ABSTRACT

Polyelectrolyte Induced Domains in Cationic Lipid Bilayer Membranes: A Deuterium Nuclear Magnetic Resonance Perspective

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The effect of membrane surface charge was studied via deuterium (2H) and phosphorus (31P) nuclear magnetic resonance (NMR), by the addition of amphiphilic cationic charge into a neutral phospholipid membrane and then subsequent neutralization of this charge through the addition of anionic polyelectrolyte. The effect of added charge was monitored by the use of a specifically deuterated phosphatidylcholine molecule which produces a predictable change in the 2H NMR quadrupolar splitting (ΔνQ) in response to changes in membrane surface charge. Addition of amphiphile cationic charge produced a decrease (increase) in the quadrupolar splitting for the α (β) deuterons. The subsequent addition of anionic polyelectrolytes onto these cationic surfaces induced the appearance of a dual component spectrum consisting of two quadrupole splittings indicative of two different charge environments. While one charge population is enriched in cationic charge the other is depleted. Therefore, the addition of polyelectrolyte induces the existence of long lived lateral
inhomogeneities or domains at the cationically charged membrane surface. The degree of separation and the composition of the domains was quantified through spectral simulations. The data show that domain formation and composition depended greatly on the identity and size of polyelectrolyte, the initial cationic surface charge, in addition to the ionic strength of the solution. The effect of anionic polyelectrolytes was also studied via $^1$H NMR of methyl-deuterated cationic amphiphiles. Although this data produced no evidence of domain formation, information was obtained which supported the results gained from deuterolabeled phosphatidylcholine. The results also indicated that the polyelectrolyte orders the headgroup of cationic amphiphiles and that salt addition reduces this electrostatic interaction. Finally, the behavior of two nucleotides as well as the macroscopic architecture of these mixed membrane systems was followed via $^{31}$P NMR. These results show that polyelectrolytes bound to these cationic surfaces become immobilized. The data also indicate that the lipids retain a bilayer arrangement, under most conditions. However, various non-bilayer phases were produced when phosphatidylcholine was replaced with phosphatidylethanolamine, in the absence and presence of polyelectrolyte. Non-bilayer phases could also be produced in the presence of double chained cationic lipids but not single chained cationic amphiphiles.
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### SYMBOLS AND ABBREVIATIONS

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<tr>
<td>$^1H$</td>
<td>proton</td>
</tr>
<tr>
<td>$^2H$</td>
<td>deuterium</td>
</tr>
<tr>
<td>$^3P$</td>
<td>phosphorus</td>
</tr>
<tr>
<td>$\Delta v_0$</td>
<td>quadrupole splitting</td>
</tr>
<tr>
<td>$\Delta \alpha$</td>
<td>chemical shift anisotropy</td>
</tr>
<tr>
<td>$\delta_i$</td>
<td>isotropic chemical shift</td>
</tr>
<tr>
<td>$T_1$</td>
<td>longitudinal, spin-lattice relaxation time</td>
</tr>
<tr>
<td>$T_2$</td>
<td>transverse, spin-spin relaxation time</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar vesicle</td>
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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>CTAB</td>
<td>cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DODAP</td>
<td>1,2-dioleoyloxy-3-(dimethylamino) propane</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-dioleoyloxy-3-(trimethylamino) propane</td>
</tr>
<tr>
<td>DC-CHOL</td>
<td>3β-[N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol</td>
</tr>
<tr>
<td>TC-CHOL</td>
<td>3β-[N-(N',N'-trimethylaminoethane)carbamoyl] cholesterol</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>POPA</td>
<td>1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoric acid</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyloxy-sn-glycerol-3-phosphatidylethanolamine</td>
</tr>
<tr>
<td>TPS</td>
<td>2,4,6-triisopropylbenzenesulfonyl chloride</td>
</tr>
<tr>
<td>TPB</td>
<td>tetrabromoethylene</td>
</tr>
<tr>
<td>PolyA</td>
<td>poly(adenosine)</td>
</tr>
<tr>
<td>OligoS</td>
<td>phosphorothioate oligonucleotide</td>
</tr>
<tr>
<td>PSSS</td>
<td>poly(sodium 4-styrene sulfonate)</td>
</tr>
<tr>
<td>PACA</td>
<td>poly(acrylic acid)</td>
</tr>
<tr>
<td>PGLU</td>
<td>poly(glutamic acid)</td>
</tr>
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1. INTRODUCTION

1.1 Overview

This thesis describes a study of domains induced by polyelectrolytes in lipid bilayer membranes. The major tool used to study these domains is deuterium nuclear magnetic resonance spectroscopy ($^2$H NMR). A domain is defined as a region of the membrane possessing a distinct composition and having sufficient dimension and duration to be of biological significance. The types of domains which may exist in lipid bilayer membranes may be subdivided into those which produce transbilayer asymmetries and those which produce lateral heterogeneities in the plane of the bilayer. The studies presented here are concerned with the latter. These lateral domains may range in size from a few lipid molecules to a square micron and may exist for times ranging from a nanosecond to the lifetime of the cell. Microdomains usually originate from lipid-lipid interactions (Mabrey and Sturtevant, 1978; Cullis et al., 1983; McElhaney, 1982). Macrodomains, on the other hand, are usually associated with either protein-lipid or protein-protein interactions (de Kruijff and Cullis, 1980; Haverstick and Glaser, 1989).

Polyelectrolytes are polymers in which the monomer segments are charged. Many biomacromonomers such as complex carbohydrates, proteins and polynucleic acids are charged. When these charged polymers bind to membranes they produce domains, although the details are as yet still unclear. The interaction between the lipids and polyelectrolytes is electrostatic in nature, although hydrophobic forces may also play a role. This interaction is of prime importance for drug delivery
in which a synthetic polyelectrolyte coats a lipid membrane which encapsulates the drug (Lasic and Needham, 1995; Winnik et al., 1995). On the other hand, the interaction of biological polyelectrolytes with lipid bilayer membranes has also been shown recently to be of great importance, as a highly efficient mode of gene transfer (Felgner et al., 1987; Leventis and Silvius, 1990; Mönkkönen and Uri, 1998).

The significance of lateral domains in biological membranes lies in their function. Lipid domains can serve to concentrate lipids which act as substrates for particular enzymes. The maximal activity of membrane associated enzymes can be controlled by the degree of enrichment of the particular lipid involved in the enzyme-substrate reaction (Yang and Glaser, 1996). The ability to determine the significance of domains rests on the particular techniques used to view domains. Some of the most recent techniques used to study the details of lateral domains are fluorescent recovery after photobleaching (FRAP), fluorescent digital imaging, differential scanning calorimetry (DSC) and $^1$H NMR (Vaz, 1992; Glaser, 1992; Tocanne, 1992; Wolf, 1992; Mitrakos and Macdonald, 1996 and 1997; Macdonald et al., 1998).

$^1$H NMR is a spectroscopic technique capable of measuring charge at the surface of membranes. The technique is not only capable of measuring the absolute charge at the surface but is also capable of measuring both lateral (Macdonald et al., 1991) and transbilayer (Marassi and Macdonald, 1993; Franzin and Macdonald, 1996) heterogeneities. The capability of monitoring membrane surface charge comes from the use of a specifically headgroup deuterated POPC (1-palmitoyl-2-oleyl phosphatidylcholine). The choline-deuterated headgroup of the POPC molecule is believed to undergo a concerted tilt in response to an electrostatic field emanating from the surface. The tilting of the headgroup of deuterated POPC produces changes in the $^1$H NMR spectrum which
can be quantified. Qualitatively similar changes in the $^2$H NMR spectrum have been noted for any of a variety of charged species which bind within the polar headgroup region of the bilayer. Thus, this $^2$H NMR technique has been dubbed the "molecular voltmeter".

This thesis begins by describing the $^2$H NMR studies of a series of cationic amphiphiles homogeneously mixed with POPC. Next, I describe the $^1$H NMR studies of domain formation in these cationically charged model membrane systems, induced by a series of anionic polyelectrolytes. I demonstrate that the $^2$H NMR spectra can be used to quantify domain size and composition. I then systematically investigate the effect on domain size and composition of polyelectrolyte identity, polyelectrolyte molecular weight, initial surface charge density and salt concentration. In addition, I have directly investigated the other components of these mixtures using $^2$H NMR for deuterated cationic amphiphiles and $^31$P NMR for polynucleotides. Furthermore, I have studied the influence of a bilayer destabilizing amphiphile (DOPE) on membrane architecture, in the presence of various cationic amphiphiles, polyelectrolytes and salt.

Chapter 1 of the thesis involves a discussion of the basic features of membrane systems, followed by a discussion of polyelectrolyte adsorption and the lateral reorganization of lipids into domains. This topic is covered in only basic outline and more detailed accounts are found in the articles by Cullis et al. (1983), Gennis (1989), Voet and Voet (1990) Welti and Glaser (1994), Tocanne et al. (1994) and Raudino (1995). Chapter 1 also covers the promising field of gene therapy through liposome delivery. Chapter 2 outlines the use of solid state NMR techniques to study bilayer membrane systems as well as a detailed account of solid state $^2$H NMR theory and more detailed discussion of the response of the choline headgroup to surface charge. An even more detailed description of the subject matter may be gained from Seelig (1977), Griffin (1981), Davis (1983),
Lindblom (1996), Roux et al. (1989) and Macdonald et al., (1991). Chapter 3 provides an account of both the synthetic and experimental techniques used in these studies. The results and discussion section will be presented in Chapter 4. The major topics to be covered in this section are (1) the "molecular voltmeter" response of cationic lipids and the "anti-voltmeter" response of polyelectrolytes, (2) factors influencing domain size and composition, (3) polyelectrolyte influence on headgroup deuterated cationic amphiphiles and (4) changes induced in membrane architecture in mixtures containing DOPE.

The primary goal of this research was to study how and why lateral domains form in biological membranes and to gain insights into the methods of both gene therapy and drug delivery technologies.
1.2 Lipids and Membranes

Biomembranes are central to the structure and function of all cells. They define inner and outer aqueous compartments and establish a permeability barrier. These membranes take on a bilayer structure for which proteins are embedded (integral) or bound to the surface (peripheral). Singer and Nicholson (1972) proposed the "fluid mosaic model" which views the bilayer as a dynamic assembly of lipids and proteins each of which are capable of lateral diffusion in the plane of the membrane as shown in figure 1.2.1. Membrane proteins may take on the role of enzymes which catalyze reactions or even as membrane bound substrates. Transmembrane proteins can take the form of channels which regulate the transport of nutrients, ions or waste products in or out of the cell. Membrane imbedded proteins may also take on structural roles such as the Band 3 protein of red blood cells. Yet other membrane associated proteins function as receptors which interact with a host of extracytoplasmic

![FIGURE 1.2.1](image)

A schematic diagram of the Fluid Mosaic model of the plasma membrane. Integral and peripheral proteins are embedded in a sea of lipids which take on a bilayer arrangement. Both glycolipids and carbohydrates are shown to reside on the exterior of the bilayer.
components.

The lipid component of biological membranes spontaneously assemble into a bilayer arrangement. Lipid molecules are amphiphilic in that they contain both a polar headgroup region and a non-polar acyl chain region. These molecules self-assemble into spherical bilayered vesicles in order to minimize the area of contact between the non-polar chains and the aqueous environment. Thus, the polar headgroup regions of the lipids are oriented towards the aqueous phase while the hydrophobic chains are sequestered towards the interior of the bilayer as shown in figure 1.2.2.

The active functions of the membrane are carried out by proteins. Most lipids have a passive function which is to provide the correct physical environment for membrane associated proteins. Some lipids such as phosphatidylinositol (PI) play more specialized roles as substrates for certain enzymes whereas lipids such as diacylglycerol (DAG) are involved in activation of membrane associated proteins (protein kinase C). There also exists a great variety of lipids which are capable of producing a number of non-bilayer arrangements. Examples of the various classes of lipids are depicted in figure 1.2.2.

The fluidity of these membranes depends mainly on the nature of the acyl chain region of the bilayer. The lipids themselves can undergo a temperature dependent transition from a viscous gel phase in which the acyl chains are fully extended, to a fluid liquid-crystalline state, where individual portions of the chains undergo a rapid trans-gauche isomerization, resulting in a reduction of bilayer thickness as well as lateral expansion of the bilayer. In biomembranes the lipids are in a liquid-crystalline state because of chain unsaturation which lowers the phase transition temperature, \( T_m \). Lipids may also undergo another type of phase transition which involves the alteration of the macroscopic assembly of the amphiphiles. For instance, a lamellar \( (L_\alpha) \) to hexagonal \( (H_\alpha) \)
1. Lipid Bilayer

- Water
- Bilayer Normal (Lipid Long Axis)
- Hydrocarbon Interior
- Polar Headgroups
- Water

2. Lipid Classes

A. Fatty Acids

\[
\text{R} - \text{C} - \text{OH}
\]

B. Triacylglycerols

\[
\begin{align*}
\text{CH}_2 - & \text{O} - \text{Cr}_1 \\
\text{CH} & \text{O} - \text{Cr}_2 \\
\text{CH}_2 & \text{O} - \text{Cr}_3
\end{align*}
\]

C. Steroids

\[
[\text{Steroid Structure}]
\]

D. Glycerophospholipids

\[
\begin{align*}
\text{R}_0 & \text{O} - \text{CH} - \text{CH}_2 - \text{O} - \text{PO} - \text{X} \\
\text{R}_3 & \text{O} - \text{CH}_2
\end{align*}
\]

FIGURE 1.2.2 (1) Self-assembly of lipids into a bilayer arrangement. The polar headgroups of the lipids are exposed to the aqueous medium while the non-polar acyl chains of the lipids are segregated to the interior of the bilayer, preventing direct contact with water. (2) The generalized structures of various classes of membrane lipids. R, R_1, R_2, and R_3 represent long hydrocarbon chains while X represents the variety of different headgroups of phospholipids.
Some thermotropic phase transitions exhibited by phospholipids. The gel to liquid-crystalline transition ($L_\beta$ to $L_n$) is shown, with $T_n$ representing the main transition temperature. The bilayer to hexagonal transition is shown in the second scheme with its characteristic temperature $T_{BH}$. This transition occurs for the case of phosphatidylethanolamine (PE) and some other lipids.
arrangement involves the conversion of planar bilayers into long cylinders of lipids with an inner aqueous environment is shown in figure 1.2.3. Such a conversion may be induced thermodromically or by the addition of various amphiphiles. Such a change is thought to arise because of the optimal packing of lipids which possess different molecular shapes (Israelachvili, 1975).

Lipid molecules which are incorporated in membranes can undergo a variety of whole body or internal motions. Lipids may exhibit slow motions such as lateral diffusion or transbilayer flip-flop, which occur on the order of days, to faster motions such as rotation about their long axes. Lipids may also exhibit internal motions such as methyl rotation in the headgroup region or trans/gauche isomerization in the acyl chain region. Figure 1.2.4 shows the type of motions exhibited by lipids along with their respective timescales and the types of techniques used to study them. Depending on

![Figure 1.2.4](image)

FIGURE 1.2.4 Molecular motions exhibited by lipid and protein membrane components with their respective timescales and the spectroscopic techniques used to study them.
the technique used to study the molecular motions the membrane may be pictured as static or highly dynamic.

The great diversity of lipid and protein components of biological membranes makes it challenging to study the physical properties and functional roles of lipids. To circumvent this difficulty one prepares model membranes of defined composition. Model membranes can be readily produced through simple hydration of dried lipid mixtures, sonication, extrusion or dialysis of lipid dispersions (Gennis, 1989).
1.3 Membrane Electrostatics

Membrane electrostatics play an important regulatory role in many cellular functions. For instance, membrane electrostatics control the rate of transverse transport of ions as well as the gating of channels, promotion of cell fusion and the binding of peripheral proteins to the membrane surface (McLaughlin, 1989).

Most biomembrane surfaces are negatively charged primarily due to the presence of 10-20% of anionic phospholipids. The charges arise from the phosphate or carboxylate groups of acidic phospholipids such as phosphatidylserine (PS) or phosphatidylionositol (PI) which reside at the membrane surface. When the charge at the surface is solely attributed to the charged amphiphiles the surface charge density can be calculated from:

\[ \sigma = e \sum Z_i X_i / S_i \]  

\[(1.1)\]

where \( e \) is the elementary charge, \( Z_i \) and \( X_i \) are the valence and mole fraction of the charged lipid species \( i \), and \( S_i \) is the cross-sectional area occupied by the lipid in the liquid-crystalline phase. A similar expression may be used when the surface charge is due to bound hydrophobic ions or charged proteins to neutral membranes (Seelig et al., 1988).

An electrical potential is produced by the charges at the surface and a diffuse cloud of ions responds to the electrical potential. The ions are not fixed at the membrane surface and distribute themselves in the aqueous phase in a balance between their entropic drive to randomize and the favourable electrostatic interaction at the surface. Thus, the fixed charges at the membrane surface are not electrically neutralized at the surface by the counterions. This results in a surface potential
which extends out into solution. The electrical potential at the membrane surface is then dependent on the charge density at the surface and the concentration and valency of the counterions.

The surface charge density which generates the surface potential, \( \Psi_m \), may then be determined by Guoy-Chapman theory (McLaughlin, 1977; McLaughlin, 1989; Cevc, 1990) according to the Poisson equation:

\[
\sigma^2 = 2000\epsilon_0 \epsilon_r RT \sum_i C_{i,\text{eq}} \left( e^{-Z_i \Psi_m / RT} - 1 \right)
\] (1.2)

where \( \epsilon_r = 78 \) is the dielectric constant of water, \( \epsilon_0 \) the permittivity of free space, \( R \) is the gas constant, \( T \) is the temperature, \( C_{i,\text{eq}} \) is the concentration of the \( i \)th electrolyte in the bulk aqueous phase, \( F \) is the Faraday constant and \( Z_i \) is the valency of the \( i \)th species. The effect of \( \Psi_m \) is to attract ions of opposite charge and repel ions of like charge. Thus, the concentration of oppositely charged species adjacent to the membrane surface is greater than the equilibrium concentration. On average, a diffuse cloud of counterions is distributed near the surface creating what is termed a diffuse double layer. Based on the surface potential produced, the local concentration of the ions in solution can be calculated by using the Boltzmann equation:

\[
C(x) = C_{i,\text{eq}} e^{-Z_i \Psi_m / RT}
\] (1.3)

where \( C(x) \) is the concentration of the ion at a distance \( x \) from the membrane surface and all other
symbols have been defined previously. A 60 mV surface potential can increase the local concentration 10 fold, adjacent to the surface as compared to the bulk (Gennis, 1989).

Guoy-Chapman theory predicts that the magnitude and the extension of the electrical potential from the surface is reduced at high salt concentrations and that this effect is greatly enhanced by ions with higher valency. The ionic screening of the surface charge is related to the Debye length, also referred to as the screening length, corresponding to the distance from the surface at which the surface potential drops to 1/e of its value. Although the Guoy-Chapman relationship gives good qualitative results, the quantitative method has its shortcomings which arise from some of the basic assumptions of the model, which are: (1) the membrane charges are uniformly smeared over the surface (homogeneous); (2) the ions are treated as point charges; (3) the dielectric constant of the aqueous phase is constant everywhere; (4) the repulsion of the ions is neglected as they approach the dielectric interface.
1.4 Polyelectrolyte Adsorption

Many naturally occurring macromolecules are polyelectrolytes. Clearly their interactions with membranes will involve electrostatic interactions. These electrostatic interactions (Van de Steeg, et al., 1992) are in addition to the chemical interactions (Van der Waals interaction, hydrophobic forces, hydrogen bonding etc.) of uncharged polymers. In addition to the electrostatic contribution to the Gibbs energy of adsorption, polyelectrolytes also have conformational entropy effects which contribute to their adsorption behaviour, unlike simple ions.

When polyelectrolytes adsorb to uncharged surfaces, they accumulate because of the chemical affinity for the surface. Further accumulation is opposed when the unfavourable electrostatic repulsion overcomes the chemical affinity of the polyelectrolyte for the surface. When polyelectrolytes interact with charged surfaces they may have either a positive or negative adsorption energy depending on the charged signs of the two. For the special case of a strong polyelectrolyte in low salt adsorbing to an oppositely charged surface of high surface charge density, the system lies in the “charge compensation limit”. The adsorbed polyelectrolyte chains are predicted to then form stoichiometric charge complexes with the oppositely charged surface. Experimental verifications of these predictions are numerous. For instance, stoichiometric complexes form between polyelectrolytes and oppositely charged single chain amphiphiles (Hayakawa and Kwak, 1991) as well as oppositely charged double chain amphiphiles (de Meijere et al., 1997; 1998; Shimomura and Kunitake, 1984; Okahata et al., 1985). When enough polyelectrolyte has bound to the oppositely charged surface to neutralize it, additional polyelectrolyte will change the charged sign of the surface and electrostatic repulsion will inhibit further accumulation.

The addition of simple electrolytes to polyelectrolytes bound to oppositely charged surfaces
results in the screening of segment-surface interactions and interpolyelectrolyte repulsions. These forces act antagonistically and depending on the balance of the two forces, polyelectrolyte adsorption may be enhanced or diminished (Stuart et al., 1991). At high salt concentrations polyelectrolyte desorption from oppositely charged surfaces is enhanced for various reasons. First, the electrical energy stored in the diffuse double layer, which aids in accumulating the oppositely charged polyelectrolyte to the surface, is diminished (Denisov et al., 1998). At high enough salt concentration the ions compete for lattice sites on the surface with the polyelectrolyte. Also, desorption of the polyelectrolyte from the surface produces a gain in conformational entropy of the chain. The effects of the electrolyte solution upon polyelectrolyte chain adsorption will play a role up to 2M of added salt.

When bound to surfaces, polyelectrolytes may adopt a variety of conformations. Individual portions of the polyelectrolyte chain may be adsorbed to a surface in train sections while other non-adsorbed portions may take the form of tail or loop regions. For the special case of "charge compensation" the chain conformation statistics predict that the adsorbed polyelectrolyte lies flat on the surface and this has been proven experimentally by Cosgrove et al., 1986. However, in general, polyelectrolyte chains exhibit mixtures of train, tail and loop regions. Each distinct conformation may have a different energy associated with it depending on the distribution, length and number of tail, loop or train portions. Train portions of polyelectrolytes result in a favourable Gibbs free energy of adsorption while formation of loops and tails result in conformational entropy gain. The conformation entropy becomes an increasingly important term when considering binding statistics for polyelectrolyte chains with contour lengths many times greater than their persistence length. The volume fraction profile of the polyelectrolyte, which is defined as the number of monomer units of
the polymer chain which are contained within defined regions away from the adsorbed surface, may be obtained from the summation of all possible conformations (Stuart et al., 1991).

Interactions between polyelectrolyte and oppositely charged lipid bilayers are of fundamental scientific, as well as applied biomedical research. Two types of biological polyelectrolytes and the consequences of their binding to charged bilayer surfaces will be discussed in the next two sections.
1.5 Membrane Domains


Polyelectrolyte adsorption to oppositely charged amphiphiles leads to formation of membrane domains. The individual charge-carrying amphiphiles are able to diffuse laterally within the plane of a two-dimensional lipid bilayer membrane. This permits domain formation upon polyelectrolyte adsorption when the Coulombic attraction draws the relatively mobile amphiphiles towards the relatively immobile polyelectrolyte. Models of domain formation induced upon peptide or protein binding to oppositely charged mixed neutral and charged lipid bilayer membranes account for domain formation by considering the Gibbs free energy of the system to be the sum of contributions from the favourable electrostatic free energy and the unfavourable free energy of demixing the charged from the neutral amphiphiles (Denisov et al., 1998). The latter term is dominated by the negative entropy of demixing the two amphiphiles into separate domains.

Microdomains are generally associated with lipid immiscibility, in the absence of proteins, due to the diversity of amphiphiles with different transition temperatures, T_m. Macrodomains are usually associated with protein interactions with the membrane. Lipid-protein interactions, such as the electrostatic interaction between cytochrome c and anionic phospholipids (de Kruijff and Cullis, 1980), are capable of producing microdomains which can aggregate into macrodomains as in the case of the MARCKS peptide (myristoylated alanine-rich C kinase substrate) and the Rous Sarcoma Virus (RSV) proteins (Haverstick and Glaser, 1989; Denisov et al., 1998; Yang and Glaser, 1995). Most
biological membranes possess these heterogeneities and a homogeneous membrane is thus the exception to the rule.

The existence of a variety of heterogeneous biomembranes has led to the study of the origin of these domains as well as determining their functional significance. Firstly, lipid domains can provide enzymes with unique environments where the activity of the enzyme may be optimized by distinct lipid-protein interactions. For instance, the maximal activity of protein kinase C (PKC) in phosphorylating the MARCKS peptide depends on the inclusion of the MARCKS peptide into cardiolipin and phosphatidylserine (PS) enriched domains (Yang and Glaser, 1996). The existence of domains may also lead to an increased passive transport of hydrophobic and ionic species across the bilayer membrane. This is believed to occur through pores which are created at the borders between domains because of induced "packing defects" (Gennis, 1989). It is also known that both the accessibility of lipids to phospholipases and the rate of lipid flip-flop are enhanced by the presence of these boundary "defects", yielding other plausible functions of domains. Much more experimental work needs to be completed, though, in order to produce more definitive results on the significance of domains.

The main difficulty in assessing the significance of membrane domains is to first find techniques capable of not only defining domains but also producing molecular level details in terms of size and composition. Each unique method has its particular advantages and disadvantages in studying domains. Fluorescent digital imaging is a popular method used to assess domain size and shape but fails in determining domain composition (Luan, 1995; Glaser, 1996). FRAP has been primarily used in determining diffusion coefficient of lipids and permits one to extract domain shape information. Diffraction (Blasie et al, 1985) and calorimetric techniques (McElhaney, 1982) provided
for the assessment of only global properties of domain structure through the construction of phase diagrams.

Until recently, NMR spectroscopy has been incapable of distinguishing in-plane domains. This inability to resolve domains was likely due to the timescale of the technique. NMR has a long timescale (~10^5 s) relative to other spectroscopic techniques such as electron spin resonance (ESR ~10^4 s) or infra-red (IR ~10^-10 s) spectroscopy. Thus, NMR allows for considerable exchange of lipids, through lateral diffusion, in and out of domains, leading to an averaging of the properties of two or more distinct regions of the membrane and thus the inability to observe domain structure (Bloom and Thewalt, 1995).
1.6 Gene Transfection

An important use for polyelectrolytes interacting with lipid bilayer membranes is in the field of gene transfection. Gene transfection involves the introduction of foreign genetic material inside a cell. One type of genetic material used are oligonucleotide drugs which inhibit protein production. A second type of genetic material used in transfection provides a cell with a gene sequence which is lacking. An example of the latter is the current work that is being done in cystic fibrosis and cancer treatment. The types of oligonucleotide drugs used may be subdivided into anti-sense, anti-gene nucleotides and ribozymes which are distinct in their mode of action. Anti-sense oligonucleotides hybridize to m-RNA and thus block translation, whereas anti-gene nucleotides hybridize to DNA to block transcription. Ribozymes, on the other hand, act by binding to the correct sequence in m-RNA and then degrading it (Mönkkönen and Urti, 1998). In all these instances the limiting step in gene translocation is the ability of a vehicle to transport the gene efficiently and safely to the nucleus. There are various such vehicles. One such vehicle is electroporation in which a membrane is subjected to an applied electric field which produces structural defects in the membrane, thus creating a pathway to the cell interior (Nicolaus, 1999). Another method uses viruses such as a retrovirus or adenovirus as vectors (Miller, 1990; Kotin, 1994). A third involves packaging DNA into cationic liposomes (Felgner et al., 1987; Leventis and Silvius, 1990; Gao and Huang, 1991). All of these particular methods have certain shortcomings. Electroporation, for instance, suffers from the fact that it requires sophisticated and expensive equipment. Viruses are probably the most efficient method for gene transfer however they produce immunogenic side-effects, the size of DNA that can be packaged is limited (Jolly, 1987) and their large scale production is complicated (Crystal, 1995). The use of liposomes to encapsulate genes has the advantage that it is non-toxic, lipids are biodegradable,
easy to synthesize, large quantities can be produced and the liposomes protect the DNA from nucleases. The one major shortcoming of this method is that it suffers from a low entrapment efficiency (Fraley, 1985).

Cationic liposomes have gained wide spread use in gene transfer research. Along with the advantages already mentioned for DNA encapsulation by liposomes, this method has the capability of transporting a much larger amount of DNA. With this method the genetic material is electrostatically packaged with cationic lipids. This favours the condensation of DNA which aids in transmembrane transfer. The electrostatic interaction also proves to be useful since the packages contain an excess of cationic charge to encourage binding to the anionically charged biomembranes. The cationic lipid/DNA packages are accepted to enter into the cell's interior by an endocytosis mechanism (Leventis and Silvius, 1990; Zahn et al., 1995; Farhood et al., 1995). The endosomal membrane must be destabilized by some stimulus in order to release "naked" DNA into the cytoplasm before lysosomal degradation takes place. Incorporating phosphatidylethanolamine (PE), a membrane destabilizing amphiphile, into mixtures with the cationic amphiphile encourages vesicle fusion, disruption of the endosomal membrane and consequently increases the potency of gene transfer (Felgner et al., 1994; Farhood et al., 1995). Vesicle fusion is defined as a polymorphic change in the bilayer structure that occurs when two apposed membranes come into close contact and mix with each other. It not only proves to be important for disruption of the endosomal membrane but also for release of DNA from the lipid complexes. The importance of non-bilayer phases in gene delivery is proven by the drastic increase of transfection potency in the presence of phosphatidylethanolamine. The final step in the transfection process occurs when a proportion of the "naked" DNA diffuses through the nuclear pore complex such that transcription can take place.
Finally, improved technologies for cationic liposome transfer of genetic material involves stabilizing the liposomes in order to diminish the amount of serum-induced leakage of the vesicles by direct interaction with serum macromolecules and lipoproteins (Semple et al., 1998). Part of this problem has been overcome by incorporating either saturated lipids or cholesterol into the vesicles (Sternberg et al., 1998). Polymer coats have also been suggested to sterically stabilize the liposomes in "Stealth" liposomes technologies as discussed by Lasic and Needham (1995).
2. THEORY

2.1 Solid State $^1$H and $^{31}$P NMR Studies of Membranes

NMR is the lowest sensitivity spectroscopic technique which is used for the study of biological systems. Yet this technique has gained widespread use as a powerful tool for determining molecular dynamics and structure. Solid state NMR, in particular, is ideally suited to such systems in comparison to the usual solution state NMR and diffraction techniques. Many biological macromolecules and molecular assemblies do not lend themselves to crystallization for diffraction studies. Secondly, certain macromolecular assemblies do not possess the rapid isotropic motions required for solution state NMR, such as lipids in bilayer arrangement. These anisotropic motions lead to spectral line broadening and are ideally suited to study by solid state NMR. Thus, there are no intrinsic size limitations to the technique and the degree to which motional averaging occurs may be measured by the breadth of the residual powder pattern lineshape. The important sources of line broadening in solid state NMR arise from the chemical shift ($H_{C\delta}$), scalar coupling ($H_\delta$), dipolar ($H_d$) and quadrupolar ($H_Q$) nuclear spin interactions. The various terms of the spin Hamiltonian and the size of the interactions are shown in equation 2.1 (Griffin, 1981).

$$H = H_{C\delta} + H_\delta + H_d + H_Q$$

for solids (Hz) $10^5 - 10^4$ $10^4 - 10^3$ $10^3 - 10^2$ $10^2 - 10^1$ $10^1$ - $10^0$ (2.1)

Due to the isotropic motion in liquids both the dipolar and quadrupolar terms are averaged to zero,
with only the isotropic portions of the chemical shift and scalar coupling terms remaining in the spectra. However, in solids there is little or no motional averaging and depending on the sample, either the dipolar or quadrupolar interactions may dominate the appearance of the spectrum. In the intermediate case of semisolids, such as lipids in a bilayer arrangement, molecules possess anisotropic translational and rotational motion, leading to incomplete averaging of the nuclear spin interactions.

In particular, the study of two different nuclei in solid state NMR has yielded a wealth of information concerning the state and dynamics of phospholipid bilayer membrane systems. I will discuss the detailed information and advantages that both $^1$H and $^{31}$P NMR have afforded us. For more detailed reviews of the subject matter the reader is directed to the reviews of Seelig (1977), Seelig (1978), Cullis and de Kruijff (1979), Seelig and Seelig (1980), Griffin (1981), Davis (1983) and Siminovich (1998).
2.1.1 Advantages of $^2$H NMR Spectroscopy

Deuterium is a spin 1 nucleus and therefore possesses a quadrupolar moment for which the NMR spectrum is dominated by the quadrupolar Hamiltonian ($H_Q$). As already stated, the quadrupole interaction is averaged to zero in the case of isotropic motion but for anisotropic motion the interaction is only partially averaged and the $^2$H NMR spectrum is referred to as a Pake doublet, shown in Figure 2.1.1 below. The frequency separation between the peaks of the spectrum is referred to as the quadrupole splitting ($\Delta v_Q$).

![Image of Deuterium NMR powder pattern](image)

**FIGURE 2.1.1** Deuterium NMR powder pattern of lipids in a bilayer arrangement. The quadrupolar splitting ($\Delta v_Q$) is shown as the frequency separation between the two peaks of the pattern.
The residual quadrupolar splitting is dependent on the amount of motional averaging as well as the orientation of the C-D bond vector with respect to the axis of motional averaging and the orientation of the director axis with respect to the laboratory frame of the magnetic field. Rates of molecular motion in the range of at least ~10-100 kHz are required to average the quadrupolar interaction (Watts, 1998).

The main advantage of the use of deuterium is that it behaves as an isolated nucleus. Due to the fact that $^2$H is a low abundance isotope, molecules under study must be isotopically labelled. Thus, the deuterium label may be placed anywhere along a molecule and the $^2$H NMR spectrum will consist of a few resonances which may be unambiguously identified. Furthermore because of the low abundance of $^2$H the spectra will remain essentially unaffected by homonuclear dipolar interactions. Heteronuclear dipolar interactions are also greatly reduced due to the relatively small gyromagnetic ratio ($\gamma$) of the nucleus. Thus, the dipolar broadening effects are removed from the spectra. This greatly simplifies the interpretation of both line shapes and relaxation time measurements. The analysis of relaxation times is also simplified due to the large quadrupole moment for which quadrupolar relaxation becomes the dominant route.

The time scale of various dynamic processes that occur within a membrane are well matched to the breadth of the quadrupolar interaction. Thus, through the use of spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation time measurements for fast motions (Vold and Vold, 1991), single and multiple pulse refocussing experiments (Bloom and Sternin, 1987) for intermediate rate processes, as well as the use of two-dimensional (2-D) chemical exchange experiments (Auger and Jarrell, 1990) for slow motions, $^2$H NMR is capable of following molecular motional frequencies in the range of 10 - 1000 Hz.

$^2$H NMR also has the advantage of a being a non-perturbing spectroscopic technique as
opposed to the use of bulky labels used in fluorescence spectroscopy and the nitroxide group used in electron spin resonance techniques. Thus, replacing a proton by a deuteron does not introduce any perturbation unlike the case with fluorescent dye or spin labels which are used in lateral diffusion measurements of lipids (Tocanne et al., 1994).

Finally, the most important quantity which may be measured from a $^2\text{H}$ NMR spectrum is the quadrupolar splitting ($\Delta v_Q$). This is due to the fact that the measured quadrupolar splitting is dependent upon both the C-D bond orientation and its amplitude of fluctuation. Thus, the quadrupolar splitting can provide structural information. The quadrupolar splitting is also related to the order parameter, $S_Q$, and represents the ensemble and time average fluctuations of the local geometry of the C-D bond about an average orientation. Since lipids may be specifically labelled anywhere along their acyl chain or polar headgroup regions, $^2\text{H}$ NMR can provide a panoramic view of the structure and dynamics of a lipid molecule.
2.1.2 Advantages of $^{31}$P NMR Spectroscopy

Phosphorus is a spin $\frac{1}{2}$ nucleus and its spectrum is dominated by the chemical shift Hamiltonian. Because of the relative size of the chemical shielding versus the dipolar coupling interactions between phosphorus and protons, strong broadband proton decoupling is required in order to remove the $^{31}$P-$^1$H dipolar broadening which overlaps the chemical shift anisotropy effects to produce broad featureless spectra. Thus, the decoupled spectral shape is determined solely by the chemical shift anisotropy (CSA) of the $^{31}$P nucleus which is only partially averaged in phospholipid bilayers. The residual chemical shift anisotropy, $\Delta \sigma = \sigma_1 - \sigma_\perp$, can be directly determined from the edges of the spectrum (Seelig, 1978) as shown in Fig. 2.1.2.

![Phosphorus NMR powder pattern for phospholipids in a bilayer arrangement. The chemical shift anisotropy ($\Delta \sigma$) is shown as the frequency separation between $\sigma_1$ and $\sigma_\perp$.](image-url)
and has a spread of resonances of approximately 40 ppm for the case of lipids in a liquid-crystalline phase.

A major advantage of $^3$P NMR is that most lipids in biological membranes are phospholipids and the natural abundance of the NMR active nucleus $^3$P is 100% and therefore no isotopic labelling is required as in the case of $^1$H NMR. This is a non-perturbing technique which can be used to study average orientation and fluctuation of the phosphate segment by measurement of the chemical shift anisotropy (CSA) (Seelig and Seelig, 1980). The minimum rate of motional averaging of the CSA is $-4 - 6 \text{ kHz}$ which is much slower than for $^1$H NMR.

Another advantage of $^3$P NMR is the fact that the CSA is obtained with the sign in the NMR spectrum. Unlike the $^1$H NMR powder pattern (Pake doublet) which is symmetric and consists of two overlapping Pake patterns, the $^3$P NMR lineshape is not, which proves to be useful in its ability to recognize lipid polymorphism (Cullis and de Kruijff, 1979). For instance, if the lipid geometry of the phase changes from lamellar to hexagonal the $^3$P NMR lineshape will have reversed symmetry (negative sign) and the CSA will be reduced by a factor of two. The reduction of the CSA can be understood by an additional motional averaging that the lipids experience due to lateral diffusion about a cylinder with a small radius ($\sim 20 \text{ Å}$). The lamellar versus hexagonal phase could not be decisively distinguished by $^1$H NMR. Finally, a third type of spectrum can be viewed by $^3$P NMR which results from rapid movement of the phospholipid molecules about all angles in space, an isotropic spectrum. In this case, the lipids can be arranged in a variety of phases such as micellar, inverted micellar, cubic and rhombic which each allow for effective isotropic motional averaging due to rapid lateral diffusion of the lipids. In such a case, other techniques such as freeze-fracture electron microscopy are required to yield a unique interpretation of such a spectrum (Cullis et al., 1983;
Gennis, 1989). $^{31}$P NMR can also be used to identify a simultaneous mixture of phases as well as quantitating the amounts through spectral simulation.
2.2 Theory of Deuterium Nuclear Magnetic Resonance

Deuterium has a spin quantum number of $I = \frac{1}{2}$ and consequently possesses a quadrupolar moment, which is described as an asymmetry of charge at the nucleus. The NMR spectrum for a spin system of a quadrupolar nucleus is determined by the spin Hamiltonian, $H$, which consists of the interaction terms,

$$H = H_Z + H_Q$$

(2.2)

where $H_Z$ and $H_Q$ represent the Zeeman and quadrupolar Hamiltonians respectively. The Zeeman Hamiltonian represents the interaction of the nuclear magnetic moment, $\mu$, with the magnetic field, $B_0$,

$$H_Z = -\mu B_0 = -\gamma I B_0$$

(2.3)

where $I$ is the nuclear spin operator and $\gamma$ the gyromagnetic ratio. The quadrupolar Hamiltonian arises from the electrostatic interaction between the nuclear quadrupolar moment, $eQ$, with the electric field gradient (EFG), $\overline{VE} = \overline{V}_{ij}$, at the nucleus. The electric field gradient is simply the second derivative of the electrostatic potential produced by the electrons. The quadrupolar Hamiltonian is written as,

$$H_Q = \sum Q_n V^{*}_n$$

(2.4)
where $Q_m^m$ and $V_m^m$ are the quadrupole moment and electric field gradient second rank tensor operators and the quadrupole Hamiltonian is the scalar product of the two tensors. The value $n$ describes the symmetry of the charge distribution within the nucleus or the electric field gradient, with $2n + 1$ components. For a quadrupolar distribution of charge $n = 2$ and thus the quadrupole second rank tensor has the five components ($m = 0, ±1, ±2$):

$$Q^0 = \frac{eQ}{l(l+1)} \frac{1}{2} [3I_z^2 - I(1 + 1)]$$

$$Q^{±1} = \frac{eQ}{l(l+1)} \frac{\sqrt{6}}{4} [I_y I_x - I_x I_y]$$

$$Q^{±2} = \frac{eQ}{l(l+1)} \frac{\sqrt{6}}{4} I_z^2$$

where $Q$ is the scalar quadrupole moment, $e$ is the elementary charge, $l$ is the nuclear spin ($l = 1$ for deuterium) and $I_x$ is the raising and lowering operator with $I_z = I_x ± i I_y$. Since only terms $Q_m^m$ appear in the quadrupole Hamiltonian then only the terms of $V_m^m$ need to be considered for the electric field gradient tensor operator which distinguishes the electric field gradient produced by the electron cloud and thus its five components are:

$$V^0 = \frac{1}{2} \left[ \frac{\partial^2 V}{\partial z^2} \right] = \frac{1}{2} V_z$$
where $V_i$ is the second derivative of the electrostatic potential $V(xyz)$ produced by the electrons at a point $(xyz)$. These elements represent the electrical field gradient at the nucleus. Therefore, the quadrupole Hamiltonian ($H_Q$) takes on the form, for $n = 2$, of:

$$H_Q = \sum Q_i^e V_{i}$$

(2.7)

where $H_Q = Q_2^e V_2^2 + Q_4^e V_4^2 + Q_4^e V_4^2 + Q_2^e V_2^2 + Q_2^e V_2^2$. Through multiplication of these individual terms the quadrupole Hamiltonian becomes:

$$H_Q = \frac{eQ}{4i(2I - 1)} [V_{\alpha\beta}(3l_z^2 - (l + 1)) + (V_{\alpha\beta} + iV_{\beta\alpha})(l_+ l_{-} + l_{-} l_{+}) + (V_{\alpha\beta} - V_{\beta\alpha}) + 2iV_{\beta\alpha}l_z^2]$$

(2.8)

The quadrupole Hamiltonian can be further simplified by recognizing that in a high magnetic field (7 Tesla - 300 MHz) the quadrupole interaction is approximately 200 kHz whereas the Zeeman term is 46 MHz for deuterium. Thus, the quadrupole interaction is simply a first order perturbation.
of the Zeeman interaction and since the total Hamiltonian is the sum of these two terms, then only
the terms in \( H_o \) which commute with \( H_z \) need to be considered. Since the terms containing the raising
/ lowering operator alter the wavefunction they are dropped from \( H_Q \) and the expression reduces to:

\[
H_Q = Q_2^0 V_2^0 = \frac{eQ}{a(2I - 1)} V_{yz} (3I_z^2 - I(I + 1))
\]

(2.9)

The above equation is the general formulation of the quadrupole Hamiltonian and is valid provided
that \( V_{yz} \) and the spin operator \( I \) are defined in the same reference frame. But, \( I \) is defined in the
laboratory frame whereas \( V_{yz} \) is defined in a molecular fixed frame of reference. In order to use
equation 2.9 it is necessary to rotate the molecular fixed frame into the laboratory coordinate system
through successive rotation through Eulerian angles \( \alpha \), \( \beta \) and \( \gamma \).

**Principle Axis Coordinate System of the Irreducible \( V_{yz} \) Elements**

The electrostatic field gradient tensor is a 3 x 3 matrix which is symmetric and traceless. It
may be transformed into a principal axis system in which its matrix elements are diagonal and all off-
diagonal elements are zero, in the form:

\[
V^{\text{PAS}} = \begin{bmatrix}
V_{11} & 0 & 0 \\
0 & V_{22} & 0 \\
0 & 0 & V_{33}
\end{bmatrix}
\]
where $\text{Tr } V^\text{eq} = 0$ and thus $V_{11} + V_{22} + V_{33} = 0$. By convention $V_{22} \geq V_{11} \geq V_{33}$ and $V_{11} = \text{eq}$ which is the tensor element with the largest field gradient. Another definable parameter for this system is $\eta$, the asymmetry parameter, which takes on the form $\eta = (V_{11} - V_{22}) / V_{33}$ and can have values between $0 \leq \eta \leq 1$. For the case where $\eta = 0$ the electronic structure about the nucleus has axial symmetry and $V_{11} = V_{33} \neq V_{22}$.

From the above definitions, the elements defined in equation 2.6 in terms of $V_{11}$, $V_{22}$, and $V_{33}$ for the electric field gradient tensor components, may be redefined in the principal axis system as:

$$V_{2} = \frac{1}{2} V_{33}$$

$$V_{12}^{1} = 0$$

$$V_{12}^{1} = \frac{1}{2\sqrt{6}} (V_{11} - V_{22})$$

where all the off-diagonal elements have been omitted since all $V_{i}$, where $i \neq j$, are equal to zero.

Now all that remains is to transform $[V_{i}]$ of the principal axis system to the laboratory frame (ie. $[V_{i}]^{\text{lab}}$ to $[V_{i}]^{\text{lab}}$), since it is the only term which remains in the quadrupole Hamiltonian. This transformation may be accomplished by one of two methods which both rely on the use of rotation matrices. If the $V_{ij}$ elements of $V^\text{eq}$ are expressed in Cartesian coordinates then the method described by Rose (1957) may be used. If $V_{ij}$ are expressed in spherical coordinates then the Wigner rotation matrix may be implemented as:
Applying the Wigner rotation matrix $D_{mn}(\Theta \Phi \Psi)$, where $(\Theta \Phi \Psi)$ correspond to the Euler angles of $(\alpha \beta \gamma)$, is equivalent to performing two rotations of the principal axis system, to which $\gamma = 0$.

Consider that initially the z-axis of the PAS is defined as being parallel to the magnetic field $B_0$. The angle $\phi$ is therefore defined as a rotation angle about the z-axis, i.e. in the $xy$ plane of the magnetic field whereas the angle $\theta$ is defined as a rotation about the y-axis, i.e. in the $xz$ plane. Thus, the PAS of the EFG tensor is rotated by an orientation defined by the polar and azimuthal angles $\theta$ and $\phi$ with

\[
[V^m_n]^{\text{LAB}} = \sum_{m,n=1.2} D_{mn} [V^m_n]^{\text{PAS}}
\]

FIGURE 2.2.1 Wigner rotation of the principal axis system of the electric field gradient tensor into the laboratory frame of reference.

36
respect to the magnetic field, \( B_m \) as shown in Figure 2.2.1. Therefore the goal of performing these rotations is to define the quadrupole Hamiltonian in more general terms where any orientation of the EFG can be described with respect to the laboratory frame of reference.

Multiplication of the interesting terms of the rotation matrix \( D_{mn}(\theta, \phi, \gamma) \), shown in table 2.1.1 (for a full account refer to Seelig, 1977), with the EFG elements defined in equation 2.10, \( [V^l_j]^m_n \) is equivalent to \( D_{00}V_0^0 + D_{10}V_1^0 + D_{20}V_2^0 + D_{30}V_3^0 + D_{20}V_2^1 \).

### Table 2.1.1 Elements \( D_{mn} \) of the rotation matrix \( D_{mn}(\theta, \phi, \gamma) \). For the complete listings of the Wigner rotation matrix refer to Seelig (1977).

<table>
<thead>
<tr>
<th>( D_{mn} )</th>
<th>( e^{i\delta_m} /\sqrt{3/8} \sin^2\theta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D_{10} )</td>
<td>( e^{i\delta_0} /\sqrt{3/2} \sin\theta \cos\theta )</td>
</tr>
<tr>
<td>( D_{00} )</td>
<td>( \frac{1}{2}(3\cos^2\theta - 1) )</td>
</tr>
<tr>
<td>( D_{10} )</td>
<td>( e^{i\delta_1} /\sqrt{3/2} \sin\theta \cos\theta )</td>
</tr>
<tr>
<td>( D_{20} )</td>
<td>( e^{i\delta_2} /\sqrt{3/8} \sin^2\theta )</td>
</tr>
</tbody>
</table>

Substituting the terms from equation 2.10 and table 2.1.1 into equation 2.9 and the use of the identity \( e^{i\delta_8} + e^{-i\delta_0} = 2\cos2\delta \) produces the most general form of \( H_q \):
or further simplified by substitution of $V_{33} = \text{eq}$ becomes:

$$H' = -\frac{eQ}{4(2I-1)}[3I_z^2 - I(I+1)]\left[\frac{1}{2}(3\cos^2\theta - 1)V_{33} + \frac{1}{2}\sin^2\phi \cos 2\phi (V_{11} - V_{33})\right] \quad (2.12)$$

or further simplified by substitution of $V_{33} = \text{eq}$ becomes:

$$H' = \frac{\epsilon Q}{8(2I-1)}[3I_z^2 - I(I+1)][(3\cos^2\theta - 1) + \eta \sin^2\phi \cos 2\phi] \quad (2.13)$$

in which the quadrupole Hamiltonian may be determined for any fixed molecular orientation with respect to the magnetic field. For molecules which contain C-D bonds, the electric field gradient lies along the bond axis, arising from the bonding electrons and is equivalent to $V_{33}$. In the simplest case the sample consists of a homogeneously oriented crystal in which all the molecules possess C-D bonds parallel to each other. This means that all the molecules in the sample contain a unique orientation of the angles $\theta$ and $\phi$ with respect to $B_0$. This, in general, is unrealistic for most lipid samples in which the sample consists of a "powder pattern” of orientations but it serves as a good starting point for the discussion of the energy levels and thus the spectral appearance of a quadrupolar nucleus.

**Energy Levels and the Quadrupolar Splitting**

Since deuterium is a spin $I = 1$ nucleus it contains three degenerate energy levels ($m = -1, 0$ and 1) in the absence of a magnetic field. When a deuteron is placed in a static magnetic field the degeneracy of the energy levels is lifted due to the Zeeman interaction. However, the energy
difference between any two levels separated by $\Delta m = \pm 1$ is degenerate. Nevertheless, the total Hamiltonian is a sum of both Zeeman and quadrupole interactions. The latter is a small perturbation of the former and causes a shift in the Zeeman energy states. This lifts the degeneracy of the energy differences and leads to a doublet of resonances. This can best be understood by considering the form of the energy levels of the total Hamiltonian:

$$E_n = -\gamma n B_0 + \frac{e^2 q Q}{8(2I + 1)} [3m^2 - (I + 1)][(3\cos^2 \theta - 1) + \eta \sin^2 \theta \cos 2\phi]$$  \hspace{1cm} (2.14)$$

where the first term represents the Zeeman energy term and the second the quadrupole contribution to the energy states. Since deuterium possesses three values for the magnetic quantum number (i.e. $m = 1, 0, -1$) then the three resulting energy levels are:

$$E_{+1} = -\gamma B_0 + \frac{1}{8} e^2 q Q ((3\cos^2 \theta - 1) + \eta \sin^2 \theta \cos 2\phi)$$

$$E_0 = -\frac{1}{4} e^2 q Q ((3\cos^2 \theta - 1) + \eta \sin^2 \theta \cos 2\phi)$$

$$E_{-1} = -\gamma B_0 + \frac{1}{8} e^2 q Q ((3\cos^2 \theta - 1) + \eta \sin^2 \theta \cos 2\phi)$$  \hspace{1cm} (2.15)$$

The two allowed transitions are determined by the selection rule $\Delta m = \pm 1$, which lead to the resonance frequencies:
Therefore two resonance lines are observed in \( ^1H \) NMR spectrum symmetrically displaced about \( \nu_0 \), the Larmor frequency, for a homogeneously oriented sample. The frequency spacing between the two lines is the quadrupole splitting \( \Delta \nu_Q \) and is given as:

\[
\nu_r = \frac{E_r - E_s}{h} = \nu_0 \left[ \frac{3 e^2 q Q}{8 h} \left( 3 \cos^2 \theta - 1 \right) + \eta \sin^2 \theta \cos 2 \phi \right]
\]

\[
\nu_s = \frac{E_r - E_s}{h} = \nu_0 \left[ \frac{3 e^2 q Q}{8 h} \left( 3 \cos^2 \theta - 1 \right) + \eta \sin^2 \theta \cos 2 \phi \right]
\]

(2.16)

Therefore two resonance lines are observed in \( ^1H \) NMR spectrum symmetrically displaced about \( \nu_0 \), the Larmor frequency, for a homogeneously oriented sample. The frequency spacing between the two lines is the quadrupole splitting \( \Delta \nu_Q \) and is given as:

\[
\Delta \nu_Q = (\nu_r - \nu_s) = \frac{3 e^2 q Q}{4 h} \left( 3 \cos^2 \theta - 1 \right) + \eta \sin^2 \theta \cos 2 \phi
\]

(2.17)

where \( e^2 q Q / h \) is referred to as the static quadrupole coupling constant and has a value of about 167 kHz for a C-D bond (Burnett and Muller, 1971). This is the most general form of the quadrupole splitting and it indicates how the splitting may be modified by the geometric term, i.e. inclusive of the angles \( \theta \) and \( \phi \) of the C-D bond vector with respect to the magnetic field. In many cases the EFG tensor is axially symmetric (\( \eta = 0 \)) and the equation above is further simplified to:

\[
\Delta \nu_Q = \frac{3 e^2 q Q}{4 h} \left( 3 \cos^2 \theta - 1 \right)
\]

(2.18)
where, by inspection of the equation, the quadrupole splitting may be reduced to zero by the right choice of $\theta$ in the geometric term. This angle is referred to as the “magic angle” and has a value of $\theta = 54.7^\circ$. At the same time, the largest value that the quadrupole splitting may take on occurs at $\theta = 0$, i.e. with the C-D bond aligned with the magnetic field, and has a value of 255 kHz.

**Powder Pattern Lineshapes and Polycrystalline Samples**

In the previous section both the energy levels and quadrupolar splittings were determined for a homogeneously oriented sample. As already mentioned, though, many samples including lipids are difficult to prepare as single crystals. Samples which are most often prepared and under study in $^2\text{H}$ NMR are polycrystalline powders in which the nuclear sites of the sample are randomly oriented with respect to the external magnetic field, $B_0$. The “powder” pattern lineshape in the NMR spectrum results from the average over all possible orientations of the C-D bond vectors. In other words, each orientation $\theta$, of the C-D bond vector with respect to $B_0$, produces a doublet of resonances and each of the individual doublets add together to produce an envelope of resonances, as already shown in the introduction. The intensity of each of the doublets depends on the number of nuclei present at a particular orientation of $\theta$ and $\phi$. Thus, the $^2\text{H}$ NMR spectrum is a map of frequency versus the probability of a particular $\theta$ and $\phi$. The shape of such a spectrum will be derived as follows for the simplest case which is that of axial symmetry ($\eta = 0$) i.e. only consider the angle $\theta$.

Assume that there is a uniform distribution of $N$ nuclei over the surface of a sphere with radius $r$. This means that all angles are equally probable. Thus, the density of spins on the surface is given by the expression $N/4\pi r^2$. The fraction of nuclei $dN$ contained between an area defined on the surface by the angles $\theta$ and $\theta + d\theta$, with respect to $B_0$, is determined as the latitudinal area of a zone of a
sphere, $2\pi r^2 \sin \theta d\theta$, multiplied by the surface density of the spins:

$$dN = \frac{N}{4\pi r^2} (2\pi r^2 \sin \theta d\theta) = \frac{1}{2} N \sin \theta d\theta$$  \hspace{1cm} (2.19)

where the probability distribution of the angle $\theta$ is $P(\theta) = dN/d\theta$ which is simply the fraction of the total surface area defined by $\theta - \theta + d\theta$ and from the above equation reduces to:

$$P(\theta) = \frac{1}{2} \sin \theta$$  \hspace{1cm} (2.20)

Now consider the form of the doublet resonance frequencies derived in equation 2.16 for the case of axial symmetry:

$$v_\pm = v_0 = \frac{3}{4} \frac{e^2 q Q}{h} \left( \frac{3\cos^2 \theta - 1}{2} \right)$$  \hspace{1cm} (2.21)

In order to proceed further it is instructive to introduce the "reduced" resonance frequency, $\epsilon$, which is a simple rearrangement of the previous equation and which isolates the geometric term containing $\theta$:

$$\epsilon_\pm = \frac{v_\pm - v_0}{\frac{3}{4} \frac{e^2 q Q}{h}} = \pm \frac{1}{2} \frac{3\cos^2 \theta - 1}{2} \hspace{1cm} (2.22)$$
where $1 \geq e, 2 \leq \frac{1}{2}$ and $-1 \leq e \leq \frac{1}{2}$ for the range of frequencies between $\theta = 0^\circ$ and $\theta = 90^\circ$. We may now define the fraction of spins with reduced frequency between $e$ and $e + de$ as $P(e)de$. The relationship between the two probability densities, $P(\theta)$ and $P(e)$ is:

$$P(e) = P(\theta) \frac{d\theta}{de} = \frac{1}{2} \sin \theta \frac{d\theta}{de} = -\frac{1}{2} \frac{d \cos \theta}{de}$$  \hspace{1cm} (2.23)

From equation 2.22 for the reduced frequencies $\cos \theta = \left[(e+2e+1)/3\right]^{1/2}$ and by substituting this into equation 2.23 and taking the derivative $P(e)$ becomes:

$$P(e) = \frac{1}{\sqrt{12} \sqrt{e+1}}$$  \hspace{1cm} (2.24)

The $^1H$ NMR powder spectrum is obtained by plotting the two resonances, $e_+$ and $e_-$, versus the total of the probability densities, $P(e) = P(e_+) + P(e_-)$, as shown in figure 2.2.2. Assuming that the transition probability is equivalent for each of the individual transitions then the total absorption intensity $S(e)$ is linearly related to $P(e)$.

By examination, the frequencies diverge at $e = \pm 1/2$ and this corresponds to the situation of the C-D bond oriented perpendicular to $B_{0}$, i.e. $\theta = 90^\circ$. The frequency spacing of these two most intense peaks $\Delta e = 1$ is the quadrupole splitting and thus the static quadrupole coupling constant may be determined directly for a static polycrystalline sample.

The above treatment is true for the case where the individual resonance lines of the powder spectrum are delta functions. But, what if the resonance lines are Lorentzian or Gaussian? This would mean that intensity at one particular frequency contains contributions from overlapping
neighbouring resonances. For a line centred at $\epsilon^*$ the shape of the individual resonance may be approximated by either a Lorentzian lineshape,

$$l(\epsilon - \epsilon^*) = \frac{1}{\pi \sigma} \frac{1}{1 + (\epsilon - \epsilon^*)^2/\sigma^2}$$

(2.25)

or a Gaussian lineshape:

$$l(\epsilon - \epsilon^*) = \frac{1}{\sqrt{2\pi}\sigma} \exp\left[-(\epsilon - \epsilon^*)^2/2\sigma^2\right]$$

(2.26)
where the full width at half height, \((\Delta \epsilon)_{1/2}\), for the single resonance line is equal to 2.35\(\sigma\). The total intensity \(I(\epsilon)\) at a frequency \(\epsilon\), is the integral over all contributions from all possible \(\epsilon^*\) as expressed by the convolution of the lineshape function with the probability function in:

\[
I(\epsilon) = \int_{-\infty}^{\infty} I(\epsilon - \epsilon^*) P(\epsilon^*) d\epsilon^*
\]  

(2.27)

Seelig (1977) has shown that through the use of computer lineshape simulations, using either the Lorentzian or Gaussian distributions, that varying the line width parameter produces the theoretical value for the quadrupole splitting for only very sharp lines i.e. small \(\sigma\). In all other cases the “real” quadrupole splitting is obscured by the broadening and attains a value smaller than that predicted for the case where individual resonances are delta functions. In such an instance, the true quadrupolar splitting may be determined through “de-Pakeing” the powder spectrum in order to retain the \(0 = \gamma^p\) orientation resonant frequencies for the two transitions (Bloom and Sternin, 1983). Another origin to the smoothing of the spectral lineshape, which should always be considered, is due to the overlapping of distinct Pake patterns which differ in breadth.

Finally, derivation of the powder pattern which results from a non-zero asymmetry parameter becomes much more complicated and the details may be found in Cohen and Reif (1957).

**Motions in Oriented Liquid Crystals**

The previous treatment was carried through for both single crystals and polycrystalline powders in the absence of motions by individual molecules. Liquid crystals are fluid systems which
exhibit both motion and at the same time order as is the case for lipid molecules in their macromolecular assemblies such as the lamellar or hexagonal phases. For such an instance the above equations must be modified in order to account for motion in these systems. In the liquid crystalline phase rod-like molecules align parallel to one another, along their long molecular axes, and exhibit unrestricted motion about these axes with frequencies in the range of $10^7$ - $10^{10}$ Hz. On the other hand, although angular motions perpendicular to the long axis are restricted the parallel packing of the molecules is not perfect and angular excursions do occur rapidly, although comparatively reduced in amplitude. The long axis of motional averaging is referred to as the director axis ($z'$) and the movement of the molecules about this axis are cylindrically symmetric. In the case of lipid molecules the director axis is normal to the bilayer surface.

The starting point for incorporation of these and other molecular motions is to consider the average order of the system. We begin by attaching a Cartesian coordinate system $(x,y,z)$ onto the liquid-crystalline molecule and then define the average fluctuation of these axes about the director axis, $z'$. As mentioned in the introduction, the measure of such fluctuations is contained within the order parameter, $S$. In this instance the order parameter $S_i$ is defined as:

$$ S_i = \frac{1}{2} \left( \cos^2 \theta_i - 1 \right) $$

(2.28)

where $\cos^2 \theta_i$ is represented by a time average of the fluctuations of the $i$th ($i = x,y,z$ or $1,2,3$) coordinate axis with respect to the director axis. The order parameters $S_i$ are defined as the diagonal elements of a $3 \times 3$ order matrix and thus define the fluctuations of a second-rank tensor. Since $\sum \cos^2 \theta_i = 1$, then $\sum S_i = 0$ and because $\cos^2 \theta_i$ can only take values of between 0 and 1, then all $S_i$ are
restricted to values between -1/2 and 1. Therefore, if the tensor is axially symmetric and the z-axis is defined as the axis of symmetry then its order parameter is $S_0$ and the order parameter of the other two axes are equivalent, $S_1 = S_2$. By definition, this means that $S_0 = -1/2 S_2 = -1/2 S_1$.

In deriving the equations which incorporate motions of the lipid molecules we first consider the case of a liquid crystal which is macroscopically ordered between parallel glass plates. Lipid molecules will arrange themselves in parallel layers on the supporting plates such that the director axes $z$ are all parallel to one another and perpendicular to the glass surfaces. The molecular motions of the lipids are thus anisotropic and the EFG at the deuterium nucleus is not averaged to zero and thus there remains a residual quadrupolar splitting. In the case of single crystals the angles $\theta$ and $\phi$ defined the polar and azimuthal angles of the EFG with respect to $B_0$. In a liquid crystal the unique axis becomes the director axis of motion. If the magnetic field is applied parallel to this axis then the average position of the EFG tensor is defined by the same angles as for the case of a single crystal. But, if the glass plates of the planar-oriented liquid crystals are rotated such that the director axis and $B_0$ are no longer parallel, a new angle must be defined $\beta'$. As shown in figure 2.2.3, $\beta'$ is the angle made between the director axis and $B_0$, for the more general case, while the angles $\theta$ and $\phi$ now specify the orientation of the C-D bond with respect to the director axis. What this effectively does is to allow for the incorporation of the rapid fluctuations of the molecules about $z'$ into the equation for the quadrupole splitting by expressing the electric field gradient tensor into the molecular coordinate system of $z'$. The static EFG is averaged by this motion and only the new effective field gradient tensor is detected and it is axially symmetric about $z'$. Thus, the angles $\theta$ and $\phi$ must be replaced by their time average about the principal axes of the static electric field gradient tensor, $(x,y,z)$. 

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By taking into account the definition of the order parameters in equation 2.28, for average fluctuations about $z'$, and defining the direction cosines of the $x, y, z$ axes of the EFG with respect to the director axis as:

$$
x'x = \cos \theta_1 - \sin \theta \cos \phi,
$$

$$
y'y = \cos \theta_2 = \sin \theta \sin \phi,
$$

$$
z'z = \cos \theta_3 = \cos \theta
$$

(2.29)
and substituting into the time-averaged equation for the angular dependence of the quadrupole splitting of a single crystal:

\[ \Delta v_q = \frac{3}{2} \frac{e^2 q Q}{h} \left( \frac{3}{2} \cos^2 \theta - 1 + \frac{1}{2} \sin^2 \theta \cos 2\phi \right) \]  

(2.30)

gives the equation for the quadrupole splitting which incorporates angular fluctuations of the constituent molecules, specifically for the case where \( z' \) is parallel with \( B \):

\[ \Delta v_q = \frac{3}{2} \frac{e^2 q Q}{h} (S_{33} + \frac{1}{3} \eta \{S_{11} - S_{22}\}) \]  

(2.31)

This equation may be further transformed to the more general case where the director axis \( z' \) can make any angle \( \beta' \) with respect to the magnetic field, \( B_0 \). This can be done in a similar fashion as was accomplished for the general case of orientation as in the static version, through Wigner rotation matrices. In the case of liquid crystals, the expression of the EFG tensor in the laboratory frame now involves two consecutive transformations and two sets of Eulerian angles which describe the transformation from the principal axis system in the molecule to the director system (\( \alpha, \beta, \gamma \)) and then from this director system to the laboratory coordinate system (\( \alpha', \beta', \gamma' \)). Thus, the expression for the quadrupole splitting becomes:

\[ \Delta v_q = \frac{3}{4} \frac{e^2 q Q}{h} (3 \cos^2 \beta' - 1) (S_{33} + \frac{1}{3} \eta \{S_{11} - S_{22}\}) \]  

(2.32)
which in the case of axial symmetry \( (\eta = 0) \), further reduces to:

\[
\Delta v_q = \frac{3}{4} \frac{e^2 q Q}{h} (3 \cos^2 \beta' - 1) S_{33}
\]  

(2.33)

By observation, in the most general case for \( \eta \neq 0 \), the quadrupole splitting is determined by two order parameters due to the fact that \( \sum S_i = 0 \), while for axial symmetry only one order parameter is required to determine the quadrupole splitting. The latter case is generally observed and thus equation 2.33 applies. This is true because not only is it known from crystal studies (Derbyshire et al., 1969; Barnes and Bloom, 1973; Fung, 1974) and theoretical calculations (Hoyland, 1968) that the asymmetry parameter is small \( (\eta \leq 0.05) \) and can be neglected for C-D bonds but also lipid motions are axially symmetric and thus \( S_{11} = S_{22} \).

By comparing the expressions of the quadrupole splitting for a homogeneous static crystal and a homogeneously oriented liquid crystal, it becomes obvious that the effect of motional averaging is to reduce the size of the static \( \Delta v_q \) by a factor of \( S_{33} \). For lipid bilayers, this means that \( \Delta v_q \) is reduced by \( S_{33} \) due to the angular fluctuations \( (\theta \text{ and } \phi) \) of C-D bond about the normal of the bilayer surface. By inspection of the geometric term of equation 2.33, the quadrupole splitting collapses to zero for \( \beta' = 54.7^\circ \), the magic angle, while reaching its maximum value at \( \beta' = 0^\circ \). By comparison of \( \Delta v_q \) of homogeneously oriented bilayers for \( \beta' = 0^\circ \) to the static value for a C-D bond \((255 \text{ kHz})\) the segmental order parameters may be determined for a lipid molecule (Seelig and Niederberger, 1974) by specifically deuterolabelling along the molecule. The overall effect of rapid axial rotation of a lipid about its long axis, on a timescale faster than that of \( ^2\text{H} \) NMR, is to reduce the quadrupole splitting.
of the deuterons on its acyl chain by 1/2 from the static case.

**Liquid Crystalline Powders**

Unlike the case of homogeneously oriented bilayers pressed between glass plates the most common practice for study of lipids is in random dispersions of lipids in spherical bilayer arrangements, liposomes or vesicles. In such an arrangement there is a random distribution of director axes $z'$ where all angles are equally probable as in the case of a static powder pattern and the lineshape, for such a sample, may be derived analogously. The main difference which exists is that now the resonance lines do not depend on the orientation of the nuclear sites but instead depend on the orientation of the director axes with respect to the external magnetic field, $B_0$. Once again the spectrum consists of an envelope of resonances symmetric about the Larmor frequency, $v_L$.

The angular dependence of the individual resonance positions $v_i$ can be determined for the case of random liquid crystalline powders by comparison to the original equations of resonance frequencies for a single crystal. By inspection of the differences in the equations of the quadrupole splitting, in transition from single crystals to liquid crystals, and incorporation into the equations, discussed above, the positions of the resonance lines for liquid crystals are determined by:

$$v_i = v_0 \pm \frac{2}{3} \frac{e^2 q Q}{\hbar} (3 \cos^2 \theta' - 1) (S_{33} + \frac{1}{3} \eta [S_{11} - S_{22}])$$

(2.34)

where $\theta'$ is once again the angle between the director axis and $B_0$. For a random distribution of director axes with respect to the magnetic field the probability density function is defined as $P(\theta') = \ldots$
½ sin β这样做，并且将共振频率记为:

\[ e_s = \frac{v_s - v_t}{\frac{3}{4} e^2 q Q \left( S_{33} + \frac{1}{3} \eta (S_{11} - S_{22}) \right)} = \frac{3 \cos^2 \beta - 1}{2} \]  \hspace{1cm} (2.35)

理论粉末图案因此是通过绘制频率 \( e_s \) 与 \( P(\epsilon_s) \) 的关系，正如在早先一节中所完成的一样，对于此新的值 \( e_s \)。偶极子分裂对于随机液晶分散液是通过此谱中两个最尖锐的峰的分离来定义的，对于 \( \beta = 90^\circ \)，即对于一个与磁场垂直的取向。

方程的偶极子分裂被给出为:

\[ \Delta v_q = \frac{3}{4} e^2 q Q \left( S_{33} + \frac{1}{3} \eta [S_{11} - S_{22}] \right) \]  \hspace{1cm} (2.36)

这进一步减少了对于轴对称取向 (\( \eta = 0 \)) 的情况:

\[ \Delta v_0 = \frac{3}{4} e^2 q Q S_{33} \]  \hspace{1cm} (2.37)
2.3 The "Molecular Voltmeter" Model for the Headgroup Response of Phosphatidylcholine to Surface Charge

Many studies of the surface electrostatics of bilayer membranes have come from the use of specifically headgroup deuterated 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC). In particular, the two most useful deuterolabeled positions are designated as α and β, shown in Figure 2.3.1. In the case of a purely neutral membrane in which the vesicles are composed of either 100% α or β deuterolabeled POPC the quadrupolar splittings, in the $^1$H NMR spectra, are nearly identical for the two labeling positions (±6 kHz).

![Nomenclature for the headgroup deuterolabeling positions of phosphocholine](image_url)

FIGURE 2.3.1 Nomenclature for the headgroup deuterolabeling positions of phosphocholine
In the most general sense, by depositing charge at the surface in the form of metal ions (Akutsu & Seelig, 1981; Altenbach & Seelig, 1984; Macdonald & Seelig, 1987 a,b), hydrophobic ions (Altenbach & Seelig, 1985), charged local anesthetics (Browning & Akutsu, 1982; Seelig et al., 1988), chaotropic agents (Macdonald & Seelig, 1988), charged peptides (Siel & Watts, 1985; Roux et al., 1989; Kuchinka & Seelig, 1989; Beschiaschvili & Seelig, 1990; 1991) or negatively and positively charged lipids (Scherer & Seelig, 1987; Scherer & Seelig, 1989) the quadrupolar splittings observed in the NMR spectra vary in a predictable manner. When negative charge is added to the POPC surface in the form of an anionic lipid such as 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG), the quadrupolar splitting progressively increases for the POPC-α-d₃ case and decreases for POPC-β-d₂ with increases in surface charge. On the other hand, the opposite effect is noted for the addition of cationic charge to the surface in the form of 1,2-dioleoyl-3-trimethylaminopropane (DOTAP) where the quadrupolar splitting decreases for POPC-α-d₃ and increases for POPC-β-d₂ bilayers. The variation of the quadrupolar splittings for the extreme cases of charge can differ by tens of kilohertz. The addition of neutral molecules such as cholesterol or indifferent electrolytes such as NaCl, which do not specifically bind to the surface of these membranes result in little or no change of the ¹H NMR response (Scherer and Seelig, 1987; Bechinger et al., 1988). Thus, the fact that any and all sources of surface charge produce similar changes in the quadrupolar splittings of deuterated POPC resulted in terming this ¹H NMR technique as the “molecular voltmeter”.

The next question which then arises is what is the physical situation which produces this response? The fact that there is an inverse relationship in the change of quadrupolar splittings from the two deuterolabeling positions rules out a generalized increase or decrease in the order of the choline headgroup, which would give rise to either an increase or decrease in the quadrupolar
Akutsu and Seelig (1981) first proposed that there was a conformational response of the choline headgroup of POPC to surface charge in which the entire headgroup tilted with respect to the plane of the membrane surface. The choline headgroup is thought to tilt about the Cα-O-P dihedral angle, between the hinge point of the glycerol backbone and headgroup region of the molecule. Scherer and Seelig (1989) discussed this "choline-tilt" model as a result of an electrostatic attraction/repulsion mechanism of the phosphocholine headgroup (P-N' dipole) in response to charge deposited within the headgroup region. The zwitterionic headgroup of POPC possesses a large dipole moment of approximately 19 D (Shepherd & Bildt, 1978) in the form of a 'P-N' dipole which lies nearly parallel with the bilayer membrane, within 30°, for a neutral membrane. This tilt angle changes in the presence of charge at the surface such that the dipole moment of the phosphocholine group will seek to align itself with the electrical field lines emanating from the charged surface. Upon addition of cationic charge the phosphocholine dipole will move out of the bilayer plane while addition of negative surface charge will produce the opposite rotation and thus pull the dipole towards the bilayer surface. This can best be visualized by placing the surface charges close to the phosphate segment i.e., the lower dielectric medium of the headgroup region. This is reasonable since generally only charged molecules which can penetrate the interface between the glycerol backbone and headgroup region of the bilayer, produce the desired 3H NMR response. The qualitative response of the phosphocholine headgroup, to a variety of compounds, is similar as long as their charge carries the same sign. Quantitatively the sensitivity of the "molecular voltmeter" will also depend on the valency of the molecule, on the extent of binding to the surface and on both the vertical and lateral distance of closest approach to either the positive or negative end of the P-N dipole.
Further evidence of the fact that the phosphocholine headgroup undergoes only two types of conformational changes comes from the so-called $\alpha$-$\beta$ plots (Akutsu & Seelig, 1981; Altenbach and Seelig, 1984; Beschiaschvili & Seelig, 1991). In this method, the quadrupolar splitting of POPC-$\beta$-$d_5$, $\Delta\nu_5$, is plotted against the corresponding splitting of POPC-$\alpha$-$d_4$, $\Delta\nu_4$, for the same molar fraction of charge in the membrane. The effect is to show the different effects of charged molecules on the two different deuterolabeling positions. Due to the counterradirectional nature of the two positions, the slope of such a plot is always negative. However, it has been noted that the slopes of such curves fall within two charge regimes. All anions produce slopes of approximately -1.0 while cations produce slopes of -0.55, regardless of the variety of anions or cations (Beschiaschvili & Seelig, 1991).

These results are consistent with the fact that cationic charge will position itself closer to the phosphate segment of the phosphocholine molecule hence affecting the $\alpha$ segment more than the $\beta$ segment because of close proximity. On the other hand, anionic charges should be positioned closer the N end of the dipole thus increasing the conformational change of the $\beta$ segment comparatively.

To date, the precise nature of the conformational response undergone by the choline headgroup is not certain and the only evidence supporting it comes from $^1$H NMR. Recent simulation studies (Konstant et al., 1994) have suggested that in addition to the overall tilting of the headgroup with respect to the bilayer surface that there are also changes in the internal torsion angles which need to be considered to explain the changes in the quadrupolar splittings. Also, since this model predicts a displacement of the quaternary N of 5 Å for the extremes of added charge perhaps neutron diffraction techniques may be used to prove or refine the model. As well, recent NMR techniques used to determine $^{13}$C-$^{13}$P and $^{13}$C-$^{13}$C dipolar couplings in lipids can be used to extract both distance and conformational information (Hong, et al., 1995).
On the other hand, $^1$H NMR results have produced a quantitative model (Roux et al., 1989) of the choline tilt concept which correctly predicts the essential features of the dependence of the quadrupolar splittings on surface charge (Macdonald et al., 1991). Before getting into the details of the model we must remember that lipid molecules undergo fast rotation about their long molecular axis which reduces the quadrupolar splitting. But, since the motion is anisotropic the averaging is incomplete leaving a residual quadrupolar splitting. The starting point for the model begins with the relationship between the quadrupolar splitting and the conformation of the headgroup as a two-step model first proposed by Akutsu and Seelig (1981). The model assumes that the polar headgroup of a lipid possesses two types of motion so that an orientational order parameter $S'$ can be expressed as the product of the two parameters, which are characteristic of each motion:

$$|S'| = \frac{\Delta v_i}{\Delta v_0} = \left| S'_x S'_y \right| = \frac{1}{2} \left( \cos^2 \theta_i - 1 \right) S_f$$  \hspace{1cm} (2.38)

where $\Delta v_i$ is the observed quadrupole splitting at the $i$th deuteration position while $\Delta v_0$ is the static quadrupole splitting constant. $S_f$ represents the order parameter of the molecular axis with respect to the director axis and characterizes the degree of off-axis "wobbling" about the motional axis. This order parameter has values which range between 0 and 1. A value of zero indicates that the angular fluctuations about the motional axis are so large that no orientation is preferred while a value of $S_f = 1$ indicates a narrow distribution about the average angle. The other order parameter $S'_i$ is referred to as the geometrical order parameter and is sensitive to the average angle $\theta_i$ between the C-D bond and the lipid's long molecular axis and thus also provides us with information on
molecular conformation. This internal order parameter for the choline headgroup reflects the statistical distribution of an instantaneous orientation about a preferred orientation, averaged over time and all the molecules in the sample. Therefore it is this average orientation of the choline moiety which changes in this model.

Roux et al. (1989) formalized this expression by introducing an "equilibrium" angle of tilt of the choline group which resulted from two offsetting forces. The headgroup experiences a torque which results from the dipole aligning itself with the electrical field while a countertorque resists this due to intra- and intermolecular steric interactions. The discussion of this model and the extensions introduced to it by Macdonald et al. (1991) begin by consideration of figure 2.3.2.

FIGURE 2.3.2 Orientation of the phosphocholine dipole moment with respect to the C-D bond and electric field emanating from a charged membrane surface.
The choline headgroup is represented by the PN vector which is assumed to be rigid body hinged about the C-O torsion angle. The vector E represents the electric field emanating from the surface as well as both the axis of motional averaging and the normal to the bilayer surface. Although the electrical field produced by the surface charges consists of both radial and axial elements, due to the fast molecular axis rotations a net field will only be experienced in the direction parallel with this motion axis ie. normal to the membrane surface. The final vector to be noted is D which represents the C-D bond. The three angles defined in figure are γ, θ and φ which represent the angles between PN and D, D and E and finally PN and E, respectively.

The total torque exerted on a point charge q located at N about the hinge position is given by:

\[ \tau = \mathbf{PN} \times \mathbf{F} \]  

(2.39)

where \( \mathbf{F} \) is the electrical field force vector. The magnitude of the torque is shown to be:

\[ |\tau| = q PN \sin \phi / (2 \epsilon_0 \epsilon_r) \]  

(2.40)

where \( \phi \) is the surface charge density, \( PN \) is the length of PN, \( \epsilon_0 \) is the permittivity of free space and \( \epsilon_r \) is the dielectric constant of water. The surface charge density, for a binary mixture of charged and neutral lipids, is defined as \( XZeIS \) where \( X \) is the mole fraction of the charged species with valence \( Z \), \( e \) being the elementary charge, and \( S \) the cross sectional area occupied by the lipid, assumed to equivalent for double chained lipids such as PC, PG and DOTAP (68 Å²).
For a purely neutral surface charge the PN vector is known to lie within 30° of the bilayer surface and this represents the equilibrium situation where all forces acting on the headgroup sum to zero. When a torque is exerted on the headgroup due to an electrostatic field the preferred torsion angle φ will be resisted by an internal rotation potential, modelled as a countertorque of magnitude:

$$|\tau| = K \sin (\phi - 60)$$  \hspace{1cm} (2.41)

where \( K \) is a force constant. Under all surface charge states an equilibrium is met where the forces cancel each other, such that:

$$\tau + \tau_i = 0$$ \hspace{1cm} (2.42)

By substituting equations 2.40 and 2.41 into equation 2.42 the equilibrium position of the choline headgroup is determined to be:

$$\sin \phi = (1 + C^2)^{1/2}$$ \hspace{1cm} (2.43)

where \( C = \sigma AK^1 + \cos 60 / \sin 60 \) and \( A = q PN / 2e_i e_s \) and \( \phi = 60° \) at equilibrium. Thus for \( \sigma < 0 \), \( \phi \) is shown to increase while the opposite is true for \( \sigma > 0 \). The resistance to changes in \( \phi \) become effective as it approaches 90° due to intermolecular hard core repulsions.

Now that there is an expression for the dependence of \( \phi \) on the surface charge density the next step is to derive the dependence of \( \Delta V \), on \( \phi \). This can be accomplished by relating the angle \( \theta \), the angle between the C-D bond vector and axis of motional averaging, in equation 2.38 to the angle \( \phi \). The addition theorem for spherical harmonics (Rose, 1957) provides the desired relationship:
\[ P_2(\cos \theta) = P_2(\cos \phi) P_2(\cos \gamma) + 3 \sin \phi \cos \phi \sin \gamma \cos \gamma \cos \phi + \frac{3}{4} \sin \phi \sin \phi \sin \gamma \cos (2\alpha) \quad (2.44) \]

where \( P_2(x) = \frac{1}{3} (3x^2 - 1) \) and all angles are defined in figure 2.3.2. Through modelling of X-ray crystallography data, Pearson and Pascher (1979) determined that the angle \( \gamma = 90^\circ \). Thus, equation 2.44 reduces to:

\[ \frac{1}{2} (3\cos^2 \theta - 1) = -\frac{1}{4} (3\cos^2 \phi - 1) + \frac{3}{4} \sin^2 \phi \cos (2\alpha) \quad (2.45) \]

Substituting equation 2.45 into 2.38 produces the relationship between the quadrupole splitting and \( \phi \):

\[ \Delta v_i = \Delta v_0 \left[ \frac{3}{4} \sin^2 \phi \cos (2\alpha) - \frac{1}{4} (3\cos^2 \phi - 1) \right] S_i \quad (2.46) \]

By further substitution of equation 2.43 into the above equation the relationship is made between the quadrupolar splitting and the surface charge density, such that:

\[ \Delta v_i = \Delta v_0 \left[ \frac{\left( \frac{3}{4} \cos (2\alpha) + \frac{3}{4} \right)}{\left( \frac{3}{4} \cos (2\alpha) + \frac{3}{4} \right) - \frac{1}{2}} S_i \right] \quad (2.47) \]

The angle \( \alpha \), which is defined in figure 2.3.2 as the dihedral angle of the C-D bond in the coordinate
system of the headgroup vector PN, is fixed by the molecular geometry and does not change with rotation of the choline headgroup and is thus treated as a constant. Therefore \( \cos(2 \alpha) \) may be estimated by substitution of the quadrupole splitting and \( \phi = 60^\circ \) for the case of neutrality for both the \( \alpha \) and \( \beta \) segments. Macdonald et al. (1991) chose a value of \( S_e = 0.25 \) for both positions and determined two values for \( \cos(2 \alpha) \) (Marassi, PhD thesis) due to the fact that the sign of the quadrupole splitting is not expressly written in equation 2.38 and can be taken to be either positive or negative. The values of \( \alpha \) were tabulated and chosen for both deuterolabeling positions such that the correct experimental response of the \(^{2}H\) NMR quadrupole splitting was replicated for the specific membrane surface charge. Finally, through substitution of the remaining constants into equation 2.47, Macdonald et al. (1991) were able to reproduce all the main features of the quadrupole splitting dependence on surface charge through this model. The reproduced features included the counterdirectional effect of positive versus negative charge on the quadrupolar splittings from either of the individual deuterolabeling positions as well as the opposite effects in change of quadrupole splittings for POPC-\( \alpha \)-d2 and POPC-\( \beta \)-d2 for the same surface charge. The greater sensitivity of the quadrupole response to positive versus negative charges as well as the non-linear dependence of change in quadrupole splittings at extreme surface charge densities were also reproduced as well as the greater sensitivity of \( \Delta V_\alpha \) versus \( \Delta V_\beta \).
3. MATERIALS AND METHODS

3.1 Materials

Solvents. Reagent grade pyridine was dried by refluxing over calcium hydride followed by sodium hydride. Reagent grade diethyl ether and tetrahydrofuran (THF) were dried over sodium metal using benzophenone as an indicator. Chloroform (HPLC grade) used for the synthesis of DC-CHOL was dried over phosphorus pentoxide and then distilled onto molecular sieves.

Reagents and Chemicals. 3-Dimethylamino-1,2-propanediol, 2,4,6-trisopropylbenzene-sulfonyl chloride (TPS), tetraphenylboron (TPB), cholesteryl chloroformate, N,N-dimethyl-ethylenediamine, iodomethane-d$_4$, and deuterium depleted water were purchased from Aldrich (Milwaukee, WI). Oleoyl chloride was obtained from Sigma (St. Louis, MO).

Phospholipids, Amphiphiles and Polyelectrolytes. Non-deuterated phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) without further purification. 1-palmityl-2-oleyl-sn-glycero phosphatidic acid (POPA) was dried by evaporation with dry pyridine prior to use in synthesis. Non-deuterated DOTAP (1,2-dioleoyl-3-trimethylaminopropane) was obtained from Avanti Polar Lipids while CTAB was purchased from BDH (Toronto, ON). Poly(adenylic acid) potassium salt (PolyA, MW 7 000 000, degree of polymerization N = 18 000) and poly(L-glutamic acid) sodium salt (PGLU, MW = 80 000, N = 550) were obtained from Sigma (St. Louis, MO). Poly(acrylic acid) sodium salt (PACA, MW = 30 000, N=320) and poly(sodium 4-styrene sulfonate) (PSSS, MW = 70 000, N = 340) were purchased from Aldrich. Poly(styrene sulfonate) sodium salt, molecular weight kit, (MW = 780 000, 100 000, 35 000 and 4 600 with N = 3790, 485, 170 and 22)
was purchased from Polysciences, Inc. (Warrington, PA). OligoS, a phosphorothioate oligonucleotide (MW = 7183, N = 21, 5'-GCCGAGGTCCATCGTACGC-3'), was a gift from ISIS Pharmaceuticals (Carlsbad, CA).
3.2 Synthesis of Choline Deuterated Phospholipids

The phosphatidylcholine derivative of 1-palmitoyl-2-oleoyl-sn-glycero phosphatidic acid was prepared in the L isomer form. The headgroup deuterated POPC was synthesized by coupling the specifically deuterated choline headgroup with POPA using TPS as the condensing agent (Aneja et al., 1970). Tetraphenylborate (TPB) was used as an organic transfer agent to solubilize the choline salt in pyridine prior to its coupling with POPA.

Choline-o-d<sub>4</sub> TPB

Choline-o-d<sub>4</sub> TPB was prepared according to the method of Harbison and Griffin (1981) as shown in Scheme 2.1. The first step involves the reduction of N,N-dimethylglycine ethyl ester with lithium aluminum deuteride (LiAlD<sub>4</sub>). 2g (48 mmol) of LiAlD<sub>4</sub> are suspended in 100 mL of dry THF. The mixture was stirred until homogeneous. 10 mL (70.7 mmol) of N,N-dimethylglycine ethyl ester was dissolved in 50 mL anhydrous THF and was then added dropwise to the mixture over the course of 45 min. The mixture was then gently stirred and refluxed for an hour. The reaction was quenched by the slow addition of 2 mL of distilled water, followed by 2 mL of 15% w/v NaOH and then 6 mL of distilled water, in order to decompose excess LiAlD<sub>4</sub>. The solution was then stirred for approximately 1 hour until the evolution of hydrogen gas ceased. The inorganic salts were removed by filtering the mixture through a sintered glass funnel and washing them with 300 - 400 mL of diethyl ether. The combined filtrate was then reduced to approximately 100 mL by rotary evaporation and 15 mL (240 mmol) of iodomethane were added and the mixture was stirred in the dark overnight at room temperature. The solvent was then removed by rotary evaporation and the
residue taken up in 100 mL of distilled water and then washed with 100 mL of diethyl ether. The aqueous layer was then divided evenly between two 150 mL centrifuge bottles and to each was added 75 mL of a NaTPB solution created by adding 34g of NaTPB to 150 mL of water. The result is a white precipitate of choline-α-d$_2$ TPB salt which was subsequently centrifuged for 20 min at 5000 rpm (4000Xg). The solvent was decanted and the precipitate was washed 4x with 100 mL of distilled water. The choline salt was then dried azeotropically with toluene/ethanol (20/75 v/v) and then recrystallized twice from hot acetonitrile. The choline-α-d$_2$ TPB salt was then collected as translucent hexagonal crystals which were dried under high vacuum and stored at -20 °C. Yield: 18g (70%).

$^1$H NMR in DMSO-d$_6$, 200 MHz: $\delta = 3.10$ ppm, singlet, 9H methyl choline protons; $\delta = 3.35$ ppm, triplet, 2H β-choline protons; $\delta = 5.26$ ppm, broad multiplet, 1H choline OH; $\delta = 6.80$ ppm, multiplet, 4H TPB para protons; $\delta = 6.90$ ppm, multiplet, 8H TPB meta protons; $\delta = 7.20$ ppm, multiplet, 8H TPB ortho protons.

Scheme 2.1
Choline-β-d, TPB

Choline-β-d, TPB salt was produced by a combination of methods by Aloy and Rabaut (1913) and Harbison and Griffin (1981) as illustrated in Scheme 2.2.

Cyanoethylbenzoate. 7g of NaCN are weighed out directly in a flask and then dissolved in 25 mL of distilled water. The solution was stirred for 20 min while being cooled in an ice bath at 0 °C. 11 mL (146 mmol) of 37% (v/v) formalin in water were added dropwise and low temperatures were maintained by replenishing the ice, since the reaction is exothermic. To the clear solution 16 mL of benzoyl chloride were added dropwise and the mixture was stirred vigorously for 2 hrs. Cyanomethylbenzoate forms oily droplets in solution and it was extracted with 5x 100 mL portions of diethyl ether. The ether solution was then washed with 300 mL of 0.1 M NaOH in order to remove benzoyl alcohol. The ether was then removed by rotary evaporation and the product purified by vacuum distillation (125 °C under reduced pressure) to yield a colourless oil. The product crystallized as long needles at -20 °C. Yield: 20g (84%).

\(^1\)H NMR in CDCl\(_3\) 200 MHz: \(\delta = 5.10\) ppm, singlet, 2H methylene protons; \(\delta = 7.45\) ppm, triplet, 2H meta benzyl protons; \(\delta = 7.62\) ppm, triplet, 1H para benzyl proton; \(\delta = 8.10\) ppm, doublet, 2H ortho benzyl protons.

Choline-β-d, TPB. 4g (95 mmol) of LiAID\(_4\) were suspended in 100 mL of dry THF and stirred until there was a homogeneous mixture. 9.5 g (59 mmol) of cyanomethyl benzoate were dissolved in anhydrous THF and added dropwise to the LiAID\(_4\) solution. The mixture was then gently refluxed for 3 hrs. Excess LiAID\(_4\) was then decomposed by the addition of 4 mL of water, followed by 4mL of 15% NaOH and then 4 mL of water. The mixture was stirred and allowed to sit for 1 hr, until the evolution of gas ceased. The insoluble inorganic salts were filtered by suction through a
sintered glass funnel and washed with approximately 300 mL of diethyl ether. The filtrate was then reduced to 150 mL, by rotary evaporation, and 50 mL of 10% NaOH (w/v) were added followed by 15 mL (240 mmol) iodomethane and the solution stirred in the dark overnight. The solution was evaporated to a small volume, diluted to 100 mL with water and washed with 2x 100 mL portions of diethyl ether in order to remove benzyl alcohol. To the aqueous layer was added NaTPB solution (34g of NaTPB in 150 mL of water). The white precipitate of choline-β-dj TPB was poured into a sintered glass funnel and washed with copious amounts of water. The product was then dried and purified as described above. Yield: 3.8g (15%).

$^1$H NMR in DMSO-d$_6$, 200 MHz: $\delta = 3.10$ ppm, singlet, 9H methyl choline protons; $\delta = 3.80$ ppm, multiplet, 2H α-choline protons; $\delta = 5.30$ ppm, singlet, 1H choline OH proton; $\delta = 6.80$ ppm, multiplet, 4H TPB para protons; $\delta = 6.90$ ppm, multiplet, 8H TPB meta protons; $\delta = 7.20$ ppm, multiplet, 8H TPB ortho protons.

Scheme 2.2
Headgroup Deuterated Phosphatidylcholine

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was produced by coupling either of the desired specifically deuterated choline TTP salts with POPA, as shown in scheme 2.3. 800 mg (1.12 mmol) of POPA were dried by rotary evaporation with 50 mL of anhydrous pyridine. POPA was then dissolved in a further 50 mL of dry pyridine in a round bottom flask flushed with argon. The flask was submerged in a water bath kept at 40 °C and the solution stirred for 30 min until POPA was fully dissolved. 900 mg (3 mmol) of TPS were added to the flask and once the solution was clear yellow 870 mg (2 mmol) of the choline TPB salt was added, with either of the deuterolabels. The mixture was stirred and the temperature kept at 40 °C for 4 hrs. The excess TPS was then decomposed by the addition of 3 mL of water. The pyridine was then immediately removed by rotary evaporation and the product further dried by adding toluene and azeotropically removing water. The product was then taken up in approximately 50 mL of trichloroethylene (TCE) and then vacuum filtered in order to remove any insoluble materials. The solid materials were then washed with further amounts of TCE to remove any trapped product. This process was repeated 2 times. The combined washings were evaporated and the residue taken up in 40 mL of (1/1 v/v) TCE/methanol and then transferred to a 150 mL centrifuge tube. The lipid was then washed using the theoretical upper phase procedure of Bligh and Dyer (1959) and McMurray (1975). The procedure encompassed adding 10 mL of water, 20 mL of TCE and 10 mL of 0.9% (w/v) aqueous NaCl to the centrifuge tube, making sure to mix the contents thoroughly after each addition. The mixture was then centrifuged for 15 min at 4000 rpm and the upper aqueous layer was removed by vacuum aspiration and the lower organic phase was further washed with 3x 15 mL portions of TCE/methanol/0.9% aqueous NaCl (3/48/47) mixture. The lower organic phase was then dried down by rotary evaporation and the lipid taken up
in a (1/1 v/v) chloroform / methanol mixture. The product was then passed through 100g (capacity 0.5 - 0.8g of lipid/100g resin) of Amberlite mixed-bed ion exchanger (BDH, Toronto, ON). The Amberlite is prewashed with methanol and the solvent is exchanged with CHCl₃ / MeOH (1/1 v/v). The lipid is applied to the column and eluted. 125 mL fractions are collected and the elution of POPC is monitored with TLC on silica gel plates (Kieselgel 60) using the solvent system of chloroform/methanol/acetonitrile/acetic acid/distilled water (10/4/4/2/1 v/v). The TLC plates were visualized in an iodine tank. POPC had an Rₑ of 0.25 in this system while POPA has a value of 0.8.

The POPC fractions were pooled and the solvent removed and POPC was further purified by acetonitrile precipitation (Kates, 1972). In this step a 1-2 mL chloroform solution of POPC was added to a 15 mL centrifuge tube with 10 mL of acetonitrile, gently warming it to dissolve POPC. The centrifuge was then cooled to -20 °C and the POPC was precipitated. The mixture was centrifuged at -20 °C for 10 min at 6000 rpm. The supernatant was decanted and the procedure repeated twice. The final product was characterized by TLC (already described) ³H NMR and ¹H NMR. Yield: for POPC-α-d₂, 530 mg, 74%; for POPC-β-d₄; 600 mg, 86%.

³H NMR in deuterium depleted water, 300 MHz: Δν₂ = 6.4 kHz and 5.8 kHz for 100% POPC-α-d₂ and POPC-β-d₄, respectively.

¹H NMR in CDCl₃, 200 MHz: δ = 0.84 ppm, triplet, 6H acyl methyl protons; δ = 1.24 ppm, singlet, acyl methylene protons; δ = 1.60 ppm, multiplet, 4H acyl β-methylene protons; δ = 2.30 ppm, multiplet, 4H acyl α-methylene protons; δ = 3.37 ppm, singlet, 9H choline methyl protons; δ = 3.60 ppm, singlet, 2H choline-β protons; δ = 3.95 ppm, multiplet, 2H glycerol-3 protons; δ = 4.15 ppm, multiplet, 2H glycerol-1 protons; δ = 4.35 ppm, singlet, 2H choline-α protons; δ = 5.20 ppm, multiplet, 1H glycerol-2 proton; δ = 5.35 ppm, triplet, 2H olefinic protons.
Scheme 2.3
3.3 Syntheses of Cationic Amphiphiles

DODAP and DOTAP

1,2-Dioleoyl-3-di and trimethylaminopropane were synthesized and purified as described by Leventis and Silvius (1990) and shown in scheme 3.1. 150 mg (1.3 mmol) of 3-dimethylamino-1,2-propanediol were weighed out directly in a round bottom flask. The flask was evacuated with argon and 150 µL of dry pyridine, 900 mg (3.2 mmol) of oleoyl chloride were added in 50 mL of dry diethyl ether and the solution stirred for 24 hrs in the dark, at room temperature. The reaction mixture was quenched with methanol and concentrated by rotary evaporation. The residue was redissolved in 50 mL of hexane and then washed with 3 portions of 50 mL of 0.1M KOH in 1/1 v/v methanol/water at 0°C, followed by a single wash with 0.1 M aqueous NaCl. The hexane layer was concentrated by rotary evaporation and the residue dried azeotropically with toluene. The product was taken up in 10 mL of 99/1 hexane/acetic acid and applied to a silica acid column (100 g column, Silica Gel 60, Rose Scientific, Edmonton, AL). The product was eluted successively with 400 mL of 20% diethyl ether in hexane (v/v), 3x 200 mL of chloroform and 5x 100 mL fractions of 5% methanol in chloroform (v/v). The fractions were monitored for product using TLC and the solvent system of chloroform/methanol (90/10 v/v). 1,2 dioleoyl-3-dimethylamino propane (DODAP) eluted in the 5% methanol fractions and had an Rf value of 0.70 while oleoyl chloride ran with the solvent front. The fractions containing DODAP were then concentrated and DODAP was converted to its active hydrochloride form by adding an equimolar amount of HCl to a methanolic solution containing the product. The methanol was removed under a stream of argon and the product was dried under vacuum overnight. Yield: 530 mg, (63%).
\(^1\)H NMR in CDCl\(_3\), 200 MHz: \(\delta = 0.9\) ppm, triplet, 6H oleoyl methyls; \(\delta = 1.3\) ppm, broad singlet, 40H oleoyl methylene protons; \(\delta = 1.65\) ppm, multiplet, 4H oleoyl \(\beta\)-methylene; \(\delta = 2.05\) ppm, multiplet, 8H \(\alpha\)-methylene to double bond; \(\delta = 2.28\) ppm, singlet, 6H amino methyls; \(\delta = 2.35\) ppm, triplet, 4H, oleoyl \(\alpha\)-methylprotons; \(\delta = 2.47\) ppm, triplet, 2H, C1-propyl protons; \(\delta = 4.1\) and 4.35 ppm, doublet of doublets, 2H, geminal coupling (J = 12 Hz) and vicinal coupling with C2 proton (J = 3.6 Hz and 6.1 Hz), C3-propyl protons (inequivalent \(H_a\) and \(H_b\)); \(\delta = 5.2\) ppm, multiplet, 1H, C2-propyl proton; \(\delta = 5.35\) ppm, triplet, 4H oleoyl olefinic protons.

**DOTAP.** 500 mg (0.73 mmol) of DODAP were added to 100 mL of dry diethyl ether. To the mixture was added 0.48 mL (7.7 mmol) of methyl iodide and the flask sealed and the solution stirred in the dark for 36 hrs. The solvent was then removed by rotary evaporation and the product purified by two acetone precipitations. The lipid was then chromatographed on a Bio-Rad AG 1-X4 anion exchange resin prepared in the chloride form (Bio-Rad, Mississauga, ON) in order to convert from the iodide form. The product was put on the column in a minimal amount of 1/1 v/v of chloroform/methanol and was eluted from the column with 300 mL of the same solvent system. The purity of DOTAP was monitored as described for DODAP. The \(^1\)H NMR spectrum was identical to that of DODAP with the exception of the shift of the 6H amino methyl protons from \(\delta = 2.28\) ppm to \(\delta = 3.55\) ppm for the 9H amino methyl protons. The purity of the product was determined based on the absence of the \(\delta = 2.28\) peak. Yield: 285 mg (56%).

**DOTAP-\(\gamma\)-d\(_5\).** The procedure was identical to the synthesis of DOTAP above. The amino functionality was quarternized with an excess of methyl-d\(_5\) iodide to produce DOTAP with one deuterated aminomethyl group. The \(^1\)H NMR spectrum was identical to that of DOTAP with the exception that the peak at \(\delta = 3.55\) ppm contained only 6H amino methyl protons.
Scheme 3.1
DC-CHOL

DC-CHOL was synthesized and characterized according to the method of Gao and Huang (1991) as shown in scheme 3.2. 2.25 g (5 mmol) of cholesteryl chloroformate in 20 mL of dry chloroform was added dropwise to a flask containing 2 mL (18 mmol) N,N-dimethylethylenediamine in 20 mL of dry chloroform submersed in an ice bath kept at 0 °C. The mixture was stirred and allowed to react for 1 hr and the solvent was then removed. The final product was recrystallized twice from absolute ethanol at -20 °C and dried under vacuum, yielding a compound which chromatographed as a single spot on TLC ($R_f = 0.58$ in 65/35 v/v chloroform/methanol eluent).

Yield: 0.56 g (22%).

$^1$H NMR in CDCl$_3$, 200 MHz: $\delta = 2.18$ ppm, singlet, 6H amino methyl protons; $\delta = 2.36$ ppm, triplet, 2H C1-ethyl protons; $\delta = 3.21$ ppm, multiplet, 2H C2-ethyl protons; $\delta = 5.18$ ppm, broad triplet, 1H amido proton; $\delta = 5.35$ ppm, broad triplet, 1H olefinic cholesterol proton.

$TC-CHOL-\gamma-d_2$ 820 mg (1.63 mmol) of DC-CHOL were reacted with 830 mg (5.77 mmol) of methyl-d$_3$ iodide in 50 mL of dry diethyl ether for 24 hrs in the dark at room temperature. The solvent was removed by rotary evaporation and the product was then purified by two acetone precipitations and run through an anion exchange column (chloride form) as described above for DOTAP. Finally $TC-CHOL-\gamma-d_2$ was recrystallized from absolute ethanol and dried under vacuum. The purity of the compound was monitored by TLC and $^1$H NMR. Yield: 220 mg (26%). The $^1$H NMR spectrum was identical to DC-CHOL with the exception of the shift in the aminomethyl peak (6H) of $\delta = 2.18$ ppm to $\delta = 3.35$ ppm.
Scheme 3.2
CTAB-γ-d₄

CTAB-γ-d₄ was synthesized by the methylation of hexadecylamine with methyl-d₄ iodide as described by Semchyschyn et al. (1996).
3.4 Sample Preparation

Preparation of Multilamellar Vesicles (MLVs)

Lipid mixtures of the desired composition were prepared by combining the appropriate volumes of chloroform stock solutions of either POPC-α-d₃, POPC-β-d₃ or non-deuterated POPC or DOPE with either of the cationic amphiphiles, DC-CHOL, CTAB, DODAP or DOTAP. Typically, the lipid mixtures contained 10 mg of either of the zwitterionic phospholipids along with the varying amounts of cationic amphiphiles, in order to achieve the prescribed lipid molar ratio. The solvent was removed under a stream of argon and the mixture was dried overnight under vacuum. The dried lipid mixtures were then rehydrated in 200 μL of deuterium depleted water. The hydration process consisted of gentle warming and vortexing, followed by five cycles of freeze-thawing in order to ensure homogeneous mixing. The mixtures were then transferred to 5 mm diameter NMR tubes for measurement.

Preparation of MLVs Containing Polyelectrolytes

The dried lipid mixtures were prepared as described above, but were hydrated by adding the desired quantity of PolyA, OligoS, PSSS, PACA or PGLU in deuterium depleted water from stock solutions, plus sufficient deuterium depleted water, and/or NaCl in deuterium depleted water to bring the final volume up to 200 μL. The mixtures were once again gently warmed and vortexed and subjected to five freeze-thaw cycles to ensure total mixing of the polyelectrolytes in the MLV dispersions.
3.5 UV Difference Assay of Polyelectrolyte-Membrane Binding

Dried lipid samples were prepared as described above and a sufficient amount of any of the different polyelectrolyte stock solutions were added to achieve the desired anion/cation ratio. Further deionized water and/or NaCl solution was added to bring the final volume of the mixture to 300 μL. The samples were then hydrated and equilibrated as described above, then centrifuged at 13,000 rpm for 1 hr to pellet the lipid/polyelectrolyte mixtures. Approximately 200 μL of the supernatant was removed, diluted and passed through a Centricon-500 microconcentrator (Amicon, Oakville, ON) to remove any unpelleted lipid, by centrifugation at 4,000 rpm for 15 min. The filtrate, containing any unbound polyelectrolyte chains was further diluted until its UV absorbance fell into the concentration regime where Beer’s law was obeyed, as measured using a Hewlett Packard 8452A Diode Array spectrophotometer. The polyelectrolyte concentration in the original supernatant was then calculated from a standard curve, and the amount bound was calculated from the difference with respect to the initial concentration.
3.6 Solid State NMR Measurements

$^1$H NMR Spectroscopy

$^1$H NMR spectra were recorded on a Chemagnetics CMX300 NMR spectrometer operating at 45.98 MHz, using a Chemagnetics wideline probe, equipped with a 5 mm solenoid coil. The quadrupole echo sequence (Davis et al., 1976) was employed ($90\degree x - \tau - 90\degree y - \tau - \text{acq}$) using quadrature detection with complete phase cycling of the pulse pairs and a $90\degree$ pulse length of 2.0 $\mu$s, an interpulse delay of 30 $\mu$s, a recycle delay of 100 ms, a spectral width typically between 50 - 100 kHz and a 2K data size.

Longitudinal ($T_1$) and Transverse ($T_2^*$) Relaxation Times. The longitudinal relaxation time, $T_1$, was measured by the combined inversion recovery and solid echo sequence ($180\degree x - t - 90\degree x - \tau - 90\degree y - \tau - \text{acq}$) by varying the time $t$, typically between 1 $\mu$s to 30 ms. The transverse relaxation times ($T_2^*$) were obtained from the echo intensity as a function of the separation $\tau$ between the two $90\degree$ pulses in the quadrupolar echo sequence. $1/T_2^*$ corresponded to the slope in a semilogarithmic plot of the normalized intensity at the peak of the echo versus the time $t = 2\tau$. All measurements were performed at room temperature.

Temperature Dependence Studies. The temperature of the sample was controlled by passing air first through a coil cooled in an ice bath and then through a sidearm resistance heater before entering the probe. The temperatures were set at the low end of the temperature studies followed by heating the sample progressively by 10°C and recording the spectrum. The temperature was equilibrated for each spectrum for approximately 15 min before acquisition.
$^{31}P$ NMR Spectroscopy

$^{31}P$ NMR spectra were recorded on the same spectrometer operating at 121.25 MHz, using a Chemagnetics double-resonance magic-angle spinning (MAS) probe but without sample spinning. For hydrated MLV samples, the Hahn echo sequence (90°x - $\tau$ - 180°y - $\tau$ - acq) with complete phase cycling of the pulses and high power proton decoupling during acquisition was employed as described by Rance and Byrd (1983). The 90° pulse length was 6.0 μs, the echo spacing was 40 μs, the recycle delay was 2 s, the spectral width was 100 kHz, and the data size was 2K. $T_1$ relaxation times were measured using a standard inversion-recovery protocol with Hahn echo detection, with $\tau$ typically varied from 1 μs - 2s. $T_2$ relaxation times were measured from the dependence of the signal intensity on the length of the delay in the Hahn echo sequence, typically between 30 μs - 30 ms.

$^{31}P$ NMR spectra of dry powders of PolyA and OligoS were recorded using a single-contact cross-polarization technique combined with Hahn echo detection. In this instance the $^1H$ 90° pulse length was 4.9 μs, the contact time was 3.0 ms and the spectral width was 250 kHz.

Pake Pattern Spectral Line Shape Simulations

$^1H$ and $^{31}P$ NMR Pake pattern line shapes were simulated using a computer program, written in our laboratory, based on the tiling method introduced by Alderman et al. (1986). The simulation variables include either the quadrupolar splitting, $\Delta\nu_q$, or the chemical shielding tensorial components, $\sigma_{11}$, $\sigma_{22}$, $\sigma_{33}$, and the line width parameter, $\Gamma$, and the intensity of a given Pake pattern. The program, however, does not include provisions for $T_1$ asymmetry effects. This can sometimes lead to a less than perfect appearance of the spectral shoulders of the experimental Pake pattern.
De-Pake-ing of $^1$H NMR Spectra

$^1$H NMR spectra were 'de-Paked' as described by Sternin et al. (1983) for the 90° orientation of the Pake doublet.
4. RESULTS AND DISCUSSION

4.1 $^2$H NMR Response of Phosphatidylcholine Headgroup to Binary Mixtures with Cationic Amphiphiles

The use of cationic liposomes in the transfer of genes has become a popular method of gene therapy. Cationic lipids are known to enhance the transport of genetic material across the plasma membrane due to their ability to electrostatically condense DNA as well as targeting biomembranes by binding to their typically anionic surface. Cationic lipids are very rare in nature though and this has necessitated the synthesis of various lipids (Felgner et al., 1987; Behr et al., 1989; Leventis and Silvius, 1990; Gao and Huang, 1991; Deshmukh and Huang, 1997). Although in recent years the mechanism of gene transfer has been determined, no definitive answers have been determined as to how the molecular structure of different amphiphiles lead to different propensities as gene transfer agents, in order to design improved transfection agents. Obviously the role played by electrostatics in these techniques must be pivotal.

$^2$H NMR of choline deuterated phosphatidylcholine is an approach for monitoring membrane surface electrostatics. It has been used to characterize the binding of charged ligands to membrane surfaces as well as to examine lateral phase separation of charged lipids and also to resolve differences between two surfaces of a lipid bilayer membrane (Seelig et al., 1987; Macdonald, 1995; Macdonald, 1997). This technique is sensitive to the detailed topography of surface charge distribution.
4.1.1 $^1H$ Magnetic Resonance Spectroscopy

In the studies presented here, the membrane electrostatic surface response was determined for three different cationic amphiphiles which have all previously been used in gene transfection studies. The structures of the three cationic amphiphiles and a polyelectrolyte (polyA) are displayed in figure 4.1.1. Each of the three cationics is shown to contain either a tertiary or quaternary amino headgroup, where the charge is located, attached to a hydrophobic moiety. In the case of CTAB (cetyl trimethyl ammonium bromide) the hydrophobic group is a single chain C16 group. In DC-CHOL (3β[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol) the hydrophobic group is a sterol ring structure. DODAP (dioleoyl dimethyl amino propane), on the other hand, has a hydrophobic moiety consisting of a double acyl chain group. The cationic lipids may be arranged in the order CTAB > DC-CHOL > DODAP in terms of their hydrophilic-lipophilic balance (HLB) (Griffin, 1949). This is an estimate of the polarity of a molecule obtained by summing up all the individual chemical groups in terms of their hydrophilic versus lipophilic contributions as detailed by Davies and Rideal (1963).

Each of the cationic amphiphiles produces a cationic surface charge when mixed with the zwitterionic POPC as detected by $^1H$ NMR of choline deuterated POPC. The fundamental spectroscopic observations are shown in figure 4.1.2, for the specific case of CTAB. The left-hand column of spectra were obtained with POPC-$\alpha$-d$_2$, whereas the right-hand column represent the spectra obtained with POPC-$\beta$-d$_2$. All spectra consist of a $^1H$ NMR Pake doublet, which is characteristic of liquid-crystalline lipids arranged in a bilayer arrangement. As already discussed in the introduction, the quadrupolar splitting is measured as the frequency separation between the two maxima in the spectra. The central resonance line (0 Hz) displayed in these spectra is due to the
FIGURE 4.1.1  Structures of the three cationic amphiphiles employed here and the polyelectrolyte PolyA (i.e. polyadenylic acid). From top to bottom, CTAB, DODAP, DC-CHOL and PolyA.
FIGURE 4.1.2  Effect of cationic surface charge on the $^3$H NMR spectrum of POPC-$\alpha$-d$_4$ (left) and POPC-$\beta$-d$_4$ (right) in the presence of (from top to bottom) 0% CTAB, 7.5 mol% CTAB and 15 mol% CTAB.
residual amount of deuterium present in the water used to make up the MLV sample.

For the case of a neutral membrane surface (i.e. 100% POPC membranes), top spectra, the quadrupolar splittings from both POPC-α-d₂ and POPC-β-d₂ are rather similar. When increasing the mole fraction of CTAB in these POPC vesicles, to 7.5 and then 15 mol %, as shown in the middle and bottom spectra, the quadrupolar splitting from POPC-α-d₂ decreases while that from POPC-β-d₂ increases. This counterdirectional change in the quadrupolar splittings from the two deuterolabeling positions is characteristic of the "molecular voltmeter" response of phosphatidylcholine to the presence of surface charge (Seelig et al., 1987). The direction of change observed here is diagnostic of the accumulation of positive surface charges. Since only a single quadrupolar splitting is observed in each instance, one may conclude that CTAB is homogeneously distributed both within the plane of the membrane and also amongst all the MLVs of the entire sample. Consequently, on the timescale of ²H NMR, all POPC molecules experience the same average surface charge environment. Qualitatively, all three of the different cationic amphiphiles display the same effects when mixed with either POPC-α-d₂ or POPC-β-d₂.

Quantitatively the particular surface charge density of the cationic amphiphile may be directly determined from the quadrupolar splitting by calibrating the relationship between the two as shown in figure 4.1.3. Here, quadrupolar splittings for both POPC-α-d₂ and POPC-β-d₂ are plotted as a function of the mole fraction of added cationic amphiphile for each of CTAB, DC-CHOL and DODAP. In each instance, for the defined range of mole fractions, the relationship between the quadrupolar splittings and mole fraction of cationic lipid is essentially linear. On a mole-to-mole basis, though, CTAB induces the greatest response (slopes of -29.1 kHz / mol and +25.6 kHz / mol for POPC-α-d₂ and POPC-β-d₂, respectively). However, mole fractions of CTAB greater than about
FIGURE 4.1.3 Surface charge dependence of the $^1$H NMR quadrupolar splittings from POPC-α-d$_2$ (open symbols) and POPC-β-d$_2$ (closed symbols) on the mole fraction of added cationic amphiphile: CTAB (circles), DODAP (squares), DC-CHOL (triangles). The quadrupolar splittings are plotted as the difference between the value measured for a given mixture and the value measured for 100% POPC vesicles.
15% lead to disruption of the bilayer due to its surfactant properties. DC-CHOL produced the slightest response of the three cationic amphiphiles with slopes of -12.8 and 4.0 kHz/mol for POPC-α-d₁ and POPC-β-d₂, respectively. Levels of up to 50 mol% were used for DC-CHOL above which broad ²H NMR spectra were produced, indicative of an inhomogeneous distribution of DC-CHOL within the plane of the membrane. On the other hand, DODAP produced well-defined spectra over the widest range of molar ratios indicating that it remains homogeneously mixed in POPC membranes. DODAP also produced an intermediate response of the "molecular voltmeter" in that the calibrated slopes for the POPC-α-d₁ and POPC-β-d₂ responses were -21.2 and 11.5 kHz/mol, respectively.

Further evidence of the distinctness of the ²H NMR response of the three cationic amphiphiles is obtained when a correlation is made between the quadrupolar splitting between POPC-α-d₁ and POPC-β-d₂ for a given level of added charge as shown in figure 4.1.4. The linearity of such an α-β correlation plot suggests that the phosphatidylcholine headgroup undergoes a concerted conformational change in response to surface charge, as already discussed. It has been generally noted that most cationic species yield slopes of approximately -0.50 in such correlation plots (Scherer & Seelig, 1989; Beschiaschvili & Seelig, 1991). For instance, it is calculated from figure 4.1.4 that DODAP displays a slope of -0.53. However, CTAB produces a slope of -0.88, while DC-CHOL displays a slope equal to -0.32. There appears to be a correspondence between the slope of an α-β plot and the location or depth of a given charge relative to the plane of the choline headgroup of POPC (Beschiaschvili & Seelig, 1991; Rydall & Macdonald, 1992). For instance, aqueous ions, which bind superficially to the membrane surface in general produce a slighter response than hydrophobic ions which are known to penetrate well into the bilayer proper (Beschiaschvili & Seelig, 1991; Rydall & Macdonald, 1992).
FIGURE 4.1.4  α-β correlation plots for headgroup deuterated POPC in binary mixtures with cationic amphiphiles. The quadrupolar splittings from POPC-α-d₄ and POPC-β-d₄ obtained under identical conditions of cationic amphiphile concentration are plotted with respect to one another for CTAB (circles), DODAP (squares), DC-CHOL (triangles). The best linear fit to a given data set is shown as a solid line.
The sensitivity of the "molecular voltmeter" response depends on a number of factors other than the location of the charged group relative to the plane occupied by the phosphocholine group of POPC. One factor is whether the charge is cationic or anionic. Another is the statistical probability that POPC and the charged species will encounter one another. Since all three amphiphiles under consideration are cationic, they each display calibration constants greater than comparable anionic species (Beschiaschvili & Seelig, 1990). Likewise, all three are sufficiently hydrophobic that one expects them to penetrate deeply into the membrane proper. However, the location of their charge relative to that of phosphocholine will depend on the length of any polar spacer between the hydrophobic portion of the molecule and the charge carrier. In general, if the charged portion of the molecule fails to penetrate the polar headgroup region of the membrane it will not be sensed by the "molecular voltmeter" (Rydall & Macdonald, 1992). The data obtained from the α-β correlation plot in figure 4.1.4 indicates that differences exist in the actual location of the charged group between the three cationic amphiphiles. Yet, the three cationic amphiphiles differ in other respects. For instance, CTAB is the only amphiphile which will be entirely charged at physiological pH. DODAP and DC-CHOL, bearing only a tertiary amino headgroup with pKa's in the range of 8.5-9.0, will only be 90% charged at physiological pH. Another difference exhibited by the cationic amphiphiles is the cross-sectional area that they occupy in the plane of the bilayer. The single chained CTAB amphiphile will be expected to occupy only half the area as a double chained lipid like DODAP. Consequently, at a given molar ratio CTAB produces a higher surface charge density than by DODAP and thus a greater response. Also, on a 1:1 encounter basis of the cationic amphiphile with POPC, the lateral separation between the charge of CTAB and choline headgroup of POPC is much reduced in comparison to DODAP which can further contribute to increased
sensitivity of the "voltmeter" (Beschischvili & Seelig, 1990). Finally, cholesterol and its derivatives are notorious for demixing (Vist & Davis, 1990; McMullen & McElhaney, 1995). This suggests the possibility that, even at lower molar ratios, POPC might not have the same statistical encounter probability with DC-CHOL as it does with other cationic species which mix ideally, such as CTAB or DODAP. Now whereas these $^3$H NMR results cannot decisively distinguish between the possibilities it has been shown that the charge location can profoundly influence the efficacy of transfection agents. For instance, Farhood et al., (1992) investigated a series of cationic cholesterol derivatives similar to DC-CHOL in which the derivatives containing a succinyl spacer arm between the substituted ethylenediamine and ring structure of the lipid were the most effective as transfection agents.
4.2 $^2$H NMR Evidence of Polyelectrolyte Induced Domain Formation in Mixed Cationic Amphiphile + POPC Membranes

The three cationic amphiphiles CTAB, DC-CHOL and DODAP are of interest because of their roles as agents of transfection of genetic material. Their interaction with DNA or RNA will be primarily electrostatic in nature, although hydrophobic contributions must also be considered to the overall interaction energy. It is of interest, therefore, to examine the $^2$H NMR response of cationic liposomes to the addition of DNA. In order to model the interaction of DNA to these surfaces a model polyelectrolyte is used which possesses both the anionic sugar-phosphate backbone of RNA as well as the hydrophobic bases, in single stranded form, polyadenylic acid (ie. polyA). The monomer structure of polyA was shown in Figure 4.1.1 and this particular polyelectrolyte is large and has a degree of polymerization of approximately N = 18000. The effects of added polyA on the $^2$H NMR spectra of choline deuterated POPC mixed with cationic amphiphiles is illustrated in figure 4.2.1, using DODAP as the model cation. The top row of spectra show the $^2$H NMR spectra for POPC-$\alpha$-d$_2$ mixed with DODAP while the bottom row of spectra show the corresponding series of spectra for POPC-$\beta$-d$_2$. The details regarding the composition of the lipid mixtures, with each of the different cationic amphiphiles, along with the added amounts of polyA are displayed in table 4.2.1.

Referring back to figure 4.2.1, the left column of spectra represent the controls of the binary mixture of lipids in the absence of any added polyA. The quadrupolar splittings are altered in a manner expected for the presence of cationic surface charge relative to 100% POPC lipid bilayers. The middle column of spectra shows the effect of adding polyA to the cationic lipid bilayers. The
FIGURE 4.2.1. 3H NMR spectra of POPC-rh-1 (top row) and POPC-β-1 (bottom row) in mixtures with DODAP. The left column represents control spectra in the absence of polyA. The middle column shows the results of adding polyA. The right column represents simulated 3H NMR spectra for the corresponding spectra in the middle. The membrane compositions, amounts of added polyA and simulation results are listed in Tables 4.2.1 and 4.2.2.
TABLE 4.2.1  Experimental $^1$H NMR data for deuterated POPC + cationic amphiphile + polyA mixtures. $\Delta \nu^f$ and $\Delta \nu^b$ represent the quadrupolar splittings for both the free and bound domains, respectively.

<table>
<thead>
<tr>
<th>Membrane Composition</th>
<th>Calibration Constant $m$ (kHz/mol)</th>
<th>PolyA / Cation Charge Ratio</th>
<th>$\Delta \nu^f$ (kHz)</th>
<th>$\Delta \nu^b$ (kHz)</th>
<th>control (kHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90/10 POPC-$\alpha$-$d_2$ / CTAB</td>
<td>-35.0</td>
<td>0.50</td>
<td>3.20</td>
<td>1.00</td>
<td>2.90</td>
</tr>
<tr>
<td>90/10 POPC-$\beta$-$d_2$ / CTAB</td>
<td>25.6</td>
<td>1.00</td>
<td>6.60</td>
<td>8.60</td>
<td>8.06</td>
</tr>
<tr>
<td>90/10 POPC-$\alpha$-$d_2$ / DODAP</td>
<td>-31.0</td>
<td>0.50</td>
<td>3.70</td>
<td>1.60</td>
<td>3.30</td>
</tr>
<tr>
<td>80/20 POPC-$\beta$-$d_2$ / DODAP</td>
<td>12.5</td>
<td>0.75</td>
<td>6.80</td>
<td>9.00</td>
<td>8.00</td>
</tr>
<tr>
<td>80/20 POPC-$\alpha$-$d_2$ / DC-CHOL</td>
<td>-20.0</td>
<td>0.50</td>
<td>5.00</td>
<td>4.10</td>
<td>4.7</td>
</tr>
<tr>
<td>70/30 POPC-$\beta$-$d_2$ / DC-CHOL</td>
<td>8.5</td>
<td>0.75</td>
<td>6.00</td>
<td>6.30</td>
<td>6.2</td>
</tr>
</tbody>
</table>

- $m_c$ is the calibration constant used in equation 4.3 as obtained from data in Figure 4.1.3
- $\Delta \nu^f$ is equal to the quadrupolar splitting in the absence of polyelectrolyte

The most obvious noted change is the appearance of a second overlapping spectral component. In comparison, there was no change in the quadrupolar splittings of 100% POPC-$\alpha$-$d_2$ or POPC-$\beta$-$d_2$ MLVs exposed to polyA, or the appearance of a second spectral component. Consequently, there is nominal DNA-lipid bilayer interaction in the absence of an electrostatic attraction.

The right column of spectra are simulations of the $^1$H NMR spectra in the middle column.
The simulations each consist of a superposition of two spectral components, each of which has a quadrupolar splitting and line width factor corresponding to that observed experimentally. Hence, the only variable is the intensity contributed by a particular component to the overall spectrum. This strategy has successfully reproduced the $^2$H NMR spectra obtained for the overlapping Pake doublets, in the presence of polyA. One concludes that polyA addition produces two distinct POPC populations which are in slow exchange with one another on the time scale of $^2$H NMR experiment, delimited by the difference in their quadrupolar splittings. As will be discussed in a moment, analysis of the $^1$H NMR quadrupolar splittings and spectral intensities provides complete information regarding the degree of phase separation and the composition of each of these separate lipid domains.

The results shown in figure 4.2.1 and table 4.2.1 were obtained with one particular amount of added anionic charge from polyA, yielding the overall anion/cation charge ratio listed in table 4.2.1. As will be shown in section 4.3.1, it was determined that by increasing the amount of added polyelectrolyte (ie. polyA) that the intensity of the spectral component with the smaller quadrupolar splitting increased for POPC-$\alpha$-$d_2$ while conversely the intensity of the spectral component with the larger quadrupolar splitting increased for POPC-$\beta$-$d_2$. This helps to identify which spectral component corresponds to the polyA-associated POPC versus polyA-free POPC. Due to the fact that $T_1$ and $T_2$ relaxation times show no significant differences between the two spectral components, the spectral simulations may be used to obtain the fraction of the total POPC contained within either domain.

By focussing on the polyA-free POPC component, one observes that its quadrupolar splitting always reports a diminished cationic surface charge relative to the initial control splitting (ie. more neutral) regardless of whether one studies the $^2$H NMR spectra from POPC-$\alpha$-$d_2$ or POPC-$\beta$-$d_2$. The
results therefore indicate that the polyA-free domain is depleted with respect to cationic amphiphile.

On the other hand, the polyA-associated POPC component has a quadrupolar splitting that always reports a surface charge more cationic than the control values, whether one examines the $^1$H NMR spectra from either POPC-α-d$_1$ or POPC-β-d$_1$. This result is contrary to previous results concerning ternary mixtures of cationic + anionic + zwitterionic charged species (Marassi & Macdonald, 1992) wherein the quadrupolar splittings report the expected neutralization of net surface charge. An interpretation of this “anti-voltmeter” effect of anionic polyelectrolytes on cationic surfaces will be presented in the next section. These results are not simply localized to the use of polyA but will be shown to be a generalized response to a variety of anionic polyelectrolytes added to cationic amphiphile containing lipid bilayers. Moreover, a similar “anti-voltmeter” response is obtained when the electrostatic mirror image experiment is performed by adding cationic polyelectrolytes to anionic amphiphile containing bilayers (Crowell & Macdonald, 1997; Crowell & Macdonald, 1998). Therefore, this property seems to be common for polyelectrolytes of sufficient size acting on oppositely charged surfaces under conditions of low salt.

4.2.1 QUANTITATION OF DOMAIN SEPARATION AND COMPOSITION

The $^1$H NMR spectra obtained in such instances may be analyzed to reveal both the degree of domain separation and the composition of the various domains. The global composition of zwitterionic ($X_z$) or cationic ($X_c$) lipid is defined in mole fractions according to:

$$X_z^l + X_c^l = 1$$  \hspace{1cm} (4.1)
Each of such lipid populations may be subdivided into those which are polyelectrolyte-bound (superscript b) and those which are polyelectrolyte-free (superscript f), according to:

\[ \begin{align*}
X_i^b &= X_i^b + X_i^f \\
X_i^f &= X_i^b + X_i^f 
\end{align*} \] (4.2)

The ratio of POPC in the polyelectrolyte-bound versus polyelectrolyte-free domains \((X_i^b/X_i^f)\) at any given level of added polyelectrolyte is equal to the intensity ratio of the two components in the corresponding \(^1\)H NMR spectrum as obtained directly from spectral simulations. The details for polyA are listed in table 4.2.2.

In general, the cationic amphiphile composition of the polyelectrolyte-free domain is obtained from the quadrupolar splitting of the corresponding component in the \(^1\)H NMR spectrum via:

\[ \Delta v^f = \Delta v_0 + m \left( \frac{X_i^f}{X_i^b + X_i^f} \right) \] (4.3)

where \(\Delta v_0\) is the quadrupolar splitting measured for 100% POPC and \(m\) is a calibration constant for either the \(\alpha\) or \(\beta\) deuteron labeling position for a particular cationic amphiphile. This equation is readily rearranged to yield directly the desired quantity \(X_i^f\) in terms of known and/or experimentally measured quantities:

\[ X_i^f = X_i^f \left( \frac{\Delta v_f - \Delta v_0}{m - \Delta v_f + \Delta v_0} \right) \] (4.4)
Simulated H NMR data for deuterated POPC + cationic amphiphile + polyA mixtures

<table>
<thead>
<tr>
<th>Membrane Composition</th>
<th>Fraction POPC &quot;free (bound)&quot;</th>
<th>Mole Fraction Cationic Amphiphile &quot;free (bound)&quot;</th>
<th>Predicted Δν° (kHz)</th>
<th>Cation/Anion Charge Ratio in polyA &quot;bound&quot; Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \frac{X_f}{X_f} )</td>
<td>( \frac{X_f}{X_f + X^b} ) (1 - ( \frac{X^b}{X_f + X^b} ))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90/10 POPC-α-d₂ / CTAB</td>
<td>0.75 (0.25)</td>
<td>0.09 (0.13)</td>
<td>2.00</td>
<td>0.90</td>
</tr>
<tr>
<td>90/10 POPC-β-d₂ / CTAB</td>
<td>0.28 (0.72)</td>
<td>0.04 (0.12)</td>
<td>8.60</td>
<td>0.87</td>
</tr>
<tr>
<td>90/10 POPC-α-d₂ / DODAP</td>
<td>0.75 (0.25)</td>
<td>0.09 (0.14)</td>
<td>2.20</td>
<td>0.86</td>
</tr>
<tr>
<td>80/20 POPC-β-d₂ / DODAP</td>
<td>0.43 (0.57)</td>
<td>0.10 (0.26)</td>
<td>8.80</td>
<td>0.99</td>
</tr>
<tr>
<td>80/20 POPC-α-d₂ / DC-CHOL</td>
<td>0.69 (0.31)</td>
<td>0.16 (0.27)</td>
<td>4.10</td>
<td>0.83</td>
</tr>
<tr>
<td>70/30 POPC-β-d₂ / DC-CHOL</td>
<td>0.55 (0.45)</td>
<td>0.25 (0.35)</td>
<td>6.20</td>
<td>0.71</td>
</tr>
</tbody>
</table>
The results of applying this analysis, for polyA with the three different cationic amphiphiles, are listed in table 4.2.2. They demonstrate that the polyA-free domain is depleted with respect to cationic amphiphiles relative to the initial global composition. Finally, the amount of cationic amphiphile contained within the polyelectrolyte-bound phase is obtained by simple substraction according to:

\[ X_{b} = 1 - X_{r} - X_{n} - X_{t} \]  
(4.5)

The results of such calculations are likewise listed in table 4.2.2. They indicate that the ratio of cationic to anionic charge within the polyelectrolyte (polyA) -bound domain is approximately 1:1, indicating a neutral complex if it is assumed that every single phosphate monomer of polyA is able to interact with a cationic amphiphile. Note that all four undetermined quantities in equation 4.2 are obtained without direct reference to the quadrupolar splittings measured for POPC-α-d, or POPC-β-d, in the polyelectrolyte-bound domain, which behave in an apparently contradictory fashion.

In binary lipid mixtures, such as the initial cationic surface produced in the absence of polyA, it is a straightforward process to relate the observed quadrupolar splitting for either of the deuterolabeling positions to the mole fraction of charged lipid in a similar fashion to equation 4.3. In ternary mixtures of zwitterionic + cationic + anionic amphiphiles, however, the observed quadrupolar splitting \((\Delta v_{\alpha})\) is altered by an amount which is the sum of the perturbations due to the cationic and anionic species taken individually in binary mixtures (Marassi & Macdonald, 1992), according to:

\[
(\Delta v_{\alpha} - \Delta v_{0}) = (\Delta v_{+} - \Delta v_{0}) + (\Delta v_{-} - \Delta v_{0}) = mX_{+} + mX_{-}
\]  
(4.6)
where $\Delta v$, once again is the quadrupolar splitting for 100% POPC. $\Delta v$, and $\Delta v_-$ are the respective quadrupolar splittings of POPC in binary mixtures with the cationic species of mole fraction $X$, or the anionic species of mole fraction $X_-$, while $m_+$ and $m_-$ are the corresponding calibration constants.

In order to apply this equation one must have knowledge of the calibration constants in the cognate binary mixtures.

Polyelectrolytes, such as polyA, exhibit little if any binding to 100% POPC lipid bilayers so that an independent determination of $m_-$ is not readily achieved. To analyze the quadrupolar splittings of polyelectrolyte-bound domains in terms of compositions one must resort, therefore, to certain assumptions. What if one assumes that POPC does not directly respond to the presence of polyA, for instance, and only detects its presence indirectly through the polyelectrolyte effect on the local distribution of cationic amphiphiles? If this situation physically exists then the calibration constant, $m_-$, is equal to zero and the effective mole fraction of anionic charge ($X_-$) drops out of the equation.

The validity of this assumption may be proven as follows. If the calibration constant $m_-$ is equal to zero then equation 4.6 reduces to equation 4.3 and the effective mole fraction of cationic charge ($X_+$) in the polyelectrolyte (polyA)-bound domain is obtained directly from the quadrupolar splitting of the corresponding spectral component. By comparing the cationic amphiphile composition determined directly from the quadrupolar splittings to that determined using equations 4.1-4.5 the validity of this assumption should be proven. This was accomplished for polyA and for three other polyelectrolytes, in which DODAP was used as the cationic amphiphile in each instance. The results are shown in figure 4.2.2. The raw data obtained for the other polyelectrolytes will be presented and discussed in a later section. But, what these results show is the 1:1 correlation between
FIGURE 4.2.2 Comparison of the two methods of calculating the mole fraction of DODAP bound in the polyelectrolyte-bound domain. Method 1 employs equations 4.1-4.5 and utilizes only the quadrupolar splitting of the polyelectrolyte-free domain plus the intensity ratios of the two components in the $^1$H NMR spectrum. Method 2 employs equation 4.6 as applied to the quadrupolar splitting of the polyelectrolyte-bound domain with the assumption that the calibration constant for the polyelectrolyte is zero. The dashed line shows the result expected for a 1:1 correspondence between the two methods. Open symbols: POPC-$a$-$d$; Closed symbols, POPC-$b$-$d$; PolyA (diamonds), PSSS (squares), PACA (circles) and PGLU (triangles).
the two methods of calculating $X_b$, once again indicating the validity of assuming that $m_i$ is equal to zero in equation 4.6. The results also show that this "anti-voltmeter" response is not specific to polyA, but instead seems to be a generalized effect of all polyelectrolytes.

What these results show is that the presence of polyelectrolyte is somehow masked from direct detection by POPC and whose presence is detected only indirectly through its influence on the local lipid composition. In the following section is a model of the polyelectrolyte-lipid bilayer interaction which explains the physical origin of the polyelectrolyte "masking" effect.

4.2.2 POLYELECTROLYTE BINDING TO CHARGED BILAYER SURFACE

The $^1$H NMR results reported above permit a proposal for the arrangement of a polyelectrolyte such as polyA at the surface of a lipid bilayer containing a mixture of cationic + zwitterionic amphiphiles, as shown schematically in figure 4.2.3. The anionically charged polyelectrolyte (polyA) is drawn to the membrane surface through electrostatic interaction. Upon binding, the adenosine bases penetrate into the acyl chain region of the lipid bilayer, as indicated by infrared (Mal’tseva et al., 1983) and NMR (Budker et al., 1990) evidence. This leaves the sugar-phosphate backbone located within the polar interface region. At this point, the intercalated polyelectrolyte is pictured to assume a two-dimensional self-avoiding random walk configuration where the individual nucleotide units occupy sites on a lattice.

The region of the lipid bilayer occupied by the polyelectrolyte defines a distinct domain through a combination of two effects. First, electrostatic interaction between the anionic monomer units and cationic amphiphiles tends to draw the two together such that the lipid composition in the vicinity of the polyelectrolyte will become more cationic than that of the bulk. If these coulombic
FIGURE 4.2.3 Two-dimensional schematic representation of polyelectrolyte (black circles) induced domain formation in mixed POPC (closed circles) + cationic amphiphile (open circles) lipid bilayers.
forces of attraction are sufficiently strong, then the cationic amphiphiles screen the charge of the anionic polyelectrolyte from POPC. Second, the effective lateral diffusion coefficient of individual lipids will be reduced because of the well known archipelago effect (Saxton, 1993) within these domains. This should inhibit the POPC lipids, which are fortuitously trapped within the polyelectrolyte-bound domain, from exchanging with and averaging over the bulk lipid population in order to produce long lived domains on the $^2$H NMR timescale. The dimensions of a random-coil polymer permit the inclusion of considerable amounts of the zwitterionic lipid, the details of which should depend on a number of factors including the hydrophobicity of the polymer, the global cationic/zwitterionic amphiphile in the lipid bilayer, the stiffness of the polymer backbone, the polymer molecular weight, the polyelectrolyte's linear charge density and the ionic strength of solution.

This model explains all the $^2$H NMR observations including the appearance of distinct POPC populations, the enrichment of cationic amphiphile in the polyelectrolyte-bound domain, the concomitant depletion of cationic amphiphiles in the polyelectrolyte-free domain and the “anti-voltmeter” response observed in the former. This “anti-voltmeter” response can be rationalized because of the overwhelming preference of the charged polyelectrolyte for the oppositely charged amphiphile, for which direct access to POPC will be blocked thus removing its statistical probability of interaction with the polyelectrolyte. Instead, POPC trapped within the polyelectrolyte-bound domain will simply encounter an environment which is locally enriched in cationic charge, leading to the “anti-voltmeter” response. Alternatively, this response can be explained in terms of the plane of binding of the oppositely charged molecules. For instance, should the anionic phosphates of polyA bind in a plane located above the headgroup region of the POPC molecules this would have the overall result of decreasing the sensitivity of the “molecular voltmeter” to the presence of the
polyelectrolyte. However, this scenario is less likely than the first given the evidence of hydrophobic penetration of the nucleotide bases of polyA into the membrane interior. Also, it would be difficult to conceive that such a superficial binding would lead to domain formation.
4.3 Factors Influencing Polyelectrolyte Binding and Domain Formation in Deuterated POPC Bilayers

The investigations reported in the previous section encompass a narrow set of circumstances for which there are many other key experimental variables which demand further investigation. Although it has been shown that a variety of polyelectrolytes are capable of producing laterally segregated domains the detailed composition and size of domains so produced should depend on a variety of factors, a few of which will be examined in the following sections. As well, a more detailed account of polyelectrolyte binding and conformation at the oppositely charged surface of bilayer membranes will be presented from the point of view of the zwitterionic POPC molecule. As will be documented later on, interactions between anionic polyelectrolytes and cationic amphiphiles were also studied directly from the perspective of both molecules.

Before embarking on the detailed information determined from choline-deuterated POPC there are some general properties of cationic bilayers exposed to anionic polyelectrolytes that need to be mentioned. The general properties of these molecules will be presented specifically for bilayers composed of DODAP and POPC exposed to the three anionic polyelectrolytes shown in figure 4.3.1. The figure shows the repeating units of the three polyelectrolytes, PSSS (poly sodium styrene sulfonate), PACA (poly acrylic acid) and PGLU (poly glutamic acid).

Pure aqueous mixtures of DODAP and POPC spontaneously assemble into lipid bilayers at all proportions examined in these studies. This point is demonstrated in the top left $^3$P NMR spectrum of figure 4.3.2 for a 20/80 (mol/mol) mixture of DODAP + POPC, in which the line shape is
FIGURE 4.3.1 Chemical structures of the three anionic polyelectrolytes (PSSS, PACA, and PGLU) employed here. The degree of polymerization (N) is 340 for PSSS, 320 for PACA and 550 for PGLU.
FIGURE 4.3.2 NMR spectra of mixed DODAP + POPC lipid bilayers in the absence (top row) and presence (bottom row) of PSSS (1:1 anion/cation charge ratio). From left to right, spectra correspond to $^3$P NMR spectra of DODAP + POPC (20/80), $^1$H NMR spectra of DOTAP-γ-d$_2$ + POPC (20/80), $^1$H NMR spectra of DODAP + POPC-α-d$_2$ (10/90) and $^1$H NMR spectra of DODAP + POPC-β-d$_2$ (20/80).
diagnostic of lipids in a bilayer arrangement (Seelig, 1978; Cullis & de Kruijff, 1979).

When any of the three anionic polyelectrolytes are added to the aqueous medium with which the DODAP + POPC mixtures are hydrated, the dispersions so produced exhibit colloidal properties markedly different from those observed in the absence of polyelectrolytes. Specifically, the MLVs formed for the pure lipid mixtures are finely dispersed and difficult to centrifuge, due to intervesicular charge repulsion between the cationic surfaces, in accordance with classical DLVO theory of colloidal stability (Dejaguin & Landau, 1941; Verwey & Overbeek, 1948). In the presence of polyelectrolytes, the MLVs clump together and are readily centrifuged, indicative of particle flocculation or bridging of vesicles by polyelectrolytes (Pefferkorn, 1995). These are the macroscopic manifestations of the interactions between these particles.

At the point where enough polyelectrolyte has been added to neutralize the cationic lipid surface charge, the lipids retain a bilayer arrangement as demonstrated in the bottom left $^3$P NMR spectrum in figure 4.3.2. Also, shown separately from the point of view of amino deuterated cationic lipid (DOTAP-$\gamma$-d$_2$) and deuterated POPC, both in the absence and presence of polyelectrolyte, the $^2$H NMR spectra are characteristic of fluid lipids in a bilayer arrangement. Thus, despite the binding of the polyelectrolyte to the lipid vesicles and the resultant neutralization of surface charge and vesicle flocculation, the assembled lipid molecules maintain a fluid bilayer organization.

Finally, the amount of polyelectrolyte chain binding to DODAP + POPC vesicles (10/90 mol/mol) may be quantified by an independent UV depletion assay, as described in the Materials and Methods section. The results are shown in figure 4.3.3 and indicate that all three distinct polyelectrolyte chains bind quantitatively to the surface up to the anion/cation equivalence point. Above this point all the polyelectrolytes bind further to the bilayers but at a reduced level relative to
FIGURE 4.3.3 Ultraviolet (UV) depletion assay of polyelectrolyte binding to lipid bilayers composed of DODAP + POPC (10/90 mol/mol) as a function of added PSSS (squares), PACA (circles) or PGLU (triangles). The dashed line indicates the results expected for 100% binding up to the equivalence point, followed by no further binding.
the amount of polyelectrolyte added. This method is capable of recording only those full polyelectrolyte chains which are completely unbound and remain in the supernatant of the aqueous mixtures. With respect to the results shown in figure 4.3.3, this means that above the equivalence point more anionic charge is present in the polyelectrolyte chains near the bilayer surface than there is cationic charge to neutralize it. Thus, above this point there must be competition for the cationic lipids by the individual polyelectrolyte chains.

4.3.1 Polyelectrolyte Chemistry

By closer examination of the structures of the three polyelectrolytes used in these studies, which will be compared and contrasted for their ability in forming laterally segregated domains, there are obvious differences in the hydrophobicity of their polymeric backbones. Of the three, PGLU is clearly the most hydrophilic, since its polymeric backbone consists of a peptide chain. In comparison, the ethylenic backbone of PACA or PSSS is relatively hydrophobic. When the aromatic ring of PSSS is considered, it is evident that one may arrange these three polyelectrolytes in order of increasing hydrophobicity as follows: PGLU < PACA < PSSS. Comparison of the effects of the different polyelectrolytes on DODAP + deuterated POPC bilayers permits the evaluation of the electrostatic and hydrophobic contributions to sequestration of cationic lipids into polyelectrolyte domains.

All three anionic species will be studied for their ability to induce in-plane domain formation by focusing on the detailed composition and size of domains so formed through a titration method. But before embarking on the detailed analysis of the effects of the three species the general features of figure 4.3.2 will be presented. The ²H NMR spectra of POPC-α-d₂ and POPC-β-d₂, in the presence of 10% and 20% DODAP respectively, are shown in the top row of figure 4.3.2 as the
second from right and rightmost spectra, respectively. These particular compositions were chosen to give the best resolution for each of the deuterolabeling positions. Once again the values of the quadrupolar splittings from these spectra (3400 Hz and 8200 Hz for POPC-o-d and POPC-β-d, respectively) are altered from the values measured in 100% POPC membranes in a manner consistent with accumulation of cationic surface charge. The subsequent addition of anionic polyelectrolyte, in particular PSSS, to the cationic lipid bilayers produces two distinct POPC populations, as shown in the spectra directly underneath the control spectra in figure 4.3.2. Similar two component ²H NMR spectra are produced by addition of any of the three polyelectrolytes. As already mentioned for the effect of polyA on the ²H NMR spectra, these three anionic polyelectrolytes also produce overlapping Pake doublets in which, regardless of the deuterolabeling position, one of the component Pake doublets exhibits a quadrupolar splitting greater than the control value, while the second exhibits a splitting less than the control. This once again suggests that one POPC population is enriched while the other is depleted in cationic charge, relative to the global molar fraction of cationic charge present in the homogeneous membrane in the absence of polyelectrolyte.

The spectra in the top and bottom row of the column secondmost from the left are representative of the ²H NMR spectra obtained for aminomethyl-deuterated DOTAP, in the absence and presence of PSSS, respectively. The main observable, in these spectra, is the absence of a two component spectrum for the addition of PSSS. The reasons for this and a more detailed discussion of the effect on a series of deuterated cationic lipids will be deferred to a later section.

Referring back to the results obtained with deuterated POPC, the particulars regarding the amount and composition of the polyelectrolyte-induced domains are obtained from a detailed examination of the two ²H NMR quadrupolar splittings and relative intensities of the two Pake
Figure 4.3.4 consists of a series of $^2$H NMR spectra from lipid bilayers composed of mixed DODAP + POPC-$\alpha$-d$_2$ (10/90). The four experimental spectra of the left column were obtained upon addition of PSSS in the amounts (from top to bottom): 0, 0.75, 1.00 and 2.00 equivalents of PSSS anions to DODAP cations. The corresponding computer-simulated spectra are arrayed in the right column.

From figure 4.3.4 it is evident that, for vesicles produced by a mixture of POPC-$\alpha$-d$_2$ with DODAP, addition of PSSS produces two-component $^2$H NMR spectra across the series, except for the highest level of PSSS. By following the series it becomes evident that the Pake doublet with the smaller of the two quadrupolar splitting grows in intensity with subsequent addition of PSSS. Simultaneously, the second Pake doublet, which possesses the larger quadrupolar splitting, has its intensity decrease with added PSSS. This titration series therefore unequivocally identifies the narrower Pake doublet as arising from POPC-$\alpha$-d$_2$ associated with added polyelectrolyte. Finally, when an excess of anionic charge from PSSS is added to overcome the cationic surface charge, only a single quadrupolar splitting can be discerned and its value corresponds closely to that of the control measured in the absence of PSSS.

It may be difficult to evaluate, by inspection, which of the two spectral components is increasing or decreasing in intensity across such a series. As well, the overlap of two such Pake doublets tend to reduce the true quadrupolar splittings by broadening of the peaks. These problems were overcome by the use of computer-simulated spectra, which aided in determining both vital quantities by careful comparison to experimental spectra. For instance, it was determined, through the simulations presented in the bottom row of figure 4.3.4, that in going from the 0.75 to 1.00 addition of PSSS that the narrower Pake doublet increased from approximately 30% to 45% of the
FIGURE 4.3.4  $^1$H NMR spectra of mixed DODAP + POPC-$\alpha$-d$_1$ (10/90) bilayers as a function of added PSSS in amounts corresponding to, from top to bottom, 0, 0.75, 1.0 and 2.0 equivalents of PSSS anionic charge to DODAP cationic charge. The left column of spectra were obtained experimentally, while the right column of spectra are the corresponding computer simulations.
FIGURE 4.3.5  $^1$H NMR spectra of mixed DODAP + POPC-$\beta$-d$_2$ (20/80) bilayers as a function of added PSSS in amounts corresponding to, from top to bottom, 0, 0.75, 1.0 and 2.0 equivalents of PSSS anionic charge to DODAP cationic charge. The left column of spectra were obtained experimentally, while the right column of spectra are the corresponding computer simulations.
The results for the observed changes in the $^1$H NMR spectrum for the case of lipid bilayers composed of DODAP + POPC-β-d$_2$ (20/80) are illustrated in figure 4.3.5. As previously, the four spectra, from top to bottom, where obtained upon addition of PSSS in the following amounts: 0, 0.75, 1.00 and 2.00 equivalents of PSSS anions to DODAP cations. Once again, the experimental spectra are arrayed in the left column, while the corresponding computer-simulated spectra are arrayed in the right column. Fundamentally the same results are obtained as observed with POPC-α-d$_2$ as far as the presence of two overlapping Pake doublets across the series is concerned followed by the observation of a single Pake doublet at an addition of excess PSSS. However, for the case of POPC-β-d$_2$, it is the Pake doublet with the larger quadrupolar splitting that increases in intensity with added PSSS, while that with the smaller splitting decreases in intensity. There is a third, minor component having a narrow quadrupolar splitting which contributes less than 5% of the total spectral intensity, which is observed only for the case of PSSS added to bilayers containing POPC-β-d$_2$. Since no such component was observed in the presence of PACA or PGLU, there is no reasonable explanation for its origin. Regardless, the same major points are noted for the results obtained for POPC-β-d$_2$ as for POPC-α-d$_2$, indicating that either method could be used for arriving at the detailed results of domain size and composition as outlined in the mathematical model.

The treatment of the data begins by accumulation of the raw data determined by spectral simulation. First, the detailed dependence of the quadrupolar splittings of the two spectral components, polyelectrolyte-bound and polyelectrolyte free, are shown in figure 4.3.6 for all three polyelectrolytes investigated (PSSS, PACA, PGLU) and for the two different mixed bilayers, DODAP + POPC-α-d$_2$ (10/90) (figure 4.3.6A) and DODAP + POPC-β-d$_2$ (20/80) (figure 4.3.6B).
FIGURE 4.3.6 ³H NMR quadrupolar splittings of the component Pake doublets in the ³H NMR spectra of mixed DODAP + POPC bilayers as a function of the amount of added polyelectrolyte: PSSS (squares), PACA (circles) and PGLU (triangles). Open symbols refer to the polyelectrolyte-free domain, while solid symbols refer to the polyelectrolyte-bound domain. In panel A the bilayers were composed of DODAP + POPC-n-d (10/90), while in panel B the bilayers were composed of DODAP + POPC-β-d₂ (50/50).
First, when looking at the figures it should be noted that it was not possible to resolve two Pake doublets for polyelectrolyte:DODAP ratios less than 0.50. However, over the range at which two spectral components could be resolved, it is evident that with increasing levels of polyelectrolyte the quadrupolar splitting from both POPC-α-d₁ and POPC-β-d₁ contained within the polyelectrolyte-free domain tends towards a more neutral surface charge. In other words there is a progressive depletion of DODAP from the polyelectrolyte-free domain as polyelectrolyte is added. Concomitantly, the quadrupolar splittings from POPC-α-d₁ and POPC-β-d₁ in the polyelectrolyte-bound domains is characteristic of high cationic surface charge. This is consistent with an enrichment of DODAP in the polyelectrolyte-bound domains. It is also evident from both figures that the quadrupolar splittings for the polyelectrolyte-bound domains revert back toward the values characteristic of the initial cationic surface charge in the absence of polyelectrolyte, with increasing levels of added polyelectrolyte. Although there may be some slight differences between the responses of the different polyelectrolytes here, a proper analysis and comparison of the polyelectrolytes requires taking into consideration the intensities of the two populations as well as the quadrupolar splittings.

The first quantity that may be evaluated by such data determined from the ¹H NMR spectra is the fraction of total DODAP (X⁺/X⁻) or POPC (X⁺/X⁻) bound in the polyelectrolyte domain as a function of the polyelectrolyte:DODAP charge ratio for the three polyelectrolytes as calculated by equations 4.1-4.6. As shown in figure 4.3.7 A and B there is a near linear dependence of the fraction of bound lipid on the level of added polyelectrolyte for both DODAP and POPC and for all of the distinct polyelectrolyte chains. Figure 4.3.7A shows the results determined for bilayers composed of DODAP + POPC-α-d₁ (10/90) and indicates that regardless of the particular identity of polyelectrolyte that each equivalent of polyelectrolyte anionic charge binds approximately 0.75
FIGURE 4.3.7 Fraction of polyelectrolyte-bound DODAP (X₁⁺X₁⁻) or POPC (X₁⁺X₁⁻) as a function of the amount of added polyelectrolyte: PSSS (squares), PACA (circles) and PGLU (triangles). Open symbols refer to POPC and show the fraction of the total intensity of the relevant ²H NMR spectrum in the spectral component identified as corresponding to the polyelectrolyte-bound domain. Solid symbols refer to DODAP and show the results of applying equations 4.1-4.5 as described earlier. In panel A the bilayers were composed of DODAP + POPC-α-d₂ (10/90), while in panel B the bilayers were composed of DODAP + POPC-β-d₂ (20/80). The solid line shows the result expected for 1:1 polyelectrolyte/lipid reference binding.
equivalents of DODAP cationic charge. On the other hand figure 4.3.7B shows the results for bilayers composed of DODAP + POPC-β-d_{2} (20/80) and this time indicates that each equivalent of polyelectrolyte anionic charge binds virtually 1.0 equivalent of DODAP cationic charge, independent of the detailed chemical structure of the polyelectrolyte. This result is more reminiscent of the results obtained for polyA as discussed earlier in mixtures with POPC and the three different cationic amphiphiles. In polyelectrolyte-surfactant complexes, there is a critical micellar surface charge that must be exceeded before the entropic cost of forming a 1:1 surface-bound charge complex is outweighed by the coulombic forces of attraction (Dubin et al., 1989). This effect might explain the apparent nonstoichiometric anion:cation ratios for the DODAP + POPC (10/90) case. As well other 3H NMR experiments (Crowell & MacDonald, 1998) and centrifugation techniques (Denisov et al., 1998) have also provided evidence that the affinity of cationic polyelectrolytes (polylysine) decreases markedly as the mole fraction of anionic lipid (PG) decreases in PC vesicles.

For POPC, the fraction trapped within the polyelectrolyte-bound domains always lags behind the amount of DODAP and can be related to the fact that POPC is not actively bound in these domains as is DODAP, instead it is fortuitously trapped.

The second quantity that can be evaluated from the 3H NMR data is the DODAP composition of the polyelectrolyte-free and polyelectrolyte-bound domains as shown in figure 4.3.8. Whereas the data for the quadrupolar splittings for POPC-α-d_{1} and POPC-β-d_{1} were nearly mirror images of one another, the data presented in figure 4.3.8 indicate how evaluation of the NMR data produces similar results for both cases, indicating the validity of use of either of the deuterolabeling positions for information. The data in figure 4.3.8A and B both indicate the progressive depletion of DODAP out of the polyelectrolyte-free domain with added polyelectrolyte even though the specifics of DODAP
FIGURE 4.3.8  Composition of the polyelectrolyte-free and polyelectrolyte-bound domains in mixed DODAP + POPC bilayers as a function of the amount of added polyelectrolyte: PSSS (squares), PACA (circles) and PGLU (triangles). Open symbols are for polyelectrolyte-free domains, while solid symbols are for polyelectrolyte-bound domains. A, DODAP + POPC-α-d$_2$ (10/90); B, DODAP + POPC-β-d$_2$ (20/80).
composition vary for the two cases due to differences in initial surface charge density. This effect will be discussed in more detail in section 4.3.3. Also shown in figure 4.3.8A and B is that there is an enrichment of DODAP within the polyelectrolyte-bound domains. The DODAP composition of this domain is not constant with increasing polyelectrolyte but rather approaches the initial composition as the membrane surface becomes saturated with polyelectrolyte. Now, whereas no differences could be discerned, between the three different polyelectrolytes, with respect to the mole fraction of bound lipid the distinct behaviour of the polyelectrolytes is now quite evident in figure 4.3.8. The polyelectrolyte least capable of segregating DODAP into distinct polyelectrolyte-bound domains is PGLU, the most hydrophilic of the three polyelectrolytes. At the same time, the most hydrophobic polyelectrolyte, PSSS, exhibits the greatest tendency to segregate DODAP and exclude POPC in its polyelectrolyte-bound domain. Thus, the propensity of the polyelectrolyte to segregate cationic amphiphiles and thus its ability to form distinct domains correlates with the potential of the polyelectrolyte to penetrate into the bilayer interior.

This behaviour has several plausible origins. The ability of certain polyelectrolytes to form lateral domains on oppositely charged bilayer surfaces may be related to their hydrophobic portions. Certain polyelectrolytes like polyA, which contain hydrophobic bases, are known to intercalate between membrane lipids. This can have the effect of producing a barrier or “fence” for lipids trapped within the dimensions of the polyelectrolyte chain. This results in a tortuosity effect whereby lipids can no longer diffuse in a direct line and thus have reduced diffusion coefficients. Consequently, exchange between the bound and free domains would be reduced and we would observe long lived domains.

Another possible origin to this behaviour is the aggregation of multiple numbers of chains in
order to form "superdomains". This is a reasonable assumption due to the fact that this aggregation of chains, in the plane of the bilayer, would reduce the hydrophobic mismatch between lipids (Israelachvili and Mitchell, 1975; Israelachvili, 1977) and proteins (Killian, 1998). This favourable clustering of polyelectrolyte chains would reduce the number of packing defects in the bilayer and would maximize the amount of hydrophobic interactions within the bilayer. The sheer size of this in-plane aggregation should produce long lived domains. The residence time of a lipid within this aggregated domain would be long enough such that exchange between the bound and free domains would be limited. Thus, POPC will have a reduced ability to exchange and average out the behaviour of distinctly charged lateral environments, resulting in the observance of domains.

Finally, it may also be the case that both of these effects act simultaneously. At present, though, the data collected for the three polyelectrolytes used in these studies do not provide direct evidence as to whether any of these explanations are operable.

The $^2$H NMR data presented here have, however, provided an expansion to the model of polyelectrolyte binding to lipid bilayers, as shown in figure 4.3.9. In the top portion of the figure is the situation of the mixed bilayer in the absence of polyelectrolyte. Lateral diffusion of the lipids within the plane of the bilayer averages out the local fluctuations from the global average composition. Thus, every POPC molecule experiences the identical DODAP composition during the time course of the NMR experiment and hence produces a single well-defined quadrupolar splitting.

When the polyelectrolyte is added in an amount below the global anion/cation equivalence point, there is quantitative binding of polyelectrolyte to the bilayer surface as deduced from the $^2$H NMR data, provided that there is sufficient initial surface charge density. This indicates that the
PolyE = 0

PolyE < DODAP

PolyE > DODAP

FIGURE 4.3.9 Schematic representation of a mixed cationic + zwitterionic lipid bilayer exposed to anionic polyelectrolytes. In the absence of polyelectrolyte, the cationic and zwitterionic lipids mix homogeneously. When the polyelectrolyte is added in an amount below the anion/cation equivalence point, there is a cationic lipid charge for each anionic polyelectrolyte charge and the polyelectrolyte lies flat on the surface. When the added amount of polyelectrolyte exceeds the anion/cation equivalence point, the polyelectrolyte can no longer lie flat and the domain structure disappears.
polyelectrolyte lies flat along the bilayer surface under these circumstances, as shown in the middle scheme in figure 4.3.9. Within the region defined by the polyelectrolyte there is an enrichment of DODAP, maintained over time. Concomitantly, there is a depletion of DODAP from other regions. Thus, the $^1$H NMR spectrum of choline-deuterated POPC reports two separate surface charge environments corresponding to the distinct domains defined by the presence or absence of polyelectrolyte.

When anionic polyelectrolyte is added in an amount above the anion/cation equivalence point, binding is no longer quantitative and individual polyelectrolyte chains and monomeric units must compete for cationic surface charges. Since the data from figure 4.3.3 indicate that there is an excess of chains binding to the surface above this point then there are fewer cationic charges than anionic charge from the monomeric units. This means that the polyelectrolyte can no longer lie flat along the surface but instead contacts the surface only intermittently, in trains or loops, as shown schematically at the bottom of figure 4.3.9. Since interpolyelectrolyte charge repulsion will cause the polyelectrolytes to distribute more or less evenly over the bilayer surface, the DODAP molecules likewise will be more evenly be distributed over the surface. This situation produces a single common environment for all amphiphiles and consequently a single quadrupolar splitting for choline-deuterated POPC with a value approximating that of the initial conditions in the absence of polyelectrolyte.

4.3.2 Polyelectrolyte Molecular Weight

There are a variety of distinct polyelectrolytes, which differ not only with respect to chemical structure but also molecular size. Biopolyelectrolytes may range from small proteins, such as mellitin with 26 amino acid residues, to large proteins, such as myelin basic protein, having 170 amino acid
residues, or in the case of DNA, from small plasmid DNA with only about $10^2$ bases to DNA strands of $10^4$ base pairs. The size of these polyelectrolytes is expected to be an important factor in controlling the composition and size of the induced domains, which in turn will affect the domain's functional properties. The degree of enrichment with anionic lipids, for example, can influence the activity of membrane-bound enzymes (Robinson et al., 1980; Gennis, 1989). In the case of DNA it is known that the efficiency of DNA entrapment (Monnard et al., 1997) and transfection (van der Woude et al., 1995) decreases with higher molecular weight DNA fragments.

In the studies presented here, the properties of domains induced by four different molecular weight chains of the anionic polyelectrolyte PSSS in lipid bilayers consisting of mixtures of either POPC-α-d$_2$, or POPC-β-d$_2$, with the cationic lipid DODAP are examined. The focus of these experiments is to study how the details of the polyelectrolyte induced domain properties vary with respect to the molecular weight of PSSS.

The type of analysis followed for the effect of the four different molecular weight chains on domain formation is similar to that already produced in the previous section for the distinct polyelectrolytes and begins with the results shown in figures 4.3.10 and 4.3.11. The $^1$H NMR spectra of POPC-α-d$_2$, and POPC-β-d$_2$, are shown in figure 4.3.10 and 4.3.11, respectively. The results presented here and throughout this study are for bilayers composed of 40% DODAP. The top spectra of figures 4.3.10 and 4.3.11 are the control spectra in the absence of polyelectrolyte. The change in quadrupolar splittings to -3.0 and 11.0 kHz, for POPC-α-d$_2$, and POPC-β-d$_2$, respectively, is indicative of the “voltmeter” response for the accumulation of cationic charge at the bilayer surface, relative to the control values for 100% POPC vesicles. Since there is only one quadrupolar splitting obtained for either case, the lipids are homogeneously mixed on the timescale of the $^1$H NMR
FIGURE 4.3.10 $^1$H NMR spectra of mixed DODAP + POPC-α-d$_4$ (40/60) bilayers with added PSSS (N=3790) in amounts corresponding to, from top to bottom, 0, 0.50 and 0.75 equivalents of anionic charge from PSSS to cationic charge from DODAP. The left column corresponds to the experimental spectra, the middle column to the corresponding de-Pakeed spectra and the right column to simulated spectra obtained using parameters determined from de-Pake-ing.
$^3$H NMR spectra of mixed DODAP + POPC-$\beta$-d$_2$ (40/60) bilayers with added PSSS (N=3790) in amounts corresponding to, from top to bottom, 0, 0.50 and 0.75 equivalents of anionic charge from PSSS to cationic charge from DODAP. The left column corresponds to the experimental spectra, the middle column to the corresponding de-Paked spectra and the right column to simulated spectra obtained using parameters determined from de-Pake-ing.
The sign of the quadrupolar splittings cannot be determined in these experiments, only their absolute values. The assignment of the value of -3.0 kHz to the quadrupolar splitting of POPC-α-d₂ in the presence of 40% DODAP arises from the fact that the calibration curve relating the quadrupolar splitting to DODAP concentration passes through a value of 0 Hz at approximately 25% DODAP. If a positive value is assumed for the control splitting, for 100% POPC-α-d₂, then the negative sign for the quadrupolar splitting of 40% DODAP follows. This effect is only observed for POPC-α-d₂ in these studies and delicate determination of the quadrupolar splittings is required when values become close to 0 Hz.

The consequences of adding the largest molecular weight chain of PSSS, used in these studies, to these bilayers is illustrated by the middle and bottom spectra in the left column of figures 4.3.10 and 4.3.11. One observes a second overlapping Pake pattern which increases in intensity as PSSS is added to the MLVs. In this instance, for both POPC-α-d₂ and POPC-β-d₂, the spectral component with the larger absolute quadrupolar splittings (outer quadrupolar splittings) grows in intensity as PSSS is added, identifying it as the POPC component associated with PSSS. The effect still indicates the counterclockwise nature of the α and β deuteron labeling positions of POPC due to the fact that in almost all instances the quadrupolar splittings for both spectral components, for the case of POPC-α-d₂, are negative. Thus, this indicates that the outer quadrupolar splitting is in fact smaller due to its sign and thus represents a polyelectrolyte-associated domain which is in fact more cationic.

In many cases of overlapping Pake pattern sub spectra it can be exceedingly difficult to extract the desired quadrupolar splittings and spectral intensities by examination, particularly when one intensity is much lower than another or the quadrupolar splittings are poorly resolved. A useful
The approach is to "de-Pake" the Pake powder patterns using the method devised by Sternin et al. (1983). This mathematical manipulation removes the spectral broadening due to the distribution of orientations of the lipid long axes in spherical bilayers, while leaving the sharp doublet of resonances from which the true quadrupolar splittings are obtained. Examples of such spectra are shown in the middle columns of figures 4.3.10 and 4.3.11. There is an enhanced resolution in the two POPC populations and one may not only derive the relevant quadrupolar splittings but also the relative intensities of the signals from the two POPC populations. The right-column of spectra in figures 4.3.10 and 4.3.11 are the corresponding computer simulations of the experimental spectra in the left-hand columns, as produced by using the quadrupolar splittings and relative intensities derived from the de-Paked experimental spectra. This approach is successful in reproducing the experimental spectra, even for cases of poorly resolved quadrupolar splittings or low intensity of one component.

It is interesting to note that both the PSSS-bound and PSSS-free populations of POPC display single well-defined quadrupolar splittings rather than distributions. In the case of the PSSS-free population this means that the lateral diffusion coefficient of POPC is rapid enough that each POPC can sample a wide range of local charge environments such that the net charge experienced becomes the mean value. In addition, for the PSSS-bound population, the charge environment within that domain must be identical for each PSSS chain, in order to produce a thermodynamic optimum of lipid composition. More specifically, if the POPC molecules are not at least somewhat free to laterally diffuse within the PSSS-bound domain, and if different such domains contain different ratios of DODAP to POPC, then a distribution of quadrupolar splittings would be observed in the $^1$H NMR spectra, reflecting different charge environments. This would lead to spectral broadening, which is clearly not the case here. As well as POPC being able to laterally diffuse within each of the separate
domains there must also be a lack of exchange of POPC between the domains which would result in either exchange broadening or a third "boundary" POPC population, which was never the case. It is also possible that two separate lamellar phases coexist, one of which contains PSSS and the other of which does not, with no exchange between the two. But other studies have shown that a single lamellar phase exists in which DNA molecules are homogeneously distributed amongst the lamellae (Cevc, 1996). Also, fluorescent digital imaging techniques have indicated that polyelectrolyte-bound domains begin as isolated chains which then laterally aggregate in order to produce "superdomains" about a micron in diameter, on the same bilayer. (Yang and Glaser, 1996; Denisov et al., 1998).

The quadrupolar splittings and relative spectral intensities for PSSS-bound and -free domains are shown in figures 4.3.12 and 4.3.13, for the four different PSSS molecular weights investigated here. Once again, as the case for other polyelectrolytes, the quadrupolar splittings for the free and bound populations change in opposite directions and the quadrupolar splittings for POPC-α-d_4 and POPC-β-d_4, are mirror images of one another, indicating their counterdirectional nature for similar surface charge. For both deuterolabeling positions of POPC, it is shown that the greatest effects on the quadrupolar splittings are caused by the highest molecular weight PSSS chain while the least change is induced by the lowest molecular weight PSSS. In all cases, at high levels of added PSSS the quadrupolar splittings of the bound domains return towards the initial values measured in the absence of PSSS.

In figure 4.3.13 it is evident that the amount of POPC trapped within the PSSS-bound domain does not vary linearly with added PSSS. At low levels of added PSSS, a separate PSSS-bound component cannot be resolved. Only at a PSSS/DODAP charge ratio of 0.5 does the existence of two POPC populations arise in the 2H NMR spectrum. It is also evident that lower molecular weight
FIGURE 4.3.12  $^1$H NMR quadrupolar splittings from POPC-α-d$_4$ (panel A) and POPC-β-d$_2$ (panel B) of the PSSS-bound and PSSS-free sub-spectra in the $^1$H NMR spectra of mixed (40/60) DODAP + POPC cationic bilayers as a function of the amount of added PSSS: N = 3790 (circles), 485 (squares), 170 (triangles), 22 (diamonds). Open symbols refer to the PSSS-free domain, while closed symbols refer to the PSSS-bound domain. The quadrupolar splittings are plotted as the difference versus the values measured in the absence of PSSS.
Fraction of PSSS-bound lipid as a function of the PSSS / DODAP anion / cation equivalence ratio: N = 3790 (circles), 485 (squares), 170 (triangles), 22 (diamonds). A: POPC-α-d, B: POPC-β-d. The fraction of PSSS-bound POPC, X, was obtained from the relative intensities of the corresponding 2H NMR sub-spectra. The fraction of PSSS-bound DODAP, X, was obtained using equations 2.1-2.5 as already described previously. The solid line shows the result expected for a 1:1 PSSS / DODAP complex.
PSSS traps more POPC within a domain at a given PSSS/DODAP charge ratio than higher molecular weight chains. For instance, the only PSSS chain which traps all the POPC into PSSS-bound domains at the 1:1 charge ratio is the N = 22 case.

The data shown in these two figures is the raw data determined for the different molecular weight chains of PSSS, through their de-Paked spectra, and they indicate that differences do exist in the domain properties formed by the different chains of PSSS. This data was analyzed, as described earlier for the domains of PSSS, PACA and PGLU, in order to determine information regarding domain composition and size due to PSSS molecular weight.

The results of such an analysis begin by referring back to figure 4.3.13, which contains the details of the charge stoichiometry within the PSSS-bound domain. Figure 4.3.13A and B illustrates the fraction of DODAP bound as a function of the global ratio of added PSSS anionic to DODAP cationic charges, for both POPC-α-d1 and POPC-β-d1, respectively. The solid line in the figure represents the results expected for a 1:1 anion/cation stoichiometry within the PSSS-bound domain. The PSSS-bound domains contain a near stoichiometric charge ratio regardless of the particular molecular weight of PSSS chain, until saturation is approached. Identical conclusions are reached with either POPC-α-d1 or POPC-β-d1. This was also observed for the three polyelectrolytes discussed in the previous section, but at much lower surface loadings of polyelectrolyte (i.e. 20/80 DODAP + POPC vesicles).

Consider next the DODAP composition of the domains for the four molecular weight chains. Figure 4.3.14A and B illustrates the manner in which the DODAP composition depends on the global ratio of added PSSS anions to DODAP cations, for POPC-α-d1 and POPC-β-d1, respectively. All different PSSS molecular weight chains qualitatively produce similar results in that their PSSS-bound
domains are enriched in DODAP while PSSS-free domains were depleted. Also, the degree of enrichment of the PSSS-bound domains decreases with increasing levels of PSSS. However, there are quantitative differences in figures 4.3.14A and B in that the higher molecular weight PSSS chains produce a higher degree of DODAP enrichment in the PSSS-bound domain. This means that the higher molecular weight PSSS chain produces a more compact domain on a per monomer basis.

The $^2$H NMR results presented here may also be used in order to assess the surface area occupied by a domain on a single polyelectrolyte chain basis. The surface area occupied by a polyelectrolyte chain ($A_d$) is the sum of the surface areas of the constituent lipids plus the polyelectrolyte itself, according to:

$$A_d = \sum N_i A_i$$

(4.7)

where $N_i$ is the number of species “$i$” within the domain, each occupying a surface area $A_i$.

The number of DODAP bound per single PSSS chain equals,

$$N_{\text{dodap}} = \frac{X_i^a}{X_i^f} \times \frac{N_{\text{pss}}}{Q}$$

(4.8)

where $Q$ is the PSSS anion to DODAP cation equivalents ratio, $X_i^f = X_i^f + X_i^n$ is the global DODAP mole fraction (ie. equal to 0.4 in this case), and $N_{\text{pss}}$ is the degree of PSSS polymerization.

The number of POPC bound per single PSSS chain may be expressed in a similar manner:
FIGURE 4.3.14 Composition of the PSSS-free and PSSS-bound domains in mixed (40/60) DODAP + POPC bilayers as a function of the PSSS / DODAP anion / cation ratio. $N = 3790$ (circles), 485 (squares), 170 (triangles), 22 (diamonds). Open symbols are for PSSS-free domains, while solid symbols are for PSSS-bound domains. A, DODAP + POPC-$\alpha$; B, DODAP + POPC-$\alpha$. 
To complete the domain size calculation, appropriate values of the surface area occupied by DODAP, POPC and DODAP must be chosen. For POPC, a surface area of 68 Å$^2$ is accepted for the liquid-crystalline state. To a first approximation, it may be assumed that both POPC and DODAP occupy similar cross-sections and that these are not altered by the presence of the polyelectrolyte.

For PSSS, the surface area occupied will depend on the degree of penetration into the hydrophobic region of the bilayer and the average orientation relative to the bilayer normal. These are both unknown. However, a rough estimate of the surface area occupied by a PSSS chain is obtained by regarding it as a chain of length $N_{\text{PSSS}} \times 2.55$ Å (monomer-monomer spacing) (Borochov and Eisenberg, 1994) and width 2.42 Å (for the aromatic ring). Therefore the surface area occupied by a monomer segment of PSSS ($A_{\text{mon}}$) is about 6.2 Å$^2$ whereas $N_i$ equals the degree of polymerization. There is some discussion in the literature regarding the correct choice for the monomer-monomer spacing (Borochov and Eisenberg, 1994; Kassapidou et al., 1997). However, the calculation shows that the area occupied by the PSSS chain never exceeds 5% of the total domain area, so the monomer-monomer spacing is a moot point. Since it appears that the bulk of the domain area is occupied by the amphiphiles, and to a first approximation DODAP and POPC occupy similar surface areas, then the essential features of the domain size may be apprehended simply by examining the numbers of DODAP and/or POPC per PSSS chain.

Table 4.3.1 lists values of $N_{\text{DODAP}}$ and $N_{\text{POPC}}$ calculated according to equations 4.8 and 4.9 as a function of $Q$ for the different molecular weight chains of PSSS. Generally, $N_{\text{DODAP}}$ is close to the
<table>
<thead>
<tr>
<th>Anion/Cation</th>
<th>DODAP</th>
<th>POPC</th>
<th>Total</th>
</tr>
</thead>
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<tr>
<td>PSSS (3790)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>4140 ± 320</td>
<td>4040 ± 400</td>
<td>8180 ± 720</td>
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<tr>
<td>0.75</td>
<td>3800 ± 140</td>
<td>4280 ± 220</td>
<td>8080 ± 360</td>
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<td>1.00</td>
<td>3420 ± 60</td>
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<td>460 ± 5</td>
<td>420 ± 5</td>
<td>880 ± 10</td>
</tr>
<tr>
<td>0.75</td>
<td>460 ± 10</td>
<td>510 ± 5</td>
<td>970 ± 15</td>
</tr>
<tr>
<td>1.00</td>
<td>445 ± 5</td>
<td>605 ± 5</td>
<td>1050 ± 10</td>
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<tr>
<td>PSSS (170)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.50</td>
<td>150 ± 5</td>
<td>150 ± 5</td>
<td>300 ± 10</td>
</tr>
<tr>
<td>0.75</td>
<td>150 ± 5</td>
<td>180 ± 5</td>
<td>330 ± 10</td>
</tr>
<tr>
<td>1.00</td>
<td>155 ± 5</td>
<td>220 ± 5</td>
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</tr>
<tr>
<td>PSSS (22)</td>
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<tr>
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<td>20 ± 1</td>
<td>23 ± 1</td>
<td>43 ± 2</td>
</tr>
<tr>
<td>0.75</td>
<td>22 ± 1</td>
<td>29 ± 1</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>1.00</td>
<td>22 ± 1</td>
<td>33 ± 1</td>
<td>55 ± 1</td>
</tr>
</tbody>
</table>

* Number in parenthesis equals the degree of polymerization of PSSS
degree of polymerization of the particular PSSS chain (N_{PSSS}) and is constant with changing Q, as expected for stoichiometric electrostatic binding. The exception is the largest PSSS chain (N=3790) where the average number of bound DODAP amphiphiles decreases at higher PSSS loadings. This may reflect entanglement or finite size effects, or even bridging between lamellae. In contrast, there is a progressive increase in N_{PSSS} with progressive addition of PSSS. This may arise from the dependence of the degree of enrichment/depletion of the bound/free domains on the statistical availability of DODAP/POPC and how this changes with increasing amounts of PSSS (Crowell and Macdonald, 1998). Overall, table 4.3.1 demonstrates that, for a given initial DODAP/POPC ratio, by far the single most important determinant of either N_{DODAP} or N_{POPC} is the PSSS molecular weight.

Figure 4.3.15 demonstrates graphically that the number of amphiphiles per polyelectrolyte chain increases linearly with increasing PSSS molecular weight. Across this range of PSSS molecular weights, the N_{POPC}/N_{DODAP} ratio decreases 40% from low to high molecular weight, consistent with increased enrichment of the PSSS-induced domain with respect to DODAP at high PSSS molecular weights.

In the cases described above the surface loading with PSSS is generally quite high and the possibility of perturbations introduced by polyelectrolyte-polyelectrolyte interactions is considerable. In order to reduce the extent of chain-chain interactions experiments were conducted with 10/90 DODAP/POPC membranes and Q ~ 0.75. Since PSSS binding is proportional to the amount of cationic surface charge, under these conditions, the overall surface concentration of PSSS will be a factor of 5.33 lower than in the case of 40/60 DODAP/POPC membranes with Q ~ 1.0. As shown in figure 4.3.15, the number of amphiphiles per PSSS chain nevertheless increases in a linear fashion with the size of the PSSS chain. However, the total number of amphiphiles per PSSS chain is now...
FIGURE 4.3.15 Number of domain-entrapped amphiphiles ($N_{\text{POPC}} + N_{\text{DODAP}}$) (open symbols) and the proportion of zwitterionic to cationic amphiphiles within a domain ($N_{\text{POPC}} / N_{\text{DODAP}}$) (closed symbols) as a function of the polyelectrolyte's degree of polymerization ($N_{\text{POSS}}$). Squares: DODAP / POPC, 40/60, $Q = 1.0$, where $Q$ is the PSSS / DODAP anion/cation equivalence. Triangles: DODAP / POPC, 10/90, $Q = 0.75$. 

Total Amphiphiles per Chain ($N_{\text{POPC}} + N_{\text{DODAP}}$)

Degree of PSSS Polymerization ($N_{\text{POSS}}$)
far greater than in the 40/60 DODAP/POPC membranes. Since the DODAP cation/PSSS anion ratio is still nearly stoichiometric in the PSSS-induced domains, the additional amphiphiles are POPC, as may be ascertained from the high POPC/DODAP ratio within the domains, illustrated in figure 4.3.15. This general effect, in which a higher (lower) initial surface charge leads to a more compact (diffuse) polyelectrolyte-induced domain can be attributed to the influence of entropy of mixing on the thermodynamics of domain formation (Crowell and Macdonald, 1998). Overall, the effects of PSSS molecular weight on domain properties is qualitatively similar at both high and low PSSS loadings.

The $^2$H NMR results described above demonstrate that PSSS binding to mixed DODAP/POPC lipid bilayers induces a lateral segregation of DODAP into DODAP-rich domains. These domains and other polyelectrolyte-induced domains contain a stoichiometric ratio of PSSS to DODAP charges, so that the polyelectrolyte will essentially lie flat on the bilayer surface provided that there remains an excess of cationic charge. These are the general properties of polyelectrolyte-induced domains in lipid bilayers. The specific findings reported here are that the domains are observable with both small and large polyelectrolytes and that the domain size, on a per chain basis, is linearly proportional to the molecular weight of the polyelectrolyte.

The fact that domains are observed via $^2$H NMR even with a polyelectrolyte chain as short as 22 monomers in length suggests that the observed domains must contain multiple polyelectrolyte chains. The reasoning is as follows. The ability to observe domains via $^2$H NMR relies on a slow exchange of lipids between polyelectrolyte-poor and -rich phases. The time scale for exchange must be slower than the inverse of the difference in quadrupolar splitting between the two environments. Typical values are in the range of 1 to 10 kHz for the difference in quadrupolar splittings between the
two phases, as seen in figure 4.3.12. Thus, a lipid residence time shorter than between approximately 0.1 and 1.0 ms within a domain will correspond to fast exchange. This allows one to place a lower limit on the domain size observable via $^1H$ NMR. Assuming that the lipid two-dimensional lateral diffusion coefficient is the same in both phases and equal to a typical bulk value for liquid-crystalline phosphatidylcholine, e.g. $D_0 = 5 \times 10^{-11} \text{m}^2/\text{s}$ (Tocanne et al., 1994; Lindblom and Orädd, 1994), then the mean-square diffusion distance in any direction in a given time is calculated via the Einstein equation in two-dimensions.

$$< x^2 > = 4D_0t$$  \hspace{1cm} (4.10)

The root-mean-square diffusion distance which corresponds to diffusion times (t) between 0.1 and 1.0 ms is calculated to be between 45 and 140 nm. This means that domains which have radial dimensions smaller than this diffusion distance will not produce domains observable by $^1H$ NMR. This diffusion distance corresponds to domains containing between approximately 10,000 and 100,000 lipids, for lipids which occupy a surface area of 68 Å$^2$. By comparison of these values to those displayed in Table 4.3.1 and figure 4.3.15, for single chain dimensions, it becomes obvious that the calculated dimensions are huge.

These conclusions force us to modify the earlier conceptions regarding the origin of the separate Pake subspectra observed in the $^1H$ NMR spectra. The previous studies presented here used polyelectrolytes which were much larger than $N = 22$, the smallest of the PSSS chains used here. It was reasonably supposed that a two-dimensional random coil conformation of the polyelectrolyte at the bilayer surface might cover a sufficient surface area such that POPC trapped within the
polyelectrolyte's folds would be incapable of diffusing out of the domain so-formed on a time scale sufficiently rapid as to lead to fast exchange spectra. This supposition no longer seems tenable in light of the present results with short polyelectrolytes. Instead, it appears that slow exchange $^1$H NMR spectra are only to be expected if a domain contains multiple polyelectrolyte chains.

This conclusion must be considered tentative until lipid lateral diffusion coefficients within the domains can be determined, as it is entirely possible that lipid lateral diffusion within a polyelectrolyte domain is slower than in the bulk lipid bilayer. Employing Einstein's equation for diffusion permits an estimate of the lipid lateral diffusion coefficient necessary to produce slow-exchange $^1$H NMR spectra for the case of domains formed by the lowest molecular weight PSSS. For domains containing 55 lipids, each with cross-sectional areas of $68 \, \text{Å}^2$, the diffusion coefficient would have to lie between $3 \times 10^{-14}$ and $3 \times 10^{-13} \, \text{m}^2/\text{s}$ to produce slow exchange, i.e. a decrease between 2 and 3 orders of magnitude relative to the bulk diffusion coefficient. It is difficult to conceive how this decrease might be accomplished by so small a polyelectrolyte when there is no direct Coulombic attraction to POPC. It would also seem inconsistent with the nature of the $^1$H NMR spectra of the domain-entrapped POPC, which indicate a highly fluid environment. Finally, the fluorescence digital imaging techniques of Glaser (1992) indicate that electrostatically-induced domain formation in lipid bilayers can produce macroscopic domains with dimension approaching the micron scale.

The main difference between synthetic and biological polyelectrolytes is the range of conformations within a given population. First, synthetic polyelectrolytes are heterogeneous in length as compared to the homogeneous size distribution of biological polyelectrolytes. Second, the conformations of synthetic polyelectrolytes is dominated by electrostatics, while for biological polyelectrolytes the conformation is determined by a combination of electrostatic, steric, plus
hydrogen and covalent bonds. In a sample of biological polyelectrolytes every molecule can be considered to adopt an approximately identical conformation. On the other hand, synthetic polyelectrolytes have a distribution of conformations for which one particular conformation can be described by a statistical probability. Thus, only the average properties of the polyelectrolyte population is measured, such as the average hydrodynamic radius or radius of gyration.

Polymer physics predicts for an isolated, freely-jointed chain which lies flat on a two-dimensional surface, that its radius of gyration should increase according to the polymer’s molecular weight to the power $\frac{3}{4}$ (De Gennes, 1979). For the case of polyelectrolytes in low salt conditions, segment-segment electrostatic repulsion dominates their behaviour and they behave as rigid rods in which the radius of gyration of a single chain increases linearly with molecular weight. At high salt concentrations, when segment-segment electrostatic repulsion is screened, the polyelectrolyte can collapse into a Gaussian coil in which the radius of gyration scales according to the square root of the molecular weight.

The $^1$H NMR data provide little insight regarding the polyelectrolyte’s conformation within the domain, other than indicating that we are not dealing with isolated chains. Specifically, the domain area per chain is too small to accommodate freely gyrating rigid rods and since the domain size suggest multiple chains per domain, isolated Gaussian coils are also disregarded. Furthermore the linear dependence of the domain area per chain upon polyelectrolyte molecular weight also does not permit differentiation between close packed rigid rods or Gaussian coils which are both expected to produce a linear increase of the total domain area with polyelectrolyte molecular weight. However, there are several studies which suggest that polyelectrolytes bound to amphiphilic surfaces assume a rigid rod-like close-packed conformation (de Meijere et al., 1998; Van Gorkom et al., 1990; Lasic,
4.3.3 Initial Surface Charge

The domain sizes calculated for the three polyelectrolytes, PSSS, PACA and PGLU, as described in section 4.3.2, are listed in table 4.3.2. The data for POPC-α-d1 originated from bilayers incorporating 10% DODAP, while that for POPC-β-d1 originated from bilayers incorporating 20% DODAP, i.e. for different initial surface charges. Table 4.3.2 shows the number of amphiphiles bound as a function of added polyelectrolyte. The data demonstrate that there is a 1:1 binding stoichiometry between DODAP and polyelectrolyte charges. It is also evident that the number of POPC per polyelectrolyte increases within the polyelectrolyte-bound domain, with increasing amounts of added polyelectrolyte. In addition, the data in table 4.3.2 reveal that the total number of POPC trapped within the polyelectrolyte-bound domain is much greater for the bilayers containing 10% DODAP versus the 20% DODAP containing bilayers. This holds true for each one of the polyelectrolytes. Thus, higher initial surface charge densities produce more compact domains.

This difference in POPC content for these two cases of initial surface charge density has a thermodynamic origin. Lipid domains which form on charged surfaces, result in a balance between the reduction of the electrostatic potential in the domain phase, which favours domain formation, versus the negative entropy of demixing, which opposes domain formation (Denisov et al., 1998). If the favourable energy of the Coulombic attraction exceeds the unfavourable negative entropy term then domains form. Assuming that the Coulombic attraction is similar in both cases, the system will seek an arrangement which minimizes the negative entropy of demixing the charged amphiphiles. The system will minimize demixing by flooding the bound-domain with neutral lipid. Thus, if there is
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<th>Anion/Cation</th>
<th># DOTAP</th>
<th># POPC</th>
<th>Total</th>
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<td>1655</td>
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</tr>
<tr>
<td>PGLU (550)</td>
<td>0.50</td>
<td>375</td>
<td>2285</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>420</td>
<td>2700</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>455</td>
<td>3350</td>
</tr>
<tr>
<td>DOTAP + POPC-β-d_{2} (20/80)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSSS (340)</td>
<td>0.50</td>
<td>335</td>
<td>795</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>340</td>
<td>910</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>300</td>
<td>940</td>
</tr>
<tr>
<td>PACA (320)</td>
<td>0.50</td>
<td>320</td>
<td>775</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>320</td>
<td>910</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>295</td>
<td>940</td>
</tr>
<tr>
<td>PGLU (550)</td>
<td>0.50</td>
<td>520</td>
<td>1545</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>550</td>
<td>1875</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>530</td>
<td>2000</td>
</tr>
</tbody>
</table>

The number in parentheses after the polyelectrolyte abbreviation is the nominal monomers per chain.
more POPC initially present in the bilayer, then the bound-domain should also contain more POPC molecules in order to limit the difference between both domains. Thus, there is a greater entropic cost to forming the same compact domains for the case of 10% global composition as compared to 20% global composition. Therefore, there shouldn’t be the same degree of enrichment of cationic charge within the bound domains in bilayers composed of 10% initial surface charge versus 20%.

Specifically, the predictions are compared to experiment by comparing the POPC / DODAP ratios listed in table 4.3.2 for PSSS. For bilayers composed of 10% DODAP this ratio falls between 4 and 5, for the PSSS-bound domains. This number is reduced to 2 to 3 for the 20% DODAP case, thus producing more compact domains. A further reduction in the POPC / DODAP ratio to a value of 1 is observed in table 4.3.2 for a 40% DODAP initial surface charge, for each of the molecular weight chains of PSSS. The results for PACA and PGLU also confirm this for domain size, based on initial surface charge.

The results presented in table 4.3.2 also show the differences in domain formation based on the chemistry of the polyelectrolyte, as already discussed in section 4.3.1. For the 20% DODAP bilayers the POPC / DODAP ratio, in the polyelectrolyte-defined domains falls between approximately 2:1 and 3:1 for PSSS and PACA but increases to between 3:1 and 4:1 for PGLU. Thus, the most hydrophilic of the polyelectrolytes forms the largest domains, encompassing the most POPC molecules.

4.3.4 Ionic Strength

The 1H NMR spectra in figure 4.3.16 illustrate the effects of increasing NaCl concentration on PSSS-induced domains in vesicles initially composed of 40/60 DODAP / POPC-a-d3. In the
FIGURE 4.3.16  $^2$H NMR spectra of mixed DODAP + POPC-$\alpha$-d$_{4}$ (40/60) cationically charged lipid bilayers with 0.75 equivalents of anionic charge from added PSSS (N = 3790) plus the indicated concentration of NaCl. Salt was added progressively to pre-assembled lipid + PSSS MLVs. For the control sample (absence of salt) the quadrupolar splittings (spectral intensities) are -7.0 kHz (57%) for the PSSS-bound domain and -2.2 kHz (43%) for the PSSS-free domain. In the presence of 50 mM NaCl, these change to -6.4 kHz (69%) and -2.7 kHz (31%), respectively. Upon adding 100 mM NaCl these alter to -6.1 kHz (74%) and -3.4 kHz (26%), respectively. At 500 mM NaCl the quadrupolar splitting equal -4.5 kHz.
absence of NaCl (top spectrum) two sub-spectra are superimposed, corresponding to the PSSS-bound and PSSS-free domains. At high ionic strength (bottom spectrum) only a single quadrupolar splitting is evident and its value corresponds to that of the control measured in the absence of PSSS. Since NaCl itself has little influence on the quadrupolar splittings, at this concentration, this result indicates that there is a dissipation of the domains due to charge screening between the polyelectrolyte and lipid bilayer surface which leads to polyelectrolyte desorption.

At intermediate ionic strengths (middle two spectra) the two $^1$H NMR Pake doublets gradually coalesce with increasing the NaCl concentration, as the quadrupolar splitting of each sub-spectrum progressively reverts towards the control value. Other experiments, soon to be mentioned, with other polyelectrolytes such as polyA or PACA suggest that even at 100 mM NaCl, little if any free PSSS will be found in solution. Therefore, although domains exist at higher ionic strengths, the domain size and composition is altered. Qualitatively, the quadrupolar splittings and spectral intensities indicate that a higher ionic strength leads to a lesser degree of enrichment with respect to the oppositely charged amphiphile and a less compact polyelectrolyte-bound domain.

The results presented in figure 4.3.16 indicate that the PSSS-bound and PSSS-free domains co-exist on the same lamellae, as opposed to occupying separate lamellae. The gradual coalescence of the $^1$H NMR sub-spectra from the PSSS-bound and PSSS-free populations upon titration with NaCl indicates a complete re-equilibration between the two populations. This can only occur via a lipid exchange mechanism. If the two populations occupied separate lamellae, then such an exchange would require inter-lamellar lipid transport, an energetically unfavourable event with a time scale measured in days when not enzymatically catalyzed. If the two populations occupied identical lamellae, then such an exchange would require only intra-lamellar lipid transport, i.e., lateral diffusion.
Intra-lamellar lateral lipid diffusion is fast relative to the time required for salt addition and sample equilibration in these titration experiments (several hours).

There is a further, statistical, argument favoring the view that the PSSS-bound and PSSS-free domains occupy common lamellae. First consider the DNA-cationic liposome complexes investigated by Lasic et al., (1997) which were small liposomes (130 nm average diameter) composed of 1:1 DODAB / cholesterol. To these liposomes, DNA chains of 4.7 kilobases were added in a 0.5:1 charge ratio to which heterogeneous complexes were formed. This can be understood by assuming an approximate cross-sectional area of 68 Å² per lipid, which amounts to about 16 DNA chains per liposome. The probability of significant number density fluctuations away from the global average is profound resulting in a heterogeneous mixture. On the other hand, for MLVs with an average diameter of 1 μm consisting of 40/60 DODAP / POPC mixtures such as employed here, adding PSSS in a 0.5:1 anion:cation ratio produces a global polyelectrolyte chain / liposome ratio of 80,000 for the case of the PSSS 22-mer. The probability of number density fluctuations so large as to produce distinct liposomes having all versus none of the polyelectrolytes, and thereby giving rise to the observed ²H NMR spectra, seems remotely small.
4.4 Influence of Polyelectrolyte Binding on Deuterated Cationic Amphiphiles

The previous results have focused on the consequences of anionic polyelectrolyte-cationic amphiphile interactions through the use of $^2$H NMR of choline-deuterated phosphatidylcholine. The goal of the studies reported here is to probe directly the consequences of the complexation of three cationic amphiphiles to anionic polyelectrolytes via $^2$H NMR. The three are CTAB, DOTAP and TC-CHOL ($\beta$-[N-$N$-$N$-$N'$-trimethylaminoethane]carbamoyl cholesterol, each deuterated at their quaternary methyl positions. In these studies the three specifically deuterated cationic amphiphiles are mixed into lipid bilayer membranes containing phosphatidylcholine and allowed to interact specifically with polyA. The $^2$H NMR spectra of the three deuterated cationic amphiphiles are examined and compared as a function of their mole fraction in mixtures with POPC, the amount of added anionic polyelectrolyte and the ionic strength.

4.4.1 $^2$H NMR of Quaternary Methyl-Deuterated Cationic Amphiphiles in Lipid Bilayers

The structures of the three cationic amphiphiles used in this study along with their deuterolabels are shown in figure 4.4.1.

The $^2$H NMR spectra obtained upon incorporation of the three cationic amphiphiles into non-deuterated POPC bilayer membranes is shown in figure 4.4.2. Each cationic amphiphile produces a Pake pattern lineshape which is indicative of lipids undergoing fast axial motional averaging. Previous $^3$P NMR experiments indicate that all the mixtures of cationic amphiphiles with POPC, at the particular mole fractions specified in these studies, produce fluid lipid bilayers as opposed to some
FIGURE 4.4.1 Structures of the three cationic amphiphiles employed here. From top to bottom, CTAB-γ-d₉, DOTAP-γ-d₃, and TC-CHOL-γ-d₃.
FIGURE 4.4.2 $^2$H NMR spectra of mixed deuterated cationic amphiphile + POPC bilayers in the absence of polyelectrolyte. From left to right, spectra correspond to: CTAB-$\gamma$-d$_3$ + POPC (15/85), DOTAP-$\gamma$-d$_3$ + POPC (70/30) and TC-CHOL-$\gamma$-d$_3$ + POPC (70/30).
other architecture. The spectra in figure 4.4.2 indicate that each of the cationic amphiphiles incorporate into the bilayer and experience considerable motional averaging but that in each case the orientational order parameter at the position of the quaternary methyl deuteron labels is not so small that the motional averaging is effectively isotropic. The quadrupolar splittings measured for these three cationics reflects a combination of differences in configuration and order at the level of the trimethylammonium group. Note that if the three cationic amphiphiles are arranged such that the boundaries between their respective hydrophobic and hydrophilic regions are aligned, then the CTAB quaternary methyls lie close to this boundary, while the polar regions of DOTAP and TC-CHOL extend far from this boundary. If these three cations adopt equilibrium locations within the lipid bilayer such that the boundary between their hydrophobic and hydrophilic regions are similar, then the extension of the cationic trimethylamino groups into the aqueous bathing medium increases in the order CTAB < DOTAP = TC-CHOL. The difference in the orientational order parameter that would result between the three cationic amphiphiles would be sufficient to explain the differences in the quadrupolar splittings observed in figure 4.4.2.

The quadrupolar splittings measured for each of the cationic amphiphiles depends on the composition of the lipid bilayer membrane, as shown in figure 4.4.3. In the case of CTAB-γ-d, the quadrupolar splitting is large initially, but decreases progressively with increasing mole fraction of CTAB relative to POPC. CTAB can only be added in amounts below approximately 15-20% before the micellization properties of the surfactant begin to isotropically narrow the 2H NMR spectra. It seems as though incipient micellization is a factor in determining the concentration dependence of the quadrupolar splittings for CTAB-γ-d.

Increasing the mole fraction of either DOTAP-γ-d or TC-CHOL-γ-d, relative to POPC
Mole Fraction of Cationic Lipid

FIGURE 4.4.3 \(^1\)H NMR quadrupolar splittings from lipid mixtures of the three deuterated cationic amphiphiles + POPC with increasing mole fraction of cationic lipid in POPC membranes: CTAB-\(\gamma\)-d\(_3\) (triangles), DOTAP-\(\gamma\)-d\(_3\) (circles) and TC-CHOL-\(\gamma\)-d\(_3\) (squares).
increases the quadrupolar splitting from a value of about 500 Hz to 1000 Hz. These values are reminiscent of those determined for the trimethylaminocholine deuterons in DMPC (Macdonald, et al., 1991). The headgroup of phosphatidylethanolamine undergoes a conformational response to changes in surface charge density because of the large dipole moment that it bears. Since the cationic charge in DOTAP and TC-CHOL is a monopole, there should not be any such response of the trimethylammonium headgroup of these cationic amphiphiles to surface charge. Thus, the effect of added cationic amphiphile on the quadrupolar splittings as reported in figure 4.4.3 is more than likely due to changes in orientational ordering and dynamics.

The values of $T_2^\text{m}$ obtained for various cationic amphiphiles mixed with POPC, at different proportions, are listed in table 4.4.1.

**Table 4.4.1** $^2$H NMR $T_2^\text{m}$ relaxation times for cationic amphiphiles mixed with POPC

<table>
<thead>
<tr>
<th>POPC/X (mol/mol)</th>
<th>CTAB-$\gamma$-d$_4$ (ms)</th>
<th>DOTAP-$\gamma$-d$_4$ (ms)</th>
<th>TC-CHOL-$\gamma$-d$_4$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95/5</td>
<td>0.88</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>90/10</td>
<td>0.65</td>
<td>1.25</td>
<td>1.90</td>
</tr>
<tr>
<td>85/15</td>
<td>1.07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>70/30</td>
<td>-</td>
<td>1.12</td>
<td>1.54</td>
</tr>
<tr>
<td>50/50</td>
<td>-</td>
<td>0.93</td>
<td>1.36</td>
</tr>
</tbody>
</table>

The results were obtained from quadrupolar echo intensity decay curves and were mono-exponential for all the cationic amphiphiles studied. The $T_2^\text{m}$ values increased in the order, CTAB < DOTAP < TC-CHOL, at comparable molar compositions. As the content of CTAB increased in these mixtures
the values of $T_2^{ex}$ also increased. But, the results for DOTAP and TC-CHOL differed from this behaviour in that $T_2^{ex}$ decreased with increasing mole fraction of cationic amphiphile. The relative values of $T_2^{ex}$ for the three cationic amphiphiles have two possible origins. First, they could reflect differences in orientational order associated with differences in the location of the trimethylamino deuterons. Second, they could reflect differences in the lateral diffusion coefficients of the three cationic amphiphiles within the plane of the bilayer (Bloom and Sternin, 1987). Reinl and Bayerl (1993) have demonstrated the relationship between reduced lateral diffusion of lipids and increased $T_2^{ex}$. If this is so in these studies, this suggests that the diffusivity of the cationic amphiphiles increases in the order: TC-CHOL < DOTAP < CTAB. It is interesting to note that this is the reverse order of the cross-sectional area occupied by the three lipids when incorporated into bilayers. However, both lipid packing and headgroup interactions with water and other lipids are major determinants of diffusivity in bilayers (Tocanne et al., 1994; Lindblom and Orådd, 1994). Since the cationic amphiphiles are expected to differ from one another in both respects, it becomes difficult to determine the origin of the different values of $T_2^{ex}$.

4.4.2 Effect of Polyelectrolytes on $^2$H NMR of Deuterated Cationic Amphiphiles

The two anionic polyelectrolytes used in these studies are polyA and PACA, which have already been shown to induce domain formation in mixed bilayers, as demonstrated by the $^2$H NMR results of choline deuterated POPC. When beginning these studies it was highly desirable to establish whether domain formation could also be observed from the perspective of these quaternary methyl-deuterated cationic amphiphiles. The $^2$H NMR spectra of these cationic amphiphiles are shown in figure 4.4.4 in the absence (top row) and presence (middle row) of polyA. The spectra were obtained
FIGURE 4.4.4: 2H NMR spectra of mixed cationic amphiphile + POPC lipid bilayers in the absence (top row) and presence of polyA (middle row) and in the presence of polyA + salt (bottom row). From left to right the spectra correspond to: CTAB-γ-d₄ + POPC (10/90), DOTAP-γ-d₃ + POPC (30/70) and TC-CHOL-γ-d₄ + POPC (30/70). The bottom row of spectra show the effect of 250 mM NaCl in the case of CTAB-γ-d₄ and 800 mM NaCl for both DOTAP-γ-d₃ and TC-CHOL-γ-d₄ mixtures.
from lipid bilayers containing POPC mixed with (from left to right): 10% CTAB-γ-d₄, 30% DOTAP-γ-d₄, and 30% TC-CHOL-γ-d₃. In each case, the effect of adding polyA is to increase the quadrupolar splitting. There is no evidence for separate polyA-bound or polyA-free components. At all ratios of added polyA anionic charges to cationic amphiphile charges, only a single Pake pattern component was observed in the ²H NMR spectrum from each of the methyl-deuterated lipids. Even low temperature experiments, which aid in resolving such differences, were of no avail in these cases.

The question then arises as to why distinct free and bound populations of cationic amphiphiles were not observed, when free and bound POPC molecules were differentiated. Since the cationic amphiphiles associated with the polyelectrolyte are electrostatically bound, then it seems unlikely that there is fast exchange between free and bound cations especially when zwitterionic POPC has been already shown to be in slow exchange between its bound and free forms. A reasonable explanation is that the difference in quadrupolar splittings between the free and bound cationic amphiphiles is so small that they cannot be resolved spectroscopically. In the case of choline-deuterated POPC, the quadrupolar splitting response was sensitive to surface electrostatic charge, a quantity which was vastly different for the polyelectrolyte-bound and -free phases. For methyl-deuterated cationic amphiphiles the quadrupolar splitting response reflects local ordering effects, which do not widely differ between the bound and free forms of the cationic amphiphiles.

Figure 4.4.5 shows the effects of adding increasing amounts of polyA and PACA on the quadrupolar splittings, for the different cationic amphiphiles. In all three cases, not only does the quadrupolar splitting linearly increase up to the 1:1 added anion / cation charge ratio but there is also no further change observed beyond this point. These results suggest the formation of a 1:1 stoichiometric complex between the anionic and cationic charged groups, as already deduced from
POLYELECTROLYTE - CATION CHARGE RATIO

FIGURE 4.4.5  $^1$H NMR quadrupolar splittings of mixed cationic amphiphiles + POPC bilayers as a function of added polyelectrolyte: CTAB-$\gamma$-d$_3$ + POPC (10/90) (triangles), DOTAP-$\gamma$-d$_3$ + POPC (30/70) (circles), TC-CHOL-$\gamma$-d$_3$ + POPC (30/70) (squares). Open symbols refer to the addition of polyA while closed symbols refer to the addition of PACA. The quadrupolar splittings are plotted as the difference between the values measured for a given mixture of the cationic membranes with polyelectrolyte and the value measured in the absence of polyelectrolyte.
the deuterated POPC studies. As will be discussed shortly, TC-CHOL is the exception. The absolute change in quadrupolar splitting, at the apparent neutralization point decreases in the order: CTAB > DOTAP > TC-CHOL. The largest observed change in quadrupolar splittings for the two extreme cases of 100% free to approximately 100% bound cationic amphiphiles is merely 600 Hz, for CTAB. This gives definitive proof for the non-observance of distinct domains via the use of methyl-deuterated cationic amphiphiles. This maximum observed change is smaller than the minimum requirement of about 1 kHz required to spectroscopically distinguish domains for choline-deuterated POPC.

Figure 4.4.5 also shows that polyA and PACA have virtually identical effects on the quadrupolar splitting of each cationic amphiphile, implying that the precise structure of the polyelectrolyte is less important than the fact of its charge. Buser et al, (1995) have reported that polyelectrolyte binding is essentially independent of the chemical nature of lipids and polyelectrolytes.

The values of $T_{2,\gamma}$ obtained for the addition of polyA to the deuterated cationic amphiphiles are provided in table 4.4.2.

<table>
<thead>
<tr>
<th>Anion/cation</th>
<th>90/10 POPC/CTAB-$\gamma$-$d_1$ (ms)</th>
<th>70/30 POPC/DOTAP-$\gamma$-$d_1$ (ms)</th>
<th>70/30 POPC/TC-CHOL-$\gamma$-$d_1$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.65</td>
<td>1.13</td>
<td>1.54</td>
</tr>
<tr>
<td>0.5</td>
<td>0.53</td>
<td>1.86</td>
<td>2.00</td>
</tr>
<tr>
<td>1.0</td>
<td>0.81</td>
<td>2.46</td>
<td>3.32</td>
</tr>
<tr>
<td>1.0+250 mM NaCl</td>
<td>0.66</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0+800 mM NaCl</td>
<td>-</td>
<td>1.29</td>
<td>1.07</td>
</tr>
</tbody>
</table>
Increasing the amount of added polyA likewise causes an increase in the values for $T_2^{\alpha}$, relative to the value obtained in polyA's absence. This behaviour is similar for all three cationic amphiphiles. It is interesting to note that no measurable difference in the longitudinal relaxation time $T_1$ could be obtained upon addition of polyA. Similar trends have been reported by Reinl and Bayerl (1993) for the electrostatic association of phosphatidylglycerol with myelin basic protein. The fact that $T_1$ is altered while $T_2$ remains unchanged indicates that the changes in $T_2^{\alpha}$ produced by the polyelectrolyte is due to changes in the spectral density of slow motions, such as lateral diffusion.

The fact that both the quadrupolar splitting and $T_2^{\alpha}$ increase, by electrostatically coupling polyA to the bilayer surface, suggests that polyA increases the order of the cationic amphiphile's head group. PolyA is known to intercalate between membrane lipids and its size, rigidity and electrostatic interaction could contribute to a decrease in the amplitude of motion of the cationic amphiphile head group. The increase in the order parameter should be manifest in both the quadrupolar splitting and $T_2^{\alpha}$ (Halle, 1991).

Another potential source for increased $T_2^{\alpha}$ relaxation times, when polyA is coupled to the cationic amphiphiles, is slower lateral diffusion. Reinl and Bayerl (1993) have demonstrated that a reduction of the lipid lateral diffusion coefficient by one order of magnitude leads to a twofold increase in $T_2$, which is more or less what is observed in table 4.4.2 for polyA. Thus, both electrostatic binding to polyA and the archipelago effect (Saxton, 1993), in which the tortuosity of the diffusion path increases due to the presence of the polyelectrolyte, should tend to decrease the diffusion coefficient of the bound cationic amphiphile.
4.4.3 Effect of Ionic Strength on 1:1 Cationic Amphiphile-PolyA Complexes

The effect of salt addition on the $^1$H NMR spectra of the deuterated cationic amphiphiles, for 1:1 charge complexes formed with polyA, is shown in the bottom row of figure 4.4.4. The bottom row of spectra in figure 4.4.4, from left to right, are the results of the addition of 250 mM NaCl to a 10/90 (mol/mol) CTAB-$\gamma$-d$_4$/POPC mixture and 800 mM NaCl to 30/70 DOTAP-$\gamma$-d$_1$/POPC and TC-CHOL-$\gamma$-d$_7$/POPC vesicles, respectively. The most obvious effect of the added salt, to the 1:1 charge complexes formed between polyA and cationic amphiphile, is to reduce the size of the quadrupolar splitting. But, by comparison of the top and bottom rows of spectra in figure 4.4.4 it is obvious that these quadrupolar splittings do not return back to the values measured in the absence of polyA.

The titration curves, for increasing amounts of added salt to the 1:1 charge complexes formed between polyA and the three different cationic amphiphiles, are shown in figure 4.4.6. There is a progressive decrease in the quadrupolar splittings with increased additions of salt. The response is approximately linear with salt concentration, until eventually leveling off. This effect is the opposite to that noted for the titration of polyA to the binary mixtures of lipids. Since the salt has no direct effect on the quadrupolar splittings of the deuterated cationic amphiphiles, as shown in figure 4.4.6, then the effect of salt can be attributed solely to its influence on the polyelectrolyte interaction with the membrane surface. Since the effect of polyA is reversed by salt, it is most likely that salt acts to screen the electrostatic interaction of polyA to the cationic surface. Thus, NaCl behaves as an indifferent electrolyte rather than competing for binding sites with the polyelectrolyte.

The greatest change in quadrupolar splittings, noted for the addition of salt, is measured for CTAB-$\gamma$-d$_4$, while the smallest change is obtained with TC-CHOL-$\gamma$-d$_7$. This simply reflects the fact
FIGURE 4.4.6. $^1$H NMR quadrupolar splittings resulting from the addition of NaCl to mixtures consisting of CTAB-$\gamma$-d$_3$ + POPC (10/90) (triangles), DOTAP-$\gamma$-d$_3$ + POPC (30/70) (circles) and TC-CHOL-$\gamma$-d$_3$ + POPC (30/70) (squares). Closed symbols refer to the addition of NaCl to these lipidic samples in the absence of polyA and the open symbols correspond to the addition of salt to 1:1 charge complexes of the cationic lipid mixtures + polyA. The quadrupolar splittings are plotted as the difference between the value measured when polyA is added in a 1:1 charge ratio to the cationic amphiphile with salt and the value measured for the same situation in the absence of salt.
that the addition of polyA to CTAB-γ-d₄ containing vesicles produced the greatest change while for
TC-CHOL-γ-d₄ it was the least. However, the salt concentration required to achieve the maximum
reversal of the original polyA effect is greater for DOTAP-γ-d₃ than for CTAB-γ-d₄ or TC-CHOL-γ-
d₄. But, before coming to any conclusion as to the strength of interaction between the cationic
amphiphile and polyA it should be noted that CTAB is initially present at 10 mole% as opposed to
the 30 mol% levels for DOTAP and TC-CHOL.

A direct measure of the amount of bound polyelectrolyte is obtained via a UV difference
binding assay. Figure 4.4.7 displays the results for the amount of polyA binding, to membranes
composed of one of the three cationic amphiphiles, as a function of salt concentration. In figure
4.4.7A each of the cationic amphiphiles was present at 10 mole%, which eliminates differences due
to initial surface charge densities. In each instance polyA was added in an amount to neutralize the
surface charge, if 1:1 binding occurred. Each case shows a sigmoidal increase in the amount of free
polyA with increasing salt concentration. Enough salt is added, in each instance, to remove all of the
bound polyelectrolyte from the membrane surface. One also observes that the amount of salt required
to remove polyA from the charged membrane surface, in its entirety, decreases in the order CTAB
> DOTAP > TC-CHOL. These results indicate that TC-CHOL binds polyA with the least affinity.
This is also evident from the fact that not all of the added polyA adsorbed to membranes composed
of TC-CHOL.

Figure 4.4.7B shows the effects of added salt on polyA binding to cationic lipid bilayers
containing both 10 and 30 mol% of either TC-CHOL or DOTAP. The comparison with CTAB could
not be made, at both proportions in the membrane, since higher amounts of it solubilize lipid bilayers.
The same sigmoidal dependence of polyA binding is noted for both initial surface charges. The
FIGURE 4.4.7  Ultraviolet (UV) difference assay of polyA desorption from mixed cationic amphiphile + POPC bilayers as a function of added salt. Each of the preparations contained a 1:1 ratio of anionic charge from polyA to the cationic charge from the particular amphiphile. (A) CTAB + POPC (10/90) (triangles), DOTAP + POPC (10/90) (circles) and TC-CHOL + POPC (10/90) (squares). (B) DOTAP + POPC (10/90) (open circles) and (30/70) (closed circles), TC-CHOL + POPC (10/90) (open squares) and (30/70) (closed squares).
difference for the two cases is that the curves shift to a higher salt concentration at higher surface charge density. For the case of 30 mole% cationic lipid, the difference between TC-CHOL and DOTAP is magnified, clearly indicating that DOTAP binds polyA with a greater affinity. Approximately 200 mM more NaCl is required to fully screen the mutual electrostatic attraction between the cationic bilayers and polyA for the case of DOTAP over TC-CHOL.

It is interesting to note the difference between the results gained by ²H NMR and the UV binding assay. The linear dependence of the change in quadrupolar splittings, for added salt, is in direct contrast to the sigmoidal dependence of the UV results. This can be understood by realizing that the UV assay monitors the desorption of entire polyelectrolyte molecules and thus the cooperativity of binding and desorption of the polyelectrolyte can be monitored. On the other hand, ²H NMR monitors events at the level of individual amphiphiles and thus the monomer segments of the polyelectrolyte. Increasing the salt concentration should reduce the number of pairwise interactions, producing incremental changes which can be sensed by ²H NMR. When the incremental changes reach the point where entire polyelectrolytes desorb from the surface then they will appear in the supernatant where they can be measured by UV spectrophotometry.

4.4.4 Conclusions

These studies were aimed at not only gaining insight into the state of the cationic amphiphile, when mixed in ternary mixtures of lipid and polyelectrolyte, but also at differentiating aspects of the behaviour of several cationic amphiphiles in their role as agents of gene transfection. The cationic amphiphiles must each fulfill at least two requirements for their application in gene transfer. First, they must bind DNA with sufficient affinity in order to neutralize its charge and also condense DNA
to reduce the energy barrier to transmembrane transport. The strong electrostatic binding also ensures that the maximum amount of DNA can be transported. At the same time, the cationic amphiphile must not bind DNA so tightly that it will not dissociate from the complexes when endocytosed, since it would fail to become utilized by the cell. Thus, it seems that the fine tuning of the strength of this interaction would offer a route towards increased efficiency of transfection.

In short, the UV binding assay results have demonstrated that the three different cationic amphiphiles studied here differ with respect to their strength of interaction with DNA. CTAB appears to bind DNA with the greatest avidity, since it is most resistant to the effects of salt, whereas TC-CHOL exhibits the lowest affinity.

The quantitative results obtained from $^2$H NMR also demonstrate the differences in the behaviour of the three different cationic amphiphiles. The effects of DNA binding on the quadrupolar splittings and the transverse relaxation times of the cationic amphiphile's quaternary methyl deuterons fall off in the order CTAB > DOTAP > TC-CHOL. Evidently the size of the DNA induced effects correlates with the strength of DNA binding. From the results already presented for the same three cationic amphiphile's effect on the headgroup of choline deuterated POPC, it was determined that CTAB produced the most compact domains with the greatest enrichment, while DC-CHOL produced the most diffuse domains. It is reasonable to consider that the more compact domains would produce quantitatively larger effects on the cationic amphiphile's orientational order. Thus, both $^2$H NMR perspectives point to the conclusion that the strength of interaction between DNA and the three cationic amphiphiles decreases in the order CTAB > DOTAP > TC-CHOL.

One may understand the physical basis for the strength of interaction between cationic amphiphiles and DNA by considering the depth of binding. For monovalent amphiphiles, the strength
of electrostatic binding by polyelectrolytes may be greater when the amphiphile’s charge is located
deep with the polar region of the lipid bilayer surface. Consider that when a polyelectrolyte like DNA binds a charged surface that there must be some loss of the waters of hydration of the charged moieties. It may be that this occurs more readily when the targeted charges are located in the lower dielectric constant medium.

Several lines of evidence indicate that the cationic charge of CTAB penetrates deeper into
the bilayer’s polar region while TC-CHOL extends farthest into the aqueous surroundings. First, the
cationic charge of CTAB is located immediately above the hydrophobic region of the molecule whereas for DOTAP and TC-CHOL this charge is separated from the hydrophobic portions by a polar spacer arm. Second, the larger quadrupolar splitting of the membrane-bound quaternary methyl
deuterated CTAB indicate a greater local order. This is consistent with a greater depth of penetration
of the charged groups into the bilayer interior. Finally, the sensitivity of the “voltmeter” response,
to the cationic amphiphiles, decreases in the order CTAB > DOTAP > TC-CHOL. The results are
interpreted to arise from the fact that the most sensitive “voltmeter” response from choline-deuterated
POPC, to a given surface charge density, corresponds to the charged species with the greater depth
of penetration into the membrane proper.
4.5 $^{31}$P NMR Observations of the Morphology of Complexes with Cationic Amphiphiles

DNA-cationic liposome packages can be transferred across a cellular membrane by an endocytic mechanism (Leventis and Silvius, 1990; Zabner et al., 1995; Friend et al., 1996). After entering the cell, the endosomal membrane is destabilized by some stimulus and the genetic material is released for uptake by the cell's transcription mechanism. The morphology of the DNA-amphiphile complex is a major factor in determining the efficiency of transmembrane transfer. The morphology of such complexes is known to depend on factors such as DNA size, the size of liposomes and the identity of the cationic amphiphile and "helper" lipids (Gershon et al., 1993; Sternberg et al., 1994; Gustafsson et al., 1995; Mok and Cullis, 1997; Harries et al., 1998; Battersby et al, 1998).

In the latter case, gene transfection technologies favour the use of the bilayer destabilizing amphiphile, phosphatidylethanolamine (PE) (Felgner et al., 1994; Farhood et al., 1995). It is believed that PE produces non-lamellar architectures, in mixtures with cationic amphiphiles, which helps to destabilize the target membrane's lipid bilayer, thereby reducing the energy to transmembrane transport of DNA.

Different cationic amphiphiles are known to display different transfection efficiencies (Farhood et al., 1992; Egilmez et al., 1996; Deshmukh and Huang, 1997) for different cell lines. Even though the role of the cationic lipid is to electrostatically bind both DNA and the target membrane it is also possible, in theory, that these amphiphiles may also influence the morphology of the package.

It has been shown that the efficiency of DNA entrapment (Monnard et al., 1997) and transfection potency (van der Woude et al., 1995) decrease with higher molecular weight DNA.
Even though DNA seems to play a more passive role in altering the morphology of these “packages”, clearly the interactions between DNA, the cationic amphiphile and helper lipid all contribute to the final morphology of the complex.

The $^3$P NMR studies reported here will focus on the morphology of the complexes formed by mixtures of these molecules. The size of the DNA molecule will be examined by comparing two different single chain species, one a 21 nucleotide oligomer and the other an 18,000 nucleotide polymer. The effects of two different cationic amphiphiles is also studied, CTAB and DOTAP. Finally, the role of the “helper” lipid is studied by comparing the two zwitterionic lipids, PC and PE.

$^3$P NMR is capable of distinguishing between various membrane architectures (Cullis and de Kruijf, 1979). As well, the effects of complexation on nucleotide chain dynamics may be examined under differing conditions of the global anion / cation ratio as well as the ionic strength.

4.5.1 UV-assay of OligoS and PolyA Binding to DOTAP/POPC mixtures

The structures of the chemical species used for these studies is shown in figure 4.5.1. The results for the binding of the two different sized nucleotide chains, OligoS and PolyA, to cationic MLVs composed of 30/70 DOTAP / POPC (mol/mol) was quantified by a UV-difference assay. The results are shown in figure 4.5.2.

The binding of both nucleotide chains is quantitative up to the anion / cation equivalence point. Just above this point, the binding of OligoS essentially ceases. However, polyA continues to bind to the surface up to a 2:1 anion / cation charge ratio. This indicates that polyA is capable of binding to the cationic membrane surface with only a fraction of its monomer units in actual contact with the surface. This means that the unbound charge of the nucleotide chain must extend outwards
FIGURE 4.5.1 Structures of the zwitterionic amphiphiles (POPC and DOPE) and nucleic acid chains (PolyA and OligoS) employed here.
FIGURE 4.5.2 UV-difference assay of OligoS (circles) and polyA (squares) binding to DOTAP / POPC (30/70) mixtures.
from the surface. Thus, polyA must undergo a change from a "pancake" conformation below the equivalence point, to that of a "brush" above this point. For the bound OligoS, the "pancake" conformation persists at all times.

The different behaviour exhibited by polyA and OligoS may be attributed to the conformational flexibility of the chains. For relatively stiff polynucleotide chains it is useful to consider the persistence length of the chain, which is a measure of the segments of the chain to continue in the same direction. When the chain is short in comparison to its persistence length then it behaves as a rigid rod. If the chain is several times longer than its persistence length then it retains overall chain flexibility and it behaves like a random coil polymer, in solution. The persistence length for double-stranded DNA can be greater than 50 nm, which corresponds to approximately 150 base pairs (Merchant and Rill, 1997). Even though the persistence length of single-stranded nucleotide chains may be slightly shorter than double-stranded, it is clear that OligoS is more like a rigid rod while polyA should possess much more chain flexibility. Consequently, polyA is more capable of adopting a "brush" conformation, above the equivalence point. In such an instance the chain will maximize the number of nucleotide anion versus cationic amphiphile charge pairs and as the amount of added polyA increases the number of anion/cation pairs should decrease on a per chain basis. Eventually, further binding should cease when the favourable Coulombic attractions are overbalanced by the entropic cost of conformational adaptation. However, OligoS cannot adapt conformationally and so this surface crowding point is reached much earlier.

4.5.2 Nucleotide Mobility

Various $^{31}P$ NMR spectra of OligoS (left column) and polyA (right column) are shown in
figure 4.5.3. The lower spectra were obtained for the dry powders of the nucleotide chains and are characteristic lineshapes for static phosphorothio- and phospho-diesters, respectively. All the data concerning the isotropic chemical shifts, static chemical shift tensor components and asymmetry parameters listed in table 4.5.1, for the two nucleotide chains.

The middle row of spectra represent the addition of the two nucleotide chains to 100% DOTAP MLVs in a 0.5:1 anion/cation charge ratio, in order to ensure quantitative binding of the nucleotide chains. Due to the absence of phospholipids in these mixtures, the $^{31}$P NMR signal originates solely from the nucleotide chains. These spectra are essentially identical to the corresponding dry powders, indicating the immobilization of the bound nucleotide chains as a result of charge pairing. There is, however, a slight decrease in the chemical shift anisotropy for the bound nucleotide chains, as shown in table 4.5.1. This implies some segmental librations within the bound nucleotide chains. But, this change may also be interpreted as an alteration in the electron density distribution about the phosphorus atom, due to ion-pairing. This effect would also manifest itself in alteration of the chemical shift tensor components.

The top row of spectra in figure 4.5.3 were obtained by addition of the two nucleotide chains to 100% DOTAP MLVs in a 1:1 charge ratio. These spectra once again indicate the immobilization of the nucleotide chains when bound to the bilayer surface. The spectrum for OligoS is essentially identical to that at the 0.5:1 charge ratio. However, for polyA there is a more drastic change in the spectrum as compared to its 0.5:1 addition. The chemical shift does not only narrow considerably but the highest intensity peak shifts towards the isotropic chemical shift value for polyA. Thus, polyA enjoys more motional freedom at charge equivalence. It is difficult to distinguish whether the spectrum represents a superposition of two populations with different mobilities or a single
FIGURE 4.5.3 Cross polarization $^{31}$P NMR spectra of OligoS (left hand column) and polyA (right hand column) in the form of a dry powder (bottom spectra), mixed with fully hydrated 100% DOTAP in an anion/cation charge ratio of 0.5:1 (middle spectra) or mixed with fully hydrated 100% DOTAP in an anion/cation charge ratio of 1:1 (top spectra). The isotropic chemical shifts, chemical shift tensor components and asymmetry parameters for the various spectra are listed in Table 4.5.1.
Table 4.5.1: $^{31}P$ NMR isotropic chemical shifts and static chemical shift tensor elements for OligoS and PolyA as dry powders or bound to hydrated 100% DOTAP.

<table>
<thead>
<tr>
<th>Nucleotide / DOTAP</th>
<th>$\delta_0$</th>
<th>$\sigma_{11}$</th>
<th>$\sigma_{22}$</th>
<th>$\sigma_{33}$</th>
<th>$\sigma_{33} - \sigma_{11}$</th>
<th>$\eta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OligoS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Powder</td>
<td>56</td>
<td>136</td>
<td>98</td>
<td>-68</td>
<td>204</td>
<td>0.31</td>
</tr>
<tr>
<td>0.5 : 1</td>
<td>56</td>
<td>128</td>
<td>82</td>
<td>-35</td>
<td>163</td>
<td>0.51</td>
</tr>
<tr>
<td>1 : 1</td>
<td>56</td>
<td>125</td>
<td>80</td>
<td>-40</td>
<td>163</td>
<td>0.47</td>
</tr>
<tr>
<td>PolyA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Powder</td>
<td>0</td>
<td>96</td>
<td>31</td>
<td>-115</td>
<td>211</td>
<td>0.56</td>
</tr>
<tr>
<td>0.5 : 1</td>
<td>0</td>
<td>81</td>
<td>20</td>
<td>-105</td>
<td>186</td>
<td>0.58</td>
</tr>
<tr>
<td>1 : 1</td>
<td>0</td>
<td>63</td>
<td>0</td>
<td>-92</td>
<td>155</td>
<td>0.68</td>
</tr>
</tbody>
</table>

All chemical shifts are in parts per million (ppm) referenced to 85% $\text{H}_3\text{PO}_4$. Anisotropic chemical shift tensor elements are defined such that $|\sigma_{11}| > |\sigma_{22}| > |\sigma_{33}|$ where $\sigma_0 = (\delta_0 - \delta_3)$ and $\delta_3$ and $\delta_0$ are the observed isotropic and anisotropic chemical shifts. The asymmetry parameter is defined as $\eta = (\sigma_{22} - \sigma_{11}) / \sigma_{33}$.

population. Either way, polyA is probably prevented from binding all the cationic surface charges due to its size and consequent entanglement effects.
4.5.3 $^3$H NMR of DOTAP-$\gamma$-d$_3$ in Complexes with OligoS and PolyA

Figure 4.5.4 shows a series of $^3$H NMR spectra of 100% DOTAP-$\gamma$-d$_3$ in complexes with either OligoS (left column) or polyA (right column). The top spectrum in each column was obtained in the absence of added nucleotide chains. The value for the two quadrupolar splittings is approximately 1.2 kHz for each case and is similar to the values reported for the mixtures of this cationic lipid with POPC, discussed in the previous section.

Adding OligoS and PolyA to the 100% DOTAP-$\gamma$-d$_3$ MLVs produces no change in the quadrupolar splitting, either at 1:1 (middle row) or 2:1 (bottom row) anion/cation charge ratios. However, there is an obvious decrease in the intensity at the centre of these spectra upon addition of nucleotide chains. This can be accounted for by the fact that the neutralized MLVs were more readily concentrated than the highly charged MLVs. The concentration of the vesicles, by centrifugation, will remove small vesicles which contribute to a central isotropic resonance frequency.

From figures 4.5.3 and 4.5.4 a rather contradictory picture of the OligoS or PolyA complexes with 100% DOTAP arises. The bound nucleotide chains are virtually immobilized, except for librations at the level of individual nucleotide segments, yet the cationic lipids retain virtually full mobility.
FIGURE 4.5.4  $^2$H NMR spectra of fully hydrated 100% DOTAP-$\gamma$-d$_1$ mixed with OligoS (left hand column) or polyA (right hand column). The top spectra were obtained in the absence of either OligoS or polyA, the middle spectra at an anion/cation charge ratio of 1:1 and the bottom spectra at an anion/cation charge ratio of 2:1.
4.5.4 $^3$P NMR of Complexes of OligoS or PolyA with DOTAP/POPC Mixtures

Figure 4.5.5 contains $^3$P NMR spectra of complexes of mixed 30/70 DOTAP/POPC MLVs as a function of added OligoS (left column) or polyA (right column). The top spectrum in each column is identical and was obtained in the absence of nucleotide chains. The lineshape is characteristic of phospholipids in a bilayer architecture where the lipids exhibit rapid anisotropic motional averaging about their long molecular axes (Seelig, 1978; Cullis and de Kruijff, 1979). The spectrum is motionally narrowed ($\Delta \sigma = \sigma_{\text{axial}} - \sigma_{\text{iso}} = 40$ ppm) from the static case and is also axially symmetric ($r = 0$).

The addition of either polyA or OligoS to the cationic lipid bilayers does not alter the overall architecture of the bilayer, as shown by the series of spectra in figure 4.5.5. Below the anion/cation equivalence point there is evidence of a broad resonance, centered at 56 ppm, for the addition of OligoS. Its frequency is centered at the isotropic chemical shift of OligoS. There is no similar signal at the isotropic chemical shift of polyA (0 ppm) below the 1:1 anion/cation ratio. Above the equivalence point, though, a much narrower resonance line begins to grow at the isotropic chemical shift of OligoS. This also occurs for polyA, but only at anion/cation charge ratios of approximately 2:1. This narrow resonance is attributed to nucleotide chains which are free in solution.

The fact that the bound nucleotide chains are not observed in these spectra is not unreasonable. The full breadth of the $^3$P NMR spectra of the bound nucleotide chains, in figure 4.5.3, are nearly 5 times greater than that from the phospholipids. This greatly reduces the signal to noise ratio for the bound nucleotides and its signal is dwarfed by that of the bilayer pattern of the phospholipids. In fact the signal to noise of the bound nucleotides was so low that a cross-polarization technique was used to enhance the signal.
\textbf{FIGURE 4.5.5} \textsuperscript{31}P NMR spectra of mixed DOTAP / POPC (30/70) cationic MLVs as a function of added OligoS (left hand column) or polyA (right hand column). The anion/cation charge ratio is indicated in the figure.
These spectra confirm the results from the UV-assay, in that signals corresponding to free nucleotide chains only appear above the anion/cation equivalence point. But, remember that the UV-difference assay reports on the binding of the entire polyelectrolyte while NMR techniques report on the average properties of individual nuclei, phosphorus in this case. Thus, with the $^{31}\text{P}$ NMR technique used here it is possible to distinguish between monomer units of the nucleotide bound to the membrane surface and individual unbound segments which have a higher degree of mobility. In particular polymers bound to surfaces are known to form tail and loop regions which retain considerable mobility. It may be reasoned then that the broad resonance observed at the isotropic chemical shift of OligoS, below the anion/cation equivalence point, could arise from a proportion of mobile chain ends. It may also be reasoned that the nucleotide chain mobility was enhanced in the presence of POPC relative to the 100% DOTAP case.

The next question to be addressed is whether or not the amount of free OligoS can be quantitated from these spectra. The quantitation can be accomplished if the $^{31}\text{P}$ NMR intensity from the phospholipid POPC, the internal standard, can be directly related to that of OligoS. Separate relaxation time experiments ($T_1$ and $T_2$) were run for both POPC MLVs and free OligoS. Longitudinal relaxation times ($T_1$) of 930 ms and 900 ms were determined for POPC and free OligoS, respectively. This indicates that if there was any signal intensity saturation, due to rapid repetition of the pulse sequence, it would be equivalent for both signals. In order to prove this a $^{31}\text{P}$ NMR spectrum was acquired for OligoS added to 100% POPC MLVs, where no OligoS binding should occur. The pulse delay between sequences was increased from 2 s to 10 s to disallow any saturation effects and there was no change in relative intensities of the two distinct signals. The transverse relaxation time ($T_2$) was then determined to be approximately 10 ms for both OligoS and POPC.
Thus, any transverse relaxation between the $\tau$ delays of the echo sequence would be minimal. Finally, the ratio of integrated intensities of OligoS to POPC resonances was about 90% of that expected, from the known composition. Therefore, it is reasonable to determine the amount of free OligoS by comparing its resonant intensity to that of the internal standard of POPC.

An example of this quantitation can be seen by focusing on the result obtained for an addition of 1.5:1 anion/cation of OligoS to the membrane surface, in the left column of figure 4.5.5. The isotropic resonance at 56 ppm is about 1/3 the intensity expected for the case of 100% free OligoS. But, there is a broad signal underlying the narrow isotropic resonance of free OligoS which cannot be separated. This broad signal is attributed to the relatively mobile segments of bound OligoS, determined below the equivalence point. At the equivalence point, the broad signal accounts for 1/5 of the total intensity expected if there was no OligoS binding to the bilayer. Thus, assuming that no more than 1/5 of the bound OligoS contributes to the integrated signal intensity at 56 ppm, then 1.2 equivalents of OligoS are calculated to be bound for the 1.5:1 anion/cation ratio. Even though this agrees well with the UV results one appreciates the approximate nature of this calculation.

This quantitation procedure would seem to be less reliable for determining the amount of free polyA for various reasons. First, when polyA was added to 100% POPC MLVs in a 3:7 phosphorus ratio, the isotropic resonance attributed to free polyA was only 1/3 the intensity expected, given that no polyA binding should occur in the absence of cationic amphiphile. Since, the $T_1$ and $T_2$ relaxation times of the isotropic polyA and POPC resonances were comparable, they could not be used to explain the loss in signal intensity from polyA. Secondly, when a calibration experiment was performed relating the $^{31}P$ intensity of polyA to its concentration in aqueous solution, the relationship was not linear. In fact the observed intensity fell below the expected value by about 50% at a
concentration of 10 mg/ml, which is the concentration range pertinent to these studies. A possible explanation for this behaviour is that long DNA chains are known to form anisotropic phases, in solution, at much lower concentrations than shorter chains (Merchant and Rill, 1997). This effect would lead to line broadening and a differential intensity loss. In either case, it becomes obvious that quantitating free polyA would be a much more difficult endeavour.

4.5.5 UV-assay of Ionic Strength Effect on Nucleotide Binding to Cationic Liposomes

The results of polyA desorption from cationic liposomes in the presence of added salt have already been described and are shown in figure 4.5.6 along with the new data for OligoS. The nucleotide anion to amphiphile cation charge ratio was kept at 1:1, for both cases. The most obvious characteristic in figure 4.5.6 is the sigmoidal increase in chain desorption, for both OligoS and polyA, as ionic strength increases. Progressive addition of salt reduces the Coulombic attraction between the oppositely charged species by a charge screening mechanism. The concentration of NaCl required to reduce the number of bound chain by half is approximately 400 -500 mM for both OligoS and polyA. Note also that a nearly quantitative amount of the nucleotide chains remain bound to the surface at physiological ionic strength (150 mM).

However, there is a clear distinction between the salt induced desorption of OligoS and polyA. PolyA shows a much steeper transition from the bound to unbound states while the ultimate ionic strength needed to achieve quantitative desorption is much lower. This result may be understood by taking into consideration chain length effects which prevent 1:1 anion/cation charge pairing. The 31P NMR spectra in figure 4.5.3 support this notion. Also, high salt will reduce intersegmental charge repulsions, leading to a more compact chain conformation, which favours the
FIGURE 4.5.6 UV-difference assay of the salt induced desorption of OligoS (circles) or polyA (squares) from DOTAP/POPC (30/70) mixtures. Each mixture contained a 1:1 anion/cation charge ratio. The polyA data were originally reported in Figure 4.4.7.
unbound state. Since the shorter OligoS chain has a greatly reduced ability to change its conformation, it should be less influenced relative to polyA.

4.5.6 $^3$P NMR of Ionic Strength Effect on Nucleotide Binding to Cationic Liposomes

The $^3$P NMR spectra of OligoS (left column) and polyA (right column) complexed to 30:70 DOTAP/POPC MLVs in a 1:1 charge ratio are shown in figure 4.5.7 along with the different ionic strengths of NaCl. Under all conditions the lipids retain an overall bilayer arrangement. As the concentration of salt increases so does the intensity of the narrow resonance at the isotropic chemical shift position of the corresponding nucleotide chain. These resonances correspond to free OligoS and polyA. The salt concentration at which these isotropic peaks reached their maximum intensity corresponded to 800 and 500 mM NaCl, for OligoS and polyA, respectively. This $^3$P NMR data confirms the UV results regarding nucleotide chain desorption by salt.

The integrated intensity obtained from the isotropic resonance of OligoS, at 800 mM NaCl, was 92% of that expected for quantitative desorption. The difficulties for quantifying the amount of free polyA have already been mentioned. To circumvent this problem, a control sample was prepared containing only polyA and POPC in the same phosphorus containing ratio as in figure 4.5.7, with 500 mM NaCl added. All polyA should be in the unbound state under these conditions. By using these relative intensities of polyA and POPC as control values, it was deduced that polyA is entirely desorbed from the surface of the 30:70 DOTAP/POPC MLVs. Therefore, both the $^3$P NMR and UV data agree both qualitatively and quantitatively.
FIGURE 4.5.7  $^3$P NMR spectra of salt induced desorption of OligoS (left hand column) and polyA (right hand column) from DOTAP / POPC (30/70) mixtures. Each mixture contained a 1:1 anion/cation charge ratio. The NaCl concentration (mM) is shown in the figure.
4.5.7 \(^{31}\text{P} \text{NMR of DOPE mixed with DOTAP or CTAB}\)

DOPE is commonly mixed with cationic amphiphiles in order to increase the efficiency of transfection. This enhancement is believed to arise from the ability of DOPE to assume an inverted hexagonal \(H_2\) arrangement when incorporated into macromolecular lipid assemblies, which aids in local destabilization of a cell’s membrane. This ability can be important for direct fusion with either the plasma membrane or the endosomal membrane in an endocytic mechanism of gene transfer (Farhood et al., 1995; Wrobel and Collins, 1995). In either instance the ability of DOPE to form non-bilayer phases is pivotal to this enhancement. Replacement of DOPE with a bilayer forming lipid like DOPC has been shown to produce “packages” which are inefficient in promoting transfection. The ability of DOPE to promote non-bilayer phases is ascribed to its inverted cone shape, which is due to a combination of a small headgroup and unsaturation in its acyl chains (Cullis and de Kruijff, 1979). In order to investigate the effects of nucleotide chains on the architecture of the transfection packages (cationic amphiphile + DOPE vesicles) the \(^{31}\text{P} \text{NMR spectra of DOPE mixed with either DOTAP or CTAB were examined first, as shown in figure 4.5.8.}\)

The spectra are identical and were obtained with 100% DOPE. The spectral line shape of the \(^{31}\text{P} \text{NMR spectra in the top row of figure 4.5.8 is indicative of lipids in an inverted hexagonal arrangement (H\(_2\)). The sign of the CSA is reversed and reduced in magnitude by a factor of 2 (\(\Delta \sigma \sim 22 \text{ ppm}\), as compared to the pattern obtained for lipids in a bilayer arrangement (L\(_\alpha\)).\)

The left hand column of spectra were obtained for mixtures of DOTAP with DOPE. With 10 mole% added DOTAP the mixture retains a hexagonal arrangement. At 20 mole% DOTAP, the spectrum shows a superposition of hexagonal (H\(_2\)) and probably cubic (Q\(_\alpha\)) phases, the latter being a high local curvature phase. Eventually at 50 mole% DOTAP the bilayer arrangement exists solely.
FIGURE 4.5.8  $^{31}$P NMR spectra of DOPE mixed with various mole% DOTAP (left hand column) or CTAB (right hand column), as indicated in figure.
The right hand column of spectra were obtained for mixtures of CTAB and DOPE. With only 10 mole% CTAB the amphiphilic mixture reverts completely to a bilayer arrangement. The line shape is altered slightly from a spherical bilayer arrangement of lipids and is indicative of an alignment of vesicles in a magnetic field (Seelig et al., 1985). At 30 mole% CTAB, the mixture is entirely bilayer in architecture with no induced orientation in the magnetic field. Eventually at 70 mole% CTAB the spectrum is dominated by a micellar isotropic resonance.

The changes in architecture of the lipid self-assembly observed by $^3$P NMR can be understood in terms of the “shape” model of Israelachvili (1975, 1977). In this model different classes of lipids exhibit distinct shapes which act as building blocks for different amphiphilic architectures. POPC, for instance, which contains a comparable size headgroup and acyl chain region is considered to have an overall cylindrical profile which preferentially assembles into a bilayer architecture. DOPE, however, has a much smaller head group and possesses unsaturation at both acyl chains. This yields an inverted cone profile for DOPE which preferentially assembles into an inverted hexagonal arrangement, $H_3$. CTAB, with its single alkyl chain and large charged head group, is considered to possess a cone shape and prefers to assemble into a micellar architecture. Finally, DOTAP possesses the same large head group as CTAB but with two acyl chains, possesses an overall cylindrical profile and is thus known to stabilize bilayer arrangements.

When DOTAP and DOPE are mixed, the bilayer tendencies of DOTAP eventually overcome the hexagonal tendencies of DOPE at higher levels of added DOTAP. When CTAB and DOPE are mixed, the cone shape of the former complements the inverted cone shape of the latter and a bilayer arrangement dominates at only 10 mole% CTAB. This may indicate that the cone shape of CTAB tapers more fully than the inverted cone shape of DOPE such that CTAB’s shape has the ability to
dominate the overall architecture at lower mole fractions of CTAB.

The ability of cationically charged lipids to stabilize DOPE into a bilayer architecture has been shown previously (Mok and Cullis, 1997), and is reminiscent of the behaviour of anionically charged lipids (Cullis and de Kruijff, 1979).

4.5.8 Effects of Nucleotide Binding on the Morphology of DOTAP/DOPE Mixtures

The top row of $^{31}$P NMR spectra in figure 4.5.9 indicate that pure lipid mixtures of 30/70 DOTAP/DOPE produce predominantly lamellar bilayer architectures. Although not obvious, there is a fraction of the $^{31}$P NMR intensity attributed to a hexagonal arrangement of lipids in these spectra. Addition of OligoS promotes a conversion of the predominantly bilayer arrangement of lipids to a predominantly hexagonal-type spectrum with a residue of an overlying isotropic signal, as shown in the left column of figure 4.5.9. The isotropic lipid resonance disappears at higher levels of added OligoS. When an excess of OligoS is added another narrow resonance begins to appear at the isotropic chemical shift of OligoS, while at the same time the lipids retain a hexagonal architecture.

The right hand column of spectra in figure 4.5.9 show the results for polyA added to the 30/70 DOTAP/DOPE mixtures. There is an analogous conversion to a hexagonal arrangement, through an intermediate isotropic phase, with free polyA eventually appearing at its isotropic resonance position.

The results demonstrate that nucleotide chains cause a conversion to a hexagonal architecture. DOTAP is known to stabilize the bilayer architecture by virtue of its cationic headgroup. Binding of the anionic nucleotide chains to the lipid/water interface neutralizes the cationic surface charge, in effect reducing the size of the DOTAP head group. This allows the DOPE to then exert its
FIGURE 4.5.9 $^3$P NMR spectra of DOTAP / DOPE (30/70) mixtures as a function of added OligoS (left hand column) or polyA (right hand column) at the indicated anion/cation charge ratio.
influence in order to restore a hexagonal arrangement of lipids. Complete charge neutralization is not required for this conversion, since the lipids are predominantly hexagonal at only 50% neutralization. The behavior of the two nucleotide chains are slightly different in that polyA is less effective at causing this transition. In both instances, though, the $^3$P NMR results obtained here demonstrate that complexes of nucleotide chains with DOTAP/DOPE mixtures form non-bilayer phases while complexes with DOTAP/POPC only produce bilayer phases (figure 4.5).

The conversion to the non-bilayer phase is more readily induced by OligoS than by polyA, a state of affairs which may have to do with entanglement effects, i.e. the difficulty of the larger polyA chain to bring all its charges to bear on the lipid surface. It may also be possible that the effect is due to difficulty in packing the larger polyA chains into the internal aqueous tubes present in the H$_2$ lipid phase, relative to the shorter OligoS chains. Certainly, the $^3$P NMR spectral line shapes are less well defined in the case of polyA versus OligoS. It seems reasonable that if non-bilayer phases are important for gene transfection, then the fact that polyA does not readily induce this transformation may explain the reduced transfection efficiency of large polynucleotide chains (van der Woude et al., 1995).

Finally, the spectra presented in figure 4.5.9 indicate some differences in chain dynamics of OligoS, when present within a hexagonal array of lipids versus a bilayer arrangement. Below the equivalence point addition of OligoS to 30/70 DOTAP/POPC mixtures there was a broad resonance centered at 56 ppm which was attributed to tail segments of bound OligoS. However, the comparable spectra in figure 4.5.9 show almost no evidence of this resonance below the equivalence point. This result indicates that OligoS chain exhibits little or no motional freedom when bound to lipids in a hexagonal arrangement. This is probably due to the reduced dimensionality of the cylindrical aqueous...
spaces of the \( H_3 \) phase relative to the lamellar aqueous spaces of the bilayer phase.

4.5.9 Effects of Nucleotide Chain Binding on the Morphology of CTAB/DOPE Mixtures

Figure 4.5.10 shows a series of \(^{31}P\) NMR spectra of 30/70 CTAB/DOPE mixtures to which have been added either OligoS (left column) or polyA (right column) in the indicated anion/cation charge ratios. The top spectra in the figure indicate the same results shown in figure 4.5.8, that is that the addition of 30 mole% of CTAB to DOPE vesicles causes a conversion to a bilayer architecture. Addition of either of the distinct nucleotide chains does not produce a change in overall architecture at any charge ratio. There is no question as to the binding of the nucleotide chains to the cationic surfaces, since there is no free nucleotide present in the spectra until after the anion/cation equivalence point.

Evidently, charge neutralization is not a sufficient condition to induce non-bilayer phases in CTAB/DOPE mixtures. Presumably, the complementary shapes of the cone profile of CTAB and the inverted cone profile of DOPE is sufficiently powerful to maintain a bilayer architecture even without the added influence of inter-headgroup charge repulsion. This then suggest that CTAB/DOPE mixtures should be less efficient in transfection, due to their inability to produce non-bilayer phases.
FIGURE 4.5.10  $^{31}$P NMR spectra of CTAB / DOPE (30/70) mixtures as a function of added OligoS (left hand column) or polyA (right hand column) at the indicated anion/cation charge ratio.
4.5.10 Effect of Salt on OligoS Binding to DOTAP/DOPE and CTAB/DOPE Mixtures

Figure 4.5.11 shows a series of $^{31}$P NMR spectra for increasing ionic strengths in complexes of OligoS added in a 1:1 anion/cation charge ratio in mixtures consisting of 30/70 CTAB/DOPE (left column) and 30/70 DOTAP/DOPE (right column). Increasing the NaCl concentration causes desorption of OligoS, since its narrow isotropic peak appears and increases in intensity. The CTAB/DOPE mixtures produced a bilayer $^{31}$P NMR line shape at all salt concentrations. Thus, the cone/inverted cone complimentary of CTAB/DOPE dominates the behaviour of the mixtures, with or without salt or bound OligoS. On the other hand, the DOTAP/DOPE mixtures produced an inverted hexagonal line shape at all salt concentrations. This indicates that removal of OligoS from the surface of DOTAP/DOPE mixtures does not cause the reversal of the macromolecular assembly of lipids back to the original bilayer phase, L$_c$, in the absence of OligoS binding. Thus, both NaCl and OligoS are capable of screening the inter-headgroup repulsion of DOTAP charges.
FIGURE 4.5.1 $^{31}$P NMR spectra of salt induced desorption of OligoS from CTAB / DOPE (30/70) mixtures (left hand column) and DOTAP / DOPE (30/70) mixtures (right hand column). OligoS was present in a 1:1 anion/cation charge ratio. The relevant NaCl concentration (mM) is indicated in the figure.
5 FUTURE DIRECTIONS

The studies presented here have constituted the first demonstration, by deuterium ($^2$H) nuclear magnetic resonance (NMR), of the formation of segregated domains of cationic lipids induced by anionic polyelectrolytes. The data presented in this thesis has shown how domain size and composition are altered as a function of polyelectrolyte identity and molecular weight, initial surface charge and the amount of salt added to these mixtures. $^1$H and $^3$P NMR have also provided insights into the fluidity and mobility of the zwitterionic and cationic lipids, as well as the polyelectrolyte used in these mixtures. Finally, $^3$P NMR was used as a tool to determine how the architecture of these “DNA-lipid” packages alter as a function of zwitterionic and cationic lipid and as the molecular weight of polynucleotide chains. This can prove to be a good tool in determining the potential efficacy of these “packages” for gene transfer. Although the investigations provided here give detailed information regarding the interaction of cationic lipids with anionic polyelectrolytes, other experimental variables require further investigation. There are at least four main topics which can be investigated in greater detail.

The first field of research should involve studying, in more detail, the location of the polyelectrolytes when electrostatically bound to these cationic surfaces. For instance, although our results indicate that penetration of the polyelectrolyte into the hydrophobic region of the lipid bilayer plays a role in domain formation, we do not know the depth of their penetration or the dynamics of their side chains. The degree of association of the polyelectrolyte with the membrane surface and its mobility can be determined from a variety of experiments. First, consider using a zwitterionic lipid
such as POPC, which is deuterated along the length of its acyl chains. In this manner the order profile of the hydrophobic region of the bilayer composed of deuterated POPC and some cationic lipid may be determined, from top to bottom. By direct comparison of such results with those obtained in the presence of a polyelectrolyte, the perturbation of the bilayer may be determined. By studying the degree to which the order parameters are altered, both the depth and strength of penetration of the polyelectrolyte into the bilayer proper may be determined. The obvious control for these sets of experiments would be to find a polyelectrolyte which has no side groups and contains its charge on its backbone.

A more direct method of studying the polyelectrolyte would be to specifically deuterate the polyelectrolyte on its chain or on its side group. In this instance two $^2$H NMR control experiments should be run. The first would be on the polyelectrolyte in powder (static) form and the other in solution (isotropic). When the polyelectrolyte is electrostatically bound to the cationic membrane surface at least two $^2$H NMR populations should be observed. One for monomer units of the polyelectrolyte which are bound and the other which are unbound in tail or loop regions. The quadrupolar splittings for these two cases should differ greatly. Since the splittings should be easy to identify then through spectral simulation amounts of polyelectrolyte bound and free could be quantified, depending on their $T_2$ values. Quantification may also be accomplished through the isotropic peak. These experiments would help to identify portions of the polyelectrolyte which retained a degree of mobility within the “charge-compensation” limit. Another interesting variable here would be to synthesize a deuterated “block” polymer which contains portions of its chain which are charged and others which are not. This should provide information as to the degree of hydrophobic association of the polyelectrolyte with the surface. Will the non-charged portions of the
polyelectrolyte still adsorb to the surface? If so then a third population may arise in the $^2$H NMR spectrum. The existence of such a population or the size of its quadrupolar splitting should provide insight into the relative strength of the hydrophobic contribution to binding.

A second field of experiments would involve studying cationic lipids in more detail. Different cationic lipids have recently been synthesized in order to improve the efficiency of transfection "packages". The use of polycationic lipids has shown definite promise. Their ability to increase efficiency is believed to correlate with their ability to condense DNA to a greater degree thus reducing the energy barrier for transport across a plasma membrane. Through $^2$H NMR of headgroup deuterated POPC, the amount of charge that these lipids bring to the surface of a membrane can be determined relative to monocationic lipids. It is possible that these lipids produce a higher surface charge density which enhances binding to DNA. $^{31}$P NMR can provide information on the state of the polynucleotide when bound to these polycationic species. The resulting size of the chemical shift anisotropy (CSA) can provide information about the relative size and mobility of the complex.

Another interesting cationic lipid for study would be a POPC molecule with an ethyl group bound to its phosphate region. If this molecule was headgroup deuterated it could produce a "voltmeter" response to charge as does another charged lipid, phosphatidylglycerol. If its response is sensitive to charge then it should be possible to observe domains directly through the cationic lipid. Will the effect of added anionic polyelectrolyte charge be directly accounted for, unlike the case of membranes containing deuterated zwitterionic POPC?

The third area of study involves determining the lipid lateral diffusion coefficient of POPC, within the polyelectrolyte-bound domain. The conclusions made in this thesis regarding domain size from $^2$H NMR was based on a diffusion coefficient for POPC that was in a bulk phase. Even though
the $^2$H NMR results indicate that the lipids are highly mobile within the bound domain, the effective diffusion rate should be slowed by the “archipelago” effect (Saxton, 1993) of the polyelectrolyte bound to the membrane surface. The diffusion coefficients of lipids in bilayers can be determined by a $^{31}P$ NMR 2D-EXSY technique (Picard, et al., 1998). This technique should aid in producing a more reliable lower limit to the relative size of these domains.

The final area of study involves determining the presence of domains, by some independent method, as well as the size of these domains. Fluorescence digital imaging techniques, run for single vesicles, should provide definitive detection of domains in the plane of the bilayer. Another method which may distinguish domains would be some x-ray or neutron scattering experiment which can study changes in bilayer thickness. Differences in bilayer thickness would be expected to be present between polyelectrolyte-bound and -free phases. An NMR approach that may also provide insight would be a 2D $^2$H NMR EXSY experiment. If lateral domains are present in the same bilayer then the two populations in the $^2$H NMR spectrum should be capable of exchanging with one another. This is in contrast to the situation where the domains actually exist on separate lamellae. Thus, the existence of a third POPC population in the spectrum, which is intermediate between POPC lipids in their “bound” and “free” forms, would be proof of exchange of lipids between two domains on the same lamellae. This should be the case for large domains, in which lipids from both domains exchange at the boundary between them. It may also be possible that, given enough time to exchange, only a single POPC population will be observed in the spectrum. This would indicate that the domains are relatively small and that all POPC molecules can exchange in and out of both domains many times in order to average out the properties of both.
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