQ}{8} [(3\cos^2\theta - 1) + \eta\sin^2\theta \cos2\varphi]$$ (20) 

$$h \nu^- = (E_o - E_{+1}) = \gamma B_o - \frac{3e^2qQ}{8} [(3\cos^2\theta - 1) + \eta\sin^2\theta \cos2\varphi]$$ (21) 

The interpretation of this result in terms of the observed spectrum is quite simple. One would
with a frequency difference between the two lines of

\[
\Delta v_Q = \frac{3e^2 qQ}{4h} [3\cos^2 \theta - 1] + \eta \sin^2 \theta \cos 2\phi
\]  

(22)

where \(\Delta v_Q\) is the quadrupolar splitting, and the term \(e^2 qQ/h\) is the static quadrupolar coupling constant. Now that the transition frequencies are known it remains to discuss the appearance of the spectrum. Is it two sharp resonance lines, two very broad ones, or somewhere in between? The deuterium spectrum lineshape is very distinctive, and it is capable of providing additional information.

The conclusion that NMR spectra of \(I = 1\) nuclei would consist of two resonance lines equally spaced about the Larmor frequency represents the less complicated extreme. It refers to homogenously oriented samples where all nuclei have the same orientation in the magnetic field, as is the case for single crystals. This is often an ideal, but unreasonable situation. For deuterium studies of lipid bilayers it is not possible. Instead, hydrated lipids are present as lyotropic liquid crystals, either in a lamellar or hexagonal phase. They are very fluid yet have a distinctive ordering, implying that conventional solution NMR techniques would be inadequate. The result is that a spectrum of such a sample (containing a single deuterium nucleus) would reflect the fact that the nuclei are present in all possible orientations, a so-called powder-type spectrum. The question then becomes one of determining what the effect of orientation is on the sample.

The simplest case, and the only one to be covered here, is that of axial symmetry, where
there are $N$ nuclei distributed over the surface of a sphere of radius $r$, giving the number of nuclei per unit area as $N/4\pi r^2$. In order to determine the fraction $dN$ oriented at an angle $\theta + d\theta$ with respect to $B_0$, one has to multiply the spin surface density by the area of the zone of the sphere, $2\pi r^2 \sin \theta d\theta$:

$$dN = \left(\frac{N}{4\pi r^2}\right) 2\pi r^2 \sin \theta d\theta = \frac{1}{2} \sin \theta \tag{23}$$

This gives a probability density $p(\theta)$ of

$$p(\theta) = \frac{1}{2} \sin \theta \tag{24}$$

It is known that there will be a variation in the intensity of the spectrum as a function of the orientation angle. Knowing the expressions for the dependence of the resonance frequency the variation of resonance frequency can be shown as

$$v_{+/−} = \gamma B_0 \hbar +/− \frac{3e^2 qQ}{4\hbar} \left[(3\cos^2 \theta − 1)/2\right] \tag{25}$$

A more clear means of viewing the orientation dependence can be shown by introducing a reduced resonance frequency $\xi_{\pm}$

$$\xi_{\pm} = \frac{v_{+/−} − (\gamma B_0 \hbar)}{(3/4)(e^2 qQ/\hbar)} = +/− \frac{(3\cos^2 \theta − 1)}{2} \tag{26}$$

where $1 ≥ \xi_+ ≥ −1/2$ and $−1 ≥ \xi_- ≥ 1/2$. (Seelig, 1977). By substituting into the probability...
which indicates that there are two resonances. By plotting \( p(\xi) \) versus \( \xi \) one obtains a plot that resembles a deuterium spectrum devoid of broadening interactions. This is seen in figure 3.

An actual experimental deuterium spectrum is shown in figure 4 and it is a characteristic Pake doublet, symmetric about the deuterium resonance frequency. There are two key features that deserve comment. First is the location and spacing of the two points of highest intensity. The separation between these points is referred to as the quadrupolar splitting, \( \Delta v_Q \), and it is this feature that is often measured and used as the main source of information for the sample. The maximum possible value of \( \Delta v_Q \) for aliphatic \( ^2H \) is 127.5 kHz, a value known as the static quadrupolar splitting. In addition, these peaks correspond to nuclei whose orientation is perpendicular to the applied magnetic field; i.e.: \( \theta = 90^\circ \). The second feature of note is the location and intensity of the “shoulders” of the spectrum. These are the areas of lowest intensity and correspond to nuclei which are oriented parallel to the applied magnetic field; i.e.: \( \theta = 0^\circ \).

Other regions of the spectrum vary in intensity as a function of the orientation of the nuclei (or more specifically the \( C-^2H \) bond vector), and it can be shown that the intensity follows

\[
I(\nu) = \frac{1}{2}[ (V_{\|} - V_{\perp}) (V_{\perp} - V_{\parallel}) ]^{-1/2}
\]  

Comparing figures it is apparent that there is a significant difference between what would be expected and what is observed. The experimental spectrum exhibits a certain degree of breadth not present in the theoretical calculation. The source of this broadening lies in the
Figure 3: Plot of probability density as a function of the reduced resonance frequency, illustrating the theoretical powder pattern for a $^2$H nucleus. Dashed lines indicate the individual components of the two allowed transitions and the solid lines indicate the sum of the two components.
Figure 4: An example of a typical experimental $^2\text{H}$ NMR spectrum. The quadrupole splitting, $\Delta v_Q$, is the separation between maxima of the signal. These maxima correspond to $\text{C}^\text{2H}$ orientations perpendicular to $B_0$, while the well defined shoulders result from $\text{C}^\text{2H}$ orientations parallel to $B_0$. 
produce a lineshape that can be approximated as Gaussian or Lorentzian. There are several sources to this broadening, an important one being the longitudinal relaxation time, \( T_2 \), which is a reflection of the particular environment of the nucleus under observation.

The discussion up to this point is valid for either a single crystal or for a polycrystalline one, but neither of these accurately reflects the state of lipid membranes. The reason for this is that lipid membranes are often in a liquid-crystalline state and this is the case for the measurements made here. Being liquid crystals implies that these molecules have both fluidity and order, the latter being the result of having long rod-like molecules aligned with their long axes parallel. Such order is not present in a powder sample. The fluid nature of liquid crystals arises because there are still several motions available to the molecules that are not available to powder samples.

Of those motions that are permitted, the most important one is the rapid rotation of the molecule about its long molecular axis (often denoted as \( z' \)), this axis being perpendicular to the plane of the membrane. There are also minor motions perpendicular to \( z' \) that are permitted, though these are generally much more restricted. The effect of rotation about the \( z' \) axis is to partially average the dominant interactions, namely the quadrupolar interaction. This motional averaging, because it results from rotations that are fast on the \(^2\text{H} \) NMR time scale, results in the EFG not being completely averaged. That portion not averaged out is manifest as the residual quadrupolar splitting, which is the value that is measured experimentally.

In order to be able to accurately account for this motion, adjustments need to be made to the expression for \( \Delta v_Q \) seen previously. This is best done by defining an average order of the
molecule-based coordinate axis (x, y, z) as seen in figure 5. Here the angle $\theta$ represents the angle between $z'$ and the C-2H bond axis, $\phi$ is the angle in the xy plane between the x-axis and the C-2H bond axis and $\beta$ is the angle between the external magnetic field, $B_o$, and the director axis, $z'$.

Since one is attempting to describe the average molecular order, it is appropriate to introduce the fluctuating order parameter, $S_f$. This parameter is a measure of the average molecular fluctuations about the director axis and is therefore a reflection of the order of the system. It is a second rank tensor that can be expressed as

$$S_f = (1/2) (3\cos^2\theta - 1)$$

(29)

with $\theta$ defined as above. The limits of the range of values for $S_f$ can be easily determined knowing that $\Sigma \cos^2\theta_i = 1$ and that $0 \leq \cos^2\theta_i \leq 1$. This implies that $\Sigma S_i = 0$ and that $-1/2 \leq S_i \leq 1$.

In order to incorporate these molecular fluctuations into the expression for the quadrupolar splitting it is necessary to express the EFG tensor in terms of the new molecular coordinate system. In the same manner as discussed earlier, this can be accomplished using rotation matrices (Rose, 1957). Once the transformations and substitutions are made one obtains a new expression for the quadrupolar splitting:

When comparing this expression to that seen in equation (23) it becomes apparent that the effect of motional averaging is to reduce the static quadrupolar splitting by the factor $S_f$, this
Figure 5: Representation of the orientation of the director axis ($z'$) of a lipid molecule with respect to the C-$^2$H bond axis and the external magnetic field, $B_0$. 
being a function of the different angles discussed above. In particular, when $\beta$ is equal to $54.74^\circ$, $\Delta v_Q$ collapses to zero. This fact precipitated the naming of this angle as the "magic angle."

\[ \Delta v_Q = \frac{(3/2) \left( 3e^2 q Q \right) (3 \cos^2 \beta - 1)}{h} S_f \]  

1.7 The Molecular Voltmeter Technique

The study of structural characteristics of lipid molecules and membranes has often been a difficult task due to the lack of techniques capable of determining membrane structural information. Despite the fact that the chemical structures of most lipid molecules have been known for some time, it wasn’t until 1974 that the first crystal structure of a phospholipid was obtained, that being for phosphatidylethanolamine (Hitchcock et al., 1974). Those for phosphatidylcholine (Pearson & Pascher, 1979) and phosphatidylglycerol (Pascher et al., 1987) have also been solved. The most important information garnered from these studies are the facts that the head group dipoles are aligned at approximate right angles to the hydrocarbon chains and that there are two different head group conformations. The problem with these studies was a lack of information about the structures when the lipids are in an aqueous environment. Neutron diffraction studies on model oriented membranes using deuterated lipids were able to show that the head groups are aligned approximately parallel (within $30^\circ$) to the plane of the membrane (Büldt et al., 1978; Büldt & Seelig, 1980 and Mischel et al., 1987).
membranes, one has to use another technique. Solid state $^2$H NMR has become one of the best methods to accomplish this, especially when one is concerned with membrane surface electrostatics. One reason for the widespread use of $^2$H NMR in such studies is that the replacement of $^1$H with $^2$H is essentially non-perturbing and a wide variety of deuterated lipids are available commercially, while many others can be synthesized. When considering electrostatics, it has been well documented that phosphatidylcholine (PC) is extremely sensitive to membrane surface charge (Seelig et al., 1987; Scherer & Seelig, 1989; Roux et al., 1989; Macdonald et al., 1991; Marassi & Macdonald, 1991; Rydall & Macdonald, 1992, Marassi & Macdonald, 1992). These observations have all been based on the use of PC that has been selectively deuterated on the choline portion of the head group, allowing for the known conformational changes in PC head group to be manifest through the observed quadrupolar splitting ($\Delta v_Q$).

The ability to use the PC headgroup as a means of garnering structural and electrostatic information is the result of its profound response to membrane surface charge. In a recent review on the use of $^2$H NMR in studying membrane electrostatics (Macdonald, 1997), an example was presented that illustrates the response of the PC headgroup to membrane surface charge, which is presented here in figure 6. This figure illustrates the two most important features of $^2$H NMR spectra of the PC headgroup selectively deuterated at the alpha and beta positions (labeling positions are illustrated in Experimental section). First, there is a profound change in the measured quadrupolar splitting upon the addition of surface charge, in this case by the addition of either positive (DOTAP) or negative (POPG) lipid. Second is the observation
Figure 6: $^3$H NMR spectra indicating the response of the PC headgroup to membrane surface charge as a result of adding either POPG (anionic) or DOTAP (cationic). Spectra on the left are for POPC-α-d$_2$ and spectra on the right are for POPC-β-d$_2$. 
Figure 7: Schematic representation of the “choline tilt” model, indicating the changes undergone by the headgroup as a response to membrane surface charge. The choline headgroup dipole is shaded. Predicted orientations as a result of the illustrated membrane surface charge are indicated.
positions. Upon the addition of negative surface charge \( \Delta v_Q \) increases for PC labelled at the alpha position, while \( \Delta v_Q \) decreases for PC labelled at the beta position.

A possible explanation for this counter-directional response was proposed to be that the PC headgroup undergoes specific conformational changes in response to membrane surface charge (Brown & Seelig, 1977, Akutsu & Seelig, 1981). A general change in the order parameter \( S_r \) was ruled out since such a change would affect the magnitude of the quadrupolar splitting, but not the direction in which it changed. In fact, a change in \( S_r \) would act in the same fashion regardless of the labeling position. It would not be counter-directional. In addition, the source of the surface charge appears not to be critical in the response. Merely the presence of surface charge is all that is necessary. Such a universal response to membrane surface charge has resulted in its being termed the “molecular voltmeter.”

In order to be able to infer structural and electrostatic information based on the experimentally observed quadrupolar splittings one must have a reasonable explanation of what causes the conformational change of the PC. To date this is best provided by the “Choline Tilt” model of Scherer & Seelig (1986), which is presented schematically in figure 7. The key points in understanding the model are the relative orientation of the P-N vector, and the effect of surface charge on this orientation. The reason for the high sensitivity of PC to surface charge is the positively-charged quaternary nitrogen at the end of the head group. In hydrated membranes with no net surface charge the P-N vector is aligned to within 30° of the membrane surface. If the surface acquires a net negative charge, either by the inclusion of negatively-charged lipids or the adsorption of negatively-charged electrolytes, then the positively-charged choline nitrogen is
positive charge; the head group will be pushed away from the surface. These changes in the choline tilt are indicative of the P-N dipole attempting to align with the electrical field gradient emanating from the membrane surface (Roux, et al., 1989).

Knowing that this happens is one thing, but using $^2$H NMR to observe it is another. When PC is selectively deuterated at either the $\alpha$ or $\beta$ position (see Experimental), the spectrum that is observed is a reflection of the orientation of the C-$^2$H bond vector with respect to the external magnetic field. With no net surface charge the values of $\Delta v_Q$ are nearly the same for both positions. When there is either a net positive or negative surface charge the PC reorients, which therefore reorients the different C-$^2$H bond vectors and changes the observed $\Delta v_Q$. However, the $\alpha$ and $\beta$ deuterated positions change their $\Delta v_Q$s in opposite directions. For increasing negative charge the $\alpha$-labelled PC $\Delta v_Q$ increases, while for the $\beta$-labelled PC $\Delta v_Q$ decreases. The opposite is true for a net positive surface charge. This counter-directional change allows one to confirm the role of electrostatics in a given interaction, and to be able to quantitate the interaction to some extent.

It must be pointed out that the choline tilt model is just that: a model. It has yet to be proven conclusively. It was observed that in mixtures of PC + anionic lipid + cationic lipid, such that the overall membrane surface charge was neutral, the quadrupolar splitting was not the value expected for pure PC membranes (Marassi & Macdonald, 1992). This suggests that the PC molecules don’t actually sense the overall membrane surface charge, but instead sample individual encounters with charged lipid molecules. Each of these produces a distinctive change in the conformation of particular PC molecule, and the $^2$H NMR spectrum reports the average of
such encounters. Furthermore, other factors have been seen to have an effect on the quadrupolar splitting that mimics an electrostatic effect. Such effects include hydration (Bechinger & Seelig, 1991; Ulrich & Watts, 1994) and hydrostatic pressure (Bonev & Morrow, 1995).

As mentioned previously, the molecular voltmeter effect has been used to measure the binding of metal ions to neutral and charged membrane surfaces containing PC. In addition, studies of the binding of larger electrolytes have been carried out, including anaesthetics (Boulanger et al., 1981), peptides (Roux et al., 1989; Beschiaschvili & Seelig, 1991), synthetic polyelectrolytes (Crowell & Macdonald, 1997) and nucleic acids (Mitrakos & Macdonald, 1996). It is also possible to use unilamellar vesicles and observe trans-bilayer inhomogeneities (Marassi et al., 1993). These can be used to measure such things as the partitioning of charged across bilayers in response to transmembrane potential (Franzin & Macdonald, 1996) as well as the fusion of small unilamellar vesicles with large ones (Franzin & Macdonald, 1997).

1.8 Objectives

The ability of $^2$H NMR to study membrane surface electrostatics presents a host of possible interactions to investigate. This thesis will be generally concerned with the interactions of positively-charged polyelectrolytes with lipid membranes with a net negative surface charge. Several different polyelectrolytes will be surveyed to determine if there is an interaction that is observable by means of $^2$H NMR. Included in this survey will be some conventional polyelectrolytes, such as poly(lysine), as well as a representative from a relatively new class of molecules called starburst dendrimers.

In addition, the interaction of one polyelectrolyte with lipid membranes will be
shown to have a profound and unique interaction with negatively-charged lipid membranes. This interaction is indicative of the formation of distinct domains that are a function of the relative amounts of cationic and anionic species present. It is possible to characterize these domains in terms of their exact composition, leading to the ability to make some conclusions about the nature of the interaction.
2.1 Materials

Solvents. Anhydrous pyridine was obtained by pre-drying over calcium hydroxide, then by refluxing over calcium hydride, followed by the addition of sodium hydride and further refluxing. HPLC grade THF was purified through the dropwise addition of ferrous sulphate (approximately 20 mL per litre of THF) to remove peroxides. Dryness was ensured by first pre-drying THF over potassium hydroxide, followed by reflux over sodium metal. Benzophenone was used to indicate the degree of dryness. All other solvents used were HPLC grade.

Reagents and Chemicals. 2,3,5-trisisopropylbenzene sulfonyl chloride (TPS), purchased from Aldrich (Milwaukee, WI), was recrystallized from n-pentane containing 1% thionyl chloride (freshly distilled). It was then dried overnight by vacuum dessication. Deuterated NMR solvents were from MSD Isotopes (Montreal, PQ). 4-(2-Hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) was from BDH (Toronto, ON). All other chemicals were from Aldrich.

Phospholipids and Polymers. All non-deuterated lipids were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Deuterated phospholipids were synthesized as subsequently outlined. Poly(vinylbenzyltrimethylammonium chloride) (PVTA) (MW 40000, N 190) and ethylene diamine core starburst dendrimer generation 6 (MW 58,000, size 72 Å, designated SD 72) were from Polysciences Inc. (Warrington, PA). SD 72, which is insoluble in aqueous solution, was converted to its protonated form by the addition of ethanolic HCl. Poly(ethyleneimine) (as the HCl salt) (PEI)
poly(lysine) (as the HBr salt) (PLYS)(MW 150,000, N 650) were from Aldrich.

2.2 Synthesis of Deuterated Phosphatidylcholines

1-Palmitoyl-2-oleyl-sn-glycero3-phosphocholine (POPC) was specifically deuterated at either the α (POPC-α-d2) or β (POPC-β-d2) position of the choline segment of the headgroup. The structure of POPC with the deuterated positions labeled, is shown below.

\[
\begin{align*}
R - O - P - O - CH_2 - CH_2 - & + \quad CH_3 (CH_3)_3 \\
\alpha & \beta
\end{align*}
\]

The choline deuteration was carried out by modifying the methods of Harbison and Griffin (1984) and Aloy and Rabout (1913). Details of the syntheses will follow. The deuterated cholines are isolated as the TPB salts, which are insoluble in water and soluble in pyridine. This permits precipitation of the choline-TPB salt from water, after which it can be readily dissolved in pyridine for the remainder of the synthesis. Once deuterated, the headgroup was coupled to POPA, using TPS as the condensing agent (Aneja, et al, 1970).

2.2.1 Synthesis of Choline-α-d₂ TPB. The synthesis was carried out as outlined in Scheme 2a using anhydrous tetrahydrofuran (THF). All glassware used in this and subsequent reactions is dried overnight at 180°C prior to use.
The synthesis commenced with reduction of the N,N-dimethylglycine ethyl ester with LiAlD₄ under argon. This reaction was carried out in a three-neck 250 mL round bottom flask equipped with a sidearm addition funnel and a refluxing condenser. 2 g (48 mmol) of LiAlD₄ were first suspended in 100 mL of anhydrous THF and stirred until homogenous. 10 mL (70.7 mmol) of N,N-dimethylglycine ethyl ester (dissolved in 50 mL of anhydrous THF) were added dropwise to the LiAlD₄ suspension over the course of 45 minutes. The mixture was stirred and gently refluxed for 1 hour, followed by the slow addition of 9 mL of distilled H₂O (dH₂O) to decompose any unreacted LiAlD₄. The solution was stirred for approximately one hour, or until gas evolution ceased. Inorganic salts were trapped by the addition of a large amount of Na₂SO₄, followed by 10-15 minutes of stirring. The inorganic salts were removed by vacuum filtration.
washing was done with 300-400 mL of diethyl ether. The filtrate contained product (I), and its volume was reduced to approximately 100 mL by rotary evaporation.

The product (I) was then methylated by adding 240 mmol of iodomethane and stirring overnight at room temperature. The flask was covered with tin foil to prevent unwanted decomposition of the iodomethane. Once the reaction was complete the solution was evaporated to a small volume by rotary evaporation and taken up in approximately 100 mL of dH₂O and washed with 100 mL of diethyl ether. Now the product must be converted from the water-soluble iodine form to the water-insoluble TPB form. This was accomplished by the addition of 34 g of sodium TPB in 150 mL of dH₂O. This yields choline-α-d₂ TPB (II) as a fine white precipitate which is isolated through centrifugation for 20 minutes at 4000xg, and is then washed 3-4 times with 100 mL of dH₂O. The salt was dried azeotropically with ethanol/toluene (75/20 v/v). Typically the product was recrystallized twice from hot acetonitrile to produce large, translucent hexagonal crystals. Yield: 19 g (75%). ¹H NMR (200 MHz) was used to characterize the product (refer to Table 1).
\[
\begin{array}{|c|c|c|}
\hline
\delta (\text{ppm}) & \text{observed peak, area} & \text{assignment} \\
\hline
3.10 & \text{singlet, 9H} & \text{choline methyl} \\
3.35 & \text{triplet, 2H} & \beta-\text{CH}_2\text{-choline} \\
5.20 & \text{multiplet, 1H} & \text{choline OH} \\
6.80 & \text{multiplet, 4H} & \text{TPB para protons} \\
6.90 & \text{multiplet, 8H} & \text{TPB meta protons} \\
7.20 & \text{multiplet, 8H} & \text{TPB ortho protons} \\
\hline
\end{array}
\]

2.2.2 Synthesis of Choline-\(\beta\)-d\(_2\) TPB. The synthesis was carried out as outlined in Scheme 2b using anhydrous tetrahydrofuran (THF). The first step in making choline-\(\beta\)-d\(_2\) is the synthesis of cyanomethylbenzoate (I). This was carried out in a 250 mL round bottom flask by dissolving 7 g (143 mmol) of NaCN in 25 mL of dH\(_2\)O and stirring while cooling to 0°C in an ice bath. 11 mL of a 37% formalin solution (146 mmol formaldehyde) were added dropwise to the cyanide solution, followed by the dropwise addition of 16 mL (143 mmol) of benzoyl chloride. This mixture is stirred vigourously for 2 hours at room temperature. The product cyanomethylbenzoate (I) was isolated by extracting with 5 x 100 mL of diethyl ether, pooling the ether layers and washing with 300 mL of 0.1 M NaOH. Rotary evaporation and vacuum distillation (160°C at 10 torr) yielded cyanomethylbenzoate as a clear, colourless solution that crystallized as long needles when placed in the freezer. Yield: 20 g, 86%. \(^1\)H NMR (200 MHz) in chloroform-d was used to characterize the product: \(\delta = 5.10\) ppm, singlet, \(^3\)H CH\(_2\); \(\delta = 7.50\) ppm, multiplet, 5H benzyl protons.
Scheme 2b: Synthesis of Choline-β-d₂

The next step is the synthesis of ethanolamine-β-d₂ from cyanomethylbenzoate. 4 g (95 mmol) of LiAlD₄ is suspended in 100 mL of anhydrous THF in a 250 mL three neck round bottom flask and stirred until homogenous. 9.5 g (59 mmol) of cyanomethylbenzoate in 50 mL of anhydrous THF were added dropwise to the LiAlD₄, through a sidearm addition funnel, over the course of one hour. The mixture is gently refluxed for 3 hours. Excess LiAlD₄ was decomposed by the addition of 4 mL of dH₂O, followed by 4 mL of 15% (w/v) NaOH and 12 mL of dH₂O. Insoluble inorganic salts were trapped with Na₂SO₄, as in the synthesis for choline-α-
The methylation of ethanolamine-β-d₂ was carried out by the addition of 50 mL of 10% (w/v) NaOH to the above filtrate, followed by 15 mL (240 mmol) of iodomethane. The reaction was stirred overnight in the dark and then rotary evaporated down to a small volume. The residue was diluted to approximately 100 mL with dH₂O, at which point the by-product benzyl alcohol had to be removed. This was accomplished by performing 3-5 extractions with 100 mL of diethyl ether. Complete removal of the benzyl alcohol is monitored by ¹H NMR. Now the product must be converted from the water-soluble iodine form to the water-insoluble TPB form, just as in the synthesis of choline-α-d₂ TPB. Yield: 3.5 g (14%). ¹H NMR (200 MHz) was used to characterize the product (refer to Table 2).

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<th>δ (ppm)</th>
<th>observed peak, area</th>
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</tr>
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</tr>
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</tr>
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<td>multiplet, 4H</td>
<td>TPB para protons</td>
</tr>
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<td>6.90</td>
<td>multiplet, 8H</td>
<td>TPB meta protons</td>
</tr>
<tr>
<td>7.20</td>
<td>multiplet, 8H</td>
<td>TPB ortho protons</td>
</tr>
</tbody>
</table>

2.2.3 Synthesis of POPC. Both POPC-α-d₂ and POPC-β-d₂ were synthesized in the same fashion from their respective headgroups, as outlined in Scheme 2c. It was essential that
all glassware and reagents be extremely dry. The reaction was carried out by first dissolving 800 mg of POPA (1.12 mmol) in 50 mL of anhydrous pyridine and stirring in a 250 mL round bottom flask at 35°C until solution became clear. The flask was dried under argon to ensure dryness. 900 mg (3 mmol) of TPS are added to the POPA solution and stirred a further 20 minutes. The TPS to be used must be recrystallized from n-pentane containing 1% thionyl chloride and dried in a dessicator overnight before using. 870 mg (2 mmol) of the deuterated choline TPB were then added and the mixture is stirred at 35°C for 4 hours, at which time the excess TPS was decomposed by the addition of 2 mL of dH₂O. The pyridine solution was then rotary evaporated to dryness, leaving a residue that was dried azeotropically with approximately 50 mL of toluene. Insoluble materials were separated from the product (III) by dissolving the POPC in 50 mL of trichloroethylene (TCE) and vacuum filtering with three Whatman #1 filter papers and washing extensively. The TCE filtrate was rotary evaporated to dryness and the POPC residue was then dissolved in 40 mL of TCE/methanol (1/1 v/v) and transferred to a 150 mL centrifuge tube. At this point the POPC was extracted from the solution using the techniques of Bligh and Dyer (1959) and McMurray (1975), as outlined below. To the 40 mL of solution are added in
addition the solution was shaken vigourously. Phase separation was accomplished by centrifuging for 15 minutes at 4000xg at room temperature. The upper aqueous phase was aspirated and discarded, while the lower organic phase was washed three times with 15 mL portions of TCE/methanol/0.9% NaCl (3/48/47). This was rotary evaporated to dryness.

Further purification of the lipid was achieved by dissolving it in chloroform/methanol (1/1 v/v) and passing it through an Amberlite mixed-bed ion exchanger. Approximately 100 g of the resin was prewashed with methanol, followed by exchange with 500 mL of chloroform/methanol (1/1 v/v). Elution of POPC was monitored by TLC (Kieselgel 60) using chloroform/acetone/methanol/acetic acid/dH2O (10/4/4/2/1) as the solvent system (Spanner, 1973). Iodine was used to visualize the TLC plates. It was often necessary to remove unreacted POPA, which was accomplished by dissolving the lipid residue in chloroform and passing it through a silicic acid column (approximately 50 g of resin) and eluted with chloroform/methanol (1/1 v/v). If the by-product lyso POPC was suspected to be present it was removed by passing the lipid through a carboxymethyl cellulose column (Whatman CM52) of 100 mL bed volume and eluting with chloroform/methanol (96/4 v/v) as described by Comfurius and Zwall (1977). All fractions containing POPC were pooled and dried.

The last purification step involved acetone precipitation (Kates, 1972). The POPC residue is first dissolved in chloroform, and the volume of this solution is reduced to approximately 1 mL. Acetone is added to a final volume of approximately 10 mL, and gentle heating and vortexing is used to ensure all of the POPC dissolves. This was placed in the freezer overnight to allow precipitation of the POPC as a white fluffy powder, after which it was
Characterization of the final product was done by TLC as described previously, as well as by \(^1\)H and \(^2\)H NMR spectroscopy. TLC should show one spot with an \(R_f\) identical to that of pure POPC, which is approximately 0.25. \(^2\)H NMR spectroscopy is used to measure the quadrupolar splitting. For POPC-\(\alpha\)-d\(_2\) and POPC-\(\beta\)-d\(_2\) the splittings are 6.4 kHz and 5.8 kHz respectively. Refer to Table 3 for characterization of POPC by \(^1\)H NMR (200MHz). Yield: POPC-\(\alpha\)-d\(_2\): 610 mg (85%); POPC-\(\beta\)-d\(_2\): 620 mg (87%).

<table>
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<td>3.25</td>
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</tr>
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<td>glycerol-(\text{CH}_2)-O-C</td>
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<td>5.20</td>
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<td>glycerol (\text{R}_2)-(\text{CH}_2)-O</td>
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</tbody>
</table>

### Table 3

\(^1\)H NMR (200 MHz) assignment of deuterated POPC in chloroform-d

#### 2.3 Preparation of Lipid Vesicle/Polyelectrolyte Samples

All vesicles used in this work were multilamellar vesicles (MLVs). Their preparation typically involved mixing 6-10 mg of deuterated POPC from a stock chloroform solution with an
a desired lipid composition. The samples were gently vortexed to ensure a homogenous mixture, followed by evaporation under a stream of argon and storage in a dessicator for 12-16 hours. Once dry, the lipids were dispersed in approximately 100-150 µL of buffer, typically 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2ethanesulphonic acid) or 10 mM citrate at a controlled pH. Samples were gently vortexed and heated to approximately 40°C, followed by 4-7 cycles of freezing and warming to ensure homogenous mixing. For those samples containing polymer the process was nearly identical, the only difference was that the polymers used were made in stock solutions of the appropriate buffer and pH, allowing for hydration of the dried lipid with either the polymer solution alone, or a combination of polymer and buffer solutions, to achieve a total added volume of 100-150 µL. All polymers were used as supplied by the manufacturer with the exception of the starburst dendrimer SD72. This molecule is uncharged and is shipped in methanol solution. In order to ensure it was positively-charged prior to use protonation was accomplished by addition of a slight molar excess of methanolic HCl, precipitating SD72 as the HCl salt. This precipitate was isolated and dissolved in aqueous solution buffered to pH 5.6 in sodium citrate buffer. This was based on the pKa of SD72 being approximately 6.7. Samples were transferred to 5 mm glass NMR tubes for measurements.

2.4 Solid State NMR Measurements

2.4.1 $^2H$ and $^{31}P$ Measurements. All solid state spectra were recorded on a Chemagnetics CMX300 NMR spectrometer. $^2H$ NMR spectra were acquired with the spectrometer operating at 45.98 MHz, using the quadrupolar echo sequence (90x - τ - 90y - τ -
the pulse pairs (Griffin, 1981). A single channel wideline Chemagnetics probe was used, equipped with a 5 mm solenoid coil. Typical experimental conditions were as follows: 90° pulse length of 2 μs, an interpulse delay of 30 μs, a recycle delay of 100 ms, a spectral width of 100 kHz and a data size of 2K.

Static $^{31}$P spectra were acquired with the spectrometer operating at 121.25 MHz using a Hahn echo sequence (Rance & Byrd, 1983) with proton decoupling during acquisition. Measurements were performed with a 2-channel Chemagnetics magic angle spinning (MAS) probe capable of holding the same 5 mm sample tubes used for the $^2$H measurements. Typical experimental conditions were as follows: 90° pulse length of 6.5 μs, an echo spacing of 40 μs, a recycle delay of 2 s, a spectral width of 100 kHz and a data size of 2K.

2.4.2 Low Temperature Measurements. It was observed that temperature effects were significant for samples containing POPC-β-d$_2$, with better resolution achieved at lower temperatures. Accordingly, many of the spectra for POPC-β-d$_2$ samples were run at 5°C. These measurements were made by using temperature controlling unit and blowing air over the sample. The temperature was controlled to within 0.1°C, and samples were equilibrated at 5°C for 15 minutes prior to beginning the measurements.

2.4.3 Transverse ($T_2$) Relaxation Times. $T_2$ measurements were carried out varying the interpulse delay in the quadrupolar sequence in a stepwise manner to a maximum value appropriate for the particular sample.
Analysis of the $^2$H spectra was facilitated through the use of simulation of the Pake pattern lineshapes. These simulations were accomplished by use of a computer program based on the tiling method of Alderman et al. (1986). Simulations were carried out with several available variables, namely the quadrupolar splitting ($\Delta v_0$), the linewidth parameter $T_2$, and the intensity of given Pake patterns.

2.6 Measurement of Polyelectrolyte Binding

The degree of binding of one particular polyelectrolyte, PVTAl, was determined by means of a difference assay based on UV absorbance. Samples were prepared as detailed previously, except that non-deuterated POPC was used. The samples were brought to a final volume of 500 mL. The samples were then centrifuged at approximately 4000xg for 15 minutes to separate the lipid from the supernatant, which was decanted and diluted such that its UV absorbance fell in a concentration range where the Beer-Lambert relationship was obeyed. The UV measurements were carried out on a Hewlett-Packard 8452A Diode Array Spectrophotometer.

2.7 Differential Scanning Calorimetry (DSC) Measurements

Select samples were subject to DSC measurements on a Perkin-Elmer DSC7 differential scanning calorimeter operating at a scan rate of 5-7°C per minute. Transition endotherms were obtained by heating from -20°C to 40°C. Sample preparation differed somewhat from that used for NMR samples in that buffers/polyelectrolytes were made up in a mixture of dH$_2$O/glycerol (1/1 v/v). This succeeded in suppressing the intense solid/liquid phase transition endotherm
for lipids.
3.1 Calibration for Mixtures of POPC-α-d₂ and POPC-β-d₂ With POPG

In order to be able to properly characterize and quantify any interactions observed between polyelectrolytes and lipid vesicles it was necessary to have a sound knowledge of how both POPC-α-d₂ and POPC-β-d₂ behaved in the presence of different amounts of POPG. The best way to accomplish this was to measure the experimental quadrupolar splittings for different molar mixtures of the two lipids and mathematically determine the relationship between quadrupolar splitting and mol% of POPG. Similar information has been previously determined for mixtures of dimyristoyl PC (DMPC) with dimyristoyl PG (DMPG) (Marassi et al., 1991). However, a useful advantage of using POPC vice DMPC is that the gel-to-liquid-crystalline-phase-transition temperature for POPC is approximately -7°C, while for DMPC it is approximately 24°C. This allows for the acquisition of spectra of liquid-crystalline samples at room temperature.

In carrying out the calibrations the quadrupolar splittings were measured on samples that ranged from zero to 70 mol% POPG in 10% increments. The splittings were plotted versus POPG content and the data were fit mathematically to an appropriate curve such that interpolation was possible. All measurements were initially made at room temperature (22°C - 25°C) and calibration plots are presented in figure 8. It should be noted that the measured splittings are an average. This is due to the fact that the splitting of the pure POPC-α-d₂ and POPC-β-d₂ samples varied somewhat with different syntheses. For POPC-α-d₂ the splitting
Figure 8: Room temperature calibration curves, plus polynomial fittings of the data, for mixtures of POPC + POPG. Upper curve is for POPC-α-d$_2$/POPG MLVs while the lower curve is for POPC-β-d$_2$/POPG MLVs.
Based on early measurements with POPG contents in the vicinity of 20% - 30% (mol/mol) it was expected that the relationship between $\Delta v_Q$ and POPG content would be approximately linear. As can be seen from the data only the beginning of the plots could be considered linear. For POPC-$\alpha$-d$_2$ the plot rises steeply, with an initial slope of approximately 11 kHz/10 mol%, only to reach a maximum at 70 mol% where the quadrupolar splitting reaches 11.0 kHz. For POPC-$\beta$-d$_2$ the plot initially drops steeply, with an initial slope of approximately -13.0 kHz/10 mol%, after which it gradually begins to flatten out, but not to the same extent as seen with POPC-$\alpha$-d$_2$. In fact, up to 70 mol% POPG it is safe to say that a minimum $\Delta v_Q$ has not been reached and it is obvious that there would be a further lowering of $\Delta v_Q$ were the POPG content increased further.

The implication of these results is twofold. First, up to 20 mol% POPG, POPC-$\beta$-d$_2$ is more sensitive than POPC-$\alpha$-d$_2$, as evidenced by the higher magnitude of the slope. Second, POPC-$\beta$-d$_2$ is still sensitive to changes in surface charge density even as high as 70 mol% POPG, while POPC-$\alpha$-d$_2$ is essentially insensitive to any further increase in surface charge density. This observation is most likely related to the relative steric hindrance of the two different locations of $^2$H. For POPC-$\alpha$-d$_2$ the deuterons are closer to the membrane surface and the bulky phosphate groups than is the case for POPC-$\beta$-d$_2$. Therefore, it would be expected that the deuterons of POPC-$\beta$-d$_2$ are less hindered and are able to adopt a wider range of conformations than the deuterons of POPC-$\alpha$-d$_2$.

One last aspect of the calibration studies involved temperature. It was observed
of $\Delta v_0$ (Marassi et al., 1991) and that it seemed to be greater with POPC-\(\beta\)-d\(_2\) than with POPC-\(\alpha\)-d\(_2\). Accordingly, selected samples were run at lower temperatures (5°C - 15°C) to ascertain whether any advantage could be taken of acquiring spectra at lower temperatures. For samples containing POPC-\(\alpha\)-d\(_2\) no significant difference was noticed. However, with samples containing POPC-\(\beta\)-d\(_2\) it was observed that at 5°C the observed values of $\Delta v_0$ were approximately 0.8 - 1.0 kHz greater than at room temperature. The relevant calibration curve is shown in figure 9. All of the data for the three calibration curves could be described empirically by second or third order polynomials of the form $y = A + Bx + Cx^2 + Dx^3$, and a summary of the relevant coefficients is presented in Table 4.

<table>
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<th>Lipid</th>
<th>Temperature</th>
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<th>B</th>
<th>C</th>
<th>D</th>
<th>Correlation</th>
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<td>-</td>
<td>0.998</td>
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Figure 9: Calibration curve, plus polynomial fitting of the data, for POPC-β-d₂/POPG MLVs at 5°C.
Multilamellar Vesicles (MLVs)

At the outset of this research there had not been a great deal of work done using $^2$H NMR to study and/or characterize the interaction of polyelectrolytes with lipid membranes. Two notable exceptions are the work by Roux et al. (1989) and Carbone & Macdonald (1996). The former studied the binding of pentalysine to lipid vesicles with several different techniques, one being $^2$H NMR. The latter used $^2$H NMR to study the binding of the poison cardiotoxin to lipid vesicles with an overall net negative surface charge. Though $^2$H NMR was not a useful tool in the pentalysine work, the fact that the binding of the positively-charged cardiotoxin to negatively-charged lipid membranes could be studied using $^2$H NMR hinted at the potential of being able to study (and possibly model) the binding of other positively-charged polymers to negatively-charged lipid membranes.

Anticipating the ability to spectroscopically observe positively-charged polyelectrolytes binding to negatively-charged lipid vesicles, four different polyelectrolytes were selected for study. These are polylysine (PLYS), poly(methylene-co-guanidine) (PMGU), poly(ethyleneimine) (PEI), poly(vinylbenzyltrimethylammonium chloride) (PVTA) and the starburst dendrimer SD72. Their basic structures are shown in figure 10. A summary of the quadrupolar splittings measured for the linear polyelectrolytes is presented in Table 5.
Figure 10: Structures of the various polyelectrolytes surveyed. The repeating monomer unit is shown for PLYS, PEI, PMGU and PVTA, while a partial two-dimensional representation of the spherical SD72 molecule is illustrated.
<table>
<thead>
<tr>
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with it being a large peptide (like cardiotoxin) it would interact with lipid membranes in a way that would be visible via $^2$H NMR. With this molecule there are relatively long side chains and the positive charge is located well away from the polymer backbone. In figure 11 are shown three representative spectra illustrating that a minor interaction could be observed. The top spectrum is from a control sample of 70/30 POPC-α-$d_2$/POPG, and the two lower spectra are for samples that contain PLYS/POPG ratios of 0.2 and 1.0 respectively. There appears to be a small effect for PLYS/POPG = 0.2, as the quadrupolar splitting decreases from 9.5 kHz to 9.1 kHz. However, the quadrupolar splitting then increases to 10.1 kHz in going to PLYS/POPG = 1.0, yet no evidence of two populations was seen. Similar results were observed when using POPC-β-$d_2$/POPG vesicles. Despite similar observations by Roux et al. (1989) with pentalysine, that there was no significant interaction with this much larger peptide was surprising.

The next compound investigated was PMGU, a polymer that has no side chains whatsoever and with potentially more than one protonation site. A neutral pH of 7.4 was selected for study with the knowledge that typical pKa values for the guanidine group are in the vicinity of 10. At the outset the presence of positive charge on the polymer was of primary importance rather than the number of positive charges. Since this polyelectrolyte has no side chains (unlike PLYS) it has the ability to bind to a membrane in a manner such that the backbone can lie on the membrane surface. Furthermore, this hints that the potential for a stronger electrostatic interaction than with PLYS is possible.

In attempting to study any possible interaction of PMGU with lipid membranes, vesicles with a composition of 70/30 POPC-α-$d_2$/POPG were used with several different ratios of
Figure 11: Representative $^2$H NMR spectra obtained for mixtures of 70/30 POPC-α-d$_2$/POPG plus the addition of PLYS. Upper spectrum is a control with a quadrupole splitting of 9.5 kHz. The middle spectrum has a PLYS/POPG ratio of 0.2 with $\Delta v_Q = 9.1$ kHz while the lower spectrum has PLYS/POPG = 1.0 with $\Delta v_Q = 10.1$ kHz.
9.5 kHz at room temperature. Some representative spectra of what was observed are presented in figure 12. What becomes immediately apparent is that there is a significant interaction in terms of what $^2$H NMR is able to observe. Upon the addition of PMGU in an amount such that the PMGU/POPG ratio is 0.33, $\Delta v_Q$ decreases from 9.5 kHz to a value of 8.4 kHz, a difference of 1.1 kHz. The middle spectrum has a PMGU/POPG ratio of 2.0 and $\Delta v_Q$ decreases further to 7.6 kHz, while with increasing amounts of PMGU $\Delta v_Q$ decreases to approximately 7.4 kHz as shown in the lower spectrum with PMGU/POPG = 5.0. The direction of this change is indicative of a molecular voltmeter response. Confirmation of this was achieved by performing experiments with vesicles containing POPC-β-d$_2$ and noting an increase in $\Delta v_Q$ with respect to the control values.

Having observed such a strong and easily measurable effect, it would have been possible to do a more detailed study of the interaction of PMGU with lipid vesicles. As noted previously however, the molecular voltmeter response has been well documented and it was felt that the potential for discovering something novel in this particular interaction was rather low. Accordingly, this polyelectrolyte was temporarily abandoned in the hope that, upon surveying additional polyelectrolytes, a previously undiscovered manifestation of polyelectrolyte/lipid interaction would be identified.

The next polyelectrolyte to be investigated was PEI, a molecule that is ideally a straight chain polymer with no side chains. The exact pKa of this molecule was not known, but it was expected to be in the vicinity of 7 or 8, which posed a slight problem in terms of being able to perform the experiments at physiological pH. Initially a few samples were prepared in solution
Figure 12: Representative $^2$H NMR spectra obtained for mixtures of 70/30 POPC-$\alpha$-d$_2$/POPG plus the addition of PMGU. Upper spectrum is a control with a quadrupole splitting of 9.5 kHz. The middle spectrum has a PMGU/POPG ratio of 2.0 with $\Delta \nu_Q = 7.6$ kHz while the lower spectrum has PMGU/POPG = 5.0 with $\Delta \nu_Q = 7.4$ kHz.
of pH = 7.4, suffered by HEI ES, but no interaction was observable. With the assumption that the working pH was too close to the pKa of the molecule it was decided to also make a few measurements with a pH of 5.6, one that should be well below the pKa. These samples were prepared in a buffer of sodium citrate.

In testing for an observable interaction two different vesicle compositions were used, these being 60/40 POPC-α-d₂/POPG and 80/20 POPC-β-d₂/POPG. Some representative examples are shown in figure 13. From these there are two general observations that can be made. The first is that there is really only a small observable interaction. For the POPC-α-d₂/POPG samples there is approximately a 0.6 kHz decrease in the observed quadrupolar splitting from a control value of 10.2 kHz when the PEI/POPG ratio is 0.4. Second is the fact that, at PEI/POPG ratio of 5.0, it appears that there are two components present in the spectrum with 80/20 POPC-β-d₂/POPG vesicles, though they are not resolvable. This may indicate the presence of vesicles with two different compositions, or that there are two regions of distinct composition present in one vesicle population. However, further experimentation failed to reveal any additional information about this interaction, though there is potentially more to be garnered from further study of this polyelectrolyte.

A very different sort of polyelectrolyte is the starburst dendrimer SD72. As can be seen from its structure, it is a spherical molecule of very large molecular weight and is formed by the stepwise addition of units of ethylenediamine to a core of ethylenediamine/methyl methacrylate. This allows for the precise control of molecular diameter and surface charge density, important characteristics for a wide variety of reasons. A recent interest in these compounds lies in their potential use as delivery agents of drugs or nucleic acids to target cells. It is known that these
Figure 13: Representative $^2$H NMR spectra obtained for mixtures of 60/40 POPC-$\alpha$-d$_2$/POPG (left) and 80/20 POPC-$\beta$-d$_2$/POPG (right) plus the addition of PEI. The 60/40 POPC-$\alpha$-d$_2$/POPG control has $\Delta v_Q = 10.2$ kHz while the lower spectrum has a PEI/POPG ratio of 0.4 with $\Delta v_Q = 9.6$ kHz. The 80/20 POPC-$\beta$-d$_2$/POPG has a control POPC-$\alpha$-d$_2$/POPG = 3.4 kHz while the lower spectrum has PEI/POPG = 5.0 with $\Delta v_Q = 2.6$ kHz and a shoulder with an approximate $\Delta v_Q = 3.5$ kHz.
mechanism by which transfection occurs is not known. There must be some sort of interaction with a cell membrane in order to facilitate delivery of the genetic material in the cell, and it is possible such an interaction could be visible via $^2$H NMR.

Samples were made of 70/30 POPC-$\alpha$-d$_2$/POPG and 70/30 POPC-$\beta$-d$_2$/POPG and representative spectra for the former are shown in figure 14. What these spectra demonstrate is that the observed response appears to be that of the molecular voltmeter and this was confirmed by the results obtained with the POPC-$\beta$-d$_2$/POPG samples. What is unusual about this particular response is that it occurs with such a relatively low cation/anion charge ratio. Typically with other polyelectrolytes there is not a significant observable effect until polyelectrolyte/POPG ratios are in the realm of 0.2. With SD72 the first notable changes, though small, occur with a molar cation/anion charge ratio of approximately 0.03, nearly an order of magnitude difference. This is even more surprising knowing that the likelihood of all of the charges on the surface of the dendrimer molecule being able to pair up with a negatively-charged POPG molecule is very minimal due to the spherical nature of the molecule. Furthermore, the change in quadrupolar splitting appears to be approximately linear up to a molar cation/anion ratio of 0.24, at which point the quadrupolar splitting is 7.8 kHz. This relationship can be seen in figure 15. Yet another interesting observation is that the quadrupolar splitting does not appear to go below 7.8 kHz for vesicles of this composition. In addition, there appears to be another splitting present in the last spectrum of figure 14 where the cation/anion ratio is 1.56. The reason for this is not known, but could be the subject of further study.

One additional polyelectrolyte was investigated, that being PVTA. This molecule is
Figure 14: Representative $^2$H NMR spectra obtained for mixtures of 70/30 POPC-$\alpha$-d$_2$/POPG plus the addition of SD72. The upper left spectrum is a control with $\Delta v_Q = 9.5$ kHz. The middle left spectrum has 13.5 $\mu$g of SD72 added (global cation/anion = 0.011) with $\Delta v_Q = 9.35$ kHz. Lower left spectrum has 75 $\mu$g SD72 (+/− = 0.063) with $\Delta v_Q = 8.95$ kHz. Top right spectrum has 125 $\mu$g SD72 (+/− = 0.104) with $\Delta v_Q = 8.5$ kHz; middle right spectrum has 200 $\mu$g SD72 (+/− = 0.167) with $\Delta v_Q = 7.9$ kHz and the lower right spectrum has 1.6 mg SD72 (+/− = 1.33) with $\Delta v_Q = 7.8$ kHz.
Figure 15: Variation of quadrupole splitting with global cation/anion ratio for 70/30 POPC-α-d$_2$/POPG MLVs with added SD72. Details on the linear regression are included.
backbone with side chains that extend well away from the molecular backbone. The main structural difference between the two is that PVTA would be expected to be more hydrophobic due to its completely aliphatic backbone and the aromatic portion of the side chain. An advantage in terms of handling the molecule is that the positive charge is permanent, so concerns regarding a specific pH are minimized.

As with most of the other polyelectrolytes investigated, work with PVTA was carried out using vesicles of 70/30 POPC-α-d₂/POPG composition. Some representative spectra are shown in figure 16. These spectra have a very striking feature in that there are very definitely two different quadrupolar splittings present, one of which is higher than the control splitting of 9.5 kHz, the other being lower than the control splitting. This appeared to indicate the presence of two distinct types of vesicle composition, either because of the formation of different domains within one type of vesicle or because of the formation of two different vesicles, presumably one with PVTA bound and one free of PVTA. Regardless, this observation was novel and was worthy of further detailed investigation. The remainder of the work was aimed toward the elaboration and characterization of the interaction of PVTA with lipid vesicles of varying composition.
Figure 16: Representative $^2$H NMR spectra for 70/30 POPC-α-d$_2$/POPG MLVs with added PVTA. The upper spectrum is a control with $\Delta v_Q = 9.5$ kHz. The middle spectrum is for a PVTA/POPG ratio of 0.44 with $\Delta v_{Q1} = 8.4$ kHz and $\Delta v_{Q2} = 10.4$ kHz. The lower spectrum is for a PVTA/POPG ratio of 1.0 and $\Delta v_Q = 10.8$ kHz.
Having made an interesting observation surrounding the interaction of PVTA with lipid vesicles was significant because such an observation hadn’t been made previously. However, it was possible that the interaction was such that the bilayer structure of the vesicles was distorted or even changed to one that is hexagonal. Though an overall change in the morphology in the vesicle structure would be an interesting subject to study, the potential of being able to observe two different populations within a given sample and to still maintain a bilayer structure is a far more exciting prospect. Furthermore, even if there is no macroscopic change in lipid architecture, a knowledge of the temperature range where a given phase is stable is essential in terms of being able to understand the interaction that is taking place. These considerations precipitated the need to determine what, if any, change in the overall vesicle architecture accompanied the binding of PVTA. To do this there are two techniques available, these being DSC and $^3$P NMR. The former has the capability of determining the phase transition temperature of vesicle samples, while the latter technique is able to directly determine lipid architecture.

As a technique that measures relative heat flow, DSC is capable of determining the temperatures where the samples under investigation undergo a phase transition. The pertinent phase transition when discussing lipids is the gel-to-liquid-crystal phase transition. For pure POPC the phase transition temperature should be approximately $-5^\circ C$ (DeKruijff et al., 1973; Graham et al., 1985; Marassi, 1993). In order to ascertain if any phase transition temperature
endotherms measured illustrated one major and important fact. The phase transition
temperature of pure POPC, pure POPG, POPC/POPG mixtures and POPC/POPG/PVTA
mixtures are all approximately -7°C, which is illustrated in figure 17. Where relevant, this is in
agreement with previously reported values, indicating that all measurements made at both 5°C
and room temperature were observing lipids in the liquid-crystalline state.

Based on the 3H spectra obtained there was no indication that the bilayer structure of the
lipid vesicles was disrupted in any manner, but it is possible to obtain such spectra and not have
samples in a bilayer arrangement. 31P NMR has the distinct advantage of being sensitive to the
macroscopic phase state of the phospholipids, able to differentiate between bilayer, hexagonal
isotropic and cubic phases (Seelig, 1978; Cullis & DeKruijff, 1979).

Figure 18 shows three representative 31P spectra for samples composed of 50/50
POPC/POPG with different amounts of added PVTA. The top spectrum is a control spectrum
that is characterized by a residual chemical shift anisotropy (Δσ) of 45 ppm. This is a
measurement of the frequency separation between the peak maximum and the shoulder
minimum. This value is consistent with what has been observed previously for pure POPC
bilayers (Wohlgemuth et al., 1980; Tamm & Seelig, 1983). The middle spectrum has PVTA
added such that the PVTA/POPG ratio is 0.4 and Δσ = 52 ppm. The last spectrum has a
PVTA/POPG ratio of 2.0, with Δσ = 53 ppm. Though it appears that there is only one resonance
present, these spectra are actually superpositions of two resonances, one each from the
phosphorus atoms of POPC and POPG. Under static conditions the two are unresolvable and
appear as a single resonance.
Figure 17: An example of a typical DSC endotherm obtained. This example is for a sample of 50/50 POPC-a-d2/POPG MLVs and it has a breadth characteristic of an equimolar lipid mixture.
Figure 18: Representative $^{31}$P spectra for 50/50 POPC-$\alpha$-$d_2$/POPG samples. The upper spectrum is a control with a residual chemical shift anisotropy ($\Delta\sigma$) of 45 ppm. The middle spectrum has a PVTA/POPG ratio of 0.4 and $\Delta\sigma = 52$ ppm while the lower spectrum has a PVTA/POPG ratio of 2.0 and $\Delta\sigma = 53$ ppm.
It is known that membrane surface charge has an impact on the value of $\Delta \sigma$. Generally, an increase in negative surface charge density serves to decrease $\Delta \sigma$ for POPC, while increasing positive surface charge density tends to increase $\Delta \sigma$. This can be seen with the results presented here, with an increase in $\Delta \sigma$ of 8 ppm as the membrane surface charge density becomes more positive with the addition of progressively more PVTA.

The key feature of all of these spectra is the spectral line shape as it indicates that the control sample is in a liquid-crystalline bilayer arrangement. In addition, it is apparent that those samples with PVTA added are also in a bilayer arrangement with very little change in the line shape. Had any portion of these samples been present in a hexagonal $H_\text{II}$ phase there would be a component of the spectra with $\Delta \sigma$ reduced by half and opposite in sign. There is no evidence of this whatsoever, leading to the conclusion that all interactions of PVTA with POPC/POPG vesicles do not alter the bilayer nature of the samples. The small isotropic resonance present is the result of a very small population of vesicles whose size is small enough to permit tumbling rapid enough that the chemical shift anisotropy is averaged to zero.

### 3.3.2 UV Measurements of PVTA Binding

Having knowledge indicating that there is a binding of PVTA to negatively-charged lipid vesicles it remained to be determined to what extent binding occurred. This was accomplished by performing measurements using UV-Visible Spectrophotometry. Once it was determined that PVTA could be observed spectrophotometrically at several wavelengths, a concentration range over which the relationship between concentration and absorbance is linear was found, in accordance with the Beer-Lambert relationship.
linearly with concentration up to approximately 4 mM, giving a reasonable working range of concentration for use in the measurements. The results of this study can best be viewed as the fraction of bound PVTA as a function of the PVTA/POPG ratio and are shown in figure 19 for vesicles with a composition of 50/50 POPC/POPG. The most important feature of this data is the fact that essentially all of the added PVTA is bound to the lipid vesicles up to a PVTA/POPG ratio of 1.1. After this point the fraction of bound PVTA falls off with increasing PVTA/POPG. This is a strong indication that the global cation/anion ratio is approximately 1:1, which is not unexpected given that both PVTA and POPG are monovalent. Additionally, this fact leads one to suspect that the nature of the binding is such that any equilibrium lies far toward the bound state, indicating either a very strong electrostatic interaction or a combination of this with some other binding interaction. Unfortunately, this type of data is not able to distinguish whether or not there are distinct domains that either have PVTA bound, or are PVTA-free, on the surface of a given membrane. It is still possible that there are vesicles that are completely saturated with PVTA while there are some that have none at all. This question will be addressed further at a later point.

3.3.3 Domain Formation as Sensed by the Molecular Voltmeter Technique

The key question to be answered after discovering the ability to resolve two different quadrupolar splittings within the same sample centers on the identifying the source of this observation. As discussed previously, other techniques have been able to ascertain that polyelectrolyte binding to lipid membranes has the potential to create regions, or domains, on
Figure 19: Fraction of bound PVTA as a function of the initial PVTA/POPG ratio, as determined by UV-Visible Spectrophotometry measurements.
rest of the membrane surface and have a lifetime that allows their being observed. It is also known that when such binding takes place it is largely electrostatic. Fluorescence studies indicate that the domains where the polyelectrolyte is bound are enriched in the lipid molecule that has the opposite charge to the polyelectrolyte (Luan & Glaser, 1994; Luan et al., 1995; and Yang & Glaser, 1995). Such interactions are garnering more and more attention as the binding of a variety of proteins to membranes is being investigated. Studies of the binding of the protein Src have determined that amino-terminal basic residues are critical for binding through electrostatic interaction with acidic phospholipids (Sigal, et al., 1994), while binding of the MARCKS protein has been observed to be dependent upon both electrostatic and hydrophobic factors, the former being more important (Kim et al., 1994). More general discussions of this topic as it relates to rather specific protein-lipid interactions are available (Buser et al., 1995; McLaughlin & Aderem, 1995).

Suspecting that the $^2$H NMR spectra reveal the presence of two distinct domains, one where PVTA is bound (PVTA-bound) and one where there is no PVTA bound (PVTA-free), it must be determined which signal corresponds to which domain. This can be easily done with a data set illustrating the effect of progressively increasing the amount of added PVTA and noting which signal increases in intensity and which one decreases. The one that increases with increasing PVTA should correspond to the PVTA-bound phase.

It will become obvious, when viewing the representative spectra for different lipid vesicle compositions, which signal corresponds to which domain, but one particular data set that makes the choice clear is presented in figure 20. It is based on the use of spectral simulations to
Figure 20: Plot of the relative intensity of the two resonance signals for 70/30 POPC-β-d₄/POPG MLVs as a function of the PVTA/POPG ratio. Inner Splitting represented by squares (■) and the outer splitting is represented by circles (○).
discussed in more detail at a later time. What is important at this time is the fact that this figure shows the relative intensity of the two overlapping spectral components as a function of added PVTA, with lipid vesicles having an initial composition of 70/30 POPC-β-d₂/POPG. From this plot it is clear that, for POPC-β-d₂ samples, the inner resonance corresponds to the PVTA-bound phase. The opposite is true for POPC-α-d₂ samples where the outer resonance corresponds to the PVTA-bound phase.

Preliminary data collected with PVTA suggest the possibility of two distinct domains being present at the same time, with a lifetime that is long on the NMR time scale. Armed with the knowledge that regions where the polyelectrolyte are bound will be enriched in the counterion relative to a control sample, such regions will be referred to as PVTA-bound. The remaining regions, where there is no polyelectrolyte bound, will be referred to as PVTA-free. To be able to thoroughly investigate this possibility, a complete set of data, illustrating two different populations in as many situations as possible, should be collected for analysis.

In attempting to assess the validity of the hypothesis that two distinct domains coexist several data sets were collected. All measurements made on POPC-α-d₂/POPG membranes were carried out at room temperature (approximately 23°C), while all of the measurements made on POPC-β-d₂/POPG membranes were done at 5°C. Data was collected for vesicles with 10%, 20%, 30%, 40% and 50% POPG. However, limits in resolution prevented the use of both the 90/10 POPC-α-d₂/POPG data and the 50/50 POPC-β-d₂/POPG data. For ease of presentation, only a select few spectra from each data set will be shown, and each will be shown with its corresponding spectral simulation. These simulations allow one to accurately estimate the
beginning with the POPC-α-\textsubscript{d} \textsubscript{2} samples, figure 21 shows three different spectra and their corresponding simulations, which are representative of those obtained for 80/20 POPC-α-\textsubscript{d} \textsubscript{2}/POPG vesicles. The top spectrum is a control spectrum with $\Delta v_Q = 8.45$ kHz. This lineshape is characteristic of liquid-crystalline lipids in a bilayer arrangement. The corresponding spectral simulation consists of a single spectral component, indicating that all the POPC molecules in the entire MLV preparation experience identical surface charge environments. The second spectrum, with a PVTA/POPG ratio of 0.44, has two measurable quadrupolar splittings, $\Delta v_{Q1} = 8.0$ kHz and $\Delta v_{Q2} = 9.3$ kHz. This indicates that, relative to the control, there are two distinct regions. One is depleted in POPG, accounting for the quadrupolar splitting less than the control value, while the other is enriched in POPG, accounting for the quadrupolar splitting greater than the control value. It is consistent with previously discussed results that the POPG-enriched region is the result of PVTA binding and drawing in additional POPG molecules. By simulating the spectrum it can be seen that the outer splitting contributes 67% to the overall spectrum, while the inner splitting contributes 33%, confirming the presence of two distinct surface charge environments. The third spectrum, with PVTA/POPG = 1.0, has only one resolvable quadrupolar splitting of 9.25 kHz. However, it appears that there may be a trace of another quadrupolar splitting at approximately 8.0 kHz. If this is in fact the case, then the outer quadrupolar splitting contributes 87% to the total spectrum, while the inner contributes 13%. Regardless, the simulation shows that one spectral component clearly dominates the spectrum. It is important to point out that those situations where clear resolution of the two resonance
Figure 21: Representative $^2$H NMR spectra and corresponding simulations for 80/20 POPC-α-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta v_Q = 8.45$ kHz. The middle spectrum/simulation are for a PVTA/POPG ratio of 0.44 with $\Delta v_{Q1} = 8.0$ kHz and $\Delta v_{Q2} = 9.3$ kHz. The lower spectrum/simulation are for a PVTA/POPG ratio of 1.0 with $\Delta v_{Q1} = 8.1$ kHz and $\Delta v_{Q2} = 9.0$ kHz.
spectral simulations.

Of note is the fact that once sufficient PVTA has been added there is essentially no trace of a quadrupolar splitting corresponding to a region that is free of the polyelectrolyte. All of the lipid molecules are in regions where PVTA is bound. If indeed all of the membrane surface is bound by PVTA, then there should no longer be any distinction between PVTA-bound and PVTA-free domains. The entire surface should be homogenous in terms of lipid distribution. This being the case, the value of the quadrupolar splitting should return to a value characteristic of the starting composition, prior to the addition of PVTA. However, this is not what is observed and a suitable explanation for this observation is currently under investigation. It is of note that in mirror-image experiments using positively-charged membranes and negatively-charged polyelectrolytes the quadrupolar splitting does in fact come back to a value near the control value. This is discussed in more detail elsewhere (Mitrakos & Macdonald, in press).

Representative spectra and simulations for 70/30 POPC-α-d₂/POPG vesicles are shown in figure 22. The control sample has $\Delta v_Q = 9.5$ kHz. At a PVTA/POPG ratio of 0.44 there are two very well resolved quadrupolar splittings, with $\Delta v_{Q1} = 8.4$ kHz and $\Delta v_{Q2} = 10.4$ kHz. The outer splitting contributes approximately 60% to the overall spectrum while the inner contributes about 40%. When PVTA/POPG = 1.0 there is only one quadrupolar splitting visible (10.8 kHz), though the optimist can detect a small population with a quadrupolar splitting of 8.5 kHz. If this is in fact the case, the dominant outer splitting contributes approximately 95% to the total spectrum, leaving approximately 5% as the contribution for the inner quadrupolar splitting.
Figure 22: Representative $^2$H NMR spectra and corresponding simulations for 70/30 POPC-$\alpha$-$d_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta v_Q = 9.5$ kHz. The middle spectrum/simulation are for a PVTAPOPG ratio of 0.44 with $\Delta v_{Q1} = 8.4$ kHz and $\Delta v_{Q2} = 10.4$ kHz. The lower spectrum/simulation are for a PVTAPOPG ratio of 1.0 with $\Delta v_{Q1} = 8.3$ kHz and $\Delta v_{Q2} = 10.8$ kHz.
Figure 23: Representative $^2$H NMR spectra and corresponding simulations for 60/40 POPC-α-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta v_Q = 10.2$ kHz. The middle spectrum/simulation are for a PVTA/POPG ratio of 0.4 with $\Delta v_{Q1} = 9.9$ kHz and $\Delta v_{Q2} = 11.5$ kHz. The lower spectrum/simulation are for a PVTA/POPG ratio of 1.0 with $\Delta v_{Q1} = 9.8$ kHz and $\Delta v_{Q2} = 11.4$ kHz.
d2/POPG with three representatives of spectra and simulations shown in figure 23. Here the control splitting is 10.2 kHz and again the lineshape is characteristic of lipids in a liquid-crystalline bilayer arrangement. The second spectrum represents a sample where PVTA/POPG = 0.4, and again there are two well resolved quadrupolar splittings present, these being $\Delta v_{Q1} = 9.9$ kHz and $\Delta v_{Q2} = 11.5$ kHz. The relative contributions of the outer and inner resonances are 54% and 46% respectively. The last spectrum, where PVTA/POPG = 1.0, has one dominant quadrupolar splitting of 11.4 kHz, with a smaller component having an approximate $\Delta v_Q = 9.8$ kHz.

One feature in particular that stands out in this last set of data is the fact that the inner resonance appears to be significantly broader than the outer one. One possible explanation for this effect is that the spin-spin, or transverse, relaxation time ($T_2$) is significantly longer for the outer resonance than for the inner. This was investigated by performing the standard quadrupolar echo sequence discussed previously with an incremented delay prior to signal acquisition. By measuring the decay of the signal over time it is possible to get a measure of $T_2$. A typical example of a result of a $T_2$ measurement is shown in figure 24. To determine the values of $T_2$ it is necessary to plot the natural logarithm of the signal intensity versus twice the value of the incremented delay, $\tau$. The plot should be linear with the slope being equal to $1/T_2$ according to the equation $A(2\tau) = A(0)exp(-2\tau/T_2)$, where $A(0)$ is the signal amplitude at time zero and $A(2\tau)$ is the signal amplitude at time $2\tau$. An example of such a determination is given in figure 25, which calculates the $T_2$ values for the PVTA-bound and PVTA-free phases of 80/20 POPC-\(\alpha\)-d2/POPG membranes. By measuring the $T_2$ values for the control sample and for a
Figure 24: A typical experiment for the measurement of the transverse relaxation time, $T_2$. This example is for an 80/20 POPC-α-d$_2$/POPG sample with PVTA/POPG = 1.5. The delay prior to signal sampling in the quadrupolar acquisition sequence was incremented by 50 μsec.
Figure 25: An example of the plot required to calculate $T_2$ for the experiment shown in Figure 23, as well as the data for the 80/20 POPC-α-d$_2$/POPG control sample. The difference in $T_2$ values is well illustrated, with $T_2$ for the PVTA-free phase (■) being 0.833 msec, while for the PVTA-bound phase (●) it is 1.122 msec.
surface charge environments) it was determined that values of $T_2$ were in fact different. The $T_2$ for the so-called PVTA-free phase was approximately 1.122 msec, while for the so-called PVTA-bound phase the $T_2$ was measured to be 0.833 msec, a significant difference. A summary of the values measured is presented in Table 6. The 60/40 POPC-\(\beta\)-d\(_2\)/POPG $T_2$ values were HDO dominated.

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<th>POPC-(\alpha)-d(_2)/POPG</th>
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<tr>
<td>80/20 PVTA-free</td>
<td>0.833 msec</td>
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<td></td>
<td>90/10 PVTA-free</td>
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<td>PVTA-bound</td>
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<tr>
<td>60/40 PVTA-free</td>
<td>0.745 msec</td>
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<tr>
<td></td>
<td>70/30 PVTA-free</td>
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<td></td>
<td>PVTA-bound</td>
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<tr>
<td>50/50 PVTA-free</td>
<td>0.822 msec</td>
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<td>60/40 PVTA-free</td>
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<td>PVTA-bound</td>
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As to exactly why the $T_2$ is shorter in the PVTA-bound phase no exact reason has been determined as yet. However, it is possible to narrow down the possibilities when considering the major contributors to $T_2$. A good review of relaxation phenomena can be found in Harris (1983), which shows that $T_2$ relaxation is most efficient with motions that are relatively slow on an NMR timescale. For example, the rotation of a lipid molecule about its long axis is a rapid motion, while molecular diffusion is relatively slow. Given that the $T_2$ values of the PVTA-bound phase are greater than those for the PVTA-free phase, $T_2$ relaxation in the PVTA-bound
present in this phase, or that there are fewer slow motions present. It seems unlikely that new motions are introduced, leading to the conclusion that some slow motion(s) has been reduced or eliminated.

Of the slow motions possible, two in particular stand out. The first is the previously mentioned molecular diffusion. This is a relatively slow motion whereby the molecules randomly move about the surface of the membrane and sample each particular microenvironment. In a homogenous environment one would expect uniform diffusion coefficients, while in the presence of two distinct regions one would expect the rates to differ. If the PVTA molecule binds in such a fashion that diffusion is restricted in the PVTA-bound phase, then the contribution of molecular diffusion to $T_2$ relaxation would be reduced, leading to a longer $T_2$. The second slow motion that could be reduced as a result of PVTA binding is the “undulation” of the membrane surface. Such a motion would be expected to be, on average, a uniform motion. However, if the regions where PVTA is bound are more rigid than the PVTA-free regions, then these regions would be less flexible. This would reduce this motions contribution to $T_2$ and therefore lengthen the $T_2$ value in this phase.

The last series investigated for samples containing POPC-α-d$_2$ were composed of 50/50 POPC-α-d$_2$/POPG with three representative spectra and their simulations shown in figure 26. The control value of $\Delta v_Q$ was 10.5 kHz, a value only marginally larger than that observed with 60/40 POPC-α-d$_2$/POPG. This is not unexpected considering the calibration curve has nearly plateaued by this point. The second spectrum represents a PVTA/POPG ratio of 0.36, with two nicely resolved quadrupolar splittings of 10.4 kHz and 12.0 kHz for the inner and outer
Figure 26: Representative $^2$H NMR spectra and corresponding simulations for 50/50 POPC-α-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta v_Q = 10.5$ kHz. The middle spectrum/simulation are for a PVTA/POPG ratio of 0.36 with $\Delta v_{Q1} = 10.4$ kHz and $\Delta v_{Q2} = 12.0$ kHz. The lower spectrum/simulation are for a PVTA/POPG ratio of 1.0 with $\Delta v_{Q1} = 10.3$ kHz and $\Delta v_{Q2} = 12.0$ kHz.
resonance and 53% for the outer one. The last spectrum represents a PVTA/POPG ratio of 1.0, with one dominant resonance of $\Delta v_Q = 12.0$ kHz and a trace of a resonance with $\Delta v_Q = 10.4$ kHz. It is possible to simulate this spectrum including a small (~10%) contribution from the inner resonance, leaving the outer resonance to contribute 90%. These data were the most useful in terms of their reliability because clear resolution was achieved with numerous samples, allowing the generation of very accurate simulations.

Having data sets for four different POPC-α-d₂/POPG mixtures (20% - 50%) the results of the measurements of quadrupolar splitting can best be represented as seen in figure 27. Part A displays the actual measured quadrupolar splittings, where open symbols are values for the PVTA-free phases and closed symbols are for the PVTA-bound phases. The trends in the data can be better visualized as seen in part B of the figure where the quadrupolar splittings are plotted as the difference from the control value, $(\Delta v_{Qi} - \Delta v_{Q0})$. Two key features are evident, the first being that the relative change in quadrupolar splitting increases with a higher initial POPG content. This is true for both the PVTA-free and PVTA-bound phases, with one exception. This exception is the second feature of note, that being the fact that the quadrupolar splitting of the PVTA-free phase for the 50/50 POPC-α-d₂/POPG samples is essentially unchanged from the control value, regardless of the ratio of PVTA/POPG. This is most likely tied to the fact that at this particular vesicle composition, changes in POPG content produce negligible changes in quadrupolar splitting. However, this does not account for the observation that the quadrupolar splittings for the PVTA-bound phase continue to increase to values much higher than those obtained in the calibration studies. Based on the calibration curve one would expect a maximum
Figure 27: Summary of measured quadrupole splittings for POPC-α-d₂/POPG MLVs. Circles (●) represent 80/20 mixtures, squares (■) represent 70/30 mixtures, triangles (▲) represent 60/40 mixtures and diamonds (♦) represent 50/50 mixtures. Closed symbols are for the PVTA-bound phase while open symbols are for the PVTA-free phase.
d$_2$/POPG samples. This would imply that there is some non-electrostatic component to the interaction that is manifest in the measured $^2$H NMR quadrupolar splittings. These observations will be revisited when attempting to mathematically model these interactions.

One other point of interest with respect to these data is that at progressively higher POPG contents it becomes easier to resolve differences in quadrupolar splitting and there are more situations where it is possible to clearly achieve this resolution. This fact becomes apparent once it is obvious that higher POPG contents lead to greater relative changes in $\Delta v_Q$. An obvious consequence, which will be apparent later, is that data collected for samples with higher POPG content are overall more reliable.

The data collected for samples of POPC-\(\beta\)-d$_2$/POPG were done so at both room temperature and at 5°C, as previously discussed. Those collected at room temperature were less successful at resolving different quadrupolar splittings than those collected at 5°C and consequently will not be discussed further. All data presented here were collected at 5°C.

The first set of data are for 90/10 POPC-\(\beta\)-d$_2$/POPG samples. Represented in figure 28 are three representative spectra and their corresponding simulations. The control spectrum has a quadrupolar splitting of 4.6 kHz and is indicative of lipid bilayers in liquid-crystalline environment, as are all remaining spectra. The second spectrum has a PVTA/POPG ratio of 0.8 and shows two overlapping Pake patterns with quadrupolar splittings of 4.1 kHz and 4.8 kHz for the PVTA-bound and PVTA-free phases respectively. Unlike data collected for POPC-\(\alpha\)-d$_2$/POPG samples, the quadrupolar splitting for the PVTA-bound phase, being relatively enriched in POPG, has a value lower than the control value. With POPC-\(\alpha\)-d$_2$/POPG samples
Figure 28: Representative $^2$H NMR spectra and corresponding simulations for 90/10 POPC-$\beta$-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta \nu_0 = 4.6$ kHz. The middle spectrum/simulation are for a PVTA/POPG ratio of 0.8 with $\Delta \nu_{01} = 4.1$ kHz and $\Delta \nu_{02} = 4.8$ kHz. The lower spectrum/simulation are for a PVTA/POPG ratio of 1.2 and $\Delta \nu_0 = 4.7$ kHz.
The last spectrum illustrates the case where PVTA/POPG = 1.2, with $\Delta v_Q = 4.7$ kHz, and essentially all of the lipid being in a PVTA-bound phase.

As mentioned for POPC-$\alpha$-d$_2$/POPG data, increasing the POPG content increases the ability to achieve resolution of different quadrupolar splittings, as well as increasing the number of situations where suitable resolution is possible. The same is true for POPC-$\beta$-d$_2$/POPG samples. In going to 80/20 POPC-$\beta$-d$_2$/POPG there is better resolution than seen with the 90/10 samples, and there are more instances of suitable resolution. Representative spectra and simulations are shown in figure 29. Here the control has a quadrupolar splitting of 4.0 kHz, while there were two samples where clear resolution was achieved. One example of this resolution is seen in the second spectrum where $\Delta v_{Q1} = 3.7$ kHz and $\Delta v_{Q2} = 4.45$ kHz, where PVTA/POPG = 0.55. In situations such as this one it is possible to simulate the spectrum with a high degree of accuracy. The last spectrum shown is near the end point where PVTA/POPG = 0.8 and the dominant $\Delta v_Q = 3.6$ kHz, with a small contribution from a signal with an approximate $\Delta v_Q = 4.5$ kHz. In this spectrum it is apparent that there is still a component where there is no PVTA bound, but that it is clearly overshadowed by the resonance for the PVTA-bound phase. Simulations for spectra such as these are essentially impossible to accomplish with a high degree of accuracy.

For 70/30 POPC-$\beta$-d$_2$/POPG samples the data collected were of an extremely high quality. There were numerous situations where complete resolution of the two resonances was possible, though only one is shown here. Representatives of spectra and simulations are shown in figure 30. The control had a quadrupolar splitting of 3.1 kHz, while the middle spectrum,
Figure 29: Representative $^2$H NMR spectra and corresponding simulations for 80/20 POPC-β-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta\nu_Q = 4.0$ kHz. The middle spectrum/simulation are for a PVTA/POPG ratio of 0.55 with $\Delta\nu_{Q1} = 3.7$ kHz and $\Delta\nu_{Q2} = 4.45$ kHz. The lower spectrum/simulation are for a PVTA/POPG ratio of 0.8 with $\Delta\nu_{Q1} = 3.6$ kHz and $\Delta\nu_{Q2} = 4.5$ kHz.
Figure 30: Representative $^2$H NMR spectra and corresponding simulations for 70/30 POPC-β-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta v_Q = 3.1$ kHz. The middle spectrum/simulation are for a PVT2/POPG ratio of 0.6 with $\Delta v_{Q1} = 1.7$ kHz and $\Delta v_{Q2} = 3.4$ kHz. The lower spectrum/simulation are for a PVT2/POPG ratio of 1.2 and $\Delta v_Q = 1.6$ kHz.
where PVTA/POPG = 0.6, has $\Delta v_{Q_1} = 1.7$ kHz and $\Delta v_{Q_2} = 5.4$ kHz. The resolution evident here is characteristic of the resolution achieved with most of the samples with 70/30 POPC-\(\beta\)-d\(_2\)/POPG starting composition, with an overall separation of approximately 1.5 - 1.7 kHz between the two signals. The end point displayed here is for a PVTA/POPG ratio of 1.2, a slight molar excess of polyelectrolyte. The quadrupolar splitting is approximately 1.6 kHz.

The last set of data that provided information of the PVTA/POPG interaction had an initial composition of 60/40 POPC-\(\beta\)-d\(_2\)/POPG, with three representative spectra and simulations being illustrated in Figure 31. Here the control sample had a quadrupolar splitting of 2.3 kHz. The problem encountered with these compositions, as well as those with higher POPG content, is that the resonance for the PVTA-bound phase, being much less than the control value, overlaps with the HDO signal, making it nearly impossible to obtain a measurement of the corresponding quadrupolar splitting. In the middle spectrum, where PVTA/POPG = 0.25, the two signals have quadrupolar splittings of $\Delta v_{Q_1} = 0.85$ kHz and $\Delta v_{Q_2} = 2.7$ kHz. The measurement of the inner resonance is an approximation due to the overlap with the HDO signal. At higher PVTA/POPG ratios the overlap becomes worse and an accurate estimation of the quadrupolar splitting becomes extremely difficult. The last spectrum represents a PVTA/POPG ratio of 0.8, and at this point it appears that the quadrupolar splitting for the PVTA-bound phase is starting to increase marginally (compared to its values at PVTA/POPG ratios between 0.25 and 0.8). The endpoint shown has a PVTA/POPG ratio = 1.2, with $\Delta v_{Q} = 2.45$ kHz.

As represented with the POPC-\(\alpha\)-d\(_2\)/POPG data, the results of the measurement of the quadrupolar splittings for the POPC-\(\beta\)-d\(_2\)/POPG data are shown in figure 32. Part A of the figure
Figure 31: Representative $^3$H NMR spectra and corresponding simulations for 60/40 POPC-β-d$_2$/POPG MLVs. The upper spectrum/simulation are controls with $\Delta \nu_0 = 2.3$ kHz. The middle spectrum/simulation are for a PVT/POPG ratio of 0.25 with $\Delta \nu_{Q1} = 0.85$ kHz and $\Delta \nu_{Q2} = 2.7$ kHz. The lower spectrum/simulation are for a PVT/POPG ratio of 1.2 and a signal that has no measurable quadrupole splitting and is essentially isotropic.
Figure 32: Summary of measured quadrupole splittings for POPC-β-d₂/POPG MLVs. Circles (○) represent 90/10 mixtures, squares (■) represent 80/20 mixtures, triangles (△) represent 70/30 mixtures and diamonds (♦) represent 60/40 mixtures. Closed symbols are for the PVTA-bound phase while open symbols are for the PVTA-free phase.
part B of the figure displays the same data in terms of the relative change in quadrupolar splitting from the control value, \((\Delta v_{Q1} - \Delta v_{Q0})\). There are two main features present, the first being that the relative change in quadrupolar splitting increases with a higher initial POPG content. This is true for the PVTA-bound phase while for the PVTA-free phase such a trend is evident, but it is not as obvious as for the PVTA-bound phase. The second notable feature is that the absolute change in quadrupolar splitting is significantly greater for the PVTA-bound phase than it is for the PVTA-free phase. Much like the situation observed with 50/50 POPC-\(\alpha\)-d\(_2\)/POPG samples there is the implication that there is some component of the interaction that is not electrostatic in nature, yet can be manifest in the observed \(^2\)H NMR quadrupolar splittings. This observation will be discussed in more detail when an attempt is made at proposing a model.

One last point of interest with respect to these data is that at progressively higher POPG contents it becomes easier to resolve differences in quadrupolar splitting, leading to more situations where it is possible to clearly differentiate between the two resonances. This fact becomes apparent once it is obvious that higher POPG contents lead to greater relative changes in \(\Delta v_Q\). However, unlike the POPC-\(\alpha\)-d\(_2\)/POPG samples, this trend becomes detrimental as the quadrupolar splitting for the PVTA-bound phase approaches an isotropic value, overlapping with the natural abundance HDO resonance.
Composition

Having surveyed wide ranges of lipid bilayer composition and noted that in the ternary POPC/POPG/PVTA mixtures there are two spectral components present in varying intensities, it remains to determine the relationship between the measured quadrupolar splittings and the exact compositions of the PVTA-free and PVTA-bound phases. The fact that the quadrupolar splittings from POPC-α-d₂ versus POPC-β-d₂ change in opposite directions in both the PVTA-associated and the PVTA-dissociated domains indicates that these two environments differ with respect to their surface charge, indicating the presence of domains of distinct composition that are in slow exchange with one another on a timescale delimited by the difference in their quadrupolar splittings. By referring to figures 27 and 32 it can be observed that Δν₉ from POPC-α-d₂ in the PVTA-associated domain remains virtually constant across a range of PVTA/POPG ratios, while Δν₉ from POPC-α-d₂ in the PVTA-dissociated domain decreases progressively. This suggests a more-or-less constant surface charge environment within the PVTA-bound domain coupled to a PVTA-free environment which becomes progressively depleted with respect to POPG as further PVTA is added. This increase in the quadrupolar splitting for POPC-α-d₂/POPG membranes, due to enrichment of POPG in the PVTA-bound phase, has recently been termed the “anti-voltmeter” response (Mitrakos & Macdonald, 1996; Crowell & Macdonald, 1997). This response has been observed for both cationic polyelectrolytes interacting with anionic membranes and for anionic polyelectrolytes interacting with cationic membranes. These observations are central in beginning to use the spectra and simulations as a means of determining domain composition.
surface produces a separate domain having a distinct composition but retaining an overall liquid-crystalline bilayer architecture. In order to be able to determine the composition of the different domains one needs a suitable starting place, which comes from the experimentally measured values for the quadrupolar splitting. In practice one has the option of using either the PVTA-free or a combination of the PVTA-free and PVTA-bound quadrupolar splitting as a starting point for quantifying the composition of the two domains. The end result should be the same. The bulk of the remaining calculations will be based on the quadrupolar splitting of the PVTA-free phase alone.

By starting with the quadrupolar splitting of the PVTA-free phase one has fewer assumptions to make as this domain is simply a binary mixture of zwitterionic and anionic lipid, much like a sample used for the calibration curves. The quadrupolar splitting should reflect the net surface charge of this domain, which is negative. Therefore, the composition of the two domains may be characterized through an analysis of the $^2$H NMR quadrupolar splittings as follows.

The total populations of zwitterionic ($X_z^t$) and anionic lipid ($X_{i}^t$) present globally can be expressed as mole fractions as follows:

$$X_z^t + X_{i}^t = 1$$ (31)

These can be further broken down into bound and free components, indicated by the subscripts b and f, respectively.
The proportion of bound lipid, \( \frac{X^b}{X^Z} \) and \( \frac{X^b}{X^f} \), comes from the spectral simulations. Having values for the PVTA-free quadrupolar splitting, the quadrupolar splitting of 100% POPC (\( \Delta\nu_o \)) and the slope of the calibration curve, \( m_\nu \), one can calculate the mole fraction of anionic lipid in the PVTA-free phase using equation (34) as follows:

\[
\frac{X^f (\Delta\nu^f - \Delta\nu_o)}{(m_\nu - \Delta\nu^f + \Delta\nu_o)} = X^f
\]  

(34)

where \( \Delta\nu^f \) is the quadrupolar splitting of either POPC-\( \alpha \)-d or POPC-\( \beta \)-d in that domain, \( \Delta\nu_o \) is the quadrupolar splitting for 100% POPC, \( m_\nu \) is a calibration constant relating \( \Delta\nu_o \) to the POPG content, \( X^f \) is the fraction of the total POPC in the PVTA-free domain as determined by spectral simulation, and \( X^f \) is the fraction of the total POPG in the PVTA-free domain. Since the total amount of lipid present at the outset is known, the amounts of zwitterionic and anionic lipid present in the PVTA-bound phase can be calculated.

This method assumes that the relationship between quadrupolar splitting POPG content is linear over the POPG range of interest. However, as was pointed out earlier, this is not the case, at least once the POPG content exceeds 20 mol%. This makes the calculation a bit more difficult than the case of simply substituting appropriate values. One option is to use a value of the slope that is appropriate to the region of the calibration curve in which measurements are
and that used here, is to use the actual value of the quadrupolar splitting based on the polynomial fit to the data on the calibration curve. Though more time-consuming, this method yielded results that accurately reflected the exact POPG content for each particular sample.

Once the spectra are acquired and the corresponding simulations have been carried out it is possible to calculate the amount of POPC and POPG in the PVTA-free phase. Using the appropriate calibration curve a value for the POPG content of this phase can be read directly from the plot based on its quadrupolar splitting. This is based on the assumption that the quadrupolar splitting in this domain is solely the result of an electrostatic interaction and is a direct reflection of the amount of POPG present. Knowing the relative POPG content and the relative contributions of the PVTA-free component to the overall spectrum, the absolute amounts of POPC and POPG can be simply calculated. Once these values are calculated the amounts of POPC and POPG remaining are assumed to be in the PVTA-bound phase. An example of this calculation follows.

Consider a sample of 70/30 POPC-β-d₂/POPG where the PVTA/POPG ratio is 0.5. The sample initially contains 7.89 mmol of POPC-β-d₂, 3.38 mmol of POPG and 1.69 mmol of PVTA. On average the control quadrupolar splitting is approximately 3.2 kHz. Knowing that the signals arising from the PVTA-free and PVTA-bound domains will be easily resolved, a measured value of 3.4 kHz for the PVTA-free domain can be correlated with a POPG content of approximately 27 mol%. From the spectral simulation assume that this phase contributes 65% to the overall spectrum. Therefore, the amount of POPC in this phase is 0.65 * 7.89 = 5.08 mmol. Since the POPG content is 27 mol% the amount of POPG in the PVTA-free phase is 1.88
consisting of 2.81 mmol POPC and 1.8 mmol POPG. This gives a domain with a POPG content of approximately 39% which should give a quadrupolar splitting of 2.4 kHz if the observed spectrum is simply the result of purely electrostatic interactions of a nature that can be sensed by the molecular voltmeter. Recalling the data presented previously it is obvious that there must be some other aspect to the interaction besides pure electrostatics. This will be discussed in more detail later.

Applying this analysis to the data in Figures 27 and 32 reveals that the PVTA-free domain is progressively depleted with respect to POPG as increasing amounts of PVTA are added. In fact, the amount of depletion is such that, regardless of whether one employs quadrupolar splittings obtained with POPC-α-d₂ or POPC-β-d₂, one calculates that the PVTA-bound domain contains approximately a 1:1 ratio of cationic charges from PVTA and anionic charges from POPG. This observation would be expected intuitively only if the nature of binding is very strong. With weak binding one would expect the data to imply that the PVTA-bound phase contained more PVTA than POPG. This result is in agreement with the results obtained with the UV-Visible Spectrophotometry binding measurements.

Prior to discussing the details of the results derived from the calculations of domain composition, one point should be clarified. In terms of data quality, one has to go back to the accuracy of the spectral simulations, which in turn is a function of the ability to resolve the two spectral components. In this regard the data collected for the POPC-β-d₂ samples at 5°C is of a higher quality than that collected for the POPC-α-d₂ samples at room temperature. Therefore, despite the fact that it is possible to present analogous sets of data for both sample types, it is the
The data for POPC-β-d₂ mixtures that allows more definitive statements to be made. The data for POPC-α-d₂ can occasionally be used to draw similar conclusions, but it is often only able to confirm what is apparent from the POPC-β-d₂ data. That said, a discussion of the remainder of the results can be carried out.

The most logical first representation of the data is a presentation of mole fraction of POPC and POPG as a function of the PVTA/POPG ratio. In figure 33 are shown the mole fractions of bound POPG as a function of PVTA/POPG. Figure 33 A represents the data obtained for POPC-α-d₂, while figure 33 B represents the data for POPC-β-d₂. Both of these plots have the notable feature that there is a linear relationship between the mole fraction of bound POPG and the PVTA/POPG ratio and that this relationship is just slightly less than one-to-one. This is not unexpected given the previously determined 1/1 cation/anion ratio in the PVTA-bound phase. Up to the point of there being a molar excess of PVTA, regardless of how much is added, the amount of POPG that is present in the bound phase is equal to the amount of added PVTA. No distinction is apparent between the POPC-α-d₂ versus the POPC-β-d₂ data.

The more interesting question becomes one of how much POPC is present in the bound phase as a function of PVTA/POPG. These results of these calculations are represented in figure 34 where the upper plot is for POPC-α-d₂ and the lower plot is for POPC-β-d₂. The data for POPC-α-d₂ demonstrate two features, the first being that most of the data points tend to be somewhat clustered, with no clear trend on whether or not the initial POPG content has an influence on the amount of POPC that becomes “trapped” in the PVTA-bound phase. The second feature of note is that these data are approximately linear up to a PVTA/POPG ratio of 1.0, with a slope marginally less than one. In fact, these data are very similar to those presented
Figure 33: Mole fraction of POPG in the PVTA-bound phase. Part A is for POPC-α-d₂/POPG MLVs where circles (●) represent 80/20 mixtures, squares (■) represent 70/30 mixtures, triangles (▲) represent 60/40 mixtures and diamonds (♦) represent 50/50 mixtures. Part B is for POPC-β-d₂/POPG MLVs where circles (●) represent 90/10 mixtures, squares (■) represent 80/20 mixtures, triangles (▲) represent 70/30 mixtures and diamonds (♦) represent 60/40 mixtures.
Figure 34: Mole fraction of POPC in the PVTA-bound phase. Part A is for POPC-α-d$_2$/POPG MLVs where circles (●) represent 80/20 mixtures, squares (■) represent 70/30 mixtures, triangles (▲) represent 60/40 mixtures and diamonds (♦) represent 50/50 mixtures. Part B is for POPC-β-d$_2$/POPG MLVs where circles (●) represent 90/10 mixtures, squares (■) represent 80/20 mixtures, triangles (▲) represent 70/30 mixtures and diamonds (♦) represent 60/40 mixtures.
For POPC-β-d₂ bilayers the situation is markedly different. There is a clear and significant difference in the amount of POPC "trapped" in the PVTA-bound phase, this difference being a function of the initial amount of POPG present. In going from 10 mol% POPG to 40 mol% POPG the amount of bound POPC for a given PVTA/POPG ratio increases dramatically and all four data sets appear to be linear when extrapolated to zero PVTA. For the 10 mol% case the amount of POPC bound is approximately half that seen for the 40 mol% case, which is nearly a one-to-one relationship.

A quick interpretation of this observation leads one to suspect that the amount of POPC "trapped" is determined by its relative availability, this being a reflection of the initial POPG content. When POPG represents 10 mol% of the total lipid, and the polyelectrolyte PVTA binds to the membrane surface, the amount of POPG present in the bound phase will be enough to neutralize the PVTA, i.e.: a one-to-one cation/anion ratio. The amount of POPC that gets trapped appears to be determined by how much is around. For 10 mol% POPG there is a relatively large amount of POPC present, so the percentage of this that gets "trapped" in the bound phase is relatively small. When the amount of POPC present initially is 40 mol%, the amount of POPG that gets bound by the PVTA is still in a one-to-one relationship, but the available POPC is much less when compared to the 10 mol% POPG samples. Accordingly, the percentage of total POPC bound is larger, accounting for the nearly one-to-one relationship. Such a characteristic of binding appears to be purely statistical.

Knowing the actual amounts of POPC, POPG and PVTA present in the PVTA-bound phase it is possible to determine the number of molecules of POPC that are bound per molecule
these calculations are shown in figure 35, the upper plot being for POPC-α-d₂ and the lower plot being for POPC-β-d₂. A summary of the numerical values is presented in table 7. With these data both the POPC-α-d₂ and POPC-β-d₂ results display the same trend, this being that the number of POPC molecules bound per molecule of PVTA decreases in going from low POPG content to high POPG content.

For POPC-α-d₂ samples with 20 mol% POPG, approximately 600 molecules of POPC are bound per molecule of PVTA. With the knowledge that there exists an approximately one-to-one cation/anion ratio, the smallest possible domain size has a composition of 1 PVTA molecule, 600 POPC molecules, and 190 POPG molecules. Whether or not this is of sufficient size to be observed via ²H NMR is not a certainty. An actual domain as sensed by the ²H molecular voltameter may be a conglomeration of thousands of such microdomains. In fact, this is a reasonable assumption given the results obtained from fluorescence digital imaging microscopy studies (Luan & Glaser, 1994; Luan et al., 1995; and Yang & Glaser, 1995). These studies have shown that the domains of bound polyelectrolyte are very large and take up significant portions of the membrane surface.

The data collected for POPC-β-d₂/POPG samples indicate that for 10 mol% POPG, there are approximately 800 molecules of POPC bound per molecule of PVTA, while for 40 mol% the number drops to approximately 300. However, as with the POPC-α-d₂ data, these data are
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Figure 35: Molecules of POPC bound per molecule of PVTA. Part A is for POPC-α-d₂/POPG MLVs where circles (○) represent 80/20 mixtures, squares (■) represent 70/30 mixtures, triangles (▲) represent 60/40 mixtures and diamonds (◆) represent 50/50 mixtures. Part B is for POPC-β-d₂/POPG MLVs where circles (○) represent 90/10 mixtures, squares (■) represent 80/20 mixtures, triangles (▲) represent 70/30 mixtures and diamonds (◆) represent 60/40 mixtures.
unable to provide any direct information on the actual size of the PVTA-bound domain. Only the size per PVTA molecule can be determined, and this size appears to be a function of the initial POPG content.

As noted previously, the accuracy of the data collected for POPC-\(\alpha\)-d\(_2\) samples was not of as high a quality overall as the data collected for POPC-\(\beta\)-d\(_2\). Generally the data becomes more accurate as the POPG content increases, especially for POPC-\(\alpha\)-d\(_2\) samples. The 50 mol% samples were the best resolved. For POPC-\(\beta\)-d\(_2\) this is true only up to about 30 mol% POPG since beyond that point the quadrupolar splitting of the PVTA-bound phase approaches an isotropic value. Despite these differences there is a close agreement between the two data sets in terms of calculating the number of molecules of POPC bound per molecule of PVTA. In addition, an analogous result has been observed in the mirror-image experiment involving the binding of negatively-charged polyelectrolytes to positively-charged lipid membranes (Mitrakos & Macdonald, in press).

As mentioned previously it is also possible to quantify the domain composition by using the quadrupolar splitting of the PVTA-bound phase as well as that of the PVTA-free phase. Such an analysis assumes that, in the ternary mixture of anionic + zwitterionic + cationic species, the interactions taking place are mainly electrostatic in nature and that the measured quadrupolar splittings are the result of the sum of individual perturbations produced by cationic and anionic interactions (Marassi & Macdonald, 1992). Therefore, the measured quadrupolar
where \( m_+ \) and \( m_- \) are calibration constants for relevant binary mixtures.

The value for \( m_- \) is easily measured, but the value of \( m_+ \) can not be easily measured because the interaction of PVTA with pure POPC bilayers is very weak, necessitating some assumptions. The first assumption is that the presence of PVTA is sensed only indirectly through its effect on the distribution of POPG, meaning that \( m_+ \) can be assumed to be zero. Therefore, the term containing the effective mole fraction \( X_+ \) in equation (35) drops to zero and that \( X_- \) can be calculated from \( \left[ X_-^b / (X_-^b + X_+^b) \right] \). In doing this one can then determine the composition of the PVTA-bound phase and predict a quadrupolar splitting and compare this value to that measured.

This leads to the last aspect of data presentation that needs to be addressed. It was noted previously, when working through the calculation to determine the composition of the two domains, that the quadrupolar splitting expected based on the enrichment of POPG was greater than what was observed experimentally (for POPC-\( \beta \)-d\(_2\)/POPG samples). This result was not merely the result of a poor choice of an example. For all of the data collected for mixtures of POPC-\( \beta \)-d\(_2\)/POPG, it was calculated that the experimentally measured quadrupolar splitting was less than what should have been observed based simply on the relative enrichment of POPG. Furthermore, the opposite was observed for samples of POPC-\( \alpha \)-d\(_2\)/POPG, i.e: the experimentally measured quadrupolar splitting for the PVTA-bound phase was greater than what was expected.
This result is illustrated in figures 36 and 37 where the former compares the theoretical (calculated) versus experimental quadrupolar splittings for POPC-α-d_2/POPG membranes, while the latter plot presents the same information for POPC-β-d_2/POPG membranes. For membranes of POPC-α-d_2/POPG this result became obvious once measurements were made on 50/50 mixtures, where the PVTA-bound phase had a measured quadrupolar splitting of approximately 12.0 kHz. Such a splitting seems impossible, regardless of the degree of POPG enrichment, because the maximum quadrupolar splitting expected would be approximately 11.0 kHz based on the calibration curve. Even for the 60/40 mixtures, this fact stands out with PVTA-bound phase quadrupolar splittings greater than 11.0 kHz.

A consequence of such observations is also apparent in figure 37. As the initial POPG content increases, the degree to which the experimental quadrupolar splitting differs from the theoretical value increases. This seems to indicate that the initial amount of POPG available dictates not only the potential for POPG enrichment, but also the degree to which the effect of PVTA binding (as seen via the quadrupolar splitting) is under-predicted. This under-prediction of the "anti-voltmeter" effect of PVTA binding is in keeping with the counter-directional nature of the molecular voltmeter, with the differences between experimental and theoretical quadrupolar splittings being in opposite directions. This points to the fact that whatever is producing this effect must be the same with both POPC-α-d_2/POPG and POPC-β-d_2/POPG membranes.

It was initially thought that this observation could be the result of an increase in the order parameter once PVTA becomes bound. However, such a change serves to increase the
Figure 36: Experimental versus calculated quadrupolar splittings for the PVTAd-bound phase in POPC-α-d_2/POPG MLVs where circles (●) represent 80/20 mixtures, squares (■) represent 70/30 mixtures, triangles (▲) represent 60/40 mixtures and diamonds (◆) represent 50/50 mixtures. Open symbols represent the calculated values while closed symbols represent the experimental values.
Figure 37: Experimental versus calculated quadrupolar splittings for the PVTA-bound phase for POPC-β-d$_2$/POPG MLVs where circles (●) represent 90/10 mixtures, squares (■) represent 80/20 mixtures, triangles (▲) represent 70/30 mixtures and diamonds (♦) represent 60/40 mixtures. Open symbols represent the calculated values while closed symbols represent the experimental values.
membranes because the experimental value is larger than expected, but it does not apply for POPC-β-d_{2}/POPG membranes. This is because an increase in the order parameter would increase the quadrupolar splitting, but in fact the quadrupolar splitting is reduced.

Relating this result back to equation (35), it appears that the assumption made is not completely valid. There should be close agreement between the measured and theoretical quadrupolar splittings and the fact that there is not suggests that some other factor is coming into play. Perhaps the assumption that the term containing X, drops to zero is incorrect, though this would be surprising given the results obtained in the mirror-image experiments using cationic membranes and anionic polyelectrolytes (Mitrakos & Macdonald, in press). It is more plausible that the calibration constant in the PVTA-bound phase (m.) is significantly different than it is in the PVTA-free phase. At best, such a consideration is capable of explaining the observations made. Validation of this theory is as yet undetermined.

With the body of information gathered on the formation of distinct domains one needs to question how PVTA interacts with the membrane. Does it sit on the surface or does it somehow insert into the membrane? A schematic of PVTA interacting with a lipid bilayer membrane is presented in figure 38. The hydrophobic polymer backbone and benzene ring side chains likely intercalate between the membrane lipids in order to minimize interactions with water. The cationic trimethylamino groups will likely locate at the membrane-water interface in order to maximize their interactions with the anionic phosphate groups of POPG. POPG remains in the vicinity of the PVTA due to electrostatic attraction such that there is a 1:1 ratio of PVTA cationic to POPG anionic charges in the region defined by proximity to the polyelectrolyte. The
Figure 38: Schematic representation of a possible mode of interaction between PVT A and POPC/POPG bilayers.
dimensions. POPC may be trapped within the folds of the PVTA where its effective lateral diffusion coefficient is reduced due to the well-known archipelago effect (Alderman, et al., 1986). This inhibits its ability to exchange with the bulk lipid but does not prevent other molecular motions.

3.3.5 *Determination of Domain Composition and Size*

The size of the PVTA-bound domain may be estimated by summing the cross-sectional areas occupied at the membrane surface by the phospholipids and the polyelectrolyte. Individual POPC and POPG molecules occupy surface areas of 68 Å². An estimate of the area occupied by the polyelectrolyte is obtained by regarding it as a chain of length 190 × 3.55 Å and width 2.42 Å, where 190 is the number of monomers per chain, 3.55 Å is the monomer-monomer spacing, and 2.42 Å is the width of a benzene ring. For the case of 60/40 POPC-α-d₂/POPG, the ²H NMR data indicate that each PVTA domain contains approximately 190 POPG plus 200 POPC molecules per PVTA molecule, yielding a total area equivalent to a circular area with a radius of 93 Å. This is far less than the dimensions of a fully-extended PVTA chain (675 Å) but far greater than the dimensions of a fully-collapsed PVTA chain (23 Å). A summary the average domain sizes per PVTA molecule are presented in Table 8. According to De Gennes (1979), the dimensions of a ideal polymer confined to two-dimensions should scale with molecular weight as \( M^{3/5} \). Using the ²H NMR technique described here, this scaling law is now amenable to investigation for the case of polyelectrolytes bound at a lipid membrane surface.
Table 8: Summary of Domain Size per PVTA Molecule

<table>
<thead>
<tr>
<th>Composition</th>
<th>POPC-α-d₂/POPG</th>
<th>Radius (Å)</th>
<th>POPC-β-d₂/POPG</th>
<th>Composition</th>
<th>Radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80/20</td>
<td></td>
<td>132</td>
<td></td>
<td>90/10</td>
<td>148</td>
</tr>
<tr>
<td>70/30</td>
<td></td>
<td>107</td>
<td></td>
<td>80/20</td>
<td>120</td>
</tr>
<tr>
<td>60/40</td>
<td></td>
<td>93</td>
<td></td>
<td>70/30</td>
<td>113</td>
</tr>
<tr>
<td>50/50</td>
<td></td>
<td>86</td>
<td></td>
<td>60/40</td>
<td>102</td>
</tr>
</tbody>
</table>

One last point deserves mention. How large must a domain be in order for it to be observed via ²H NMR? Intuitively, one would not expect that the small domain sizes calculated here are capable of being differentiated from the rest of the membrane surface. This is especially true when considering that the diffusion rates of lipids are relatively high in terms of the NMR timescale. In fact, the ability to detect two domains with ²H NMR must be largely determined by the lipid diffusion rates. If the lipid molecules in the PVTA-bound domain are able to diffuse out rapidly when compared to the NMR timescale, then only an average quadrupolar splitting will be observed. If diffusion is relatively slow, then two domains can be visualized. Obviously, if the domains are large, it is more likely that lipid molecules will be unable to diffuse out of the domain very rapidly. For this to be the case there would have to be many small domains clustered together. Based on the work of Glaser cited earlier, where the polyelectrolyte-bound domain takes up a large portion of the membrane surface, this seems very plausible indeed.
The results obtained from this study have provided some very important information about the interactions of cationic polyelectrolytes with anionic lipid membranes. Foremost among these is the fact that $^2$H NMR is a very useful tool for studying membrane surface electrostatics, as has been noted on numerous occasions previously for a wide variety of studies. That it is beneficial for this type of study comes as no surprise.

The fact that more than one response of the "molecular voltmeter" is also notable, having observed a straight-forward voltmeter response with polyelectrolytes such as PMGU, as well as the more interesting "anti-voltmeter" response that permits the simultaneous observation of two distinct domains. That these spectra could be accurately simulated for use in quantifying the amounts of the different components present in these domains only added to the usefulness of $^2$H NMR spectroscopy. In addition, it is also possible to calculate a minimum domain size based on the results obtained here.

So what is left? As pointed out on several occasions, there are many unanswered questions, an important one being what is the actual domain size? It seems likely that it is much larger than the minimum size calculated here, but this technique has yet to demonstrate this. Another unanswered question surrounds the lack of agreement between the experimental and theoretical values for the quadrupolar splitting of the PVTA-bound phase. This is a very important question that will be explored in the future. One last point that deserves more study is the fact that the quadrupolar splitting of the PVTA-bound phase doesn’t return to a value representative of a sample with no added PVTA once an excess amount of PVTA has been added.
V. REFERENCES


Mitrakos, P. & Macdonald, P.M. in press.


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