MONITORING DYNAMICAL AND STRUCTURAL CHANGES AT THE LIPID-WATER INTERFACE THROUGH CHEMICAL SHIFT ANALYSIS: A XE-129 NMR STUDY

By

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To my father
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The accumulation of inhalational anesthetic in lipid membranes can alter their distributive properties and energetic demands. It is hypothesized that as anesthetic molecules partition into the biomembrane, changes in the lateral pressure profile ensue, indirectly altering membrane protein structure and function. Though it is well established that this dynamic process is largely controlled by interfacial properties, the actual modifications the surface tension undergoes at this boundary is not fully understood. Here, the inhalational anesthetic xenon (Xe) is used as a nonpolar, weakly binding spin probe to investigate the anesthetic-lipid bilayer interaction by gaseous NMR spectroscopy. Fundamental kinetic and thermodynamic behaviors are studied by monitoring interaction induced chemical shift changes of thermal and hyperpolarized $^{129}$Xe in various lipidic media. The nature of xenon-phospholipid interactions and Xe exchange depend on the structure of the lipid headgroups and acyl chains, the phase state of the lipid bilayer, and the heterogeneity in both vesicle size and overall distribution of lipids with external variables. The primary focus of this work is to explore the effects of temperature and composition on solvation parameters, gas adsorption properties and molecular rearrangement at the bilayer interface via $^{129}$Xe chemical shift changes.
Thermodynamic and kinetic information are extracted by monitoring the observed chemical shift with changing external variables; results are fit to a mathematical model in order to extract pertinent parameters. Partitioning behavior as related to increasing molecular stress and changing lipid morphology. Intermediate lipid phases were probed as well. Data suggests the presence of multiple binding sites as well as moderate cooperative binding. The mole fraction partition coefficient increases with temperature and behaves ideally in a single component lipid system. The presence of nonbilayer lipids has an opposite effect on the partitioning parameters. What’s more, evidence suggests the addition of Xe promotes the formation of highly curved structures at elevated temperatures and pressures. Anodic Aluminum Oxide (AAO) substrates are utilized to stabilize bilayers in the magnetic field, facilitating the study of Xe diffusivity between phases using 2D-exchange NMR methods. Results are discussed in context of anesthetic action and the lateral pressure profile.
CHAPTER 1
INTRODUCTION

1.1 Introduction

The membrane is the main structural element of a living cell and is often viewed as a supportive substrate whose purpose is to facilitate protein function. In addition to acting as a permeability barrier, lipid membranes form the intracellular environment and define its outer boundaries; the lack of covalent interactions within these assemblies provides the basis for its highly dynamic properties. Recent studies into the effect of surface tension on interfacial dynamics of a phospholipid bilayer suggest a surface tension mediated mechanism for membrane organization and structure. As such, lateral organization of the membrane may have consequence on protein function. In the “indirect mechanism” of anesthetic action, anesthetic molecules partition into the biomembrane and modify the lateral pressure profile. According to several modern lipid theories of anesthesia, the preferential location of anesthetics at the membrane interface is of prominent importance. It has been proposed that the anesthetic’s intrinsic properties may mediate the redistribution of the lateral pressure profile and in this way facilitate anesthesia. Our interests lie in investigating the potential effect lateral heterogeneity plays in the membrane-anesthetic interaction while exploring the utility of $^{129}$Xe NMR as a probe of interfacial dynamics.

$^{129}$Xe NMR has found many applications in material science and medicine within the last twenty years due to its high sensitivity to subtle changes in its local environment and ability to be hyperpolarized. [1-7] The detection of hydrophobic cavities within proteins has prompted creative technical advancements in gas delivery and experimental applications and its use as a biomolecular probe of dynamical systems in solution has been recently reviewed. [1-6] $^{129}$Xe NMR spectroscopy has been employed to distinguish between multiple conformational states of protein...
solutions and to monitor protein-peptide binding events in solution.\cite{7} What’s more, it has been proven useful for probing specific and nonspecific binding to proteins in addition to detecting changes in cell morphology.\cite{2, 8-10} The use of hyperpolarized $^{129}$Xe as a biosensor is also very appealing due to its potential to be used to detect foreign matter (e.g. tumors, lesions) in inaccessible regions in the human body.\cite{11-13}

Xenon gas is a potent anesthetic with NMR properties that make it well-suited to the study of the basic nature of the anesthetic-membrane interactions. Similar to other inhalation anesthetics, it shows a high affinity for the amphipathic region of the bilayer and is thought to interact directly with water molecules near the lipid membrane-water interface.\cite{14} However, molecular dynamic simulations predict that a significant fraction of the gas is also located within the bilayer core, a behavior commonly identified with nonimmobilizers.\cite{15, 16} As discussed by Stimson et al., charge distribution and polarizability may be the key to understanding the distribution processes and consequent effects of small molecules on the biomembrane. Clearly, a study of these effects is needed.

Herein we study what role nonspecific, weak interactions play in membrane dynamics and how additional stress imposed by the presence of nonpolar molecules affects partitioning and binding behavior. In brief, $^{129}$Xe NMR spectroscopy is used to characterize the anesthetic-membrane interaction with modified surface tensions; changes in solubility and binding parameters with temperature and membrane composition should prove useful to those developing techniques in lung imaging and blood perfusion, and the larger anesthesiology community as a whole. Though rarely used due to its expense, xenon produces the lowest toxicity levels compared to all other anesthetic molecules. Several questions we hope to address in particular are:
• What directs the distribution of xenon between water and the membrane environment?

• Is the thermodynamic transfer of noble gas between phases governed by the hydrophobic effect and what membrane properties does it reflect?

• Is it possible to distinguish between surface and interior membrane binding by via $^{129}$Xe chemical shift measurements?

• How does packing heterogeneity affect lipid membrane structure and dynamics with changing lipid composition and applied molecular stress?

1.2 Structure of Dissertation

The work is structured as follows: Chapter 2 summarizes the basics of NMR theory and briefly describes specific NMR techniques utilized within this dissertation (e.g. magic angle spinning and 2D EXSY). Chemical shifts, chemical exchange nuclear relaxation phenomena are discussed in particular detail. The second part of this chapter introduces $^{129}$Xe NMR and key aspects of the spin exchange optical pumping process. Contributions to the observed $^{129}$Xe chemical shift are discussed in context of xenon adsorption and dissolution into solid materials and bulk solvent, respectively. Recent developments in the gas delivery methods are also presented. A general overview of the lipid environment is given in Chapter 3 in which we discuss the general structural and chemical properties of lipid molecules and bilayer membrane. The differences between bilayer and nonbilayer lipids are highlighted. Several techniques utilized in the characterization of our lipid systems are explained and their experimental results provided.

In Chapter 4 $^{129}$Xe NMR is employed to study its interaction with dioleoyl-phosphotidylcholine (DOPC) bilayers in several different vesicle morphologies. A new model is introduced to extract pertinent partitioning parameters based on chemical shift measurements; experimental results are compared to literature values for verification. These results provide the basis for Chapter 5 and Chapter 6, wherein thermodynamic and kinetic properties of the xenon-
membrane association are evaluated, respectively. Solvation thermodynamics are utilized in Chapter 5 to elucidate the temperature dependence of the transfer mechanism. Thermodynamic parameters (e.g., solvation enthalpies and entropies) are determined from chemical shift data and compared to literature values. We then treat the lipid matrix as a porous solid in Chapter 6; a number of surface models are employed to extract binding constants with the aim of differentiating between surface and hydrocarbon binding.

Furthermore, we examine the effect of varying lipid composition on xenon partitioning in a two-component lipid system (Chapter 7). Increasing the mole fraction of the nonbilayer lipid dioleoyl-phosphatidylethanolamine (DOPE) in a DOPC bilayer membrane induces changes in the membrane’s surface tension. The extent in which xenon solubility parameters vary with increasing DOPE content was examined using the approach previously employed in the DOPC system. In this chapter we also investigate the effect of temperature and changing lipid composition on membrane morphology and local deformations within bilayer structures. Lastly, preliminary data investigating the use of anodic aluminum oxide (AAO) supported membranes are presented in Chapter 8. The main obstacle in making $^{129}\text{Xe}$ NMR a useful tool in the characterization of biological and inorganic materials in solution within a reasonable experiment time and moderate physical conditions, is the low density and long relaxation time of thermally polarized $^{129}\text{Xe}$ in solution. The only way to circumvent this is through hyperpolarization (HP) techniques. Inorganic substrates, such as AAO, can be functionalized by self assembly of lipid bilayers on their surfaces, creating a convenient model of cellular membranes. In addition to verifying effective bilayer formation on the substrate via $^{31}\text{P}$ magic angle spinning, we monitor at the xenon gas-to-lipid membrane exchange via 2D EXSY. Conclusions and future applications discussed in Chapter 9.
2.1 Introduction

Nuclear magnetic resonance (NMR) spectroscopy is the predominant technique utilized in this work. This chapter focuses on providing the background necessary for understanding measurable NMR parameters and various techniques employed in our studies. Of which include: polarization, relaxation, saturation recovery, chemical exchange, spin-exchange optical pumping and factors contributing to the chemical shift and relaxation behavior of $^{129}$Xe in solution and solid materials. In light of the many books and literature reviews on this subject, we refer the reader to a number of texts for more basic,[17, 18] intermediate,[19-22] and advanced/theoretical approaches to NMR theory.[23-25]

2.2 Basic NMR Theory

The NMR phenomenon relies on the interaction of an atomic nucleus with static magnetic field. All that is required is for nuclei of elements with an odd atomic number or odd mass to possess a nuclear magnetic moment (i.e. nuclear spin). The nuclear spin ($\vec{\mu}$) is often view as a microscopic needle, having both magnitude and direction. Under normal circumstances, the nuclear spin can adopt any spatial orientation. Once a NMR active nucleus is placed into a strong magnetic field its nuclear magnetic energy levels split into multiple, non-degenerate spin states where only a discrete number of orientations are allowed. This process, called the Zeeman splitting, is caused by interactions between the nuclear magnetic moment and the magnetic field that breaks degeneracy and creates a slight shift in the atomic energy levels at thermal equilibrium. The distinct quantum energy for each state ($E_m$) is directly proportional to the strength of the applied magnetic field ($B_o$) and is characterized by the magnetogyratic ratio of the spins ($\gamma_I$), which varies between atomic nuclei:
\[ E_m = -\vec{\mu}_z \cdot \vec{B}_o = m\gamma I hB_o / 2\pi \]  

The magnetic quantum number, \( m \), denotes the total number of spin states and is given by \( m = -I, -I+1, \ldots, +I \), where \( I \) is the nuclear spin quantum number. The total number of orientations is given by \((2I+1)\). Due to restrictions described by quantum mechanics, \( \vec{\mu} \) is unable to align exactly parallel to \( B_o \). The torque experienced by the magnetic moment causes it to precess around \( B_o \) with an angular frequency, \( \nu_L \), known as the Larmor frequency.

\[ \nu_L = \omega_o / 2\pi = \gamma I B_o / 2\pi \quad \left( \text{s}^{-1} \right) \]  

The direction of the rotation is dependent on the sign of \( \gamma I \), while the angle between it and the magnetic field is based on the directional quantization, \( m \). The effect of \( \gamma I \) and natural abundance on the relative spectral sensitivity is given in Table 2-1 for select nuclei.

NMR is induced by applying a small alternating magnetic field (\( B_1 \)) of appropriate frequency (\( \nu_L \)) to flip the spin from the low energy state (\( |\alpha\rangle \)) to the high energy state (\( |\beta\rangle \)). When the applied energy is equal to \( \Delta E \), the energy absorbed by the nuclear spins causes a transition between states; those in the low spin state move to the high spin state and vice versa. The

| Nucleus | I  | Abundance (%) | \( \gamma I \) | \( |S_I / S_{1\text{H}}| \cdot 100\% \) | \( |S_I / S_{1\text{C}}| \cdot 100\% \) |
|---------|----|---------------|---------------|---------------------------------|---------------------------------|
| \(^1\text{H}\) | 1/2 | 99.99 | 26.75 | 100.000 | - |
| \(^{13}\text{C}\) | 1/2 | 99.99 | 26.75 | 100.000 | - |
| \(^{31}\text{P}\) | 1/2 | 100.00 | 10.84 | 6.650 | 4150.6 |
| \(^{129}\text{Xe}\) | 1/2 | 26.44 | -7.45 | 0.572 | 3357.4 |
irradiation energy is in the radiofrequency range (RF) and is generally applied as a short pulse (on the order of microseconds). This absorption process induces a voltage in the coil region which can be detected and amplified. The resulting signal is displayed as the free induction decay (FID). Thermal equilibrium will be restored via relaxation processes assuming no additional RF pulses are applied.

According to Eq. (2-2), all the nuclei within a sample would resonate at the same frequency if they all experience the same \( B_o \). However, this is not the case since the characteristic \( v_L \) of each nucleus is reflective of changes in its molecular environment; these differences are expressed as chemical shift values. The chemical shift (in parts per million, ppm) of a \(^{129}\text{Xe}\) nucleus is given as:

\[
\delta = \frac{v_L - v_{\text{ref}}}{v_o} \times 10^6
\]

where \( v_{\text{ref}} \) is the resonance of our gas standard and \( v_o \) is the operating frequency of the spectrometer (400 MHz). The thermal standard used for reference consists of 5.26 atm of xenon gas contained within a flamed-sealed NMR tube with 0.26 atm of oxygen gas.

### 2.2.1 Nuclear Polarization

At thermal equilibrium, the difference in the atomic energy levels prevents them from being equally populated. The population ratio between states is dependent on \( \gamma \) and \( B_o \) and can be characterized by Boltzmann statistics. As such, the occupation numbers of the \( |\alpha\rangle \) and \( |\beta\rangle \) states, denoted by \( N_\alpha \) and \( N_\beta \) for a \( I = 1/2 \) system, have direct consequence on the nuclear spin polarization \( (P) \). This in turn affects the magnetic resonance signal which is directly proportional to the \( z \)-magnetization \( (M_z) \). Specific relations are provided in Eq. (2.2)-(2.5) for clarity:
\[
\frac{N_\beta}{N_\alpha} = \exp\left(- \frac{\Delta E}{k_B T}\right) = 1 - \frac{\Delta E}{k_B T} = 1 - \left(\frac{\gamma_i h B_0}{2\pi k_B T}\right) \approx 0.9999777 \quad (2-4)
\]

\[
P \equiv \left| \frac{N_\beta - N_\alpha}{N_\beta + N_\alpha} \right| \times 100\% \quad (2-5)
\]

\[
M_z = \frac{[I] \gamma_i h}{4\pi} \cdot \frac{P}{100\%} \quad (2-6)
\]

\(T\) is the temperature (in Kelvin), \(k_B\) is the Boltzmann constant, \(h\), the Planck constant, and \([I]\) is the total number of spins per unit volume. \(N_\beta\) and \(N_\alpha\) represent relative populations of the upper and lower energy levels, respectively; consistent with Eq. (2-4), \(N_\alpha > N_\beta\). However, \(\Delta E\) is small at thermal equilibrium, so the population difference between the spin states is small (on the order of parts per million). These spin conditions generate extremely weak NMR signals and are the primary reason for the low sensitivity of NMR in standard experiments. For example, the \(\Delta E\) value for an ensemble of spin-1/2 \(^{129}\text{Xe}\) gas in a magnetic field of 9.4 Tesla (400 Mz NMR) is approximately \(7.38 \times 10^{-26}\), yielding an equilibrium spin polarization of about \(9 \times 10^{-6}\) at 300 K.

For NMR experiments suffering from low signal-to-noise, the signal can be enhanced by increasing the number of detectable nuclei, or by signal averaging. As shown in Table 2-1, the \(^{129}\text{Xe}\) isotope is only 26.4% naturally abundant. In the gaseous state, the amount of xenon can be increased by simply increasing the pressure in the NMR tube. However, the use of high pressures in biological samples can be detrimental to the system being studied and the sensitivity is often limited by solubility. Use of high pressures is somewhat limited in solid samples as well since the amount of adsorbed xenon is limited by the number of available surface sites. These limitations can be circumvented by utilizing isotopically enriched xenon. Though enrichments of \(> 86\%\) are commercially available, they are more costly and only provide a threefold increase in the NMR signal. Signal averaging is the most common technique used to increase the signal-to-
noise ratio ($SNR$)—though there are practical limitations to the improvements that can be gained. Nuclei with long relaxation times can require anywhere from a few hours to several days for adequate resolution. The $SNR$ is proportional to the square root of the number of signal transients averaged. Thus, a tenfold increase would require the averaging of one hundred free-induction-decays ($FID$).

The nuclear spin polarization may also be enhanced via brute force methods (i.e. significant decrease in the temperature, or increase in the magnetic field). Unfortunately, low temperatures have adverse effects on biological samples and the maximum magnetic field currently available, 21 Tesla (900 MHz) only enhances the $^{129}$Xe polarization by one order of magnitude ($P \approx 2\times10^{-5}$) under similar experimental conditions. Polarization enhancement may also be achieved through non-equilibrium methods, as in the case of hyperpolarized NMR,$^{[27-30]}$ dynamic nuclear polarization,$^{[31-33]}$ parahydrogen induced nuclear polarization,$^{[34-36]}$ and semiconductors.$^{[37-39]}$ The spin-exchange optical pumping (SEOP) of spin-1/2 noble gases is of particular interest.$^{[40-43]}$ Details of this particular technique will be discussed in more detail in subsequent sections.

### 2.2.2 Chemical Shift Anisotropy

As mentioned previously, chemical shifts reflect changes within the electronic environment that surrounds the nucleus. The electron density reduces the external field at the nucleus by some factor $\sigma$, and modifies the local magnetic fields experienced by different nuclei. These local fields can be either isotropic or anisotropic. If the electron density is identical in all directions, the local field is said to be isotropic—it has no affect on the shielding ($\sigma$) and thus no affect on the observed chemical shift. If the shielding is anisotropic, the local magnetic fields generated by the nucleus will depend on the molecular orientation with respect to $B_0$; these
different magnetic fields at the nucleus can change with molecular motion, causing fluctuations in the chemical shift.

The chemical shift tensor gives the orientation dependence of the chemical shift by relating the orientation of the magnetic field to the molecular frame in which the induced fields are generated:

\[
\tilde{\delta} = \begin{bmatrix}
\delta_{xx} & \delta_{xy} & \delta_{xz} \\
\delta_{yx} & \delta_{yy} & \delta_{yz} \\
\delta_{zx} & \delta_{zy} & \delta_{zz}
\end{bmatrix} = \gamma_i (1 - \tilde{\sigma}) B_o
\]  

(2-7)

where \( \tilde{\sigma} \) is the shielding tensor. When placed into its principal axis system (PAS), \( \tilde{\delta} \) takes on a simplified form:

\[
\tilde{\delta}_{\text{PAS}} = \begin{bmatrix}
\delta_{xx} & 0 & 0 \\
0 & \delta_{yy} & 0 \\
0 & 0 & \delta_{zz}
\end{bmatrix} \delta_{\text{iso}} = \left(\delta_{xx} + \delta_{yy} + \delta_{zz}\right)/3
\]  

(2-8)

The principal axis system is the reference frame in which yields the diagonal form of the chemical shift tensor and the chemical shift that would be observed if the magnetic field were along the \( x, y, \) or \( z \)-axis of the PAS. The resulting eigenvalues (\( \delta_{xx}, \delta_{yy}, \delta_{zz} \)) are termed the principal components. The eigenvectors are the direction cosines that relate the PAS to the original frame of reference (i.e., laboratory or molecular frame). The laboratory frame is typically defined by the direction of the field, while the molecular frame is defined by the local molecular symmetry.

If the molecular motion is fast on the NMR time scale, the observed (isotropic) shift will be an average of the eigenvalues: one third the trace (sum of the diagonal) of the chemical shift tensor:

\[
\delta_{\text{iso}} = \left(\delta_{xx} + \delta_{yy} + \delta_{zz}\right)/3
\]  

(2-9)
A similar expression can be generated for the shielding tensor, where \( \sigma_{xx} \geq \sigma_{yy} \geq \sigma_{zz} \) and \( \delta_{xx} \geq \delta_{yy} \geq \delta_{zz} \) by convention. The chemical shift anisotropy (CSA) is defined as the chemical shift difference between the isotropic and anisotropic states and is usually expressed as:

\[
\Delta \delta = \delta_{zz} - (\delta_{xx} + \delta_{yy})/2 \eta = (\delta_{yy} - \delta_{xx})/\delta_{zz} \tag{2-10}
\]

the asymmetry of the tensor (\( \eta \)) is given by:

\[
\eta = (\delta_{yy} - \delta_{xx})/\delta_{zz} \tag{2-11}
\]

Often times molecular rotations (in 2-dimensions) causes the shielding to show axial symmetry; this results in two of the three principle components to be equal. According to convention, the tensor element parallel to the symmetry axis is denoted by \( \delta_z \), while the two equivalent elements are designated by \( \delta_\perp \). As such, the CSA of this particular system is defined as the difference between the two (\( \delta_z - \delta_\perp \)). This particular lineshape is called a CSA powder pattern and is commonly found in the NMR spectra of solid systems.

**Magic angle spinning.** High resolution solid-state spectra can be obtained one of two ways: by magic angle spinning (MAS) or to observe aligned molecules. MAS is a tool commonly used in solid state NMR to remove the effects of CSA and heteronuclear dipolar coupling from NMR spectra by mimicking the rapid tumbling that would naturally occur in solution to average the anisotropic interactions in solid-state. As mentioned previously, if the rate of change in molecular orientation is fast relative to the magnitude of the CSA or dipole-dipole coupling (in frequency units), the molecular orientation dependence of the transition frequencies will result in an isotropically averaged value. The molecular orientation dependence of the nuclear spin interactions are discussed in more detail elsewhere. Sufficed to say, they are of the general form: \( 3\cos^2\varphi - 1 \). The orientation of the spin interaction tensor to the magnetic field (i.e.,
Figure 2-1. The placement of the magic angle spinning (MAS) rotor with respect to the external magnetic field ($B_0$).

shielding tensor, dipolar coupling tensor) is given by the angle $\varphi$. In powder samples, all molecular orientations and so all possible values of $\varphi$ are represented. Spinning the sample at a particular angle, $\varphi_R$, with respect to the magnetic field results in an averaging of $\varphi$ with time. So, provided that the spinning speed exceeds the amplitude of the anisotropic interactions, it is possible to average out the orientation dependence of the interaction anisotropy by setting $\varphi_R$ to a value of $54.7^\circ$ (the magic angle). Mathematical details of this NMR technique can be found elsewhere.\cite{20,44}

2.2.3 Relaxation Mechanisms

Relaxation processes arise from interactions between the nuclear spin and fluctuating magnetic fields due to i) dipole-dipole interactions ($T_1^{DD}$), ii.) molecular motions ($T_1^{SR}$) iii.) chemical shift anisotropy ($T_1^{CSA}$), and interactions with unpaired electrons in paramagnetic compounds ($T_1^{para}$):

$$\tau_c = \frac{1}{T_1} = \frac{1}{T_1^{DD}} + \frac{1}{T_1^{SR}} + \frac{1}{T_1^{CSA}} + \frac{1}{T_1^{para}} + \ldots$$

(2-12)
all of which have the potential to induce oscillating magnetic fields of the same frequency as \( v_L \) (Larmor resonance frequency). Dipole-dipole interactions do so via the through-space interaction of magnetic dipoles of a pair of nuclei. Fluctuating fields may also be produced by molecular motions (vibrational, rotational, or translational) or by changes in chemical shielding of other surrounding nuclei. CSA describes the orientation dependence of a chemical shift with respect to the magnetic field. This relaxation mechanism is particularly important for nuclei with a large chemical shift scale, such as \(^{31}\text{P}\). As seen in Eq. (2-12), the longitudinal relaxation time is inversely proportional to the correlation time (\( \tau_c \)) for aqueous solutions of normal viscosity. As such, slower random motions often give rise to shorter longitudinal relaxation times (\( T_1 \)) while faster molecular motions result in longer \( T_1 \) values.

The longitudinal relaxation time is the amount of time required for the longitudinal magnetization to return to 63% of its original value after perturbation. This particular relaxation is due to energy exchange between the nuclear spins and its surroundings and is characterized by an exponentially growth in the observable magnetization:

\[
M_z(t) = M_z(0) \cdot e^{-t/T_1} + M_0 \cdot \left(1 - e^{-t/T_1}\right)
\]  

(2-13)

where, \( M_0 \) is the equilibrium magnetization and \( M_z(t) \) is the magnetization along the longitudinal magnetic field at time \( t \). The spin system is considered to be fully relaxed after three to five \( T_1 \) periods. Herein, the longitudinal relaxation time was obtained by saturation-recovery methods—this essentially equalizes the populations of the \( |\alpha\rangle \) and \( |\beta\rangle \) spin states (i.e., saturation) and measures the recovery of the magnetization from zero as a function of time. The exponential growth is then fit to Eq. (2-13).

The decay of the magnetization in the \( xy \) plane is governed by the transverse relaxation (\( T_2 \)). Similar to the longitudinal relaxation, \( T_2 \) is the amount of time required for the net
magnetization in the $xy$ plane to be reduced approximately 63% of its original value. This particular relaxation process is characterized by a continued dephasing of the magnetic moments in the transverse plane with time. What’s more, $T_2$ can be monitored through changes in the NMR linewidth by the following relation:

$$
\Delta \nu_{1/2} = \frac{1}{\pi T_2}
$$

(2-14)

It should be noted that $T_2$ is always shorter than $T_1$ as the spins in the transverse plane dephase faster than they align with the static magnetic field.

**Effect of Paramagnetic Ions.** Unpaired electrons produce significantly stronger magnetic fields than nuclei. As such, the presence of paramagnetic ion in solution will significantly increase the longitudinal relaxation rate (Eq. (2-12)). In addition to their shifting ability, the use of shift reagents (i.e., contrast agents) can introduce severe line broadening in the NMR signal if present at high enough concentrations. This is consequence of the relaxation process provided by the unpaired electron. Paramagnetic shift reagents are commonly used to induce changes in both the chemical shift and relaxation behavior of select signals and molecular environments within liposomes. In the context of this work, it is used to confirm the chemical shift assignment of xenon dissolved within the lipid environment and to help resolve the relaxation properties of $^{129}\text{Xe}$ gas within two separate compartments.

### 2.2.4 Chemical Exchange

Chemical exchange is a dynamic process which causes a nucleus to reside between multiple environments. This can have large repercussions on the observable NMR parameters, making it difficult to accurately describe the environment. Here, we limit ourselves to several key points since the theory of chemical exchange effects have been treated in detail elsewhere.\textsuperscript{45-}
Let us consider the two-site chemical exchange of a $^{129}$Xe nucleus between sites $A$ and $B$, having fractional populations $f_A$ and $f_B$:

$$A \xrightleftharpoons[k_A^{-1}]{k_B} B$$  \hfill (2-15)

where $f_A k_A = f_B k_B$ at equilibrium. Two separate resonance lines will be observed at their characteristic resonant frequencies in the absence of chemical exchange (Figure 2-2). NMR evolution of this process can be described according to the modified Bloch equations (i.e., McConnell equations), the solutions of which are composed of real and imaginary parts.\[46, 48\] It is the solution to the imaginary component which yields the lineshape.

Figure 2-2. Simulated NMR spectra depicting the effect of exchange and population on NMR spectra. A) Population: $f_A = f_B = 0.50$ for equally populated exchange sites. B) $f_B > f_A$ for unequally populated exchange sites for a two-site exchange process.
Table 2-2. The effects of exchange on the properties of an NMR spectrum for various exchange rates.

<table>
<thead>
<tr>
<th>Exchange Rate</th>
<th>$\delta_{\text{obs}}$ (in Hz)</th>
<th>$T_2$ relaxation (s$^{-1}$)</th>
<th>$T_1$ relaxation (s$^{-1}$)</th>
<th>Experimental technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>$k_{\text{ex}} \ll \delta_A - \delta_B$</td>
<td>$k_{\text{ex}} \ll \frac{1}{T_{2,A}} - \frac{1}{T_{2,B}}$</td>
<td>$k_{\text{ex}} \ll \frac{1}{T_{1,A}} - \frac{1}{T_{1,B}}$</td>
<td>Linewidth measurements</td>
</tr>
<tr>
<td>Intermediate</td>
<td>$k_{\text{ex}} = \delta_A - \delta_B$</td>
<td>$k_{\text{ex}} = \frac{1}{T_{2,A}} - \frac{1}{T_{2,B}}$</td>
<td>$k_{\text{ex}} = \frac{1}{T_{1,A}} - \frac{1}{T_{1,B}}$</td>
<td>Lineshape analysis</td>
</tr>
<tr>
<td>Fast</td>
<td>$k_{\text{ex}} \gg \delta_A - \delta_B$</td>
<td>$k_{\text{ex}} \gg \frac{1}{T_{2,A}} - \frac{1}{T_{2,B}}$</td>
<td>$k_{\text{ex}} \gg \frac{1}{T_{1,A}} - \frac{1}{T_{1,B}}$</td>
<td>Spin-echo</td>
</tr>
</tbody>
</table>

**Slow exchange.** Slow exchange gives rise to two, separate, broadened signals. The increase in the full-width at half height (FWHH) of $^{129}$Xe at each site ($i = A, B$) is given by:

$$\Delta v_{1/2,i} = \frac{1}{\pi T_2} + k_{\text{ex}}$$  \hspace{1cm} (2-16)

Thus, for slow exchange, the exchange rate can be measured from the linewidth of the observed resonance (see Eq. (2-14)). In this regime, changes in the observed relative intensities are often related to modifications in relative population with varying solute, or ligand concentration as the positions of the resonance signals remain unchanged.

**Fast exchange.** Spins from sites $A$ and $B$ experience an average local field within the fast exchange regime, resulting in a single resonance representing a weighted average of the intrinsic chemical shifts at each site (see Eq. (2-17)). The increase in the linewidth due to chemical exchange is now given by:

$$\delta_{\text{obs}} = f_A \cdot \delta_A + f_B \cdot \delta_B$$  \hspace{1cm} (2-17)

$$\Delta v = \frac{4\pi^2 \cdot f_A \cdot f_B^2 \cdot (\omega_A - \omega_B)^2}{k_A}$$  \hspace{1cm} (2-18)

31
So, the smaller the frequency difference (in Hertz) the narrower the linewidth. This regime is by far the most useful for determining binding and partitioning parameters from changes in NMR parameters.

**Intermediate exchange.** As its name implies, intermediate exchange exists somewhere between fast and slow exchange conditions; its lineshape is characterized by a broad, complex spectra that can be difficult to analyze. This exchange process can result in broadening so great that peaks disappear. Analysis can be made easier by shifting the system to either fast or slow exchange.

### 2.2.5 $^{129}$Xe 2D EXSY Exchange NMR

A number of two-dimensional (2D) NMR techniques have been developed for the study of slow exchange phenomena, the most common of which are Nuclear Overhauser Effect (NOESY) and Exchange (EXSY) Spectroscopy. Since the transfer of magnetization by the NOE is identical to the magnetization exchange resulting from the physical movement of nuclei, the pulse sequence of the NOESY and EXSY experiments are the same. NOESY is used to provide information on through-space correlations through spin-lattice relaxation while EXSY is utilized to detect chemical and conformational exchange. What’s more, exchange rates are generally much faster than the cross-relaxation rate in 2D EXSY than for NOESY. EXSY is characterized by peaks of the same sign and measures correlation times between 10 ms – 1 s. The greatest amount of information is generally obtained when the exchange is slow on the NMR timescale and fast on the $T_1$ scale.

Chemical exchange is the process in which a nucleus physically moves from one molecular environment to another, resulting in a change in its chemical shift. Exchange measurements can be used to qualitatively map exchange pathways when little to no previous information is known about the dynamics of the system. It can also be utilized to quantitatively determine rate of
interparticle xenon exchange. All that is required is for the exchange process to be slow on the NMR timescale. However, if the exchange is too slow, all memory of the exchange process will be removed through relaxation processes. Similarly, the characteristic resonance of each site will be unresolved if the exchange is too fast, making the transfer process unobservable. Cross peaks due to magnetization transfer are based on chemical exchange during the mixing period of the EXSY pulse sequence.

**Thermally polarized experiment.** The standard 2D EXSY experiment consists of three \( (\pi/2)_x \) pulses. As shown in Figure 2-3A, the first two pulses are separated by evolution delay

![EXSY pulse sequence diagram](image)

**Figure 2-3.** The pulse sequence for 2D EXSY (and NOESY) experiment for thermally and hyper-polarized \(^{129}\text{Xe}\). A) The standard EXSY pulse sequence is indicated. The sample is treated with a saturating pulse train (SAT) followed by a repolarization delay (\( \tau_1 \)) which reduces the acquisition times due to long \( T_1 \)'s typical in thermally polarized \(^{129}\text{Xe}\) experiments. B) Continuous flow pulse sequence. C) Interrupted flow pulse sequence. Figure modified from Cheng and Bowers (2007).
time, $t_1$, which is the frequency labeling period. The second and third pulses are separated by the mixing time, $\tau_m$, during which the magnetization is transferred between spins; $t_1$ is the detection period. Like all 2D spectra, all peaks have two frequency coordinates: $f_1$ corresponds to the frequency experienced by the spin during the first time period ($t_1$) while $f_2$ is the frequency observed during the detection period ($t_2$). In the simplest case, spins $A$ and $B$ both contribute to the $z$-magnetization at thermal equilibrium. The equilibrium magnetization in the $I_z$ before the first pulse is given by:[23,49-51]

$$I_z = I^A_z + I^B_z$$  \hspace{1cm} (2-19)

The first ($\pi/2$) pulse produces transverse magnetization $I_y$ by tipping the bulk magnetization onto the -$y$ axis on the transverse plane. Using the product operator treatment, this can be described as follows:

$$I_y \rightarrow I^A_y - I^B_y$$  \hspace{1cm} (2-20)

$I_y$ develops as it precesses around $B_0$ with different Larmor frequencies; the final phase ($\phi$) of each precessing NMR signal increases as a function of $t_1$. It is this phase angle that determines how much magnetization remains on the $y$-axis. Only the magnetization for the first spin (spin $A$) will be considered for simplicity. The second arrow has no effect since it involves the operators of spin $B$ only. The evolution of the system during $t_1$ can be described for each spin by:

$$-I^A_y \quad \xrightarrow{\text{evolution time } (t_1)} \quad I^A_y \cos(\phi t_1) + I^A_y \sin(\phi t_1)$$  \hspace{1cm} (2-21)

The second ($\pi/2$) pulse then rotates the first term of Eq. (2-21) into the $z$-direction, creating a $z$-magnetization that has a cosine dependence on $\phi$. As shown below, the second pulse leaves the sine term unaffected:
The longitudinal magnetization evolves under two effects: $T_1$ relaxation and chemical exchange. The amplitude of the signal recorded during $t_2$ is dependent on both $\tau_m$ and the efficiency of magnetization transfer; the $z$-magnetization is proportional to the frequency $\phi$ and $t_1$ via the cosine term. If spin $A$ undergoes chemical exchange with spin $B$ during the mixing time, it carries with it the frequency label acquired during $t_1$. The effect of the mixing process is given by:

$$
-I_z^A \cos(\phi, t_1) \xrightarrow{(\pi/2), t_1^\phi} \xrightarrow{(\pi/2), t_2^\phi} -I_z^A \cos(\phi, t_1)
$$

(2-22)

$$
I_z^A \sin(\phi, t_1) \xrightarrow{(\pi/2), t_1^\phi} \xrightarrow{(\pi/2), t_2^\phi} I_z^A \sin(\phi, t_1)
$$

(2-23)

The final pulse creates the magnetization in the transverse plane which is ultimately detected during $t_2$. The $t_1$ value is sequentially increased to form the second chemical shift axis, while $t_2$ is the acquisition time. The 2D spectrum is a result of a double Fourier transformation in the $t_1$ and $t_2$ time domains. It is the $\cos(\phi t_2)$ term that modulates the relative size of the peaks at $f_1$ and $f_2$. For a simple, first-order two-site exchange process with equal relaxation rates and relative populations between sites (thermally polarized experiment); the exchange rate can be determined
Figure 2-4. An example of 2D EXSY spectra. A) A spin \( A \) in \( t_1 \) that is transformed into a \( B \) spin during \( t_2 \) will have the coordinates \(( \omega_A, \omega_B )\). A cross peak would also appear at \(( \omega_B, \omega_A )\) in the event that exchange proceeds in both directions simultaneously. B) A spin that remains unchanged during the experiment will have the same frequency coordinates in both dimensions, denoted by the diagonalized peaks \(( \omega_A, \omega_A )\) and \(( \omega_B, \omega_B )\).

directly by comparing the diagonal and cross-peak intensities. The cross-peak intensities reflect cross-relaxation, while the intensities of the diagonal peaks reflect the characteristic relaxation behavior of the nuclei in each phase. They are sensitive to phenomena that contributes to the magnetization transfer during the mixing time (e.g., relaxation processes).

**Continuous and interrupted flow experiment.** Long relaxation times and low densities of thermally polarized samples often limit the applicability of 2D EXSY to kinetic studies due to long acquisition times. Kinetic models exist that permit the extraction of relaxation and kinetic parameters from a 2D EXSY spectrum acquired at a single mixing time.\(^{[53, 54]}\) However, more accurate results can be obtained by acquiring a multiple spectra as a function of mixing time.\(^{[53, 55]}\) Recent studies have provided the analytical expressions pertinent to the application and interpretation of hyper-polarized \(^{129}\)Xe 2D EXSY.\(^{[52, 56]}\) Bowers and Cheng (2007) presented two modified 2D EXSY pulse sequences. The first sequence is for continuous flow experiments in which a stream of hyper-polarized gas circulates through the sample, continuously replenishing the hyper-polarized gas in the sample region (Figure 2-3B). The second pulse
sequence describes the interrupted flow technique wherein the gas flow is stopped for a short
time ($\tau_2$) right before the 2D EXSY sequence. This has been shown to significantly increase the
signal-to-noise ratio. Interrupting the flow during the mixing time gives the probe molecule
sufficient time to desorb from its adsorption site and accumulate in the coil region for detection.
A schematic of the interrupted flow pulse sequence is provided in Figure 2-3C for visualization.

Assuming a steady-state, two-site exchange process under continuous flow conditions, the
theoretical values of the diagonalized ($I_{AA}, I_{BB}$) intensities are expressed as:

$$I_{AA}(\tau_1, \tau_m) = I_{A0} \exp \left[ -\tau_m \left( \frac{1}{\tau_R} + k_d \frac{n_B\theta}{n_A} \right) \right]$$  \hspace{0.5cm} (2-26)

$$I_{BB}(\tau_1, \tau_m) = I_{B0} e^{-k_d\tau_m}$$  \hspace{0.5cm} (2-27)

and the cross-peak ($I_{AB}, I_{BA}$) intensities are given by:

$$I_{AB}(\tau_1, \tau_m) = I_{A0} \frac{k_d}{\tau_R^{-1} - k_d} \frac{n_B\theta}{n_A} \left( e^{-k_d\tau_m} - e^{-\tau_m/\tau_R} \right)$$  \hspace{0.5cm} (2-28)

$$I_{BA}(\tau_1, \tau_m) \approx I_{B0} \frac{k_d}{\tau_R^{-1} - k_d} \left( e^{-k_d\tau_m} - e^{-\tau_m/\tau_R} \right)$$  \hspace{0.5cm} (2-29)

where, $A$ is the $^{129}$Xe gas phase environment and $B$ is the nanotube environment; $n_B/n_A$ denotes
the ratio of total adsorption sites to gas atoms, $k_d$ is the desorption rate constant, $\tau_R$, the gas
residence time (inversely proportional to the gas flow rate), $I_{A0}$ and $I_{B0}$ are the initial
magnetizations in each respective phase at $\tau_m = 0$, while $\theta$ is the fraction of occupied sites.
According to Eq. (2-28) and (2-29), the cross-peaks are likely to have unequal amplitudes.

2.3 Introduction to $^{129}$Xe NMR

2.3.1 The $^{129}$Xe Chemical Shift

The chemical shift of $^{129}$Xe is approximately 2 orders of magnitude larger than of $^1$H (7500
ppm versus 20 ppm) and is a sensitive function of configuration of atoms that make up a physical
system. The observed $^{129}$Xe-NMR chemical shift is generally expressed as a weighted average of these configurations according to its relative adsorption probability. As such, the intermolecular contributions to the chemical shift depend on the distance and lifetime between xenon and the individual atoms that constitute the absorption sites. While highly fluid, lipid membranes possess mechanical properties similar to solid substrates. It is important to understand the potential intermolecular interactions that contribute to the observed $^{129}$Xe chemical shift in porous solids and bulk solutions.

**Xenon adsorbed onto solid surfaces.** Let us consider a solid surface that consists of chemically different target sites ($S_i$) available for xenon adsorption. The observed $^{129}$Xe NMR spectrum will not only depend on the distribution of target sites in the sample, but the relative lifetime xenon spends at each adsorption site as well.[57] Theoretically, if the lifetime of xenon on each surface site is long, the resulting $^{129}$Xe NMR spectrum would contain as many chemical shifts as there are target types ($\delta_i$); the population at each site would be proportional to its spectral intensity. However, if the lifetime of xenon at these absorption sites is short, the rapid diffusion between environments (surface and pores) will cause the intrinsic shifts of each site to coalesce, making it very difficult to extract any information about the system. If the distribution is homogenous, the observed chemical shift is then given by the following expression:

$$\delta_{\text{obs}} = \sum_{i=1}^{m} \alpha_i \cdot \delta_i$$  \hspace{1cm} (2-30)

The characteristic shift and probabilities corresponding to each Xe-$S_i$ are denoted by $\delta_i$ and $\alpha_i$, respectively.

According to studies by Fraissard et al. (1988) the chemical shift of the adsorbed xenon can be written as follows:[58]
\[
(\delta - \delta_o) = \delta_S + \delta_{Xe} + \delta_{SAS} + \delta_E + \delta_M
\]  

(2-31)

\(\delta_o\) is the gas reference at zero pressure; \(\delta_S\) denotes the contribution from the \(Xe-S_i\) interactions and reflects the geometry of the \(Xe\) geometry at the surface; the \(\delta_{Xe}\) contribution reflects \(Xe-Xe\) collisions (proportional to the local xenon density); \(\delta_{SAS}\) arises from interactions between xenon and strong adsorption sites (\(SAS\)); while \(\delta_E\) and \(\delta_M\) express the difference in the electric and magnetic fields due to the presence of charged ions. Study of the different terms of Eq. (2-31) has been helpful in revealing structural defects; determining the dimensions and internal volumes; and determining the porosity of new mesoporous solids.

**Xenon dissolved into isotropic solutions.** Often dubbed the ‘medium effect’, the total shielding of a solute molecule (relative to a reference shielding: \(\sigma_o\)) due to solute-solvent interactions can be expressed as a sum of several terms corresponding to various perturbations:

\[
(\sigma - \sigma_o) = \sigma_v + \sigma_r + \sigma_b + \sigma_a + \sigma_E + \sigma_{E'}
\]  

(2-32)

where, \(\sigma_v\) denotes the van der Waals dispersion interaction; \(\sigma_r\), the repulsive interaction, \(\sigma_b\), bulk susceptibility effects; \(\sigma_a\), the magnetic anisotropy of neighboring solvent molecules; \(\sigma_E\), reaction field induced by the permanent electric moment of the solute; and \(\sigma_{E'}^2\), the contribution due to the permanent electric dipole moment of the solvent. This expression is similar to that found in Eq. (2-31). However, both the \(\sigma_E\) and \(\sigma_{E'}^2\) contributions can be neglected due to the lack of and relatively weak permanent dipole moments of xenon and polar solvents, respectively. What’s more, shifts arising from bulk susceptibility and solvent magnetic anisotropy have been estimated to be under 2 ppm in value; this is negligible compared to the \(^{129}\)Xe NMR shifts typically observed in polymer solutions (200 ppm). Thus, the dominant contributions to the \(^{129}\)Xe chemical shift are from van der Waals dispersion and repulsive interactions.
**Effects of exchange.** According to the fast exchange model proposed by Liu et al. (1994), the chemical shift can be expressed as follows:[59]

\[ \delta_{\text{obs}} = f_d \cdot \delta_d + f_{id} \cdot \delta_{id} + f_g \cdot \delta_g \]  

(2-33)

for xenon adsorbed onto at least two, distinct environments (i.e., direct and indirect adsorption sites). \( f_d, f_{id}, \) and \( f_g \) are the fractions of xenon adsorbed directly (\( d \)), indirectly (\( id \)), and gaseous xenon (\( g \)), while \( \delta_d, \delta_{id}, \) and \( \delta_g \) are their corresponding intrinsic chemical shifts. The \( f_g \cdot \delta_g \) contribution was found to be less than one percent, resulting in the following simplification of Eq. (2-33):

\[ \delta_{\text{obs}} \approx (1 - f_{id}) \cdot \delta_d + f_{id} \cdot \delta_{id} \]  

(2-34)

Here, the fraction of indirectly adsorbed xenon (\( f_{id} \)) is proportional to the fractional coverage of the zeolite surface, and can be expressed as:

\[ f_{id} = \beta_1 \left( \frac{N}{N_S} \right) + \beta_2 \left( \frac{N}{N_S} \right)^2 + ... \]  

(2-35)

where \( \beta_1 \) and \( \beta_2 \) are the expansion coefficients, \( N \) is the number of adsorbed \( Xe \) atoms, and \( N_S \) is the total number of available sites per zeolite supercage. Liu et al. (1994) also introduced a virial expansion model in which \( \delta_d \) and \( \delta_{id} \) are a function of \( N \).[59] Fitting experimental values to:

\[ \delta_{\text{obs}} (N) = \delta_o + C_1 \cdot N + C_2 \cdot N^2 + ... \]  

(2-36)

provides information on the adsorption strength via \( C_1 \) and \( C_2 \); \( \delta_o \) denotes the effect of the zeolite surface on \( \delta_d \) in the absence of \( Xe-Xe \) interactions. More specifically, the two virial coefficients \( C_1 \) and \( C_2 \) represent coefficients of binary and three particle \( Xe-Xe \) interactions, respectively. However, the adsorption strength must be considered as a weighted average of the two adsorption sites in order to distinguish between the directly and indirectly adsorbed \( Xe \) atoms; there is no experimental method available that allows for direct measurement of the adsorption...
strength between the two. This particular method appears to provide a meaningful quantitative
description of a large number of systems—as such, we hope to use a similar formalism to
differentiate between $^{129}$Xe binding at the membrane-water interface surface and the lipid core.

2.3.2 Alkali Metal-Noble Gas Spin-Exchange Optical Pumping (SEOP)

Spin-exchange optical pumping is a two step process in which the angular momentum
from an alkali valence electron is transferred to the nucleus of a rare gas atom. This can result in
$^{129}$Xe NMR signal intensity three or four orders of magnitude higher than its thermal equilibrium
value. As mentioned previously, this is a non-equilibrium method of polarization enhancement
which involves an imbalance in the atomic ground states via circularly polarized light. Here we
present a brief, qualitative description of the optical pumping process necessary to understand the
SEOP enhancement of the $^{129}$Xe nucleus. A more in-depth look at this process is provided in
literature—it has been studied and reviewed extensively.

Optical pumping. The first step in the polarization process is the optical pumping of an
alkali metal, which uses the electronic excitation of atoms by light to transfer angular momentum
from photons to atoms. With respect to hyperpolarized gas, the most successful methods make
use of simple alkali metals. Rubidium ($Rb$) is the common alkali metal of choice for several
reasons: its low melting point and high vapor pressure ensures a high atom density at
temperatures less than 473 K, and the wavelength of its electronic transition is within range of
many commercially available, tunable light sources. Rubidium has two naturally occurring
isotopes: $^{85}Rb$ (72.2% naturally abundant, $I = 5/2$), and $^{87}Rb$ (27.8% naturally abundant, $I = 3/2$).

The electronic ground state of $^2S_{1/2}$ is split into two states, in accordance with the
previously described Zeeman splitting. A given excited level can be populated by irradiating $Rb$
metal vapor with circularly polarized light whose frequency corresponds to the absorption line of
the atom at the ground state. The rubidium $D_1$ line for $^{87}Rb$ occurs at 794 nm and is displayed
Figure 2-5. The energy level diagram of $^{87}\text{Rb}$ showing the hyperfine structures for the $D_1$ transition. $\vec{F}$ is the total angular momentum quantum number, $\vec{I}$ is the Rb nuclear spin, $\vec{J}$ is the Rb electron spin. The magnetic sublevels ($m_\text{F}$) are separated by the Zeeman interaction.

Schematically in Figure 2-5. Light is scattered by only one of the $S$ multiplet, provided that it is tuned to the lowest energy transition ($^{2}\text{S}_{1/2} \rightarrow ^{2}\text{P}_{1/2}$) and the light source is sufficiently narrow to avoid the excitation of other states. Selections rules ($\Delta m_\text{F} = \pm 1$) dictate that spin-down state can only adsorb right-circularly polarized light ($\sigma^-)$, and spin-up states left-circularly polarized light ($\sigma^+$). For example, continuous irradiation of $\sigma^+$ light only excites atoms from the spin-down sublevel of $^{2}\text{S}_{1/2}$ to the spin-up sublevel of $^{2}\text{P}_{1/2}$.

Once excited, the sublevel populations in ($^{2}\text{P}_{1/2}$)are randomized by collisional mixing (Figure 2-6). Thus the electron has equal probability of decaying into either of the ground states; excited electrons are relaxed by fluorescence or non-radiative pathways. Non-radiative
quenching of the excited $^{87}\text{Rb}$ in the presence of nitrogen and helium gas allows the electrons to return to the ground state without releasing depolarizing radiation. And so, continued irradiation of light repeats the excitation/relaxation cycle until the electrons reach a spin state with no allowable transitions. These trapped electrons are what give rise to Rubidium’s net polarization.

**Spin-exchange.** The second step of SEOP involves the transfer of spin between interacting particles. The spin exchange process between the optically pumped rubidium and $^{129}\text{Xe}$ is based on collisional polarization transfer and can occur in one of two ways: through simple binary collisions, or as short-lived, three body van der Waals molecules described by Eqs. (2-37) and (2-38), respectively.

$$Rb_\uparrow + Xe_\downarrow \rightarrow Rb_\downarrow + Xe_\uparrow \quad (2-37)$$

$$Rb_\uparrow + Xe_\downarrow + N_2 \rightarrow Rb_\downarrow Xe_\uparrow N_2 \rightarrow Rb_\downarrow Xe_\uparrow N_2 \rightarrow Rb_\downarrow + Xe_\uparrow + N_2 \quad (2-38)$$

The specific number and kind of collisions is determined by the gas pressure in the pumping cell; binary complexes dominate under high pressure conditions, while three body van der Waals molecules are more likely at low gas pressures (see Figure 2-7). Spin exchange occurs during the lifetime of the van der Waals pair and the relative lifetime of both complexes is limited by collisions with another buffer gas molecule (e.g., $N_2$). The Fermi contact interaction describes the hyperfine coupling that exists during the polarization transfer and is responsible for the spin exchange between electron and nuclear spins. The time evolution of the $^{129}\text{Xe}$ nuclear polarization can be expressed by:

$$P_{\text{Xe}}(t) = \frac{\gamma_{SE}}{\gamma_{SE} + \Delta \gamma_1} \cdot P_{\text{Rb}_\uparrow} \cdot \left[1 - e^{-(\gamma_{SE} + \Delta \gamma_1)t}\right] \quad (2-39)$$

where $\Delta \gamma_1$ is the relaxation rate of $^{129}\text{Xe}$, and $\gamma_{SE}$ is the rubidium-$^{129}\text{Xe}$ exchange rate. As shown in Eq. (2-40), $\gamma_{SE}$ is dependent only on the rubidium concentration and velocity averaged cross
Figure 2-6. Optical pumping for transitions from the ground state \( F = 2 \) level to the excited states in \(^{87}\text{Rb}\). The red line shows absorption of \( \sigma^+ \) photons while the orange, purple and blue lines show decays from each of the \(^5\text{P}_{1/2}\) magnetic sublevels. All ground state sublevels are depopulated by absorption, except for the \( m = +1/2 \) level where the population builds up.

Figure 2-7. A schematic illustration of the polarization of \(^{129}\text{Xe}\) nuclei via collision and spin exchange. A) Binary complexes form at high gas pressure. B) Three-bodied complexes between \( \text{Rb}, \text{Xe}, \) and \( \text{N}_2 \) gas occur at low pressures.
section ($\langle \nu \sigma \rangle_{Rb-Xe}$) in binary spin exchange; it is determined by the temperature dependent vapor pressure of the rubidium.

$$\gamma_{SE} = \left( \langle \nu \sigma \rangle_{Rb-Xe} + \frac{\gamma_M \zeta}{[Xe]} \right) [Rb]$$  \hspace{1cm} (2-40)

$\gamma_M$ is a constant that depends on the rate of van der Waals molecule formation and contact interaction strength between rubidium and $^{129}$Xe; $\zeta$ is a constant that depends on the polarization and isotopic composition of rubidium. The average achievable polarization, as a function of the optical pumping ($\gamma_{CP}$) and spin relaxation ($\Gamma_{SD}$) rates can be written as:

$$P_{Rb} = \frac{\gamma_{OP}}{\gamma_{OP} + \Gamma_{SD}}$$  \hspace{1cm} (2-41)

The rate of rubidium optical pumping is dependent on the time spent in the optical pumping cell and the temperature therein, the laser power, the rubidium adsorption lineshape, and the frequency overlap of the laser output. The spin relaxation rate in the interior of the pumping cell is determined by collisions between alkali-alkali metals and alkali-buffer gas molecules, as well as the strength of the external magnetic field.

The spin relaxation rate, $\Gamma_{SD}$ is the sum of the relaxation rates from all relaxation mechanisms. The main contributor to the spin relaxation of rubidium comes from the $Rb-X$ spin rotation interaction; $X$ denotes a specific nucleus such as $Xe$, $N_2$, or another $Rb$ atom. The relaxation rates due to each relaxation collision process are given in Eqs. (2-42)-(2-44), the sum of which gives the projected value of $\Gamma_{SD}$.

$$\Gamma_{Rb-Rb} = [Rb] \cdot \langle \nu \sigma \rangle_{Rb-Rb}$$  \hspace{1cm} (2-42)

$$\Gamma_{Rb-N_2} = [N_2] \cdot \langle \nu \sigma \rangle_{Rb-N_2}$$  \hspace{1cm} (2-43)

$$\Gamma_{Rb-Xe} = [Xe] \cdot \langle \nu \sigma \rangle_{Rb-Xe}$$  \hspace{1cm} (2-44)
\[
\Gamma_{SD} = \Gamma_{Rb-Rb} + \Gamma_{Rb-N_2} + \Gamma_{Rb-Xe} + \Gamma_{\text{other}}
\] (2-45)

The brackets denote the relative concentrations of each nuclei while \(\langle v_\sigma \rangle_{Rb-Xe}\) is the velocity averaged relaxation cross section for each \(Rb-X\) pair. Collisions with the cell wall and magnetic field inhomogeneity also lead to relaxation, details of which are discussed elsewhere.

2.3.3 Recent Improvements to Gas Delivery System

The gas handling system was redesigned due to severe leaks in the previous apparatus. The current system was designed to suit the needs of various types of hyperpolarized experiments which include continuous flow, direct expansion, and interrupted flow techniques. The schematic design is presented in Figure 2-8. The ballast tank was reincorporated to expand the total volume (2.0 Liters) of the system if needed; it is particularly useful for experiments requiring the condensation of hyperpolarized \(^{129}\)Xe liquid or solid. Consistent with previous designs, the gas flow was generated by a magnetically coupled gas recirculation pump (Model 51429, Thomas Ind, Sheboygan, WS) while the flow rate is controlled inline by a combination needle valve and variable area flowmeter (Model U-03217-06, Cole-Palmer, Vernon Hills, IL). The previously existing PFA tubing within the recirculation loop was replaced with copper tubing to reduce the risk of leaks in the system; copper ferrules were placed on copper tubing and PFA ferrules on PFA tubing.

To begin, a gas mixture of specified pressure is introduced to the system from one or more of the following sources: \(GS_1\), \(GS_2\), \(GS_3\). The various gas lines allow for custom mixing between different source gases, if desired. The gas output is then passed through an oxygen trap (Model 4002, Alltech Associates Inc, Deerfield, IL) and an inert gas purifier (Model 35K F-I-4R, Aeronex Inc. San Diego, CA) to reduce the amount of impurities in the gas before it is loaded into the pumping cell (not shown). Numerous valves are in place to help control the
Figure 2-8. Schematic drawing depicting the redesigned gas delivery system. GS1, GS2, GS3: gas sources 1,2,3; P: pressure gauge; RV: relief valve; V: needle valve; S: normally open solenoid valve; BP: bypass; \( \Rightarrow \): throughput valve; \( \square \): oxy-trap; GP: gas purifier; \( \blacklozenge \): recirculation pump; \( \square \): three-way valve; \( \circ \): needle valve.
expansion of the gas and the pressure is monitored using a Baratron pressure transducer (Model 722A, MKS Instruments, Andover, MA) near the input of the pumping cell. In the closed-cycle, recirculation mode, three-way valves (B-42XS4, Swagelok, Solon, OH) control whether the sample probe is bypassed or not. We find them to be useful when building up polarization via recirculation methods and when changing the NMR samples.

For clarification, the gas handling system is mounted onto an aluminum table built on the top of optical pumping system which consists of the optical polarizer, pumping cell and Helmholtz pairs. Six stainless steel supports were introduced to provide additional stability to the mounted apparatus (Figure 2-8) and create a secure frame for the laser curtain. The relative distance between the gas handling system and the 9.4 Tesla NMR magnet remained unchanged (approximately two feet) in order to prevent depolarization of hyperpolarized $^{129}$Xe gas during the transport process. Specific details of optical pumping apparatus can be found in Zook et al. (2002).\(^{60}\)
CHAPTER 3
THE LIPID ENVIRONMENT

3.1 Introduction

The cellular membrane is a semi-permeable network of glycerophospholipids, sterols and proteins. On average, a single mammalian cell is 5 nm thick and possesses an average diameter of only 20 μm. Its structural integrity is maintained primarily through non-covalent interactions between proteins and lipid molecules. Despite its small size and apparent fragility, the cellular structure is highly flexible and mechanically stable. This is largely attributed to the amphipathic nature of phospholipids and the vast number and variety of lipid species that help compose it. In fact, cells are able to control the molecular transport properties through the membrane by modifying its lipid content; changes in membrane fluidity and solvation allow the cell to regulate its functions. Biomembrane composition differs between species and can even vary between organelles within the same cell. While the actual role the lipid membrane and its heterogeneity play in common biological processes are unclear, it is increasingly apparent that lipid biophysics contributes to a large number of cellular processes.[61-64] As stated in our general introduction, our specific interests lie in elucidating the role of lipid membranes in anesthetic action. The following chapter will introduce the readers to the general structural and chemical properties of lipids—the foundation on which my dissertation research is based. Experimental results on the characterization of our lipid systems are also provided as several techniques were employed.

3.2 Lipid Structure and Assemblies

3.2.1 What Makes a Lipid?

Phospholipids are by far the most common type of lipid in the cell membrane, though what actually constitutes a lipid loosely defined. Easily distinguished by its structure, phospholipids typically possess i) a phosphate containing headgroup, ii) a semi polar backbone
Table 3-1. Approximate percentage by weight of total lipid in several cell membranes. Lipid types are characterized by the composition of their headgroup region. This is an adapted table.\cite{65}

<table>
<thead>
<tr>
<th>Membrane/Lipid</th>
<th>PC</th>
<th>PE</th>
<th>PS</th>
<th>Glycolipids</th>
<th>Sphingomyelin</th>
<th>Cholesterol</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human erythrocyte</td>
<td>17</td>
<td>18</td>
<td>7</td>
<td>3</td>
<td>18</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>Rat liver plasma</td>
<td>24</td>
<td>7</td>
<td>4</td>
<td>7</td>
<td>19</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>Myelin</td>
<td>11</td>
<td>17</td>
<td>9</td>
<td>20</td>
<td>8</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>Neurons</td>
<td>48</td>
<td>21</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>11</td>
<td>8</td>
</tr>
</tbody>
</table>

and iii) two fatty acid chains. These three regions are illustrated for several lipid species in Figure 3-1 for visualization. Phospholipids are different from sphingomyelins and glycolipids in that they possess a glycerol instead of a sphingosine backbone. The fatty acid chain can vary both in length (12-24 carbons) and degree of unsaturation; modifications in either have large consequence on the membrane’s overall fluidity. For clarification, a saturated acyl chain consists of a singly bound carbon chain while an unsaturated lipid has one or more double bonds. It should be noted that this region is largely apolar.

As mentioned previously, the lipid composition can vary considerably between species (Table 3-1). A simple modification in the headgroup region can give rise to distinct changes in the membrane’s chemical and physical environment. Contrary to the hydrocarbon region, this segment of the lipid molecule is distinctly polar. Two of the most commonly found phospholipids are the zwitterionic (no net charge) phosphatidylcholine (PC) and phosphatidylethanolamine (PE). These particular lipids will be discussed in more detail in subsequent chapters. The ‘other’ lipids listed in Table 3-1 appear in lesser quantities within the cell membrane. And while cholesterol is a primary ingredient of the lipid membrane, it is a lipid
steroid whose chemical structure is unique unto itself. Unlike other lipids, cholesterol is unable to form membranes on its own due to its pronounced rigid structure.

3.2.2 The Bilayer Phase and Self-Assembly

The greatest attribute the phospholipid brings to the cell membrane is its amphiphilicity. One part of the lipid molecule is polar (the headgroup) and thus water soluble, while the other is not. The apolar hydrocarbon chain makes it characteristically hydrophobic and only moderately soluble. When lipid molecules are placed into polar solutions they tend to aggregate—forming self-assembled structures in water. This process is often referred to as the hydrophobic effect. Contrary to common thought, hydrophobicity does not indicate repulsion between the hydrophobic molecule and water. Rather, the non-polar parts of a molecule aggregate with each other to exclude water molecules; the solubility properties are driven by an entropic effect. The water molecules that would surround the hydrocarbons can do so only at the cost of decreasing the entropy of the water shell structure, thus making it more ordered. According to the second law of thermodynamics, the more favored process would be that which increases the entropy of the water structure. This is achieved through the self aggregation of the hydrocarbons, effectively freeing the water molecules. Electrostatic interactions, hydrogen bonding, and van der Waals and dispersion forces also contribute to the effectiveness of lipid self-assembly.

The prominent self assembled lipid structure is the bilayer. We have already established that it is energetically favorable for the hydrocarbon chains to aggregate. This can be doubly effective if the acyl chains form their own domain, effectively sheltering it from the polar exterior and the hydrophilic nature of the headgroup region. This double layer of lipids possesses both mechanical and electrical properties and is viscous and permeable. It is for these reasons the bilayer is described as a one dimensional solid, two dimensional liquid structure. A pictorial representation of the bilayer membrane environment is provided in Figure 3-2. Lipid molecules
diffuse laterally though the membrane plane and occasionally flip-flop between the inner and outer leaflets. As biological membranes are notoriously complex systems, artificial cells are often tailored for more controlled studies in a bilayer setting.
A large variety of artificial membranes can be made from amphiphilic molecules for laboratory use. Commercial lipids normally come dissolved in an organic solvent, but once the lipid is dry, it can spontaneously assemble in water to form lipid bilayers. These model membranes, also known as liposomes, provide simple imitations of biological membranes and cells. Liposomes have been used in the study of lipid-lipid, lipid-protein interactions as well as shape transformations, transport and elasticity.\cite{68, 69} These model membranes form vesicles with an enclosed aqueous center. As mentioned previously, they can be tailor made to mimic the compositions of cellular membranes. The physical properties underlying the various shapes and topological transformations of lipid membranes at variable environmental conditions often affect lipid membrane properties and functions.\cite{70-72}

### 3.2.3 The Shape Concept of Lipid Polymorphism

While most phospholipids possess a bilayer structure, some adopt alternate morphologies.\cite{73} The subtle balance between favorable and unfavorable interactions at the hydrophilic-hydrophobic lipid interface allows for the stabilization of all lipid membrane architectures.\cite{74, 75} The importance of the dynamics at this boundary has been extensively reviewed and has shown that the relationship between the molecular area and molecular shape contributes largely to the overall intrinsic properties of the lipid membrane.\cite{76, 77} The shape-structure concept of lipid polymorphism is a simplified way to view and predict preferred lipid structure. By comparing the cross sectional area of the lipid headgroup to that of the hydrocarbon chain region one can determine the average molecular shape via the packing parameter.

The geometric packing parameter ($P$) is a dimensionless quantity used to characterize the preferred shape a lipid will possess at set environmental conditions.\cite{78} According to Israelachvili (1991),\cite{67} $P = \nu/\alpha d l_c$, where $\nu$ is the average volume of the entire molecule, $l_c$ is the approximate
length, and $a_o$ the average area of the polar head group at the lipid-water interface. For a bilayer structure (i.e. phase), lipids have an overall cylindrical shape, having zero intrinsic curvature and a packing parameter is equal to one (e.g. PC). However, when the average area of the phospholipid headgroup is much smaller than that of the acyl chains, inverted curved assemblies are preferred, consistent with $P > 1$ and negative intrinsic curvature (e.g. PE). When $P < 1$, the headgroup region is large relative to the total volume of the molecule and micelle-like phases are formed, possessing positive intrinsic curvature (e.g. lyso-PC). See Figure 3-2 for pictorial representation.

<table>
<thead>
<tr>
<th>Packing Parameter</th>
<th>$P&lt;1$</th>
<th>$P=0$</th>
<th>$P&gt;0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean curvature</td>
<td>positive</td>
<td>zero</td>
<td>negative</td>
</tr>
</tbody>
</table>

**Figure 3-2.** The geometric packing parameter as a function of lipid type. The intrinsic geometry of the lipid structure changes with the packing parameter. The positive and negative curvature arising from the nonbilayer lipids are thought to play a part in membrane transport dynamics and protein function. This figure has been modified from Shearman et al. (2003).

The packing parameter can be used to predict mean intrinsic curvature for various lipid structures having singular geometry. It has also been shown that the mean curvature of lipid mixtures can be expressed in terms of $P$, allowing for interpretations changing lipid membrane morphology as a function of temperature and composition. To recap, each lipid has an intrinsic molecular shape. This shape-concept allows for the prediction of spontaneous curvature,
which is fixed for set environmental conditions. However, small changes in any of those conditions (e.g., acyl chain length and/or unsaturation, headgroup type, pH, temperature, hydration, osmotic pressure, solution composition) can result in a transition between lipid phases as the preferred geometry and/or curvature is altered.

3.3 Physical and Chemical Properties of Lipid Membranes

3.3.1 Interfacial Tension and the Lateral Pressure Profile

At equilibrium, the lipid membrane is a balance act between the attractive and repulsive forces between its headgroups and acyl chains.[82] The lateral pressure profile describes the distribution of lateral stress in the membrane created by the conformational disorder and lateral repulsion existing in the in the apolar and polar lipid regions, respectively (see Figure 3-1). It is not homogenous throughout the membrane and varies with bilayer depth.[83-85] These forces are balanced by the surface tension, which lies at the phospholipid headgroup/acyl chain interface. Its existence is a direct consequence of the hydrophobic effect—the nonpolar solvent/acyl chain interaction is highly unfavorable. The surface tension is largely dependent on the lipid packing and thus changes with lipid type. This in turn can affect lipid membrane permeability,[86, 87] thus has a direct consequence on partitioning. It is this interfacial phase that makes partitioning between water-lipid systems fundamentally different from typical two-phase partitioning.[88]

The possible effects of lipid type on the lateral pressure profile and it subsequent role in protein dynamics was first introduced by Cantor.[84, 89] He proposed that the conformational balance of integral membrane proteins and anesthetic action are both affected by changes in the lateral pressure profile.[90] If we compare two lipid types, one with a packing parameter $P > 1$, the other having a $P < 1$, the cone shaped lipid (e.g. PE) is more likely to have its pressure dominated by stress in the hydrocarbon core, while a bilayer containing micellular lipids ($P < 1$) is more likely have stress nearer the interface. When incorporated in a bilayer environment, these
lipids introduce a variety of stress which may result in interdigitation at the membrane surface. Biomembranes often form self-assembled micro-domains (lipid rafts) as a way of balancing the additional strain.[91]

### 3.3.2 Lyotropic versus Thermotropic Phase Transitions

It is energetically possible to inhibit the formation of a lipid’s intrinsic curvature by forcing the lipids to remain in the lamellar phase.[92, 93] The most common example of this particular kind of liposome is a PC/PE containing lipid mixture, often referred to as a frustrated bilayer. A phase transition will occur between two dynamical states when the preferred morphology becomes more energetically favorable than the lipid bilayer arrangement. This can be brought about by modifying the environmental parameters of the lipid membrane, such as temperature and/or lipid composition. Lyotropic phase transitions are those stimulated by changes in temperature and concentration, while thermotropic transitions are those induced thermally.[94] Transitions that occur between phases of matter (gas, liquid, solid) take place at a specified temperature (e.g., melting point, boiling point). There are no intermediary phases—the molecule will exist in either the gas, liquid or solid phase. This is not so for phospholipids; it is not uncommon for several phase transitions between phases. These pre-transition states often have complex morphologies representative of deformations displayed in cellular functions.

Nonbilayer structures have been postulated to play an important role in membrane function; the formation of local regions of nonbilayer structures (i.e. lipid rafts) within the biomembrane is one such idea.[95-98] It has been proposed that transient formation of inverted structures is responsible for trans-bilayer transport of lipids and polar solutes, a possible precursor to membrane fusion. By affecting the membrane barrier and flexibility properties, the presence of nonbilayer lipids may indirectly affect protein function as well—they have been shown to increase the activity of numerous peripheral and integral membrane proteins.[99] In
addition, nonbilayer lipids can also influence protein folding and the conducting properties of channel forming peptides such as gramicidin.\textsuperscript{[100, 101]} It should be noted that no experimental methods are currently available that allow for direct measurement of the lateral pressure profile with sufficient precision. However, we can study the affect of lipid micro-domains on the partitioning of non-polar solutes. This issue will be addressed in more detail in Chapter 7.

3.4 Experimental Methods

3.4.1 Methods for Preparing and Characterizing Liposomes

Phospholipids DOPC and DOPE were obtained from Avanti Polar Lipids and used without further purification. Two types of lipid suspensions were prepared: large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs). MLVs can vary in size from 0.5 to 5 µm and often contain multiple non-spherical compartments and a non-uniform distribution of lipids, while LUVs are smaller (200-1000 nm), spherical in shape and possess a homogenous dispersal of lipids. Single component lipid suspensions were prepared as follows: 1) Lipids in organic solvent of predetermined volume were dried with a gentle stream of nitrogen, forming a thin film, then placed under vacuum to remove any residual solvent 2) a set amount of buffer solution (50 mM Hepes Buffer with 10% D\textsubscript{2}O) was added in appropriate amount, effectively hydrating the lipid vesicles and leading to the formation of MLVs, 3) these hydrated vesicles were then extruded—subjected to 7-40 complete passes through a 100 nm pore size polycarbonate filter, depending on the sample concentration. Once a single pass could be performed under a minute (and the sample appearance was as opaque as possible) one could be assured of homogenous size and distribution of lipids within solution. The extrusion methods lead to the formation of LUVs which were 100 nm in diameter. Lipid mixtures containing various mole percentages of DOPC and DOPE were produced along similar guidelines, only they were premixed in appropriate amounts while still in their organic solvent prior dehydration.
Figure 3-3. The effect of $^{31}\text{P}$ orientation and motion on the $^{31}\text{P}$ CSA for phosphodiester, representative of lipids in the membrane environment. A) A fully static spectrum in which all components are resolved due to limited motion. B) A typical powder pattern, having order with rapid axial rotation. C) The inverted hexagonal phase possessing additional disorder due to lateral diffusion around small water pores. HG denotes the oxygen (pink sphere) connected to the phospholipid headgroup region.

$^{31}\text{P}$ NMR is frequently used to probe the structural and dynamical properties of membranes. Its high natural abundance (100%) and presence in the headgroup region of each phospholipid molecule make it particularly useful in the study of lipid polymorphism. Despite its high sensitivity and large chemical shift range (100 ppm), the $^{31}\text{P}$ linewidth is often dominated by an unusually large chemical shift anisotropy which arises from the orientation dependence of the local nuclear structure. For example, the fast diffusion of small vesicles gives rise to isotropically averaged lineshapes; the more the motion is restricted, the larger the spectral broadening. In a completely static sample lipid sample (Figure 3-3A), each of the chemical shift tensors can be resolved (see Chapter 1). In the case of MLVs, the lipid rotation along its long axis (Figure 3-3B) is only partially averaged, leading to the characteristic powder pattern.
associated with the bilayer phase. The formation of nonbilayer phases is often monitored through
$^{31}$P NMR as they often result in radical changes in lipid motion (Figure 3-3C).

### 3.4.2 Verification of Vesicle Size through Dynamic Light Scattering

The particle size distribution was measured by dynamic light scattering on a PDDLS-Cool Batch +90T system (Precision Detectors, Bellingham, MA) tuned at a wavelength of 800 nm. All measurements were performed at 293 K using the viscosity and refractive index of water. The intensity autocorrelation was measured at a scattering angle of 90° and the distribution profiles were analyzed using the Precision Deconvolve software package. Newly prepared LUVs were placed in a quartz cuvette and subjected to ten separate measurements of the intensity correlation function. This technique measures particle diffusion due to Brownian motion. Differences in the solvent-vesicle interaction produce random motion of the vesicles in solution. DLS relates the diffusive properties of the liposomes to its particle size through the translational diffusion coefficient ($D$). If the viscosity ($\eta$) of the solution (i.e., water) is known, the Stoke-Einstein equation, Eq. (3-1), can be used to correlate the particle position decay; the smaller the particle, the faster the diffusion and decay.

\[
d(H) = \frac{k_B T}{3\pi \cdot \eta \cdot D}
\]  

Here, $d(H)$ is the hydrodynamic diameter, $k_B$ is Boltzmann’s constant, and $T$ is the absolute temperature. This hydrodynamic diameter is the diameter measured by the DLS via particle diffusion within a specified liquid. Ion concentration and type utilized in the experiment can potentially affect the vesicle diffusion speed if their presence change interfacial properties.

### 3.4.3 Phosphate Assays of Lipid Stock Solutions

The concentrations of the lipid stock solutions were determined through phosphate assay analysis. The lipid assay is based on phosphate content in the range of 1-100 nmole quantities of
lipid. This assay is dependent on the dephosphorylation of the lipid molecule by sulfuric acid (H$_2$SO$_4$). Once the phosphate ion is liberated and subsequently oxidized, a colored complex is formed which is quantified by absorbance spectrophotometry. To be specific, 25, 50, and 100 nmole (estimated) quantities of lipid were placed into separate glass tubes. Then the solvent was removed by evaporation using a stream of nitrogen gas. Six phosphorus standards were prepared for comparison, ranging from 0-0.0975 µmoles. H$_2$SO$_4$ was then added to all samples in order to free the phosphate ion. The samples were heated to 493 K to facilitate this process. Both the standards and samples were cooled to room temperature and mixed thoroughly via vortex prior to addition of the malachite green phosphate assay solution. After a brief incubation (10 minutes), the absorbance was read at 650 nm. All samples were prepared in triplicate to help determine error. Experimental results are presented as dilution-corrected averages of the 25, 50, and 100 nmole samples.

3.5 Results and Discussion

3.5.1 $^{31}$P NMR of Lipids

$^{31}$P NMR was performed on single component lipid samples of DOPC and DOPE. In addition to monitoring the lamellar-to-inverted hexagonal phase transition, $^{31}$P NMR was also used to verify the vesicle type in solution. As seen in Figure 3-4, aqueous suspensions of multilamellar vesicles gave rise to the characteristic powder pattern discussed in the previous sections. The broad, single lineshape observed in LUV samples is consequent of the higher vesicle mobility (Brownian motion) in solution. The full width at half-height (FWHH) for the LUV DOPC sample is much broader than those typically seen in micellar systems, which is expected. As discussed previously, the $^{31}$P NMR CSA is dependent on motional averaging of both the lipid molecule in its local environment and the macromolecular system in which it exists. For all intensive purposes, the DOPC lipid is confined to the bilayer, thus any averaging
of the $\sigma_{//}$ component of CSA must come from the diffusive nature of the vesicle. If the vesicle tumbles rapidly enough in solution, the anisotropy will reduce a single resonance according to $\sigma_{\text{iso}} = (\sigma_{11} + \sigma_{22} + \sigma_{33})/3$ or $(\sigma_{//} + \sigma_{\perp})/2$. The broader the resonance, the slower and larger the vesicle must be.

![31P NMR spectra](image)

**Figure 3-4.** The $^{31}$P NMR spectra of 50 mM DOPC for various lipid environments. A) Large unilamellar vesicle (LUVs) systems result in a broad isotropic peak. B) Multilamellar vesicles (MLVs) possess a characteristic powder pattern. The $\Delta \sigma$ between the $\sigma_{//}$ and $\sigma_{\perp}$ components determined to be 41 ppm, consistent with expected values. The presence of xenon gas had no noticeable effect on the $\Delta \sigma$ values or the shape of the MLV CSA.

### 3.5.2 Dynamic Light Scattering

We performed DLS on two separate concentrations of DOPC LUV solutions. Two samples of varying concentration (2 mM and 1 mM) were prepared using the extrusion method discussed above. The average vesicle diameter was calculated after twenty sequential runs. As seen in Figure 3-5, this gave a value of $117 \pm 20$ nm, which is within error of the 100 nm polycarbonate pore size used in the extruder. Also, the sharp slope of the correlation function is
consistent with the high degree of Brownian motion expected in vesicles of this size. Despite our interest to do so, we were unable to modify the apparatus to handle gas tight samples over the pressure range used in our studies. While modifications in bilayer thickness in the presence of anesthetics have yet to be observed, it would have been an interesting attempt. In addition, it would have allowed us to test for vesicle aggregation in the presence of anesthetic gas.

Figure 3-5. Dynamic light scattering data on a 2 mM DOPC LUV suspension. A) The volume distribution with respect to vesicle size. B) The correlation function associated with the 2 mM samples.

3.5.3 Phosphate Assay Analysis

The standard curve shown in Figure 3-5 was made by plotting the calibrated absorbance of the standard samples as a function of the relative nmoles of phosphate contained in each sample. This resulted in a nonlinear least squares fit, yielding a $R^2$ value of 0.984. The stock bottle concentrations were measured for both DOPC and DOPE and their dilution corrected values are provided in the table embedded in Figure 3-6. Approximately, 25, 50 and 100 nmol samples were measured in triplicate for each lipid sample. The dilution correction adjusts the 50 and 100 nmol values for comparison to the 25 nmol sample. This is the source of the ‘measured’ and ‘dilution corrected’ values found in Figure 3-6. The correlation ratio gives the relative
difference between the theoretical and experimentally determined values. As one can see, both concentrations of the stock solutions are within error of the predicted value. Thus, we can assume that no significant solvent evaporation has taken place and that our projected lipid concentrations are correct.

Figure 3-6. Experimentally determined values of phosphate assays. A) The titration curve of the phosphate standards. B) Extracted and dilution corrected concentration values for both DOPC and DOPE stock bottles.

### 3.6 Conclusions

From these results we can conclude that we have uniform dispersions of large unilamellar vesicles using the extrusion method described above. Both the DOPC vesicle size and the bilayer and inverted hexagonal phases have been confirmed through $^{31}$P NMR. Lastly, our projected lipid concentrations were confirmed through phosphate assay for both DOPE and DOPC stock solutions. While the phosphate assay was not performed on every lipid sample made, I am confident that in our projected lipid concentrations. Not only were the sample volumes incredibly large (1 ml) and easy to manage, the majority of the samples were prepared during the same time frame in which the phosphate assay was performed.
CHAPTER 4
CHARACTERIZATION OF XE-LIPID PARITIONING BY $^{129}$XE NMR

4.1 Introduction

Despite continued research on the pharmacological properties of general anesthetics, there is still no generally accepted molecular level mechanism.[102-105] Two prevalent theories exist: those that focus on membrane perturbation effects and those that point to specific protein activation sites. What is apparent is that accumulation of inhalational anesthetics in lipid membranes can alter their distributive properties and energetics. It is hypothesized that as anesthetic molecules partition into the biomembrane, changes in the lateral pressure profile ensue, indirectly altering membrane protein structure and function. Though it is well established that this dynamic process is largely controlled by interfacial properties, the actual modification of the surface tension at this boundary is not fully understood. Here, the inhalational anesthetic xenon (Xe) is used as a non-polar, weakly binding, spin-probe to investigate the anesthetic-lipid bilayer interaction by NMR spectroscopy. We use $^{129}$Xe NMR to study the interaction between xenon with dioleoylphosphotidyl-choline (DOPC) bilayers in several different vesicle morphologies.

Although $^{129}$Xe NMR has been used extensively in biological research, the basic kinetic and dynamic properties of the xenon-lipid membrane interaction have not been fully characterized. In this chapter, the xenon-DOPC interaction will be investigated though the Xe chemical shift. In addition to analyzing the sensitivity of $^{129}$Xe NMR to various types of DOPC vesicles, we extract the limiting chemical shift of $^{129}$Xe in DOPC using methods which are frequent in anesthetic binding studies. The limiting shift is then used to determine the buffer/lipid membrane partition coefficient. Obtaining a useful, reliable expression to extract pertinent thermodynamic and kinetic parameters is vital. The details presented in this chapter provide the
foundation for Chapters 5 and 6. We begin by providing a brief review of the uses of $^{129}$Xe gas in the study of biologically relevant media.

### 4.1.1 The Dissolution of $^{129}$Xe in Biological Media

As discussed in Chapter 1, of the prevailing hypothesis for anesthetic action involves perturbation effects experienced within the membrane due to anesthetic uptake. Anesthetics have been shown previously to depress the gel-to-lamellar phase transition temperature for various lipidic systems, $^{[106-110]}$ increase both membrane fluidity $^{[111, 112]}$ and surface area, $^{[113, 114]}$ and decrease acyl chain order. $^{[115-117]}$ While it is generally understood that the partitioning of small molecules has the potential to induce significant changes in the physical properties of the lipid membrane, it is still unclear if and how these translate to anesthesia. This is in part due to the low physiological concentration needed to induce anesthesia. However, computational studies as well as several experimental observations indicate that anesthetic molecules strongly influence the membrane surface tension. $^{[118, 119]}$ Thus, it has been recently proposed that an anesthetic’s intrinsic properties may mediate the redistribution of the lateral pressure profile, and in this way facilitate anesthetic action. $^{[84, 89]}$

Xenon is a potent anesthetic with NMR properties that make it well-suited to the study of the basic nature of the anesthetic-membrane interactions. $^{[7, 120-123]}$ Xenon gas is inherently nonpolar and chemically inert; it possesses distinct hydrophobic character and a large chemical shift range ($\approx 250$ ppm) when dissolved in simple liquids due to the high polarizability of its electronic cloud. Its relative high solubility in lipid rich media has led to a significant increase in its use in biological studies for lipid containing components. $^{[120, 124-126]}$ Enhanced sensitivity of $^{129}$Xe through spin exchange optical pumping has prompted creative technical advancements in gas delivery and experimental applications. $^{[127-129]}$
Recent studies have focused on designing efficient transport mechanisms to enhance $^{129}$Xe NMR/MRI signals in lipid rich domains in macromolecular biomedia (i.e., brain, lung). Laser-polarized $^{129}$Xe gas has been successfully delivered in vivo through both inhalation and vascular injection methods\cite{130} and has been used to study proteins in solution in an attempt to elucidate the site of anesthetic action.\cite{131} However, the high exchange rate of the xenon between two or more environments usually yields broad resonances without resolution of distinct binding sites. When an isolated xenon gas atom is dissolved in solution, its chemical shielding changes by several hundred ppm.\cite{132, 133} This gas-to-solution chemical shift, commonly dubbed the “medium effect”, is dominated by van der Waals dispersive and repulsive forces between the Xe atom and its surroundings. The interpretations of solvent shifts are typically based on the Onsager reaction field model which predicts the shift to vary linearly with the square of the function $f(n) = [(n^2 - I)/(2n^2 + I)]$, where $n$ is the refractive index of the pure solvent.\cite{134} Correlating the solvent shifts with the solubility parameter is another approach, wherein the shift is related to the cohesive energy of the solvent through its heat of vaporization and the molar volume. However, these models do not provide much information about the intermolecular interactions that give rise to these shifts.

4.1.2 Xenon and the Lipid Bilayer

Several published studies have examined the interaction of xenon in suspensions of PC vesicles of various compositions at room temperature using $^{129}$Xe and $^{131}$Xe NMR spectroscopy, with the intention of elucidating xenon’s anesthetic properties.\cite{10, 15, 123, 135, 136} It is impossible to assign a specific location of xenon within a micelle or single bilayer vesicle through direct observation of $^{129}$Xe NMR chemical shift due to fast exchange processes. The rapid exchange of xenon within the lipid phase and bulk solution generally results in a single broad resonance in the $^{129}$Xe spectrum.\cite{10, 137} However the dissolution of $^{129}$Xe into aqueous suspensions of multi-
lamellar vesicles (MLVs) often results in two distinct resonances; a broad peak representing the lipid environment is often accompanied by an additional narrow resonance associated with the solvent phase (for example, see Figure 4-1). The specifics of the exchange processes and the significance of vesicle type will be discussed in further detail below. Almost all $^{129}$Xe NMR studies in biomembranes employ MLVs since the signals representing the two phases, water and lipid, are fully resolved. Mohseni-Hosseini (1985)\[136\] did attempt to look at the size dependence of the vesicles on the gel-to–lamellar phase transition of several saturated lipids in fully hydrated solutions. By using sonication methods to create increasingly homogenous distributions of vesicle size in solution, a general trend emerged—if the vesicle size is too small, detection of the gel-to-lamellar is obscured.

One of the most informative studies of the xenon-lipid interaction was published by Xu and Tang (1997).\[14\] The intermolecular $^{129}$Xe- nuclear Overhauser effect (NOE) was utilized to determine the location of xenon in egg yolk PC (EPC). By selectively saturating different groups of protons within the lipid membrane and measuring the subsequent change in the $^{129}$Xe spectral intensity as a function of proton saturation time, the authors were able to determine the most probable location of the xenon from the build-up rate of the $^{129}$Xe-${^1}$H NOE. The effect of cholesterol was also investigated and shown to enhance xenon’s tendency to interact with the interfacial region of the model membrane. These results indicate that xenon interacts preferentially with the amphiphilic interface in PC lipid membrane, not the hydrocarbon core. The result was attributed to the high polarizability of the Xe atom. It was previously thought that an anesthetic’s hydrophobic nature would make the interaction with the acyl chain region more favorable, but it is now known that only strongly hydrophobic solutes (non-immobilizers)
partition into the membrane core.[138] Similar trends have been observed utilizing $^{19}$F NMR techniques when comparing the dissolution of fluorinated anesthetics and non-anesthetics.[16, 139]

4.2 Partition Model

4.2.1 Membrane-Buffer Partitioning

The ideal partition model is generally used to elucidate the small molecule-biomembrane interaction.[140] Often related to lipid solubility, partitioning describes the distribution of solute between two distinct phases. In a dilute solution, one can assume ideal mixing of the solute in each phase, allowing for the determination of thermodynamic parameters. Here, we relate partitioning theory to NMR fast exchange theory in order to further characterize the xenon-membrane interaction. The mole fraction partition coefficient is defined as the ratio of the equilibrium mole fraction of xenon in the lipid vesicle ($X_{\text{Xe,L}}$) to the equilibrium mole fraction of xenon in the bulk aqueous phase ($X_{\text{Xe,aq}}$); i.e.,

$$ K_p = \frac{X_{\text{Xe,L}}}{X_{\text{Xe,aq}}} \approx \frac{n_{\text{aq}}^{\text{Xe}} \cdot n_{\text{L}}^{\text{Xe}}}{n_{\text{aq}}^{\text{Xe}} \cdot (n_{\text{L}} + n_{\text{L}}^{\text{Xe}})} \quad (4-1) $$

For clarification, $n_{\text{aq}}$ and $n_{\text{L}}$ denote the total moles of water and lipid in solution and $n_{\text{L}}^{\text{Xe}}$ and $n_{\text{aq}}^{\text{Xe}}$ represent the moles of xenon in the lipid and aqueous phases at equilibrium. In the limit of low solute concentration, $n_{\text{aq}}^{\text{Xe}} \ll n_{\text{aq}}$, resulting in a minor simplification in Eq. (4-1). Assuming ideal solution conditions with an activity coefficient of unity, the equilibrium fraction of bound xenon ($X_{\text{Xe,L}}$) is expected to be directly proportional to $X_{\text{Xe,aq}}$. In order to determine the mole fraction of xenon in both the aqueous and lipid phases, the partition coefficient is related to the NMR chemical shift in the fast exchange regime, providing the basis for the determination of the partition coefficients from the observed $^{129}$Xe chemical shifts in LUVs. The same approach has been utilized in the study of drug-membrane interactions.[16, 141]
4.2.2 Determination of Partition Coefficients by NMR

Here we utilize the simple two-site exchange model, assuming rapid xenon exchange between the aqueous and lipid membrane phases; the aqueous phase consists of $Xe$ atoms located in the bulk water phase away from the vesicle while the lipid phase consists of all $Xe$ atoms “bound” to the vesicle surface and membrane interior. In the fast exchange regime, the expression for the observed chemical shift is given by the weighted average of the chemical shifts in each of the two phases. As explained in Chapter 2, if the rate at which xenon relocates between two or more environments is much faster than the reciprocal of their limiting chemical shift difference, the observed chemical shift will occur at the weighted average shift. In this particular system, xenon is either associated with the lipid environment (bound) or the bulk solution (free). [16, 142-144] Thus,

$$[4-2]$$

$$\delta_{obs} = X_{bound} \cdot \delta_{bound} + X_{free} \cdot \delta_{free}$$

where $\delta_{obs}$ is the observed chemical shift of xenon in DOPC solution, $\delta_{bound}$ and $\delta_{free}$ represent the intrinsic chemical shifts of xenon in the free and bound environments, while $X_{bound}$ and $X_{free}$ correspond to the mole fractions of bound ($n_{L}^{Xe} / n_{tot}^{Xe}$) and free ($n_{aq}^{Xe} / n_{tot}^{Xe}$) xenon. Setting the observed chemical shift of xenon in lipid-free buffer solution ($\delta_{obs}^{buffer}$) equal to $\delta_{free}$ and substituting $X_{free} = 1 - X_{bound}$ into Eq. (4-2) yields:

$$[4-3]$$

$$X_{bound} = \frac{\delta_{obs} - \delta_{buffer}}{\delta_{bound} - \delta_{buffer}} = \frac{\Delta \delta}{\Delta \delta_{max}}$$

Note that $\delta_{bound} - \delta_{free} \cong \Delta \delta_{max}$ is the difference in the intrinsic shifts of xenon in the lipid and aqueous phases. Rearrangement and substitution of Eq. (4-1) into Eq. (4-3) allows for extraction of the $\Delta \delta_{max}$ value, providing the basis for NMR binding analysis. As shown in Eq. (4-4), measuring the change in the observed chemical shift with increasing moles of lipid allows for the
determination of the mole fraction partition coefficient at fixed xenon overpressures. This expression is easily converted to concentration form by dividing by the total volume of the solution (1ml).

\[
\Delta \delta = \frac{\Delta \delta_{\text{max}} \cdot K_p \cdot n_L}{n_w + K_p \cdot n_L - K_p \cdot n_{\text{aq}}^{\text{Xe}}} = \frac{\Delta \delta_{\text{max}} \cdot K_p \cdot [L]}{55.5M + K_p \cdot [L] - K_p \cdot [Xe]_{\text{aq}}}
\] (4-4)

Similar to previous annotations, \([L]\), 55.5 M and \([Xe]_{\text{aq}}\) represent the total phospholipid, water, and free xenon concentrations in aqueous solution, respectively. The mole fraction form of the partition coefficient is then found by plotting \( \Delta \delta \) against the total lipid concentration. From this point forward, the total lipid concentration, \([L]\), will be referred to as \([\text{DOPC}]\).

For certain small-molecule lipid membrane systems, the two-site exchange model described above is an oversimplification that cannot be made to fit the data, and better compliance can be obtained using a three-site exchange model in which the membrane surface and membrane interior represent distinct, chemically inequivalent environments. The \(X_{\text{bound}} \delta_{\text{bound}}\) term in Eq. (4-2) is modified to account for the additional binding site:

\[
\delta_{\text{obs}} = X_S \cdot \delta_S + X_I \cdot \delta_I + X_{\text{free}} \cdot \delta_{\text{free}}
\] (4-5)

Here, \(X_S\) and \(X_I\) are the fractional occupancies of xenon at the membrane surface and interior, respectively, while \(\delta_S\) and \(\delta_I\) denote the theoretical limiting chemical shifts of xenon associated with each of these respective environments. Considering the nonspecific nature of the xenon-membrane interactions (no specific binding sites), and apparent fast exchange between the surface and lipid environments, the two-site model is deemed adequate for our purposes.

As shown by Kennan and Pollack (1990)[145] the solubility of xenon in water does not change significantly within the pressure range used in this study. \([Xe]_{\text{aq}}\) is estimated assuming the solubility in buffer solution is the same as the solubility in water, and that it behaves ideally.
According to Henry’s law, the concentration of xenon in solution is directly proportional to the gas pressure. So, \([Xe]_{aq} = P_{Xe} \cdot K_H\) where \(K_H\) denotes Henry’s Law constant and \(P_{Xe}\) the xenon overpressure in the gas phase above the liquid. The \(K_H\) value was taken to be 0.0437 M/atm.[146]

As required by conservation of mass, the total concentration of xenon in solution is given by
\[
[Xe]_{tot} = [Xe]_{aq} + [Xe]_{bound}.
\]

4.3 Results and Discussions

4.3.1 Spectral Properties of the Xe-Lipid Interaction

It has been shown previously that xenon typically exhibits fast exchange in and out of the hydrophobic environment in small unilamellar vesicles.[136] Spectra of MLVs in solution typically exhibit two resolved signals; a broad peak corresponding to the dissolution of xenon in the lipid environment and a narrower peak associated with free \(Xe\) atoms in the buffer solution.[10, 136] We observed two separate peaks in MLV samples formed in 50 mM DOPC. In this lipid, the broad peak associated with the lipid environment occurs downfield (higher ppm) of the solvent peak (Figure 4-1), contrary to the upfield shift observed in dimyristoyl-phosphatidylcholine (DMPC) and dipalmitoyl-phosphatidylcholine (DPPC).[10, 136] Whether this observation is a consequence of factors such as partitioning behavior, lipid packing effects, vesicle fluidity, etc, is not known since only a few lipid systems have been investigated via \(^{129}\text{Xe}\) NMR. Nevertheless, partitioning of inhalation anesthetics have been shown to depend on the acyl chain length as well as the degree of acyl chain saturation which are likely to be reflected in the observed \(^{129}\text{Xe}\) NMR chemical shift. The addition of double bonds induces chain disordering while increasing the molecular order in the vicinity of the double bond. This results in an increased area per lipid molecule toward the center of the bilayer, which in turn reduces the packing density at the lipid/water interface. Recent investigations into the phase behavior of thermotropic liquid crystals (LCs) via \(^{129}\text{Xe}\) NMR spectroscopy demonstrates the sensitivity of
The peak assignments to the aqueous and lipid environments of $^{129}$Xe in MLVs were verified independently through chemical shift analysis of $^{129}$Xe in lipid-free buffer solution and LUV samples (Figure 4-1). Note that the intrinsic chemical shift of xenon in the buffer solution (Figure 4-1A) is up-field with respect to that observed in 50 mM DOPC LUVs (Figure 4-1B). Furthermore, the observed $^{129}$Xe spectrum in LUVs consists of a single peak which lies almost directly between the two phases observed in MLVs. This is consistent with fast exchange between the lipid and aqueous phases in DOPC LUVs. However, the associated trends with increasing temperature differ from those reported by Miller et al. (1981). Namely, fast exchange is not induced with temperature and the DMPC associated peak is upfield of the water
Figure 4-2. $^{129}$Xe NMR chemical shift and lineshape behavior of 5 atm of xenon dissolved in 50 mM DOPC MLVs. While spectral overlap is observed at 313 K, it is not indicative of fast exchange. This is simply a cross-over event, in which the two resonances happen to pass through a common chemical shift range.

peak. While the resonances do overlap at 313 K, two distinct peaks re-emerge at higher temperatures (Figure 4-2).

A summary plot describing the temperature dependence of the chemical shifts is provided in Figure 4-3. The variation in the observed shift decreases monotonically with temperature for both MLV and LUV lipid dispersions. The chemical shift behavior of the lipid-free buffer solution appears rather insensitive in comparison. In addition, the temperature dependence of the LUV resonance appears as a weighted average between the two phases exhibited in the MLV data. Inspection of Figure 4-3 suggests that xenon’s affinity for the lipid phase increases with temperature; the fast exchanging LUV resonance shifts away from the MLV ‘aqueous phase’ towards the MLV ‘lipid’ phase. This will be explored in more detail in the next chapter. It should be noted that the resonance associated with the lipid phase under slow exchange conditions was
Figure 4-3. Temperature dependence of the $^{129}$Xe chemical shift in 50mM MLVs and LUVs: (○) and (●) denote MLV ‘lipid’ and ‘aqueous’ phase, (*) signifies LUVs environment. Inset: lipid-free buffer solution, (Δ), compared to the MLV ‘aqueous’ phase. Experiments performed with identical gas-to-solution volumes under $P_{Xe} = 5$ atm.

found to be dependent on the concentrations of both lipid and xenon in solution. For this lipid system, a single resonance was observed under the following conditions: [DOPC] < 25 mM, and $P_{Xe} < 5$ atm. So, the fast and slow exchange observed in PC vesicles by Saba et al. (1996)[148] and Miller (1981),[10] respectively, may be a result of both $P_{Xe} / \text{lipid}$ concentrations utilized in each study as well as vesicle size; Miller[10] dissolved $^{129}$Xe into MLV dispersions while Saba[148] performed experiments on sonicated vesicles.

4.3.2 $^{129}$Xe $T_1$ Relaxation Rates Determined by Saturation Recovery Methods

The longitudinal relaxation time ($T_1$) was measured for $^{129}$Xe dissolved in lipid-free buffer solution by the saturation-recovery method and found to be $110 \pm 34$ s at 5 atm and 298 K. The relaxation recovery of xenon in DOPC MLV solutions required a bi-exponential fitting function.
for [DOPC] ≤ 25 mM, allowing for the extraction of $T_1$ values in both the aqueous and lipid associated phases in solution assuming that the exchange is slow on the time-scale of $T_1$. Both the mono-exponential and bi-exponential fits of xenon in 25 mM MLV DOPC solution are provided in Figure 4-4B for comparison; experimental results show better compliance to the bi-exponential function. On the other hand, the relaxation recovery in LUV solutions could be fit to a single exponential, as expected for fast exchange between the two environments. The extracted

![Figure 4-4. $^{129}$Xe NMR saturation recovery curves for xenon dissolved in various solutions. A) Lipid-free buffer solution (pH = 7.4). B) 25 mM DOPC LUV solution with a mono-exponential fit (dotted line) versus bi-exponential fit (dashed line). C) Lipid-free buffer solution in the presence of 3.5 mM MnCl$_2$. D) The lipid (●) and aqueous (□) associated phases in 50 mM DOPC MLV solution under $P_{Xe} = 5$ atm of overpressure.](image-url)
$T_1$ value is comparable to those determined by Xu and Tang (1997)\cite{14} and serves to confirm the relative strength of the xenon-membrane interaction under fast exchange conditions (Figure 4-4C).

Saturation recovery measurements were also performed on a 50 mM DOPC MLV sample under similar experimental conditions in order to extract the $T_1$ values of xenon associated with the lipid and aqueous environments through independent analysis of their previously assigned resonances (Figure 4-1C). As seen in Figure 4-4D, the longitudinal relaxation rates differ significantly; the dissolved gas within the lipid environment relaxes more rapidly than that within the aqueous phase. The results are summarized in Table 4-1. There is a significant decrease between the $T_1$ values of xenon in the lipid-free buffer solution and the “aqueous” phase of the MLV solutions despite the fact that their chemical shifts are near identical (inset of Figure 4-3). This reduction may be attributed to $\text{Xe}$-surface interactions at or near the lipid/water interface and/or $\text{Xe}$ relaxation in the lipid associated phase due to exchange. The heterogeneous nature of the MLVs allow for xenon to diffuse freely through multiple bilayers, with limited contact with the aqueous phase. $^{129}\text{Xe}$ NMR methods were used to differentiate between DMPC/DHPC bicelle phases via changes in $T_1$ relaxation times.\cite{135} Contrary to previous investigations,\cite{149} no significant change was observed, suggesting that increased viscosity, changes in morphology and/or ordering has no substantial effect on the observed relaxation time.

4.3.3 Pressure Dependence of Xe Partitioning

According to the simple two-site partitioning model, variations in the observed xenon chemical shift can be analyzed according to Eq. (4-4). The observed variation in the $^{129}\text{Xe}$ chemical shift with lipid concentration is shown in Figure 4-5A, where $\Delta\delta$ denotes the difference in the observed chemical shift in aqueous suspensions of lipid vesicles (LUVs) and
Table 4-1. Experimentally determined $^{129}$Xe relaxation times ($T_1$) associated with the lipid-free buffer solution and the lipid phase in DOPC LUVs and MLVs, in units of seconds (s). Literature values for aqueous dispersions of Egg PC vesicles (sonicated LUVs) are given for comparison. Errors are reported as ± standard errors of the estimate (SSE).

<table>
<thead>
<tr>
<th>[Lipid] (mM)</th>
<th>MLVs lipid phase</th>
<th>MLVs buffer phase</th>
<th>LUVs</th>
<th>Lipid-free solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>10 ± 2 s</td>
<td>74 ± 12 s</td>
<td>54 ± 8 s</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>4 ± 1 s</td>
<td>70 ± 11 s</td>
<td>72 ± 15 s</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3 ± 1 s</td>
<td>53 ± 9 s</td>
<td>58 ± 7 s</td>
<td>110 ± 34 s</td>
</tr>
<tr>
<td>Egg PC [14]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>51 ± 4 s</td>
<td>122 ± 37 s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4-5. Concentration dependence of the observed $^{129}$Xe chemical shift as a function. A) The lipid concentration dependence of the observed chemical shift performed over a large concentration range. Similar plots were made at various loading pressures (1-10 atm) in order to get an adequate profile. These measurements were performed at 5 atm in 1 ml of solution. Data points possessing significant error is a result of inaccurate loading. B) The $Xe_{aq}$ pressure dependence of the observed chemical shift for a range of lipid concentrations: 15 mM (○), 25 mM (▲), 40 mM (□), 50 mM (●), and 100 mM (*). The open circles (○) denote the pressure dependence of the lipid-free buffer solution. This was repeated for a series of xenon gas overpressures. Figure 4-5B shows the chemical shift change as a function of $[Xe]_{aq}$ for a specified lipid concentration. Notice the distinct difference in the shape of the plots; the chemical shift appears be approaching a
plateau with increasing lipid concentration (Figure 4-5A) while the trend is distinctly linear with increasing \([Xe]_{aq}\) (Figure 4-5B). It has been suggested that a solute’s lipid solubility is limited by its saturation of the aqueous solution.\[150]\) According to this hypothesis, one might expect the \(^{129}\text{Xe}\) chemical shift to reach a limiting value at high enough xenon overpressures. However, the linear nature of the chemical shift, as seen in Figure 4-5A, makes this unlikely for the pressure range utilized in this study. The change in the \(^{129}\text{Xe}\) chemical shift with increasing lipid concentration (Figure 4-5) is similar to the trends seen in xenon-protein studies and is characteristic of nonspecific interactions. The saturation value should be comparable to the limiting shift of xenon at a specified pressure.

Xenon partitioning in lipid vesicle solutions was characterized as a function of \([Xe]_{aq}\). The partition coefficient should be independent of the amount of dissolved xenon in an ideal-dilute solution. Previous studies have shown that \(K_p\) can decrease with increasing anesthetic concentration,\[151]\) suggesting the possibility of non-ideal partitioning at higher anesthetic concentrations.\[152]\) The infinite-dilute value of the partition coefficient (\(K_p^o\)) was determined by fitting y-intercept values (\(\Delta \delta^o\)) extrapolated from Figure 4-5B to Eq. (4-4). The subsequent plot of \(\Delta \delta^o\) against [DOPC], shown in Figure 4-6 allows for the extraction of both \(K_p^o\) and \(\Delta \delta_{max}^o\) values, where \(\Delta \delta_{max}^o\) is the infinite-dilution value of the maximum chemical shift difference between the lipid and buffer phases. Similar methods were applied to investigate the concentration dependence of xenon partitioning in POPC bilayers.\[153]\) The variation of \(\delta_{obs}^{buffer}\) with increasing \([Xe]_{aq}\) in the lipid-free buffer solution likely arises from xenon-solvent interactions in solution; the downfield shift observed with increasing temperature may reflect its decreased solubility with temperature. To account for any contribution this might have on the
Figure 4-6. Determination of the infinite dilution mole fraction partition coefficient. The y-intercept values of Figure 4-1 plotted as a function of lipid concentration result in an infinite dilution partition coefficient of $390 \pm 46$ when fit to Eq. (4-4).

If the concentration of bound xenon is negligible with respect to the total lipid concentration, then the ‘-$K_p[Xe]_{aq}$’ correction term in Eq. (4-4) can be safely neglected. However, this leads to an overestimation in the partition coefficient at higher xenon concentrations (Figure 4-7A). The standard expression utilized in the determination of the binding constant ($K_a$) via NMR chemical shift data is similar to Eq. (4-4), where

$$\Delta\delta = \frac{\Delta\delta_{\text{max}} \cdot K_a \cdot [L]}{1 + K_a \cdot [L]}.$$  \hspace{1cm} (4-6)

As described by Eqs. (4-7) and $K_a \times 55.5 M \equiv K_p$ if $[Xe]_{\text{bound}}$ is low enough; changes in the binding and/or partitioning behavior are signified by a changes in the slope. A comparison of partitioning and binding profiles is provided in Figure 4-7B.
Note that the measured partition coefficient does not vary significantly with $[Xe]_{aq}$ over the pressure range used in this study—the observed slope is distinctly linear. As expected, the binding constant diverges at higher loading pressures. The binding behavior will be evaluated in further detail in Chapter 6.

The predicted limiting shift difference, $\Delta \delta_{\text{max}}$ extracted from the fits of the experimental data to both Eq. (4-4) and Eq. (4-6) are identical, and serves to validate both equations. The linear behavior of the limiting chemical shift difference allowed us to extrapolate the $\Delta \delta_{\text{max}}$. 

Figure 4-7. The pressure dependence of the partition coefficient. A) Results from fits to Eq. (4-4): (blue ▲), Eq. (4-10): (□), and Eq. (4-4) without the ‘$K_p[Xe]_{aq}$’ correction: (red ○). B) The binding model, (○), versus the partitioning model, (▲): the difference denoted by $[L]-[Xe]_{\text{bound}}$ and by $[L]+[Xe]_{\text{bound}}$, respectively (Eq.(4-9)). Errors are reported as ± (SSE), resulting from the fit of chemical shift data to Eq.(4-4).
Figure 4-8. The limiting chemical shift as a function of xenon concentration in solution. The limiting shift changes $+37 \pm 2$ ppm for every M of $[Xe]_{aq}$. The infinite dilution value of the chemical shift was found to be approximately 2.56 ppm. Errors are reported as $\pm$ (SSE).

values in order to obtain $[Xe]_{bound}$ and thus $K_p$ for all pressures. The experimentally determined $\Delta \delta_{\text{max}}$ values are provided in Figure 4-8 where it is plotted as a function of $[Xe]_{aq}$. Now that $\Delta \delta$ and $\Delta \delta_{\text{max}}$ are known for each $[Xe]_{aq}$ and specific lipid concentration, $K_p$ can be determined using Eq. (4-10), where $X_{\text{bound}} = \Delta \delta / \Delta \delta_{\text{max}}$.

$$K_p = \frac{X_{\text{bound}} \cdot [55.5M]}{[L] \cdot [L] \cdot X_{\text{bound}} + [Xe]_{aq} \cdot X_{\text{bound}}}$$  \hspace{1cm} (4-10)

As one can see in Figure 4-7A, the two different methods yield partition coefficients which are in close agreement. For purposes of comparison, we were able to refit Meier’s data to our model and determined the mole fraction partition coefficient of xenon in POPC to be $K_p = 466 \pm 78$. This can be compared to our experimentally determined result of $475 \pm 41$ for DOPC under similar experimental conditions. Small errors in the extracted $\Delta \delta_{\text{max}}$ values are propagated into
the overall errors for $K_p$ having a large effect. The smaller error in our determination of $K_p$ reflects the greater number of data points available for the fit.

While their headgroup regions are identical, these DOPC and POPC differ structurally only in their acyl chain regions (Figure 3-1); the presence of the shorter, saturated acyl chain in POPC results in a reduction in the molecular volume.\cite{154,155} Thus, POPC has a slightly smaller interfacial area, effectively reducing the number of water molecules bound per lipid molecule in fully hydrated lipids. According to theoretical predictions from Cantor, partitioning depends strongly on lipid composition and degree of unsaturation.\cite{156,157} Recent experimental work, comparing the distributive properties of ethanol in fully saturated DMPC and unsaturated DOPC, cite similar behavior.\cite{119} It should be noted that the difference in our partition coefficients for DOPC and POPC are within error of each other. According to the statistical thermodynamic estimates by Cantor, the values for these two lipids are expected to be very close.\cite{158}

4.3.4 $^{129}$Xe Relaxivity Utilized to Probe Partitioning and Vesicle Stability

Following previously established theory, NMR relaxation methods were used in conjunction with the two-site rapid exchange model to confirm the $K_p$ values determined by chemical shift analysis.\cite{159} If the exchange rate of xenon molecules between the aqueous and lipid environments is fast on the NMR timescale, the observed spin relaxation rate ($R_1 = 1/T_1$) becomes a weighted average of the intrinsic relaxation rates of xenon in each phase. According to this model, the average time ($\tau_b$) xenon spends in the ‘bound’ state must be much less than the bound relaxation time, $T_{1,\text{bound}}$.\cite{160} This results in the following expression:

$$R_{1,\text{obs}} = \frac{1}{T_{1,\text{obs}}} = X_{\text{bound}} \cdot R_{1,\text{bound}} + (1 - X_{\text{bound}}) \cdot R_{1,\text{aq}} \quad (4-11)$$

where $R_{1,\text{obs}}$ is the observed longitudinal relaxation rate of xenon in the fast exchange regime, while $R_{1,\text{bound}}$ and $R_{1,\text{aq}}$ are the spin-relaxation rates of xenon in the lipid and aqueous phases.
respectively. Saturation recovery methods were used to determine $R_{1,\text{bound}}$ and $R_{1,\text{aq}}$ for the dissolution of 5 atm of xenon within a 50 mM DOPC MLV sample. The two distinct resonances denoting the aqueous and lipid phases have markedly different relaxation times; the fit of the narrow upfield peak associated with $\delta_{\text{free}}$ yields a $T_{1,\text{aq}}$ value of $74 \pm 12$ s while the broader peak located downfield ($\delta_{\text{bound}}$) a $T_{1,\text{bound}}$ of $10 \pm 2$ s. These relaxation times provide an estimation of $R_{1,\text{aq}}$ and $R_{1,\text{bound}}$, while the analysis of the 50 mM DOPC LUV solution under similar experimental conditions allows for the approximation of $R_{1,\text{obs}}$. $K_p$ can now be calculated directly from Eqs. (4-10) and (4-11). The result of which is significantly less than expected, yielding a mole fraction partition coefficient of 66.

In an attempt to make sense of this inconsistency, we reassess the initial assumption that the relaxation time of xenon within the lipid environment is much greater than its lifetime within the MLV state. This results in a slight modification of Eq. (4-11) to account for the average binding time, $\tau_b$:

$$\frac{1}{T_{1,\text{obs}}^{\text{obs}}} = \frac{(1 - X_{\text{bound}}^{\text{bound}})}{T_{1,\text{aq}}^{\text{aq}}} + \frac{X_{\text{bound}}^{\text{bound}}}{T_{1,\text{bound}}^{\text{bound}} + \tau_b}$$  \hspace{1cm} (4-12)

Here, we utilized the $X_{\text{bound}}^{\text{bound}}$ parameter extracted from chemical shift measurements and set the longitudinal relaxation times to previously said values. The extracted $\tau_b$ value was found to be approximately 33 seconds. This relatively long lifetime within the lipid phase is somewhat understandable in a MLV environment since there is a high likelihood for xenon to diffuse through multiple bilayers, with limited contact with the aqueous phase. Substitution of Eq. (4-11) into Eq. (4-10) also allows for an estimation of $T_{1,\text{bound}}$ under fast exchange conditions. Using Kitamura et al. (1999)\textsuperscript{159} as a guide, we set $T_{1,\text{obs}}$ to 54 s, $K_p$ to 475, and $T_{1,\text{aq}}$ to 110 s.
According to this model, the predicted longitudinal relaxation time of xenon associated with the 50 mM DOPC LUV environment under 5 atm of xenon gas is approximately 24 s.

The NMR paramagnetic relaxation technique was employed to verify the stability of both the LUV and MLV DOPC solutions at elevated $^{129}$Xe loading pressures. The presence of paramagnetic ions has been shown to significantly increase the relaxation rates of nuclear spin systems in aqueous solution.\cite{161-163} This method is based on assumption that paramagnetic ions are unable to pass through the lipid bilayer environment. If there is a significant amount of vesicle rupture, the paramagnetic contribution to xenon’s relaxivity and chemical shift behavior cannot be distinguished between phases. As shown in Figure 4-9, the paramagnetic induced chemical shift change appears to be limited to upfield resonance, while the broad resonance representing xenon dissolved within the lipid phase remains unchanged. Thus, we can infer that $R_{1,bound}$ is unaffected and the paramagnetic contribution to the longitudinal relaxation rate can be expressed as:

$$R_{1,obs}^p = X_{bound} \cdot R_{1,bound}^p + (1 - X_{bound}) \cdot R_{1,aq}^p,$$

under fast exchange conditions, where $R_{1,obs}^p$ and $R_{1,aq}^p$ are the observed relaxation rates of xenon in 25 mM DOPC and lipid-free buffer solution in the presence of MnCl$_2$, respectively. The severe broadening of the ‘buffer’ resonance is an unfortunate side effect of the paramagnetic induced relaxation process.

The solubilization parameter ($X_{bound}$) can be calculated by measuring the change in relaxation rates of xenon in both lipid-free buffer solution and LUV DOPC solutions, in the presence and absence of paramagnetic shift reagent. Together, Eqs. (4-11) and (4-13) generate a usable expression for $X_{bound}$. 

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\[ X_{\text{bound}} = 1 - \left( \frac{R_{1,\text{obs}}^p - R_{1,\text{obs}}}{R_{1,\text{aq}}^p - R_{1,\text{aq}}} \right) = 1 - \left( \frac{\text{slope 2}}{\text{slope 1}} \right) \] (4-14)

where \text{slope 1} and \text{slope 2} provided in Eqs. (4-15) and (4-16) signify the relaxation rate enhancement generated by the presence of paramagnetic ion in solution.

\[
\text{slope 1} = \frac{\Delta R_{1,\text{aq}}^p}{\Delta [\text{MnCl}_2]} = \frac{R_{1,\text{aq}}^p - R_{1,\text{aq}}}{\Delta [\text{MnCl}_2]} \quad (4-15)
\]

\[
\text{slope 2} = \frac{\Delta R_{1,\text{obs}}^p}{\Delta [\text{MnCl}_2]} = \frac{R_{1,\text{obs}}^p - R_{1,\text{obs}}}{\Delta [\text{MnCl}_2]} \quad (4-16)
\]

Consistent with theory, the paramagnetic enhancement of the longitudinal relaxation rate \(R_{1,\text{obs}}^p\) increases with [MnCl2] for both lipid containing and lipid free solutions (Figure 4-10A). Though paramagnetic ions have the potential to impede xenon partitioning when present at sufficiently high concentrations, the linear nature of the relaxation behavior suggests this not to be the case.

**Figure 4-9.** \(^{129}\text{Xe}\) NMR spectra of xenon dissolved in 50mM DOPC MLVs, in the absence (red spectrum) and presence (blue spectrum) of MnCl2. The paramagnetic shift reagent is limited to the aqueous phase (upfield resonance).
Figure 4-10. The variation in the NMR parameters with increasing paramagnetic shift reagent. A) Observed longitudinal relaxation rates of $^{129}$Xe in lipid-free buffer solution ($R_{1,aq}$) and 25 mM LUV DOPC lipid suspension ($R_{1,obs}$) as a function of $[\text{MnCl}_2]$. B) The variation in the $^{129}$Xe chemical shift with increasing $[\text{MnCl}_2]$ for 10 atm of xenon dissolved in lipid-free buffer solution (referenced to $\delta_{\text{buffer}}$).

Table 4-2. Extracted mole fraction partition coefficients obtained through a variety of methods: chemical shift analysis ($K_{p,1}$), direct measurement of longitudinal relaxation rates associated with the isolated lipid and aqueous phases in MLV solution ($K_{p,2}$), and paramagnetic shift enhancement ($K_{p,3}$). Errors are reported as ± (SSE).

<table>
<thead>
<tr>
<th>$P_{Xe}$ (atm)</th>
<th>$K_{p,1}$ Eq. (4-4)</th>
<th>$K_{p,2}$ Eqs. (4-10)/(4-11)</th>
<th>$K_{p,3}$ Eq. (4-17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>475 ± 41</td>
<td>66 ± 20</td>
<td>485 ± 53</td>
</tr>
<tr>
<td>10</td>
<td>452 ± 34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Here, Eq. (4-14) is easily modified to make use of the MnCl$_2$ titration data provided in Figure 4-10A. This allows for a more accurate determination of the solubilization parameter; substitution of Eq. (4-14) into Eq. (4-10) provides a direct measurement of the mole fraction partition coefficient, yielding a $K_p$ value of 485 ± 53.

$$K_p = \frac{55.5 M \cdot \left( \Delta R_{1,aq} - \Delta R_{1,obs} \right)}{[L] \cdot \Delta R_{1,obs} + [Xe]_{aq} \cdot \left( \Delta R_{1,aq} - \Delta R_{1,obs} \right)}$$ (4-17)
These results agree with previously determine partition values using chemical shift analysis and confirms that the vesicles remain intact when exposed to high xenon overpressures.

4.3.5 Bunsen, Ostwald, and Mole Fraction Partition Coefficients

The mole fractions partition coefficients of xenon dissolved in olive oil and various lipid solutions are summarized in Table 4-3 for comparison. This has been graphed in Figure 4-12 for visualization and is listed by increasing molecular weight of the biomedia (excluding lecithin). The Ostwald solubility coefficient of xenon in olive oil is often used to estimate its concentration in lipid bilayers and is easily converted into the mole faction partition coefficient. For clarification, the Bunsen coefficient, \( \alpha \) measures the volume of dissolved gas per volume of water or lipid sample at atmospheric pressure and 273.15 K. We corrected for the sample temperature to allow for easy conversion to the Ostwald \( (L) \) coefficient, the details of which are shown in Eq. (4-18).

\[ L = \alpha \left( \frac{T}{273.15K} \right) \]  

(4-18)

\[ K_{(c)} = \frac{L_{oil}}{L_{water}} \]  

(4-19)

\[ K_p = K_{(c)} \cdot \frac{V_L}{V_W} = \frac{L_{oil}}{L_{water}} \cdot \frac{M_{oil}}{M_{water}} \cdot \frac{\rho_{water}}{\rho_{oil}} \]  

(4-20)

Here, \( V_L \) and \( V_W \) denote the partial molar volumes, while \( L_{oil} \) and \( L_{water} \) symbolize the Ostwald coefficients of oil/lipid and water, respectively. When the molar volumes were not readily
available, we utilized molecular weight and density values found in the literature.

The Meyer-Overton correlation between olive oil solubility and anesthetic potency suggests that the site of anesthetic action has similar physical properties to that of olive oil. While lipid theories assume that nonspecific anesthetic-membrane interactions is key, protein theories target specific interactions between proteins and receptors. However, neither proteins nor lipids have uniform structure; their anisotropic nature suggests different physical properties compared to a bulk, isotropic apolar liquids. A number of organic solvents have been investigated in addition to olive oil with hopes of establishing a more accurate chemical model to quantify the solubility-potency correlation. This has lead to the octanol-water partition coefficient as the most widely used model for lipophilicity. The presence of the hydroxyl

Table 4-3. The mole fraction partition coefficients of xenon in various lipidic species and solvents. LUV-large unilamellar vesicles; SUV-small unilamellar vesicles; SV-sonicated vesicles; HCS-hydrocarbon solvent. Errors are reported as ± (SSE), resulting from the fit of chemical shift data to Eq.(4-4).

<table>
<thead>
<tr>
<th>Aqueous Media</th>
<th>Vesicle type</th>
<th>Temp. (K)</th>
<th>P_{Xe} (atm)</th>
<th>Corrected K_p - value</th>
<th>Δδ_{max} (ppm)</th>
<th>Exp. method</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>LUV</td>
<td>298</td>
<td>1</td>
<td>461</td>
<td>446 ± 42</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>SUV</td>
<td>298</td>
<td>5</td>
<td>584</td>
<td>475 ± 41</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>POPC[^153]</td>
<td>SV</td>
<td>297</td>
<td>6</td>
<td>597</td>
<td>466 ± 78</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Lecithin[^146]</td>
<td>SV</td>
<td>310</td>
<td>1</td>
<td>804</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC/PA[^169]</td>
<td>SV</td>
<td>299</td>
<td>1</td>
<td>636</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PC/PA/chol.[^169]</td>
<td>SV</td>
<td>298</td>
<td>1</td>
<td>481</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2:1 PC/chol.[^170]</td>
<td>SV</td>
<td>293</td>
<td>1</td>
<td>445</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n-octane[^171]</td>
<td>HCS</td>
<td>293</td>
<td>1</td>
<td>326</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n-octanol[^103]</td>
<td>HCS</td>
<td>298</td>
<td>1</td>
<td>205</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Olive oil[^172]</td>
<td>HCS</td>
<td>298</td>
<td>1</td>
<td>945</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
group in the octanol molecule introduces a small degree of polarity within the solvent, resulting in a better correlation.\textsuperscript{173,174} Despite the improvement, this model still fails to adequately characterize the interaction of charged solutes, given that octanol can only interact through hydrogen bonding and hydrophobic interactions. As the focus of this thesis is on the partitioning behavior of a nonpolar solute, we refrain from discussing this further and refer the reader to Razak et al. (2001)\textsuperscript{175} and Escher et al. (1996)\textsuperscript{176} for additional information.

The primary difference between the hydrocarbon-water model and liposome-water interaction is that the physical properties within the apolar solvents are uniform throughout while they vary as a function of membrane depth in the lipid bilayer phase. To be more specific, the dielectric constant ($\varepsilon$) exists as a gradient, decreasing from a high value of approximately 76 in bulk water to about 2 in the lipid core; the lower the numeric value of $\varepsilon$, the greater the hydrophobicity.\textsuperscript{127,177-180} According to Ueda et al., the ‘real’ meaning of the Meyer-Overton rule is that anesthetic molecules will tend to reside in regions where the dielectric constant is near that of the lipid/water and protein/water interfaces; the best correlation is when the relative permittivity is between 10-11.\textsuperscript{181} The dielectric constants of various lipid and lipid free solutions are tabulated in Table 4-4 for comparison. An illustration is provided in Figure 4-11 for visualization. Excluding the high frequency permittivity ($\varepsilon_{\infty}$), values summarized in Table 4-4 were obtained at low frequencies. Molecular rotation can lead to a net loss in the permittivity which increases with frequency. As shown below, the dielectric constants associated with various regions within the bilayer are no longer distinguishable at high frequencies.

As mentioned previously, intermolecular $^{129}$Xe-$^{1}$H NOEs have been utilized to quantify the xenon-membrane interaction strength for different regions in the lipid bilayer; the intermolecular cross relaxation rate of xenon associated with the interfacial choline protons are
Table 4-4. The dielectric constants of apolar solvents, water, and a PC lipid system at 298 K, unless stated otherwise. The ‘bound’ water refers to water molecules in the immediate vicinity of the PC headgroup (HG) region. Here, $\varepsilon_\perp$ denotes the permittivity perpendicular to the membrane surface. The existence of a dielectric constant in the $xy$-plane, $\Delta\varepsilon_\parallel$, is consequent of the dipolarity in the HG region. Finally, $\Delta\varepsilon$ represents dielectric decrement due to molecular motion (increases with frequency), and $\varepsilon_\infty$ indicates the high frequency permittivity.

<table>
<thead>
<tr>
<th>Aqueous Media</th>
<th>$\varepsilon_\perp$</th>
<th>$\Delta\varepsilon_\parallel$</th>
<th>$\Delta\varepsilon$</th>
<th>$\varepsilon_\infty$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Bulk $^{[179,182]}$</td>
<td>76</td>
<td>6.7</td>
<td>$\sim$71</td>
<td>1.8</td>
</tr>
<tr>
<td>- Bound-to-membrane surface $^{[183,184]}$</td>
<td>20-30</td>
<td></td>
<td>$\sim$47</td>
<td></td>
</tr>
<tr>
<td>Olive Oil (293 K) $^{[127,185]}$</td>
<td>3.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Octanol (293 K) $^{[127,186]}$</td>
<td>10.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integral Membrane Protein $^{[180]}$</td>
<td>6-8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC lipid $^{[179-184,187]}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- P-N headgroup</td>
<td>30</td>
<td>20-35</td>
<td>$\sim$10</td>
<td>2.2</td>
</tr>
<tr>
<td>- Glycerol backbone</td>
<td>8-10</td>
<td>140</td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>- Lipid core</td>
<td>2-4</td>
<td>0</td>
<td></td>
<td>2.2</td>
</tr>
</tbody>
</table>

Figure 4-11. The variation in the membrane dielectric constant as a function of membrane depth ($z$) according to a simple capacitor model. The bound water (BW), lipid headgroup (HG) and hydrocarbon chain (HC) regions all serve as possible sources for the membrane dipole potential $^{[188]}$. Here, $\varepsilon_\perp$ denotes the relative permittivity, perpendicular to the bilayer normal (norm).$^{[189]}$
Figure 4-12. A graphical representation of xenon-oil/lipid partition coefficients, sorted by increasing molar mass. Bunsen and Ostwald coefficients were converted from values found in literature. References and numeric values are found in Table 4-3. The darker grey bars represent the ‘corrected’ $K_p$ values determined by $^{129}$Xe NMR chemical shift analysis. The error bars on the lighter grey regions are ± (SSE) when multiple values were available in literature. The $K_p$ values were obtained within 297-298 K and at atmospheric pressure, unless stated otherwise.

three times larger than that associated with the aliphatic region. The induced dipole on the $Xe$ atom is due its close proximity to the polar lipid headgroup which makes it more ‘adaptable’ to the interfacial region than the lipid core. And so, according to Ueda’s theory, the partition coefficient should be closer to that of 1-octanol than olive oil. However, the interfacial properties of the lipid membrane can be adjusted via changes in the surface charge density, facilitated through modifications in lipid membrane composition (e.g. lipid type, cholesterol) or external variables. As such, changes in the interfacial properties may manifest in partitioning behavior. High pressure (> 50 atm) xenon-membrane studies showed that the net effect of gas on membrane order results from a two-step process. Following a sudden increase in the xenon pressure, the presence of xenon was shown to increase the relative order of the membrane
environment, due to a membrane compression effect. However, the gas molecules were able to
diffuse and dissolve into the lipid core, effectively decreasing the order parameter once
equilibrium is established. What’s more, despite an initial increase with xenon uptake, the
relative permittivity was shown to gradually decrease as the xenon diffused through the bilayer.
Since high pressures are involved in this study, it is unclear if these effects are relevant under
physiologically conditions.

Taking a closer look at Table 4-3 (or Figure 4-12) we see that the lipid/water mole fraction
partition coefficient of xenon dissolved in various compositions of lipid vesicles spans between
445 and 636, yielding maximum and minimum values of 945 and 205 in olive oil and octanol,
respectively. This has been graphed in Figure 4-12 for visualization and is listed by increasing
molecular weight of the biomedia (excluding lecithin). Assuming the degree of hydrophobicity
is proportional to the experimentally obtained partition coefficients, neither octanol or olive oil
models fully describe the partitioning of xenon between the aqueous and lipid phases. And while
the Ostwald and Bunsen solubility coefficients for the lipid solutions are comparable to our
uncorrected NMR partitioning values under 5 atm of xenon gas, they are not entirely equivalent
to the corrected quantities. This is in part because the Bunsen and Ostwald methods for
determining partitioning essentially measure the dissolution of gas into two bulk phases (aqueous
solution and concentrated lipid) and compare them. This is reminiscent of xenon partitioning in
the MLVs, where the lipid phase is saturated by xenon (Figure 4-1). While these methods
provided a good estimation of the maximum partitioning capacity of bulk lipids, the solvent-
solute interactions may not necessarily be the same as those in smaller, homogenously dispersed
systems.
Cell membranes typically contain cholesterol, whose presence contributes largely to the control of the membrane diffusive properties. Similar to other partitioning studies, cholesterol appears to decrease the partition coefficient.\cite{192, 193} The addition of cholesterol is known to induce tighter molecular packing within the cell membrane, which has consequence on the membrane permeability—the higher the cholesterol content, the lower the uptake of anesthetic. According to the solubility diffusion mechanism, the solute molecules first partition into the hydrophobic phase and then diffuse across the lipid bilayer. Assuming xenon permeates through the lipid bilayer according to this method, we can relate the decrease in permeability to a decrease in partitioning, consistent with the observed trends. In contrast, elevated temperatures appear to enhance partitioning (lecithin at 310 K). This has been verified in the absence and presence of cholesterol.\cite{92} The amount of anesthetic associated with the lipid phase has been shown to steadily decrease with increased concentration of cholesterol.

### 4.4 Conclusions

The nature of xenon-phospholipid interactions and xenon exchange depend on the structure of the lipid headgroups and acyl chains, the phase state of the lipid bilayer, and the heterogeneity in both vesicle size and overall distribution of lipids with external variables. Our study of the dissolution of xenon into aqueous solutions of MLVs and LUVs provide a reliable basis for understanding the $^{129}$Xe exchange dynamics within DOPC lipid membranes. What is clear is that not all lipid systems exhibit the same trends. For instance, both DMPC and DPPC MLVs have been shown to start as a single peak at room temperature then split into two phases with increasing temperature.\cite{32} Miller et al. also looked at the dissolution of xenon in DMPC and saw a change in the exchange process from slow to fast with increasing temperature.\cite{10} The primary difference between these lipids those that we have studied is that DPPC and DMPC are saturated
lipids, while DOPC is unsaturated. The majority of studies investigating hydrophobic cavities of proteins in solution use a slightly more complex model to differentiate between specific and non-specific interactions. Non-specific interactions take place at the protein surface, while specific interactions consist of those inside a hydrophobic pocket. This treatment may prove to be useful in the study of lipid domains (a.k.a. lipid rafts) to determine localized effects of xenon-lipid interactions. $^{129}\text{Xe}$ NMR studies of non-specific xenon-lipid interactions in the lipid system will help to develop $^{129}\text{Xe}$ NMR as a biomolecular probe of packing effects, chemical exchange, factors affecting lateral pressure, and phase transitions in membrane model systems. Can we disentangle the contributions from the acyl chain regions and those from the lipid headgroup? Which has a larger contribution to the observed chemical shift? Now that the basic theory is established and validated, it will be possible to extend this approach to fully characterize the thermodynamic properties in other lipid systems.
CHAPTER 5
THERMODYNAMIC PROPERTIES OF PARTITIONING

5.1 Introduction

Historically, an anesthetic’s hydrophobicity was thought to dominate its interaction with biologically relevant media. The linear correlation between an anesthetic’s potency and its relative solubility in hydrocarbon solvents, as shown by Meyer and Overton,\cite{86,194} largely contributed to this view. However, it has become increasingly apparent that the predictive behavior provided by the Meyer-Overton (MO) Rule may not adequately relate lipid solubility and anesthetic potency.\cite{157,195-198} Several exceptions include the existence of non-immobilizers (molecules predicted by the MO rule to be anesthetics, but aren’t) and observed variations in solubility with temperature and lipid composition. The partitioning behavior of anesthetic molecules is traditionally thought to be driven by the hydrophobic effect. More specifically, it is believed to follow a similar thermodynamic model of partitioning as observed between aqueous solutions and hydrocarbon solvents. Herein we hope to establish whether the Meyer-Overton Rule is applicable to xenon-bilayer interactions and determine the extent the hydrophobic effect contributes to the partitioning process.

Two important thermodynamic functions are the standard enthalpy and the standard entropy. The standard entropy accounts for the relative change in membrane order, while the standard enthalpy measures the energy of the solute-solvent interaction in solution. According to general statistical mechanical theory, the solvation energy depends on the kinetic energy, the solute’s rotational and vibrational properties, and the potential energies due to nearest neighbor interactions.\cite{199-201} To our benefit, these complex functions are simplified for a noble gas containing apolar solvent as the rotational and vibrational contributions disappear due to the spherical symmetry of these small solute molecules. Unlike simple solutions, liposomes are
Figure 5-1. Thermodynamic solvation parameters. A) For 1 atm of xenon from the gas phase to water. B) From the gas phase to the solvent hexane at 298 K. Numeric values provided by Hefter et al. (2003) and Bonifácio et al. (2001).[^202] [^203]
comprised of three phases: bulk water, non-solvent water (strongly bound water molecules at the interface), and a solvent-like hydrophobic core. It may possible to monitor solute induced structural changes at the lipid membrane-water interface using these thermodynamic parameters. Does preferential hydration of the solute at the lipid-water interface direct the distributive properties of non-polar molecules with temperature or modifications in membrane order?

In this chapter we use $^{129}$Xe NMR chemical shift analysis to directly measure the thermodynamic partitioning parameters of xenon in DOPC. The extracted molar enthalpy, entropy and chemical potential are then compared with model data for xenon partitioning in several hydrocarbon solvents. We begin by presenting a more detailed picture of the hydrophobic effect as related to the solvation model. This is then expanded to include a brief summary on the theory behind partitioning thermodynamics; specific relations utilized in our own analysis are highlighted. Lastly, we discuss our results in the context of the recent literature and confirm that our analysis of experimentally determined chemical shifts (Chapter 4) is a valid approach in thermodynamic analysis of the xenon-membrane interaction.

5.1.1 The Classic Hydrophobic Effect

The hydrophobic effect describes the anomalous behavior of non-polar solutes in aqueous solutions and their tendency to form aggregates of like molecules in solvent water. Hydrophobic effects are characterized by two distinct processes: hydrophobic hydration and hydrophobic interactions. The molecular interpretation of the hydrophobic hydration is associated with structured water molecules in the presence of nonpolar solutes in aqueous solution. The dissolution process depends on the capacity of the solute to substitute for the hydrogen bonds lost by the water network. In the presence of a nonpolar molecule, the water molecules will reorganize in an attempt to minimize the loss of energy, resulting in more ordered structures, or water cages, around the solute. This leads to an enthalpic gain as well as a entropic loss. Thus,
the whole process is energetically unfavorable and the nonpolar solute will move to a friendlier
environment when available. This entropic effect is considered to be the dominant force behind a
solute’s hydrophobicity (compared to the enthalpic factor) and provides the basis for the classical
interpretation of hydrophobic interactions; non-polar groups aggregate in such a way that
minimizes their contact area with water. [207]

The thermodynamics of transfer for xenon from is gaseous phase $Xe_{(g)}$ to a bulk solvent
differs substantially depending on the molecular nature of the solvent. For example, the solvation
energy of $Xe_{(g)}$ to an apolar solvent is spontaneous and dominated by a large, negative enthalpy
and moderately small entropy; both thermodynamic parameters should be relatively independent
of temperature. The mixing of nonpolar molecules in water exhibits markedly different behavior.
The simplest thermodynamic description of the hydrophobic hydration is found in the transfer of
a nonpolar solute from a gas or liquid phase, to bulk water. This process is identified by i) a
large, negative entropy (unfavorable) associated with the release of the water molecules from
around the solute molecule, and ii) a temperature dependence of the chemical potential that
yields a large, positive heat capacity. [208, 209] The solvation entropy and enthalpy of nonpolar
solute from the gaseous phase to bulk water increase with temperature, consequent of the large
change in the heat capacity. In comparison, the solvation energy is large, positive and rather
insensitive to changes in temperature due to the compensatory nature of the entropy and enthalpy
parameters. The solvation energy profile is reaches a maximum at a critical temperature, $T_S$; it
has been shown that the transfer process is most favorable when the entropic contribution to the
solvation energy becomes negligible. [210] As such, the entropy contribution to the transfer energy
is optimal at temperatures above $T_S$. Using similar notation, $T_H$ is the temperature above which
the enthalpy of transfer becomes unfavorable, reflecting the weakening and/or breaking of the hydrogen bonded water network. These effects are illustrated in Figure 5-1 for clarity.

The change in the heat capacity ($\Delta C_p$) upon the transfer of nonpolar solute from bulk water to hydrocarbon solvent is typically large and negative in value. It increases with temperature and is thought to arise from the melting of the ordered water.\[^{[211]}\] It was once thought that the water-ordering effect was responsible for the low solubility of nonpolar solutes in aqueous solution; however it is now known that this occurs because it makes the interaction more favorable.\[^{[211]}\] The variation in the $\Delta C_p$ value with changing molecular environments has been found to be directly proportional to the number of water molecules in the first hydration shell; the larger the size of the embedded hydrophobic surface area, the higher the degree of hydrophobic interaction.\[^{[212]}\] The negative contribution to the heat capacity change arises from the solvation of nonpolar molecules within like environments and provides a measure of the strength of solvent-induced attractive forces.\[^{[213]}\] General expressions for the variation in these thermodynamic parameters with temperature and their respective relations to the heat capacity will be provided in subsequent sections. Thermodynamic parameters for the transfer of $\text{Xe(g)}$ from its gas phase to water, and several simple hydrocarbon solvents are provided in Table 5-1.

**5.1.2 Environmental Swap Energy (ESE)**

Now that we have some sense of the energies at play in gas-to-solution solvation, it is useful to describe the processes involved in the transfer of solute from an aqueous to apolar phase. According to the Ben-Naim model of solvation thermodynamics, a solute’s solubility is dependent on the reversible work required for cavity creation and the solute-induced forces upon insertion at a given temperature, pressure and chemical composition.\[^{[214, 215]}\] As mentioned earlier, water molecules tend to orient themselves around the solute, decreasing the entropy of
Table 5-1. Tabulated literature values of thermodynamic parameters of transfer for 1 atm of $Xe(g)$ to both water and hydrocarbon solutions. Here, $\Delta$ denotes experimentally obtained values of the solvation energies,\cite{171, 216}, while $B$ indicates calculated Ben-Naim\cite{217} standard enthalpy, entropy and solvation energy changes using statistical methods and $C$ denotes values determined from scaled particle theory.\cite{216}

<table>
<thead>
<tr>
<th>Gaseous phase transfer</th>
<th>Method</th>
<th>Temp. (K)</th>
<th>$\Delta \mu^\circ$ (kcal/mol)</th>
<th>$\Delta H_m^\circ$ (kcal/mol)</th>
<th>$\Delta S_m^\circ$ (cal/mol·K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Xe(g)$ : H$_2$O</td>
<td>A</td>
<td>273</td>
<td>+2.36</td>
<td>-4.05</td>
<td>-18.00</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>293</td>
<td>+1.23</td>
<td>-4.32</td>
<td>-18.90</td>
</tr>
<tr>
<td></td>
<td>[211]</td>
<td>298</td>
<td>+1.58</td>
<td>-4.71</td>
<td>-21.10</td>
</tr>
<tr>
<td>$Xe(g)$ : 44 solvents</td>
<td>A</td>
<td>273</td>
<td>-0.63</td>
<td>-1.84</td>
<td>-4.10</td>
</tr>
<tr>
<td>$Xe(g)$ : n-C$<em>8$H$</em>{18}$</td>
<td>- Ben Naim method</td>
<td>B</td>
<td>293</td>
<td>-0.86</td>
<td>-2.12</td>
</tr>
<tr>
<td></td>
<td>- Molar concentration scale</td>
<td>C</td>
<td>298</td>
<td>-0.63</td>
<td>-1.74</td>
</tr>
<tr>
<td></td>
<td>- Mole fraction scale</td>
<td>A,C</td>
<td>298</td>
<td>1.87</td>
<td>-2.04</td>
</tr>
</tbody>
</table>

The system. These solvent cages are broken upon solute removal, resulting in a loss in structured water and a positive change in the standard entropy. The removal of solute from the aqueous phase also triggers reformation of the water hydrogen-bonded network, yielding a negative change in the enthalpy. In the same way, work must be done in order to form a cavity of appropriate size within the apolar solvent (positive enthalpy) at which point the solute-solvent interaction yields negative contributions to the standard enthalpy and entropy. Thus, the energy of transfer from the aqueous to apolar phase may be viewed as:\cite{218, 219}

$$\Delta \Delta \mu^\star_{\text{solv}} = \Delta \mu^\circ_{\text{soln}} - \Delta \mu^aq_{\text{soln}} = \left( \Delta \mu^hc_{\text{cav}} - \Delta \mu^aq_{\text{cav}} \right) + \left( \Delta \mu^hc_{\text{int}} - \Delta \mu^aq_{\text{int}} \right)$$

(5-1)

where $\Delta \Delta \mu^\star_{\text{solv}}$ denotes the energy change of transfer from the aqueous ($aq$) to hydrocarbon ($hc$) phase, $\Delta \mu^hc_{\text{cav}}$, the work required to form a cavity, and $\Delta \mu^hc_{\text{int}}$ reflects changes in both solvent reorganization and the relative strength of the solute-solvent interaction. Here, the $\Delta \mu^hc_{\text{cav}}$
Figure 5-2. Graphical description of the solute transfer process. A loss of a solute from water is expected to be accompanied by an increase in entropy, while a negative change in enthalpy is accompanied with filling the cavity with water. Lipid bilayers are more organized structures than apolar solvents, and thus would require more energy to form a suitable cavity in the lipid phase. The van der Waals interaction associated with the hydrocarbon phase would not be significantly different from that observed in apolar solvents. If present at sufficiently high levels in the membrane, solute molecules may increase the average distance between lipid molecules, resulting in positive enthalpy and entropy values.\[220-222]\n
contribution to $\Delta \Delta \mu_{\text{soln}}$ is significantly greater than the solute-solvent term ($\Delta \mu_{\text{int}}$) as the energetic cost of cavity formation is higher than van der Waals interactions—it depends strongly on the molecular size and density of the solvent.\[223]\ A general scheme describing the solute transfer process is provided in Figure 5-2. While Eq. (5-1) provides valuable mathematical model for the solute transfer process, we are unable to experimentally confirm the individual contributions of $\Delta \mu_{\text{cav}}$ and $\Delta \mu_{\text{int}}$. However, the partition coefficient can be used as a structural...
tool to monitor changes in lipophilicity parameters via changes in the free energy, enthalpy and entropy of transfer and does not require knowledge of the solvent density.

**Standard States.** The partition equilibrium is influenced largely by the solute’s lipophilicity which is a measure of the capacity of a solute molecule to solvate into the hydrophobic region of the lipid bilayer. The higher the lipophilicity, the deeper the solute is embedded within the hydrophobic core. The differences in the structural properties between simple binary solutions and aqueous dispersions of lipid vesicles have been discussed previously. The inhomogeneous, anisotropic nature of the bilayer phase has the potential to exclude solute molecules due to packing restraints caused by the incorporation of solute into the acyl chain region. As such, changes in the partition equilibrium ensue—now multiple equilibrium process exist between the dissolved solute, undissolved solute near the lipid-water interface, and bulk

Figure 5-3. A simple schematic depicting the differences between several types of partitioning processes. A) The dissolution of Xe\(_{(g)}\) into single component, water (aq) or apolar solvent (solv). B) The net result of the Ostwald and Bunsen solubility ratios \(L^{\text{oil}}/L^{\text{water}}\) between two distinct phases. C) Graphical representation of the dispersion of LUVs. D) The heterogeneous environment of the MLVs that gives rise to the limiting chemical shift of xenon associated with the lipid environment: subscripts (I1) and (I2) signify the multiple interfacial phases that can exist in this kind of lipid suspension.
aqueous solution. The differences between several types of partitioning processes (bulk solution, binary solution, LUVs, and MLVs) are illustrated in Figure 5-3 for clarity.

When a gaseous solute (such as an inhalation anesthetic) is introduced to simple biphasic system, it is distributed over the gas (g), water (w) and hydrocarbon (s) domains (Figure 5-3B). At thermodynamic equilibrium the chemical potential of the gaseous solute in each phase are equal,[224] where

\[ \mu_g = \mu_w = \mu_s \]  

(5-2)

The individual chemical potentials of gaseous solute in each phase can be written as:

\[ \mu_g = \mu_g^\circ + RT \cdot \ln \left( f \cdot P_g \right) \]  

(5-3)

\[ \mu_w = \mu_w^\circ + RT \cdot \ln \left( \gamma_w \cdot X_w \right) \]  

(5-4)

\[ \mu_s = \mu_s^\circ + RT \cdot \ln \left( \gamma_s \cdot X_s \right) \]  

(5-5)

Assuming ideal gas and solution theory, the fugacity \( f \) and activity coefficients \( \gamma_w = \gamma_s \) can be approximated to unity at physiologically relevant temperatures and pressures. Here, the standard state of solute in the gas, aqueous and solvent phases are denoted by \( \mu_g^\circ \), \( \mu_w^\circ \), and \( \mu_s^\circ \), respectively; \( P_g \) signifies the partial pressure of the gas, while \( X_w \) and \( X_s \) refer to the mole fractions of gaseous solute in the aqueous and hydrocarbon solvent phases. The mole fraction form of the Ostwald solubility coefficient accounts for the relative distribution of gaseous solute between phases through population ratios. Manipulations of Eq.(5-3), (5-4), and (5-5) result in the following expressions:

\[ \Delta \mu_i = \mu_w^\circ - \mu_g^\circ = RT \cdot \ln \left( P_g / X_w \right) \]  

(5-6)
\[ \Delta \mu_2 = \mu_s^0 - \mu_g^0 = RT \cdot \ln \left( \frac{P_g}{X_s} \right) \]  

(5-7)

where the chemical potential differences \( \Delta \mu \) and \( \Delta \mu_2 \) represent the energies of transfer from the gaseous solute to the water and solvent phase, respectively. These are also known as the solvation energies, which possess both enthalpic and entropic contributions. The transfer energy of gas between the water and hydrocarbon phases can now be related by Eq. (5-8), where:

\[ \Delta \Delta \mu^0 = \Delta \mu_2 - \Delta \mu_1 = -RT \cdot \ln \left( \frac{X_g}{X_w} \right) \]  

(5-8)

The environmental swap (or transfer) energy is now represented by \( \Delta \Delta \mu^0 \), which is easily related to the mole fraction form of the partition coefficient and is equivalent to the Ben-Naim notation, \( \Delta \mu^*_{\text{soln}} \). As will be discussed in more detail in the next section, the possibility exists that \( P_g, X_w, \) and \( X_s \) are temperature dependent.

Unlike the hydrocarbon solvent, the degree of water-lipid interaction at the bilayer interface depends largely on the phospholipid headgroup. While these hydrating water molecules experience less hydrogen bonding than those in bulk solution, they have been shown to possess large dynamic orientational order.[225] The relative degree of order depends on how close the water molecules are to the lipid-water interface. For PC lipids, simulation studies suggest that approximately 22 water molecules per PC lipid[226] are required for complete hydration—but only 0.5-3 of those water molecules are actually tightly bound.[227] Furthermore, this structured effect is only thought to extend 7 Å from the outermost part of the PC headgroup. As stated previously, these waters lose their structural properties at elevated temperatures and eventually adapt the behavior of the bulk solution. This effective dehydration at the lipid interface may vary between lipid systems and have an effect on the partition coefficient.
5.2 The Partitioning Model as a Function of Temperature

5.2.1 Determining $K_p(T)$ by Chemical Shift Methods

The temperature dependence of the chemical shifts of xenon in LUVs, MLVs and lipid-free buffer solution allowed us to extract thermodynamic properties such as enthalpy, entropy, and the heat capacity corresponding to the transfer of xenon from bulk solution to the lipid. Once it was established that the partition coefficients determined from Eq. (4.6) were comparable to values obtained from Eq. (4.8), and that the “lipid” phase observed in the MLVs correlates well to chemical shift of xenon saturated by lipid, we were able to manipulate these properties in order to examine the role hydrophobic forces play in the xenon-membrane interaction. More specifically, we utilized the following equation,

$$ K_p = \frac{\beta \cdot [55.5M] \beta \cdot (\text{[Xe]}_aq - [L]) + [L]} $$

(5-9)

where $\beta = \Delta \delta / \Delta \delta_{\text{max}}$, $[L]$ is the total lipid concentration, and $[\text{Xe}]_{aq}$ is the amount of xenon in aqueous solution. As mentioned previously, higher $[\text{Xe}]_{aq}$ is required in order to obtain the two distinct chemical shifts representative of the two partitioning phases in DOPC MLVs. Thus, experiments were performed at an overpressure of 5 atm and a DOPC concentration of 50 mM. Xenon’s solubility in pure water decreases at elevated temperatures, so minor corrections were made to the $[\text{Xe}]_{aq}$ value in Eq. (5-9) utilizing solubility parameters available in literature.\[171, 228\]

A simple relation is needed to determine the change in the chemical potential ($\Delta \mu^\circ$) when 1 mole of xenon is transferred from bulk solution to the membrane:

$$ \Delta \mu^\circ = -RT \cdot \ln K_p(T) $$

(5-10)

$$ \Delta \mu^\circ = \Delta H_m^\circ - T \cdot \Delta S_m^\circ $$

(5-11)
\[ \ln K_p(T) = \Delta S_m^o / R - \Delta H_m^o / RT \]  \hspace{1cm} (5-12)

where \( R \) is the gas constant, \( T \) is the temperature, and \( K_p(T) \) the temperature dependence of \( K_p \).

The enthalpic and entropic components of partitioning are related via Eq. (5-11), where \( \Delta H_m^o \) and \( \Delta S_m^o \) are the partial molar enthalpy and entropy of transfer, both of which possess the capacity to be temperature dependent. The combination of Eq. (5-10) and (5-11) yields the van’t Hoff equation (Eq. (5-12)). Plotting \( \ln K_p(T) \) versus \( 1/T \) allows for the extraction of \( \Delta H_m^o \) and \( \Delta S_m^o \): \( \Delta H_m^o (T) \) is given by the changes in the slope.

### 5.2.2 Influence of Partitioning Units on Calculated Transfer Energies

In order to get a sense of how our extracted thermodynamic parameters compare to similar systems, we searched the literature and tabulated changes in the chemical potential, enthalpy and entropy due to the transfer of xenon from aqueous solution to several organic suspensions. The results are summarized in Table 5-1. The molar chemical potentials at each given temperature were determined by converting the volume fraction partition coefficient \( (K_v) \) to the mole fraction partition coefficient \( (K_p) \). Details of the conversion process can be found in section 4.3.3 of the previous chapter. The choice of partitioning units has been shown to affect the ESE’s.[229-231] The principle thermodynamic transfer function of \( Xe(g) \) is dependent on whether the molar concentration, molality or mole fraction partition coefficients are used. According to results published by Jung Hag Park et al. (1989), the energy of transfer \( (\Delta \mu_2) \) for \( Xe(g) \) to \( n \)-alkanes can be either positive or negative depending on which form of the partition coefficient is used (see Table 5-1).[216] According to our results, the mole fraction form of \( \Delta \mu^o \), describing the partitioning of xenon from aqueous solution to DOPC lipid vesicles, yields a numeric value of -3.6 kcal/mol at 298 K. Making use of the molar concentration partition coefficient yields a lesser
value of -1.39 kcal/mol. Careful attention must be paid when comparing partitioning values in literature. We have adopted the mole fraction partition coefficient as it does not require knowledge of the molecular volume of the lipid phase. All tabulated values have been converted to the mole fraction partition coefficient using methods presented in Section 4.3.5. We have already demonstrated the potential error introduced when the contribution of bound xenon to the partition expression is neglected when working at elevated pressures. It is likely that overlooking the contribution of xenon to the overall volume of the lipid phase will introduce a similar error.

5.3 Results and Discussions

5.3.1 The van’t Hoff Plot

The van’t Hoff plot describing the partitioning behavior of 5atm of xenon in 50 mM DOPC is given in Figure 5-4. First and foremost, there is a clear change in $\ln K_p$ with temperature—it increases monotonically until a maximum is reached, at which point the slope changes. The observed curvature reflects changes in the heat capacity. $\Delta C_p$ can be approximated by monitoring the standard molar enthalpy as a function of temperature (Eq.(5-13)).

$$\Delta C_p = \frac{\partial \Delta H_m}{\partial T}$$  

(5-13)

For clarification, the dashed line in Figure 5-4 is the van’t Hoff (Eq. (5-12)) equation fit to the first three data points ignoring $\Delta C_p(T)$ effects (curvature). The solid line is the least squares fit of our partitioning data to Eq. (5-12). [203, 232, 233] We have tabulated environmental swap energies for xenon partitioning in various binary solutions for comparison. The extracted thermodynamic parameters are listed in Table 5-2. When comparing the magnitude of the molar chemical potential of transfer between solutions, we see that it is relatively consistent over the whole range of lipid/oil species found. In addition to being all negative, and thus spontaneous, there is very little variation in the actual numeric value of the molar chemical potential. This is can be seen

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Figure 5-4. The van’t Hoff plot showing the temperature dependence of xenon-membrane partitioning for 5 atm of xenon dissolved in 50 mM of DOPC. Here, (○) signifies the maximum partitioning temperature and (●) denotes the experimentally determined values from chemical shift analysis. The solid line is the non-linear least squares fit of the van’t Hoff equation to all data points.\cite{203, 232, 233} The dashed line reflects the fit of Eq. (5-12) to the first three data points.

more clearly in Figure 5-5. What’s more, all enthalpy and entropy values are positive, in accordance with the classical hydrophobic effect. While we can confirm that xenon exhibits typical lipophilic thermodynamics, interpreting the significance of the thermodynamic functions is more complicated. As seen in Figure 5-6, the molar enthalpy of transfer decreases with increasing temperature; it remains endothermic under 317 K and is exothermic above it. The sign of $\Delta H^\circ_m(T)$ is a good quantitative measure of how the xenon distribution changes between the lipid and water phases with temperature; $T_H$ reflects the temperature at which water reorganization effects are energetically balanced with solute-solvent interactions. Our results suggest that xenon’s affinity to the lipid phase increases even though the solubility decreases in the aqueous phase. The subsequent decrease in the relative magnitude of the molar enthalpy of
DOPC* indicates parameters resulting from the fit to Eq. (5-13), POPC* are parameters obtained from the van’t Hoff plot using corrected $K_p$ values, POPC (no asterisk) are explicit values obtained by Meier (2006). Thermodynamic partition parameters were determined under 1 atm of $Xe_{(g)}$ overpressure, unless stated otherwise.

Figure 5-6. The enthalpy of solute transfer as a function of temperature for 50 mM DOPC under $P_{Xe} = 5$ atm of overpressure: the slope provides an approximation of $\Delta C_p$ (see Eq. (5-13)).
transfer with temperature means that xenon’s interaction with the lipid phase becomes increasingly favorable at elevated temperatures. The change in the molar heat capacity due to transfer (fit of Figure 5-6 to Eq. (5-13)) was found to be -601 ± 50 cal/mol-K. The large negative
change is not surprising as it is a thermodynamic signature of strong hydrophobic interactions. For comparison, the heat capacity of xenon in water is approximately 60 cal/mol-K.\textsuperscript{[236]}

As can be seen from Table 5-2, xenon transfer from bulk water to lipid membranes is driven by entropy change and is endothermic ($\Delta H^\circ > 0$) at ambient temperature. According to trends reported in literature, partitioning into liposomes can be driven by either enthalpic or entropic effects.\textsuperscript{[110, 237-242]} For example, partitioning into gel phase liposomes have been shown to be governed by changes in entropy, while lipid membranes in the liquid crystalline phase tend to be driven by enthalpy change. Experimental results suggest that more structurally organized liposomes consisting of saturated lipids, non bilayer lipids, and/or cholesterol, possess a much larger entropy contribution to the transfer processes. This can be explained on the grounds that more rigid membrane structures require more thermal energy to create a sufficient void for solute transfer. This is confirmed by the slight change in the thermodynamic parameters between H$_2$O:PC/PA and H$_2$O:PC/PA/cholesterol partitioning systems (see Figure 5-5). Notice the significant difference between the molar enthalpy and entropy values for DOPC and POPC lipid vesicles compared to other lipid/oil systems. Recall that the thermodynamic parameters of DOPC and POPC are measured under 5 and 6 atm of xenon overpressure, respectively. The thermodynamic parameters of all other solutions are determined at 1 atm of $P_{Xe}$. (see Figure 5-5).\textsuperscript{[237-242]} While xenon is a rather small solute molecule, it may perturb the membrane structure if present at sufficiently high levels in the membrane. An increase in the average distance between lipid molecules will result in larger, positive enthalpy and entropy values.

As discussed earlier, POPC has one shorter, saturated chain which increases the fluidity of the bilayer. Despite this difference, the thermodynamic parameters of transfer between water and the DOPC/POPC environments are nearly identical (utilizing the mole fraction partition
coefficient). The only thing that can be said for sure is that xenon partitioning appears to increase with molecular weight of the lipid/oil phase. These observations are similar to those observed by Simon et al. and their investigations of the halothane-lipid interaction.\textsuperscript{[243]} When comparing the $K_p(T)$ behavior in a variety of lipid systems, they found the partition coefficient to be insensitive both to acyl chain length and degree of saturation. However, a four-fold increase in $K_p(T)$ was observed at the dipalmitoyl-phosphocholine (DPPC) main phase transition temperature. This was attributed to the reduced entropic and enthalpic contributions due to tighter acyl chain packing in the gel phase which possesses more order and fewer van der Waals interactions.

Recent molecular dynamics simulations studying the role of lipid membranes on anesthesia may be helpful in the interpretation of our data.\textsuperscript{[15]} While these studies were not performed at variable temperature, it does provide a sense of the effect increased anesthetic concentration has on the bilayer structure. We recognize that the thermal changes we have introduced on our system are likely to perturb the whole system, while anesthetic doping is likely to induce changes more locally. Despite this, Stimson et al. saw an increase in membrane thickness and area per lipid with increasing xenon concentration. It also exhibited bimodal distribution between the lipid interface and the hydrophobic core. However the marked preference for the inter-leaflet space is at odds with experimental results.\textsuperscript{[14]} A slight increase in the deuterium order parameter suggests ordering of the acyl chains with increasing xenon concentration. Thus, our decrease in $\Delta S_m(T)$ at higher temperature might be partially explained in terms of the increased partitioning.

### 5.3.3 Enthalpy-Entropy Compensation

The compensatory effect of the enthalpy and entropy as described by Eq. (5-12) is shown clearly in Figure 5-7. The filled circles are the energy values extracted by combining Eqs. (5-9)
Figure 5-7. Variations in the molar enthalpy and entropy values with temperature. Closed circles (●) are the standard enthalpy and entropy values obtained from experimental data. The open circles (○) show the relative location of $T_S$ and $T_H$. and (5-12). As a reminder, the temperature dependence of the mole fraction partition coefficient is known from chemical shift data. This is then substituted into the van’t Hoff equation—changes in the slope and $y$-intercept values reflect changes in the molar enthalpy and entropy of transfer, respectively. Stronger molecular interactions between solute/solvent molecules result in a reduction in the configurational freedom of the system, which in turn decreases the entropy. Similarly, weaker interactions lead to an increase in the entropy. Evaluation of Figure 5-4 and Figure 5-7 allowed for the determination of the upper and lower limits of partitioning and the chemical potential, respectively. The maximum possible entropic contribution to the molar chemical potential, $\Delta S_m^\circ (317 \text{ K})$, is determined to be $-5.18 \text{ kcal/mol}$. This occurs when the enthalpy is equal to zero. Accordingly, the maximum enthalpic contribution occurs at 324 K, with a $\Delta H_m^\circ (324 \text{ K})$ value of $-4.89 \text{ kcal/mol}$. Just as $\Delta H_m^\circ (324 \text{ K})$ denotes the minimum in the
molar chemical potential for the transfer of xenon from the water to the lipid phase, \( \Delta S^m_m (317 \text{ K}) \) defines the upper partitioning limit. Similar to the molar enthalpy of transfer, the change in the molar entropy is also positive and changes with temperature. The reduction in the overall degree of order with increasing temperature is likely two-fold. As the temperature is increased i) the structured water molecules surrounding xenon become more like bulk solution, increasing the entropy associated with the aqueous phase, while ii) van der Waals interactions between the xenon and the acyl chains may also affect the order of xenon associated with the lipid phase. It is possible that both of these effects are manifest in \( \Delta S^m_m (T) \).

### 5.4 Conclusions

Herein we proposed a simple method to extract the thermodynamic parameters associated with the transfer of the noble gas xenon between two aqueous phases. By relating the temperature dependence of fast exchanging LUVs to the slower MLV system and assuming the resonance associated with the lipid phase (see Figure 4-1) is representative of the maximum chemical shift difference of xenon dissolved between phases, we manipulated the two-site exchange model to extract pertinent parameters. According to the mole fraction form of the partition coefficient, the energy of transfer is characterized by a positive enthalpy change and favorable entropy change; this process appears to be driven mainly by an entropic effect for all tabulated lipid dispersions. One of the main drawbacks to this technique is that it requires direct observation of the NMR resonance associated with the lipid phase under slow exchange conditions. This creates complications because the peak is very broad and highly dependent on the xenon overpressure and lipid concentration, having a direct effect on the maximum chemical shift difference between aqueous and lipid phases. As mentioned previously, the DOPC lipid
system requires an overpressure of at least 5 atm in order to successfully resolve out the lipid phase; if the lipid or xenon concentrations are too low only a single resonance will be observed.

In analyzing our data we cannot ignore the possibility that higher concentrations of xenon may perturb the lipid matrix as subtle changes in membrane structure or dynamics may be reflected in the standard molar entropy and enthalpy values. For example, changes in xenon’s distributional volume or diffusion properties may have influence on the lipid membranes lateral or perpendicular pressures with temperature. Results suggest increased xenon-membrane interaction at elevated temperatures. Furthermore, the positive entropy change that is shown to dominate the transfer process could be due to the release of water molecules at the lipid-water interface as well as increased disorder of the acyl chain region with partitioning. So, to answer the question posed in the introduction, it is our view that both preferential hydration of the xenon at the membrane-water interface and modifications in membrane order direct the distributive properties of xenon with temperature. These two effects are not mutually exclusive. What’s more, the lack of significant change in the molar chemical potentials, enthalpies and entropies of transfer of xenon between solutions (lipid-water versus oil-water) suggests good adherence to the MO rule with temperature (at low \( Xe_{(g)} \) overpressures).
CHAPTER 6
PRESSURE EFFECTS ON BINDING BEHAVIOR

6.1 Introduction

The two prevailing models used to characterize the association of small molecules to phospholipid vesicles are the adsorption isotherm (binding) and ideal partitioning models. As described in previous chapters, the partition equilibrium considers a two phase problem in which a probe molecule is distributed according to favorable solvation effects. The partition coefficient can be utilized to compare the solute’s lipid solubility by comparing its free energies in each phase with changing external variables. Binding models provide simple explanations for interactions between probe molecules (i.e., xenon) and a target site. It is the association of the anesthetic molecule to the lipid and/or protein that leads to its relative activity. The complexity of the binding model depends on whether the binding sites are independent, and/or equivalent. And though it is generally acknowledged that anesthetic binding to lipid membranes is nonspecific in nature, solutes are not uniformly distributed throughout the membrane. So, while the partitioning model provides information on the relative distribution between phases, it does not account for differences between surface and inner-membrane binding.

Recent computer simulations investigating the anesthetic potency/solubility relationship have shown that the potencies of inhalation anesthetics correlate better with their interfacial solubility. This was particularly true for nonpolar compounds, like xenon. These results are consistent with several modern lipid theories of anesthesia, which suggest that the preferential location of anesthetics at the membrane interfacial region is of importance to anesthetic action. However, there is limited experimental evidence of a two-step adsorption mechanism between anesthetic molecules and the lipid membrane, suggesting the presence of both a low capacity, high affinity adsorption site at the hydrophilic surface and a high capacity, low affinity site...
within the lipid core.\textsuperscript{[249-255]} It is the lipid core, with its higher binding capacity that is thought to correlate well with the solubility/potency relationship of apolar solutes in olive oil (Meyer-Overton rule) as it involves partitioning into the acyl chain region.\textsuperscript{[256]}

Herein, we utilize extracted binding data from the $^{129}$Xe NMR chemical shift analysis (presented in Chapter 4) to differentiate between anesthetic binding at the membrane interface and the lipid core. If observations by Xu and Tang (1997)\textsuperscript{[14]} are correct, and xenon interacts specifically with water at the lipid-water interface, we should be able to confirm it through binding analysis. The mathematical treatment of binding data is often fit according to solution and surface models. Since the lipid is considered to possess both solution and solid state properties, we use several isotherms for comparison. The Langmuir,\textsuperscript{[257]} BET,\textsuperscript{[258]} the D’Arcy and Watt\textsuperscript{[259]} isotherms will be reviewed in subsequent sections. Our primary goal in this chapter is to determine whether the lipid membrane surface is saturable and ascertain whether $^{129}$Xe NMR can distinguish between binding sites at the bilayer interface and lipid core.

\textbf{6.1.1 Specific versus Nonspecific Binding}

While specific binding implies strong, localized interactions, non-specific binding is often characterized by weaker, less localized interactions. Hydrogen bonding and electrostatic interactions are typical examples of specific binding, just as dispersion forces and hydrophobic interactions epitomize nonspecific binding. It remains unclear whether anesthetics act indirectly through the lipid membrane, or directly through to specific protein target sites. A wide variety of inhalational anesthetics are either diatomic or noble gases, which possess no permanent dipole or charge—they interact primarily through van der Waals forces. Xenon, for example, has a large electron cloud which is highly polarizable. This allows for nonspecific interactions at the protein surface, as well as low capacity, high affinity binding within hydrophobic pockets. However, identifying a specific protein molecule that possesses the necessary saturable binding and
structural stabilization associated with anesthesia has been difficult. As recently discussed by Carmody (2009), the Meyer-Overton theory of anesthesia overly emphasizes lipid solubility. While Carmody supports protein mediated theories of anesthetic action, he readdresses the potential role structured water molecules have on both protein conformations and lipid solubility. Similar to lipid membranes, anesthetics have been shown to preferentially bind to exposed protein-water interfaces in a nonspecific manner (e.g., firefly luciferase) despite inhibitory effects at lower anesthetic concentrations. If anesthetics act on all sites of intermediate polarity, regardless of protein or lipid environment, there may be credence to lipid-mediated action. Or, as suggested by Ueda and Yoshida (1999), anesthetic action may be due to simple changes in protein and lipid hydration.

6.1.2 Interfacial Membrane Partitioning

Experimental results indicate that the primary difference between non-immobilizers (molecules predicted to be anesthetics based on hydrophobicity, but are not) and anesthetics are their relative locations within the bilayer. Anesthetics associate strongly with the lipid-water interface, significantly affecting the dipole potential, while non-immobilizers partition into the hydrocarbon core, having minimal perturbing effect. Experimental results suggest that the adsorption mechanism of anesthetics in membrane systems change the hydrophilic properties of the membrane. Using microwave spectroscopy, Enders showed that volatile anesthetics decrease the Debye frequency of membrane-associated water molecules by a factor of three. Not only was the effect completely reversible, the observed behavior was identical for chemically different anesthetic species. Ueda et al. (1986) also showed a low-dose absorption mechanism for the inhalation anesthetic-membrane interaction using low frequency capacitance and conductance measurements. The lipid membrane is thought to possess an energy barrier at its surface, which prevents anesthetics from penetrating into the lipid core. At sufficiently high
anesthetic concentrations, the membrane loses its barrier properties, allowing penetration within the lipid core. Statistical thermodynamic models were utilized to evaluate the capacitance data and two binding modes were determined. All anesthetic molecules exhibited a similar surface-associated binding constant of 650 M\(^{-1}\) while the secondary binding affinity ranged between 1.8 and 7 M\(^{-1}\). Similar to recent computer simulations, the interfacial concentrations were significantly higher than those within the core region.[248]

6.1.3 Common Adsorption Types

Adsorption can be defined as the selective accumulation of a molecular species between two phases. If the molecule of interest is gaseous and it binds to a liquid or solid, the interface is normally between the gas and solid/liquid phase. Adsorption can be classified in one of two ways: chemisorptions (chemical adsorption) and physisorption (physical adsorption). Chemisorption is characterized by strong interactions between adsorbate and adsorbent, while physisorption is associated with weaker van der Waals interactions (i.e., dispersion and dipole). The stronger the interaction, the longer the contact time is between the adsorbing molecule and the surface. This is why chemisorbed gases tend to be more difficult to remove from the adsorbent, often resulting in changes in surface structure. Unlike chemical adsorption, physisorption is a much longer range interaction caused by nonspecific interactions. Though the molecular interaction is weak, accommodation of the adsorbate can also result in structural modifications of the surface structure including bilayer swelling, changes in the phase transition temperature, membrane fluidity, and/or the available lipid area. Several of these have been discussed in previous chapters.

Several factors which help to determine the strength and specificity of an intermolecular interaction are the hydrophobic and hydrophilic properties of the interacting species, in addition to their respective molecular size, symmetry, degrees of freedom and polarizability. As discussed
above, the higher the specificity, the stronger the interaction. Here we consider the interaction between dissolved xenon gas and the lipid membrane, where the inhalation anesthetic is physisorbed to the membrane. Similar to previous studies, we assume multilayer adsorption; each binding mode is taken to possess its own volume and capacity for the gas. After accommodation, the sorption is reversible when the gas overpressure is decreased. The bilayer surface will be referred as the adsorbent, while dissolved xenon in bulk solution ([Xe]_{aq}) is considered the adsorbate molecule. The sorption of xenon from bulk solution to the lipid surface is depicted in Figure 6-1. The specific models illustrated will be explained in further detail in upcoming sections.

Figure 6-1. Several examples of xenon-membrane binding models. The surface is the lipid membrane, and the adsorbent is xenon gas. A) Depicts monolayer coverage of the membrane surface, in which each binding site is discrete, identical and non-interacting. B) Assumes multilayer formation on the surface (BET isotherm). C) suggests that second and higher level adsorption occurs in the membrane core (modified BET isotherm).[249]

6.2 Binding Models

6.2.1 Adsorption Equilibrium

Due to the complex nature of the adsorption process, adsorption experiments are often presented in the form of binding isotherms. These binding models attempt to make sense of the adsorbate-adsorbent interaction through different assumptions and mathematical relations. The amount of pure gaseous species adsorbed by a unit mass of adsorbent is a function of
temperature and pressure alone. Results are most commonly expressed as a function of pressure, at constant temperature. The Langmuir and Brunauer, Emmet, and Teller (BET) isotherms are two of the most commonly used binding models and are typically used to describe gas-to-surface binding. The D’Arcy and Watt isotherm, a solution based model, is less commonly used. The base of each of these models is the simple equilibrium expression which measures the affinity of adsorbate to a given adsorbent.

A key aspect of the binding process is that the magnitude of the affinity between two substances is related to their interaction energy under a given set of conditions. The affinity is measured by the equilibrium constant ($K_a$); the larger the $K_a$ value, the higher the affinity. A general binding expression describing the binding process between the probe molecule, $Xe_{(aq)}$, and a lipid adsorption site, $L$, is given in Eq. (6-1). Its specific relation to the stoichiometric equilibrium constant ($K_a$) is provided in Eq. (6-2).

$$nXe_{(aq)} + L \xrightleftharpoons[k_d]{k_a} L \cdot Xe_n$$

$$K_a = \frac{[L \cdot Xe_n]}{[L][Xe]_{aq}^n} = \frac{k_a}{k_d}$$

Here, $[L]$ is the total lipid concentration, $[Xe]_{aq}$, the available xenon concentration in aqueous solution, and $[L \cdot Xe_n]$, the xenon-lipid binding complex in solution. If a macromolecule (i.e., lipid molecule) possesses many identical and non-interacting binding sites, the $n$-value shown above would be taken as 1, which is the basis of the Langmuir isotherm. However, if the binding of one adsorbate affects subsequent binding events, the affinity changes as a function of adsorbate concentration. Before we get ahead of ourselves, let us go over the assumptions of the most simple of isotherm models, the Langmuir isotherm.
**Langmuir isotherm.** The Langmuir isotherm was initially developed to describe monolayer formation of chemisorbed processes. Once equilibrium is established between the gas phase and the partially formed monolayer, one can measure the fractional coverage \( \theta \) of a given adsorbent surface at a specified pressure \( P_{Xe} \). To be specific, \( \theta \) is defined as \( \frac{V}{V_m} \) where \( V \) is the volume of adsorbed gas and \( V_m \) is the volume of gas adsorption sites that exist on the surface.

Under this model, dynamic equilibrium is reached when the rate of desorption \( (k_d) \) is equal to the rate of condensation \( (k_a) \) of the gas molecules, as described by:

\[
  k_a P_{Xe} \cdot (1 - \theta) = k_d \cdot \theta
\]

This allows for a simple relation between the fractional surface coverage, the gas pressure, and the dissociation constant where

\[
  \frac{\theta}{(1 - \theta)} = \frac{P_{Xe} \cdot k_a}{k_d} = P_{Xe} \cdot K_a
\]

at equilibrium. If \( P_{Xe} \) is small, the Langmuir adsorption isotherm reduces to a simplified expression between \( \theta \) and the binding constant, consistent with Henry’s Law—all isotherms should reduce to zero as the pressure goes to zero. For clarification, \( \theta \) is the amount of adsorbed gas per unit of adsorbent divided by the saturation value for monolayer coverage. As explained previously in Chapter 4 (Section 4.2), \( P_{Xe} \) is the xenon overpressure in the gas phase above the aqueous solution of dispersed vesicles. It is easily related to xenon’s solubility in water, allowing for a direct relation between \( P_{Xe} \) and \([Xe]_{aq}\).

There are two main criteria of the Langmuir isotherm. Firstly, the gaseous molecules are assumed to be adsorbed at a fixed number of well defined sites where each site has the capacity to hold a single adsorbate molecule. Furthermore, each site is considered to be energetically equivalent—so, there are no lateral interactions between gaseous molecules adsorbed onto neighboring sites. The Langmuir model is excellent at identifying ideal binding behavior but may
not adequately describe the gas-to-membrane adsorption process. We can easily confirm ideality from the n-value shown in Eq. (6-2). If there is no interaction between sites the n-value will be unity. The linearized form of the Langmuir isotherm is called the Scatchard plot and it is perhaps the most widely used expression in the analysis of binding equilibria in biological solutions. It is useful in that it can be used to identify changes in the binding behavior graphically. It will be discussed in more detail in upcoming sections.

The BET isotherm. Similar to the Langmuir isotherm, the BET isotherm assumes that the surface is homogenous and that there are no lateral interactions between adsorbed gases. However, gas adsorption is not limited to a single monolayer and dynamic equilibrium exists between each layer (i.e., the rate of adsorption to the first layer equals the rate of desorption of the second layer). Furthermore, the heat of adsorption of all layers above the first is equal to the heat of condensation. When the saturated vapor pressure is reached, the gas will condense as an ordinary liquid on the surface, creating an infinite number of layers on the adsorbent surface. This is unlikely for our experiments as the vapor pressure of xenon is approximately 58.21 atm at 298 K. The general expression for the BET isotherm consists of two parts, i) a Langmuir-type segment which accounts for monolayer of adsorption onto the surface, ii) a second component accounting for weakly adsorbed multi-layers. The full expression is given as:

\[
\frac{V}{V_m} = \frac{K \cdot [Xe]_{aq}}{1 + K \cdot [Xe]_{aq}} + \frac{[Xe]_{aq}}{1 - [Xe]_{aq}}
\]

(6-5)

where \( \frac{V}{V_m} \) is the fraction of the surface covered and \([Xe]_{aq}\) is as previously defined. Notice that \( K \) is not explicitly defined as the association constant. Instead, it is a proportionality constant that represents both the binding affinity and the specific number of binding sites needed to accommodate a monolayer of adsorbate. A shortcoming of the BET isotherm is that it fails to
predict limited adsorption. Guggenheim, Anderson, de Boer (GAB) model has been proposed to correct this.\cite{266}

**D’Arcy and Watt isotherm.** Briefly, the D’Arcy and Watt isotherm is a three parameter model that tries to account for both strong and weak adsorption as well as the interaction of the gaseous molecule with water.\cite{259} It can be described as a solution-to-solid adsorption isotherm that accounts for two types of binding interactions. Similar to the BET isotherm, the first term of Eq. (6-6) deals with monolayer adsorption onto a surface. The second term describes adsorption to weaker binding sites, while the last term accounts for multilayer formation, similar to the BET isotherm. The equation coefficients, $K$, and $[Xe]_{aq}$ are as previously defined, while $C$ is a constant proportional to the number and affinity of weakly adsorbed sites, and $D'$ and $D$ denote the number and affinity of the adsorbed multi-layers (Eq. (6-6)).

$$V = \frac{K' \cdot K \cdot [Xe]_{aq}}{1 + K \cdot [Xe]_{aq}} + C \cdot [Xe]_{aq} + \frac{D' \cdot D \cdot [Xe]_{aq}}{1 - D \cdot [Xe]_{aq}} \quad \text{(6-6)}$$

### 6.2.2 Macroscopic and Microscopic Binding Constants

Now that we have a sense of the typical models used in binding analysis, let us distinguish between macroscopic and microscopic binding constants. As noted in the models above, the explicit form of the association constant is not given. Rather, they express $K_a$ as proportionality constants. If a macromolecule has multiple identical non-interacting binding sites, it can be shown that the binding isotherm will be the same whether microscopic or macroscopic equilibrium constants are used. Microscopic binding is site specific, while macroscopic binding is not. This is seen more clearly in Figure 6-2; $K_1$ and $K_2$ represent sequential stoichiometric binding constants which describes occupancy by class, while $k_1$ and $k_2$ describe the complete adsorption/desorption cycle in a site specific manner. Distinct classes of binding sites are characterized by different binding affinities.
Figure 6-2. The difference between macroscopic and microscopic binding constants. In the case of multiple identical binding sites, the relationship between the microscopic ($k_1$ and $k_2$) and macroscopic binding constants ($K_1$ and $K_2$) is relatively simple. When both binding sites are empty (box 1 and 2), there are two possible binding sites for the adsorbate, so $K_1 = k_1 + k_2 = 2K$, where $K$ is the microscopic binding constant. When both sites are occupied, there are two sites from which the adsorbate can dissociate, leading to $K_2 = k_1k_2/(k_1+k_2) = K/2$.\[267\]

As the binding scheme becomes more complex, statistical thermodynamic methods become more useful. For a macromolecule that possesses more than one set of independent identical binding sites (having different affinities), then the degree of binding can be expressed as a summation of binding classes. The general expression is given in Eq. (6-7), where $r$ signifies the moles of bound adsorbate per mole of macromolecule, $n_i$ is the number of distinguishable sites in the $i$th class, $M$ is the number of classes, and $K_i$ is the binding constant associated with each.

$$r = \sum_{i=1}^{M} \frac{n_i \cdot K_i \cdot \left[Xe\right]_{aq}}{1 + K_i \cdot \left[Xe\right]_{aq}} + \ldots$$  \hspace{1cm} (6-7)$$

$$\ldots + \frac{n_i \left(K_i \left[Xe\right]_{aq}\right)^{\alpha}}{1 + \left(K_i \left[Xe\right]_{aq}\right)^{\alpha}}$$  \hspace{1cm} (6-8)$$

$$\ldots + K_{\text{nonspecific}} \left[Xe\right]_{aq}$$  \hspace{1cm} (6-9)
Notice how each term in Eq. (6-7) has the same form as the first term of the BET and D’Arcy Watt isotherms. Eq. (6-7) takes into account more complex binding behavior. For example, if there is another binding class that is not independent of previous binding events, an additional term can be added to account for it (e.g., Eq. (6-8) and (6-9)). This is particularly useful when cooperative binding occurs. Taken independently, the expression in Eq. (6-8) is known as the Hill equation, where $\alpha$ is the Hill coefficient. If $\alpha = 1$, the term becomes equivalent to the first binding class in Eq. (6-7). However, if $\alpha$ is less or greater than unity, the term adjusts for positive or negative cooperative binding. Cooperativity is when one binding event promotes or inhibits subsequent binding. The last term accounts for nonspecific binding (Eq. (6-9)). This is similar to the linear term in the D’Arcy and Watt isotherm as it describes weakly, non-interacting binding processes. The BET and D’Arcy and Watt isotherms describe sequential binding events to account for the formation of multi-layers. The adsorbate forms a monolayer on the adsorbent, after which multi-layers begin to form. Ueda et al. (1983) utilized a modified BET isotherm to extract the equilibrium constants arising from surface and lipid core partitioning. While the first layer was taken as monolayer adsorption, the second and higher layer adsorptions were taken to occur in the interior of the membrane instead of on top of the first. They assumed that surface adsorption sites were distinguishable and that an additional set of distinguishable binding sites are made available in the lipid core when a surface site becomes occupied. This model was too complicated to apply to our data. However, we did make use of similar assumptions by accounting for the possibility multiple, distinguishable binding sites.

6.3 Results and Discussion

6.3.1 Estimation of Kinetic Parameters

The membrane surface can be considered to be a two-dimensional array of lipid molecules, having distinct partition sites which allow for gas adsorption into the lipid core.
While it is understood that there are no specific interactions between lipid and xenon, the existence of multiple adsorption sites possessing different affinities is likely. The question is whether the lowest free energy region lies within the lipid interior or at the membrane surface. As the surface areas of both adsorbate and adsorbent are known we can start with a two-dimensional picture of the binding process. Following Ueda et al.’s lead, consider a two dimensional triangular lattice on the membrane surface. Now let us consider the interstitial site located at the center of this lattice to be a partition site, allowing for xenon to penetrate into the membrane core. As shown in Figure 6-3, this adsorption site is characterized by the surface areas of both the triangular lattice and the adsorbate molecule.

![Figure 6-3](image)

**Figure 6-3.** A graphic illustration of the two-dimensional binding model. According to this model, the number of partition sites \( S \) determines the saturation limit. The degree of saturation is dependent on the available membrane surface area\(^{269-271}\) and the relative amount of bound adsorbate. This figure was modified from Word and Smejtek (2005).\(^{272}\)

In calculating the total membrane surface area, both inner and outer membrane leaflets are considered available for binding, each exhibiting identical behavior. It should be noted that if only the outer layer is considered, the binding affinity increases by a factor of two. Firstly, the number of partition sites is determined according to the following relation:

\[
S = \frac{A_m}{A_{Xe}} = \frac{L \cdot A_L}{A_{Xe}}
\]  

\(6-10\)
\( A_m \) denotes the lipid membrane surface area, \( A_{Xe} \), the area of the partition site, \( A_L \), the membrane surface per lipid molecule, and \( L \) the total number of lipid molecules in a given experiment.\(^{[272]}\)

The number of lipid molecules per partition site is given by the \( A_{Xe} / A_L \) ratio.

Here, the fractional coverage (\( \theta \)), which was introduced in previous sections, is considered to be equivalent to the fraction of occupancy of these partition sites. The fractional coverage gives an indication of membrane saturation and provides the basis for all previously described binding models. As discussed by Word and Smejtek (2005), if lateral interactions between adsorbate and absorbent prevent nearest neighbor gas molecules from approaching each other within a distance comparable to the separation between lipid molecules, the number of lipid molecules associated with the partition site will be greater than one.\(^{[272]}\) According to this model, \( \theta \) is determined directly from the xenon-to-lipid ratio, where

\[
\theta = \frac{n_{\text{bound}}^{Xe}}{S} \cdot \frac{N_A}{n_L} = \frac{n_{\text{bound}}^{Xe}}{n_L} \cdot \frac{A_{Xe}}{A_L}
\]

This now allows us to estimate the binding parameters for the xenon-lipid interaction. It must be stated that while \( \theta \) is approximately equal to the \( r \)-value defined in Eq. (6-7). Even so, each will be specified when used.

At low solute concentrations, the partition coefficient may be related directly to the association constant according to \( K_a = K_p / 55.5M \). However, we have already observed the potential errors that can arise from this approximation. The infinite dilution value of the partition coefficient predicts an apparent binding constant (\( K_p^\infty \)) of \( 7.1 \pm 0.8 \) M\(^{-1} \) at trace levels of xenon. To determine the concentration dependence of xenon on the binding equilibrium, we generated isotherms for several lipid concentrations with xenon overpressures ranging from 1 to 10 atm. If ideality holds, and there is only a single partition site, the binding constant will remain unchanged.
Assuming a 1:1 complex, the association between xenon and the lipid membrane can be described by a simple equilibrium expression: \[ \text{Xe}_{(aq)} + L \rightleftharpoons \text{Xe} \cdot L \] where \( \text{Xe}_{(aq)} \) is the free xenon in solution, \( L \), the membrane environment, and \( \text{Xe} \cdot L \) the binding complex. Utilizing the law of mass action in conjunction with \( \Delta \delta \) and \( \Delta \delta_{\text{max}} \) values obtained from spectroscopic data (see Chapter 4 for details), the apparent binding affinity can be measured from the association constant of the complex \( (K_a) \), according to Eq. (6-2). Assuming ideal behavior, experimentally determined values of \( [\text{Xe}]_{\text{bound}} \) were extracted from the following relation:

\[
[\text{Xe}]_{\text{bound}} = [\text{Xe}]_{aq} \cdot \left( \frac{\Delta \delta}{\Delta \delta_{\text{max}} - \Delta \delta} \right)
\]  

(6-12)

As a reminder, \( \Delta \delta \) is the experimentally observed chemical shift difference between xenon in the LUVs and lipid-free buffer solution (\( \delta_{\text{LUV}}^{\text{abs}} - \delta_{\text{buffer}}^{\text{abs}} \)), and \( \Delta \delta_{\text{max}} \) is the maximum chemical shift difference between the lipid and buffer phases (Figure 4-1). The pressure dependence of the \( \Delta \delta_{\text{max}} \) value was shown previously (Figure 4-8).

The combination of Eqs. (6-12) and (6-2) yields a linearized form the Langmuir isotherm known as the Scatchard equation:

\[
\frac{r}{[\text{Xe}]_{aq}} = \frac{\Delta \delta}{[L] \cdot (\Delta \delta - \Delta \delta_{\text{max}})} = n \cdot K_a - r \cdot K_a
\]  

(6-13)

Here, \( r \) refers to the amount of bound xenon per mole of lipid, and \( n \) is the number of bound adsorbate molecules per binding site. The Scatchard plot is often used to quantitatively interpret the binding behavior of biologically relevant media in solution and is generally employed to differentiate between specific and nonspecific binding.\[^{273-275}\] Nonspecific interactions are characterized by its proportionality to the amount of adsorbate in solution, i.e. doubling the concentration of \( [\text{Xe}]_{aq} \) doubles the amount of nonspecific binding. Thus, nonspecific
interactions are often indicated by straight, horizontal lines (zero slope) in the Scatchard plot. Specific, higher affinity interactions are usually manifest by a low initial slope (negative in value). Changes in the slope reflect changes in the relative binding activity. Here, a Scatchard plot were made for fixed [L] and variable [Xe]_{aq}, (Figure 6-4). The initial slope is positive, which is an unusual feature for Scatchard plots. Positive slopes are often given as indications of positive cooperativity and unsaturated binding.\cite{278-281}

To further elucidate the quantitative binding of xenon to lipid vesicles, the experimental data was fit to the Hill equation to check for cooperativity. It is possible that the changing affinity is due to the increased presence of xenon on the surface. The Hill plot is commonly used in the evaluation of dose-response curves and allows for the extraction of both the apparent
association constant ($K_a$) and the relative degree of cooperativity. The Hill equation is shown explicitly in Eq. (6-8), while the fit of experimentally derived data to this model is provided in Figure 6-5. A fit to the Langmuir isotherm is given for comparison. As expected, the Langmuir isotherm provides a good fit at low $[Xe]_{aq}$. This section, defined by the initial slope from origin, is called the Henry’s law region and is characteristic of nonspecific binding. The apparent association constant resulting from the fit of the Langmuir isotherm (Eq.(6-7)) to the first three points was found to be $8.1 \pm 0.2$ M$^{-1}$; this is near our predetermined infinite dilution value ($K_a^\infty = 7.1 \pm 0.8$ M$^{-1}$). But, just as the Langmuir model fails to accurately fit binding at higher xenon doping, the Hill equation gives a poor fit for binding at low xenon concentrations (dashed line Figure 6-5). Combining the two models yields the following equation:

$$\frac{\theta}{1-\theta} = n_1 \cdot K_1 \cdot [Xe]_{aq} + n_2 \cdot (K_2 \cdot [Xe]_{aq})^\alpha$$

(6-14)

where $n_1 = n_2 = 1$, $\alpha$ is the Hill coefficient, and $K_1$ and $K_2$ signify the association constants stemming from the Langmuir isotherm and the Hill equations, respectively. Doing so generates an adequate model for our data, the fit of which can be seen clearly in Figure 6-5 (solid line). The extracted equilibrium constants are as follows: $K_1 = 10.8 \pm 0.8$ M$^{-1}$, $K_2 = 21.3 \pm 0.2$ M$^{-1}$, with $\alpha = 3.7 \pm 0.3$. The high $\alpha$-value suggests a large degree of positive cooperativity with increased doping, meaning that each xenon-membrane binding event facilitates further interaction.

The dose dependency of $[Xe]_{aq}$ on the nonspecific binding isotherm (Eq. (6-9)) is shown in Figure 6-6. Similar to the Hill model, the nonspecific binding isotherm fails to accurately fit binding at low xenon doping (dashed line Figure 6-6). However, adjusting for cooperativity yields an apparent association constant of $K_{nonspecific} = 13.6 \pm 0.1$ M$^{-1}$ and a Hill coefficient ($\alpha$) of
Figure 6-5. A Hill plot (dashed line) fit to experimental data resulted in an apparent binding constant of $24.9 \pm 0.3\text{ M}^{-1}$ and a Hill coefficient of $2.1 \pm 0.1$. The dotted line is the Langmuir isotherm (Eq. (6-4)) fit to the first three points, yielding a $K_a$ of $8.1 \pm 0.2$. The solid line corresponds to the fit which combines the two models (Eq. (6-14)). Errors are reported as ± (SSE).

Figure 6-6. Adsorption profiles of experimental data fit to several models. Experimental data fit to the Langmuir isotherm (dotted line: fit to first three points only) and a generalized non-specific model (dashed line) which yields a $K_a$ of $8.1 \pm 0.2$ and $11.5 \pm 0.2\text{ M}^{-1}$, respectively. The solid line is the fit to Eq. (6-15). Errors are reported as ± (SSE).
1.2 ± 0.1, indicating slight positive cooperativity with increasing \( [\text{Xe}]_{aq} \). A reduced form of the Hill equation (Eq. (6-8)) accounting for cooperative, nonspecific binding is then combined with the Langmuir adsorption isotherm through the following relation:

\[
r = \frac{n_1 \cdot K_1 \cdot [\text{Xe}]_{aq}}{1 + K_1 \cdot [\text{Xe}]_{aq} + (K_{\text{nonspecific}} \cdot [\text{Xe}]_{aq})^\alpha}.
\] (6-15)

Doing so results in an adequate fit of our binding data, as shown in Figure 6-6. The extracted binding parameters were: \( K_1 = 6.9 \pm 1.3 \text{ M}^{-1} \), \( n_1 = 1 \), \( K_{\text{nonspecific}} = 11.0 \pm 0.2 \text{ M}^{-1} \), and \( \alpha = 1.6 \pm 0.2 \). The difference between the nonspecific portion of the modified binding model shown in Eq. (6-15) and the Hill equation (see Eq. (6-7)) is the lack of terms within the denominator. This simplification is correct assuming \( K_{\text{nonspecific}}[\text{Xe}]_{aq} \ll 1 \). As suggested by the Scatchard plot (Figure 6-2), there appears to be two stages of binding—an initial Langmuir-type (i.e., identical, non-interacting sites) and another that appears consequence of increased doping.

In order to rule out multi-layer binding on the membrane surface, we attempted to fit our data to the BET and D’Arcy and Watt isotherms. While unsuccessful on reaching satisfactory fits with the BET model, accounting for an additional weak adsorption site (second term of D’Arcy and Watt isotherm) did allow for the extraction of the binding constants from Eq. (6-6). The fit yielded six parameters (see Figure 6-7): the affinity for xenon binding on the surface and its number of adsorptions sites (\( K_1 \) and \( M_1 \), respectively), a weaker interaction (\( K_2 \)) and its specified sites (\( M_2 \)), and a correction term that accounts for xenon’s affinity to and formation of multilayers. The extracted parameters are as follows, \( M_1 = 3.8, K_1 = 6.5, M_2 = 1.2, K_2 = 11.7, D' = 6.3, \) and \( D = -6.5 \). Consistent with Eq. (6-6) formalisms \( C = M_2 \cdot K_2 \approx 14 \), \( K' = M_1 \cdot V_m \), and \( K = K_1 \). Interestingly, the negative value for the \( D \) parameter changes the sign of the third term in Eq. (6-6). Though unclear why, its numeric value is identical to the \( K_1 \) term, suggesting that...
multilayer binding on the membrane surface possesses similar affinity. If this were true, the $K_1$ binding constant could be associated with the membrane surface. However, the D'Arcy and Watt model does indicate the presence of two binding modes. Similar to the Scatchard plot, we see a higher capacity, lower affinity binding site as well as a lower capacity, higher affinity site. However, adsorption isotherm analysis still does not identify which binding site corresponds to the lipid surface and which to the membrane core.

Lastly, we looked at the effect of sequential binding to see whether the nonspecific cooperative binding is due to partitioning into the lipid core or surface binding. A universally valid equation that accounts for cooperativity effects, the possibility of multiple binding sites that may or may not possess different affinities, is the stoichiometric binding equation:

$$
r(Xe) = \frac{K_1 \cdot [Xe]_{aq} + 2 \cdot K_1 \cdot K_2 \cdot [Xe]_{aq}^2 + \ldots + M \cdot K_1 \cdot K_2 \cdot K_3 \cdot \ldots \cdot K_M \cdot [Xe]_{aq}^M}{1 + K_1 \cdot [Xe]_{aq} + K_1 \cdot K_2 \cdot [Xe]_{aq}^2 + \ldots + K_1 \cdot K_2 \cdot K_3 \cdot \ldots \cdot K_M \cdot [Xe]_{aq}^M}
$$

(6-16)
Figure 6-8. Klotz affinity profile for binding of xenon to DOPC. $K_i$ is the stoichiometric binding constant and $i$ the sequential binding number. The dotted line is an approximation of ideal binding, while ($\bullet$) signifies two binding events, ($\circ$), three, ($\Delta$), four, and ($*$) seven binding events.

where, $K_1$ to $K_M$ are stoichiometric binding constants. For ideal, non-interacting sites, the macroscopic $K_i$ (Ind) values are linearly related when placed on a Klotz affinity plot.\cite{282,283} If no cooperativity exists, the plot will show a linear decrease in the stoichiometric parameter with increasing number of binding events ($i$) according to the following relation:

$$iK_{i(\text{Ind})} = (n + 1) \cdot K_1 - iK_1$$

Positive and negative cooperativity are seen graphically through changes in slope; a positive slope implies enhanced binding while negative slopes suggest some impedance. Though Eq. (6-16) provides a stepwise interpretation of the binding data, it does not indicate the number of binding events that are present ($M$). In order to account for this, we used a simple iterative procedure, varying the number of binding events in a sequentially, from $i = 2$ to $i = 7$. The variations in binding parameters are obtained by repeating the fit procedure, starting from initial
estimates. As seen in Figure 6-8, the best fit changes slightly when additional binding events are introduced. Our initial estimation of $K_1$ and $K_2$, provided from parameters acquired through Eq. (6-13), drastically improved our chances for convergence. The two binding events are shown clearly in the Klotz plot. The binding constant, $K_i$, is the average binding affinity of xenon to the lipid matrix. The first binding event to one lipid molecule is rather low affinity, while the second binding event to the lipid shows some cooperativity as well as higher affinity. Interestingly, the third binding event shows negative cooperativity and almost no affinity. However, it appears that xenon will have greater affinity for it once the binding event has occurred. This is consistent with Ueda’s theory[249] of a dysfunctional lipid interface with increased doping.

6.4 Conclusions

The goal of this chapter was to differentiate between surface and core binding via chemical shift analysis. The Scatchard plots show atypical behavior at low $r$-values in the form of a positive slope. This unusual feature is often indicative of positive cooperativity.[278-281] Various binding isotherms were prepared from the xenon-lipid binding data with hopes of elucidation this phenomenon, thereby providing additional information about the net affinity with increasing xenon loading. Langmuir type adsorption (ideal, non-interacting binding sites) is observed at low xenon concentrations while positively cooperative, non-specific behavior emerges at higher concentrations. Kreishman et al. (1985) reported similar behavior in that higher ethanol concentrations were found to perturb the membrane in a cooperative manner.[284] Computer simulations suggest that as anesthetic molecules bind to the membrane interface, the surface becomes partially dehydrated, increasing the anesthetic binding potential.[285] Interestingly, this is consistent with theoretical work investigating the distribution of xenon in a phosphocholine environment with increased doping; the population in the membrane core was more substantial at higher xenon-to-lipid ratios.[15] This dose dependent binding may also explain why $^{129}$Xe- NOE
experiments by Xu and Tan (1997)\cite{14} only observed interactions at the lipid interface—their experiments were performed at 1 atm. Lastly, it should be noted that Ostwald solubility coefficients are essentially association constants. The typical values range between 12-20 M\(^{-1}\) in lipid systems at 1 atm of \(Xe(\text{g})\) overpressure, which is in the range of our higher affinity adsorption site. Consistent with theory, we see that partition coefficients do not necessarily translate to net affinity when working at higher xenon overpressures. To be clear, we understand that there are no specific binding sites between xenon and the membrane adsorption sites. Our results clearly indicate that the collective interactions in the lipid array may change with relative amounts of anesthetic gas.
CHAPTER 7
CHANGES IN LIPID COMPOSITION: EFFECTS OF NONBILAYER LIPIDS

7.1 Introduction

There are still many open questions concerning the mechanisms by which variations in lipid composition affect membrane function and considerable interest has been focused on elucidating the structural properties and behavior of nonbilayer lipids. A particular source of debate is whether proteins modulate its interaction with the cell membrane, or if the local dynamics and structural features of the lipid membrane prompt biological action. Though there are strong advocates for both sides of this controversial topic, our interests lie in gaining insight into how variations in lipid composition affect molecular interactions and transformations within lipid membrane topologies.

Nonbilayer structures are thought to play an important role in membrane function; the formation of local regions of nonbilayer structures within the biomembrane (i.e. lipid rafts) is one such idea. In addition to being responsible for trans-bilayer transport mechanism of lipids and polar solutes, transient formations of inverted structures may be possible precursors to membrane fusion events. By affecting the barrier and flexibility properties, the presence of these lipid species may indirectly affect protein function as well. Not only have they been shown to increase the activity of numerous peripheral and integral membrane proteins (e.g. protein kinase C and rhodopsin), there is experimental evidence that they also influence the conductance of channel forming peptides as well. Not surprisingly, nonbilayer lipids have also been suggested to play a key role in the anesthesia. Chapter 7 is focused on elucidating what role, if any, the nonbilayer lipid DOPE plays in these processes by $^{31}$P and $^{129}$Xe NMR methods.

We begin by studying the effect of increasing molecular strain by nonbilayer lipids with hopes of gaining insight into the subtle energetic changes in stressed lipid bilayers. In lieu of the
$^{129}$Xe-DOPC NMR studies, similar applications of the partition model (Chapter 4) were utilized to investigate the influence of the nonbilayer lipid DOPE on the partitioning behavior of xenon between bulk solution and ‘frustrated’ bilayers. Theoretically, the lateral pressure profile should be altered with PE content and may be reflected in changes in the apparent mole fraction partition coefficient. By inducing the actual phase transition from $L_\alpha$-to-$H II$ we hope to confirm the presence of transient structures possessing similar curvature exhibited in some of these membrane processes.

### 7.1.2 Nonbilayer Lipids on Biological Processes

Though biological membranes inherently possess bilayer structure they also tend to contain substantial amounts of nonbilayer lipids. The sheer abundance ($\approx 75\%$) of phosphatidylethanolamine (PE) in the inner membrane of Escherichia Coli suggests some role in the overall functionality of the membrane, yet a precise explanation for its presence has been elusive. Numerous models describing nonbilayer behavior exist in the literature and can be explained in terms of one or more of the following factors: curvature stress, packing defects, lateral pressure profile, hydrophobic mismatch or bilayer fluidity. Despite this, only a moderate mechanistic understanding is known about nonbilayer lipid function at the molecular level. While the physics of isolated tubular membrane structures (e.g. PE) has been studied extensively and is well characterized, the mechanism by which these structures are formed from planar or spherical membrane species are not.

At equilibrium, the lipid membrane stability reflects a balance between the attractive and repulsive forces between the headgroups and hydrocarbon tails, and is often described in terms of the lateral pressure profile. In the context of lipid-protein interactions, modifications in bilayer properties (i.e., intrinsic lipid curvature, bilayer thickness, etc.) can decrease the free-energy difference between various protein conformations. As discussed by Anderson and Koeppe
(2007), the bilayer deformation energy is largely responsible for regulating the kinetic and equilibrium properties of the protein due to hydrophobic coupling between protein function and bilayer properties. Deformations in the membrane via area compression, expansion, or bending processes, help facilitate morphological changes in lipid assemblies which can affect the interfacial properties of the bilayer. For example, the protein rhodopsin has been shown to be extremely sensitive to the degree of spontaneous curvature. When reconstituted into PC lipid mixtures, this protein does not activate; its function appears to be dependent on the presence of lipids that possess the tendency to promote nonbilayer phases and/or negative curvature. A graphical representation on the effect of membrane compression and bending properties as related to integral membrane proteins is shown in Figure 7-1. Evidence suggests that nonbilayer lipids localize near membrane spanning channels and proteins in an effort to reduce the bilayer stress associated with conformational changes.

Lipid ‘rafts’ are areas of membrane that possess different composition and physiochemical properties from the rest of the lipid matrix; they have been experimentally verified to exist in both biomembranes and model membranes. Lateral heterogeneity has been induced by alcohol as well as dehyration mixtures and been shown to be caused by interactions between lipids and integral membrane proteins. Furthermore, it is hypothesized that these domain formations are precursors to the inverted hexagonal phase (HII) formation. The Lα-to-HII transition is a multistep process which involves the formation of intermediate structures having similar curvature and structure as those involved in membrane transport events (e.g. vesicle trafficking, pore formation). The pore-stalk fusion hypothesis is the most widely accepted model for this process. The stalk mechanism is based on the existence of membrane defects in the lipid membrane. In order for fusion to occur i) two membranes must be brought
into contact, ii) defects in the two membranes must align, iii) fuse and form a bridge-like configuration called a stalk, iv) internal monolayers are expelled from the contact region as external monolayers are simultaneously brought together, resulting in an elongated stalk structure which allows v) small holes to begin to form. An open fusion pore results when the bilayer formed by the external monolayers is broken down. This can be seen more clearly in Figure 7-2. While the inverted hexagonal phase itself has never been directly detected under physiological conditions, local structures with similar curvature have been observed.

The difficulty in creating a concrete molecular picture of the formation of these intermediate structures is that the structures themselves are short lived and small in size. For

![Diagram](image1.png)

**Figure 7-1.** A graphical depiction of the hydrophobic mismatch and examples of common membrane deformations associated with it. A) $d_c = d_p$. B) $d_c < d_p$. C) $d_c > d_p$. When the length of the unperturbed hydrocarbon region ($d_c$) is equal to the hydrophobic span ($d_p$) of an integral membrane, the deformation energy is 0. If $d_c < d_p$ or $d_c > d_p$ the molecular strain is manifest through modulations in membrane structure. The energy associated with expansion/compression and bending mechanisms are proportional to $\frac{1}{2} K \cdot (2C/d_c)^2$ and $\frac{1}{2} K \cdot (\nabla^2 D - H_0)^2$, respectively. Here, $C$ is proportional to the changes in area, and $D$ to the membrane’s principle curvature.\(^{[308]}\) This figure has been modified from Anderson and Kopepe (2007).\(^{[287]}\)
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Figure 7-2. Depiction of the membrane fusion intermediated according to the stalk mechanism. When two bilayers come into close proximity to each other, modifications in the surface curvature are thought to ensue, resulting in pore formation. The process is described in detail in the text. This figure was modified from Jahn, Yang and Sudhof (2003).[309]

example, the cubic phase, an intermediate structure in the inverted hexagonal phase formation, is characterized by a single isotropic peak in $^{31}$P NMR spectra and does not necessarily provide the best molecular description beyond changes in motion. Micellar phases and small unilamellar vesicles (SUVs) are also characterized by isotropic peaks. X-ray diffraction methods were recently used to verify the existence of pore-like structures on a bilayer[310], but how these morphological changes are initiated is still not completely understood. Interestingly, several solute molecules have been shown to stabilize the formation of these intermediate structures.[310, 311] Assuming xenon gas behaves in a similar manner, we expect $^{129}$Xe NMR to substantially improve our understanding of the process.

7.1.2 Energetics of the Bilayer-to-Inverted Hexagonal Transition

As mentioned in Chapter 3, bilayers can exist in a variety of physical states depending on the membrane composition and temperature. The most common change in states occurs between the gel and bilayer phase, termed the main phase transition. The gel phase is characterized by higher order and rigidity. When the temperature is increased the membrane core becomes more fluid due to a conformational change of the acyl chains. The temperature at which this occurs is
known as the main phase transition temperature \( (T_m) \). This depends largely on the length and
degree of unsaturation of the acyl chain; longer acyl chains and higher saturation increase the
packing efficiency, increasing the value of \( T_m \). Conceptually, the bilayer-to-inverted hexagonal
phase \( (L_\alpha\text{-to-}\text{H}_{II}) \) transition follows similar principles, albeit on a more complicated system.
Unlike the gel-to-bilayer transition, the \( L_\alpha\text{-to-}\text{H}_{II} \) transition involves a significant morphological
change in the bilayer structure. However, the transition temperature is still dependent on the
relative packing efficiency of the bilayer. If there is too much molecular strain at the membrane
surface or hydrophobic core, the energy is released through the formation of lipid domains or
even a phase transition.\(^{[312-314]}\)

In many cases the bilayer can be viewed as two separate, independent monolayers. The
free lateral motion of the lipids in each leaflet enables each layer to effectively relax surface
tension (caused by changes in protein/pore conformations) through the redistribution of
nonbilayer lipids. If we consider the lipid membrane as an incompressible, two-dimensional fluid
that behaves as an elastic body when bent, the curvature elastic energy can be expressed as a
summation of the bending modulus and the area expansion (see Figure 7-1).\(^{[308]}\) As mentioned in
Chapter 3, it is energetically possible to suppress a lipids’ intrinsic curvature, forcing the lipids to
remain in the bilayer phase. Doing so introduces lateral stress in the bilayer which often
manifests as changes in the membranes deformation energy (\( \mu_c \)).

The spontaneous curvature measures the tendency for monolayers to bend into nonplanar
geometries. If the headgroup layer prefers to be convex and bend outward into the water phase, it
is assigned positive curvature Figure 7-1C. However, it the headgroup layer prefers to curl
around the water in a concave fashion, it is said to possess negative curvature (Figure 7-1B).
Table 7-1. Structural and elastic properties of single component DOPC and DOPE lipids within a (1:3) DOPC/DOPE lipid mixture

<table>
<thead>
<tr>
<th>Property</th>
<th>Molecular weight</th>
<th>( V_L ) (Å³)</th>
<th>( d_c ) (Å)</th>
<th>( c_o \cdot d_c )</th>
<th>( A_o ) (Å²)</th>
<th>( \Delta A/A_o )</th>
<th>( \kappa_c / \gamma_{int} )</th>
<th>( \kappa_b / \gamma_{int} \cdot d_c^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>786</td>
<td>1292</td>
<td>25[^{[315]}]</td>
<td>-0.29[^{[316]}]</td>
<td>0.0082</td>
<td>0.05[^{[317]}]</td>
<td>2.9[^{[315]}]</td>
<td>0.12[^{[318]}]</td>
</tr>
<tr>
<td>DOPE</td>
<td>744</td>
<td>1235</td>
<td>30[^{[319]}]</td>
<td>-1.1[^{[318]}]</td>
<td>0.0065</td>
<td>0.05[^{[317]}]</td>
<td>4.4[^{[315]}]</td>
<td>0.10[^{[316]}]</td>
</tr>
</tbody>
</table>

When the spontaneous curvature is sufficiently negative, inverted and cubic phases are formed.

A comparison of structural and elastic properties of DOPE and DOPC bilayer membranes is provided in Table 7-1. Despite the small differences in their relative molecular weights and volumes, the curvature frustration energy for maintaining DOPE in the bilayer phase can range between 0.2-2 \( k_B T/nm^2 \). The spontaneous curvature (\( c_o \)) is likely to vary between -0.0107 Å⁻¹ and -0.0476 Å⁻¹ for DOPC and DOPE, respectively. The membrane properties listed in Table 7-1 are defined as follows: \( d_c \) is the thickness of the membrane hydrophobic core, \( c_o \), the monolayer spontaneous curvature, \( \Delta A/A_o \), the bilayer area expansion, \( \kappa_c \), the bilayer elastic compressibility modulus, \( \kappa_b \), the monolayer bending modulus, and \( \kappa_c / \gamma_{int} \) the interfacial tension. Molecular weights and volumes were provided by Chen and Rand (1998).\[^{[320]}\]

The \( L_\alpha \)-to-\( H_{II} \) phase transition temperature (\( T_h \)) is largely dependent on the curvature elastic energy and various models have been presented over the years discussing the stabilizing factors of the inverted hexagonal phase formation in a variety of lipid systems. In general, the difference of the chemical potential between phases (\( \Delta \mu \)) can be expressed as a summation of the curvature elastic energy (\( \Delta \mu_{curv} \)), and the interstitial chain packing energy of the inverted hexagonal phase (\( \Delta \mu_{ch} \)).\[^{[321]}\]

\[
\Delta \mu = \Delta \mu_{curv} - \Delta \mu_{ch} = (\Delta \mu_{curv} - \Delta \mu_{ch}) + (\Delta \mu_{ch} - \Delta \mu_{curv}) = \Delta \mu_{curv} - \Delta \mu_{ch} \tag{7-1}
\]
Where $\Delta \mu^{\text{bil}}$ and $\Delta \mu^{\text{HII}}$ are the chemical potentials of the bilayer and inverted hexagonal phases, respectively. The $L_\alpha$-to-$H_{II}$ transition arises primarily from reductions in the membrane spontaneous curvature. An entropic need to pack the hydrocarbon moieties as uniformly as possible is what gives rise to the chain packing energy—it is more pronounced in the inverted hexagonal phase than the lamellar phase, which means that $\Delta \mu_{ch}$ is always positive. Interstitial domains are formed in the $H_{II}$ phase and the acyl chains of the lipids to extend to different lengths to accommodate. This in turn decreases the entropy of the hydrocarbon chains and increases the chemical potential of the membrane.

\[
\mu_{\text{curv}} = \frac{1}{2} \left( N_A \kappa_c A_o \right) \left( \frac{1}{R_w} - \frac{1}{R_o} \right)^2 \tag{7-2}
\]

\[
\Delta \mu_{\text{curv}} = \left( \mu_{\text{curv}}^{H_{II}} - \mu_{\text{curv}}^{\text{bil}} \right) = -\frac{1}{2} \frac{N_A \kappa_c A_o^{\text{bil}}}{R_o^2} (<0) \tag{7-3}
\]

The general expression for the curvature elastic energy ($\mu_{\text{curv}}$) is provided in Eq. (7-2) and the difference between the inverted hexagonal and bilayer phase in Eq. (7-3); $\Delta \mu_{\text{curv}}$ must be negative in order for a stable transition to occur. The terms are defined as follows: $N_A$, Avogadro’s number; $\kappa_c$, the elastic bending modulus; $A_o$, the optimal surface area of the lipid phase; $1/R_w = c_w$, curvature of the lipid monolayer; and $1/R_o = c_o$, the intrinsic curvature of the lipid monolayer. In fully hydrated conditions $1/R_w$ is approximately equal to $1/R_o$ in the inverted hexagonal phase, facilitating the minimization of its curvature energy. Likewise, $1/R_w = c_o$ must equal zero in the bilayer phase in order for its elastic curvature to be reduced.

As shown in Figure 7-3, the inverted hexagonal phase is characterized by tubular structures formed around water channels and the hydrophobic interstitial spaces that exist between them. The acyl chains must stretch in order to accommodate the structural change which reduces the
entropy and increases the chain packing energy. However, this can be lowered by adding nonpolar oils,[322] long chain alkanes,[323] or longer chained phospholipids,[324] which act to stabilize the interstices. In theory, these should stabilize fusion intermediates as well. $T_h$ can be further reduced by increasing the PE content since the spontaneous curvature of a lipid mixture is a weighted average of the curvatures of the individual components. This chapter focuses on the potential utility of xenon to stabilize these intermediate structures and what role, if any, nonbilayer lipids play in the anesthetic-membrane interaction via $^{129}$Xe NMR.

### 7.2 Experimental

In this study we used DOPC/DOPE mixtures containing 0 to 75% DOPE. All samples were extruded to 100 nm LUVs, excluding the (1:3) DOPC/DOPE mixture which is already in the inverted hexagonal phase. While the majority of experiments were performed at relatively low xenon overpressures (2.5 atm), we were unable to detect the phase transition via $^{129}$Xe NMR at overpressures under 5 atm of thermally polarized xenon. Thus, we made to use hyper-polarized $^{129}$Xe NMR to study the lamellar to inverted hexagonal phase transition at low xenon
concentrations. A schematic of the hyper-polarized $^{129}$Xe NMR apparatus used is shown in Figure 7-4. The experimental procedure was as follows: 3300 Torr of 2% gas mixture of xenon was recirculated through the pumping cell for optimal polarization. As shown below, two valves were placed on the input and output of the sample in order to create a pressure differential over the solution. The normally closed solenoid valve was controlled by the spectrometer through a pulse sequence. Each ‘bubbling’ event consisted of ten open/close cycles of the solenoid valve, where $d_3$ is bubbling time (25 ms), and $d_1$ the delay between opening and closing of the solenoid valve (2s). Each spectrum obtained through this method resulted in a net loss of 600Torr of gas mixture.

**Figure 7-4.** Schematic of the recirculation apparatus used to introduce hyper-polarized $^{129}$Xe gas mixture to lipid sample. S: solenoid valve; V: needle valve; PC: pumping cell; $d_3$: bubbling time; $d_1$: delay between bubbling events; $p_1$: 90° pulse length; aq: acquisition.
7.3 Results and Discussion

The primary difference between DOPE and DOPC is the headgroup moiety (see Chapter 3). The substitution of the three methyl groups by hydrogen, \(N(CH_3)_3\) to \(NH_3\), has large consequences on the interfacial structure; not only does the hydrogen bonding scheme change but modifications in both the bilayer swelling limit and vesicle fluidity can occur. While the orientation of water molecules around PC membrane is characteristic of solvation shells of hydrophilic solutes (e.g. clathrate-like structures), PE posses hydrophobic-like solvation in which the water oxygens oriented towards the nitrogen.\(^{[187]}\) This may result in stronger hydrogen-bonding interactions for PE headgroups as well as stronger inter-headgroup hydrogen bonding, leading to a more restricted motion.\(^{[325, 326]}\) Thus, the PE headgroup protrudes further into the bilayer and is less fluid, allowing for the formation of hydrophobic pockets at the lipid-water interface.\(^{[327]}\)

To begin, we looked at xenon partitioning as a function of the DOPC/DOPE molar ratio utilizing the NMR exchange theory presented in Chapter 4. As was done previously, the \(\Delta\delta\) values were plotted as a function of total lipid concentration and fit to Eq. (4.2). The only difference is that the lipid composition has changed. This is shown for several lipid compositions in Figure 7-5A. All spectra were obtained at 298 K, a \(^{129}\)Xe overpressure of 2.5 atm and fixed volume. From previous results, we know that it is highly likely that xenon interacts at the membrane-water interface at low \([Xe]_{aq}\). This is further substantiated by the DOPE data. As seen in Figure 7-5B, the partition coefficient decreases with increasing DOPE mole fraction \(\chi(DOPE)\). What’s more, the association constant also appears to decrease with higher DOPE content isotherm (Figure 7-6A). The association constants were determined by plotting \([Xe]_{bound}\)
\/[Xe]_{aq}$ as a function of lipid concentration, where the bound-to-free xenon ratio was calculated from the following equation:

$$\frac{[Xe]_{bound}}{[Xe]_{aq}} = \frac{\Delta \delta}{\Delta \delta_{max} - \Delta \delta}. \quad (7-4)$$

As mentioned previously, $\Delta \delta$ is the experimentally observed ($\Delta \delta$) chemical shift difference between the fast exchanging xenon between the LUVs of various composition and the bulk water system. The predicted maximum shift difference ($\Delta \delta_{max}$) was obtained from the fit of experimental results to Eq. (4.2). The extracted $K_a$ values were found to be proportional to the mole fraction partition coefficient ($K_a = K_p/55.5M$), suggesting ideal binding and the Henry’s law region of the binding isotherm (Figure 7-6B). Numeric values for $K_a$ were found to be 7.9 M$^{-1}$, 5.8 M$^{-1}$, 4.1 M$^{-1}$, 3.7 M$^{-1}$ for 0, 0.125, 0.25 and 0.50 mole fractions of DOPE in DOPC containing liposomes, respectively. Again, it should be made clear, that both inner and outer vesicle leaflets were considered available for binding.

Figure 7-5. Influence of DOPE doping on the xenon-membrane mole fraction partition coefficient. A) Experimental data fit to Eq. (4.2) for various lipid compositions. Here, (Δ) denotes pure DOPC, (○), (7:1) DOPC:DOPE, and (●), (3:1) DOPC:DOPE lipid ratio. B) Shows the influence of DOPE doping on the xenon-membrane mole fraction partition coefficient. Errors are reported as ± (SSE).
Recent studies have shown that the membrane spanning peptide gramicidin is particularly sensitive to lipid composition.\cite{328} Gramicidin is shorter than the hydrophobic thickness of either DOPC or DOPE, which results in a hydrophobic mismatch (Figure 7-1). As explained earlier, this mismatch introduces both curvature and elastic strain. The introduction of halothane led to a 10 fold decrease in channel lifetime in DOPC bilayers; the magnitude of this effect was shown to be dependent on both lipid composition and anesthetic concentration. The lifetime of the channel was shown to be inversely proportional to the anesthetic concentration; the partitioning of halothane was reduced 3-fold in DOPE containing membranes compared to the single component DOPC lipid matrix. The tighter lipid packing of DOPE actually reduced the effect of halothane on the channel due to reduced partitioning. Theoretical predictions by Cantor (2001)\cite{158} indicate that in the limit of low solute concentration the partitioning of short \textit{n}-alkanols should decrease in the presence of both DOPE and cholesterol, consistent with our observations and those of Weinrich et al. (2009).\cite{328}
The reduced partitioning may also be explained in terms of changes in the membrane’s curvature elastic energy ($\mu_{\text{curv}}$); modifications in the membrane’s chemical potential due to increased molecular strain can have repercussions on the $K_p$ value since it is related to the difference in the chemical potential between phases. This means that $\Delta \mu_{\text{curv}}$ should be directly proportional to the changes in the spontaneous curvature, $c_o$. The net membrane curvature can be expressed as a weighted average of the curvatures of the individual lipid components. For lipid membranes containing mixtures of DOPC and DOPE, the curvature can be formulated as a weighted sum:

$$c_o^{\text{mixture}} = c_o^{\text{DOPE}}X_{\text{DOPE}} + c_o^{\text{DOPC}}(1-X_{\text{DOPE}})$$  \hspace{2cm} (7-5)$$

where $c_o^{\text{mixture}}$ is the membrane curvature of the specified lipid composition, $X_{\text{DOPE}}$ is the mole fraction of DOPE in the system, and $c_o^{\text{DOPE}}$ and $c_o^{\text{DOPC}}$ are the values for the intrinsic curvatures for DOPE and DOPC, respectively. Using the data from Figure 7-5B, we calculated the change in the energy of transfer ($\Delta \Delta \mu^0$) of xenon between the aqueous and bilayer phase as a function of both spontaneous curvature ($c_o^{\text{mixture}}$) and DOPE mole fraction. As mentioned previously, Eqs. (5-9) relates experimentally obtained chemical shift data to the mole fraction partition coefficient, while Eq. (5-10) allows for the approximation of $\Delta \Delta \mu^0$. Lewis and Cafiso showed that the energy of transfer of the membrane spanning peptide alamethicin was linearly dependent on the membrane curvature.$^{[329]}$ Our results are consistent with their observations at low DOPE mole fractions. Once the DOPE mole fraction reaches 50%, the linearity does not hold. This may be due to xenon induced curvature effects at the lipid interface. The change in the energy of transfer, as fit to the linear segment of Figure 7-7, yields a slope of 1.5 kcal/mol per mole fraction of added DOPE.
Figure 7-7. The change in the transfer energy, \( \Delta \Delta \mu \), as a function of mole fraction of DOPE and membrane curvature, \( c_{o}^{mixture} \), for various DOPC/DOPE mixtures. Errors are reported as \( \pm \) (SSE).

Figure 7-8. Variation in NMR parameters of DOPE containing lipids as a function of Xe overpressure. A) The observed \( \Delta \delta \), as a function of \%DOPE for various \( P_{Xe} \): 2.5 atm (○), 5 atm (green-▲), 8.5 atm (●), and 10 (red-□) atm. B) Normalized Bruker peak intensity with increasing xenon overpressures for various lipid compositions: 0 (○), 0.125 (▲), 0.25 (□), and 0.50 (●) mole fraction of DOPE. Note that the total lipid concentration and sample volumes were fixed to 50 mM and 1.5 ml, respectively. Errors are reported as \( \pm \) (SSE), resulting from deviations in \( P_{Xe} \).
7.3.1 Effects of Xenon Doping and Temperature

In addition to molecular stress imposed by the addition of DOPE to DOPC lipid membranes, we also studied the effect of external strain brought on by xenon doping and temperature in order to better characterize the effect of packing frustration within the bilayer phase. The general trends have been summarized in Figure 7-8A. While a steady downfield shift is observed with xenon doping, there is a distinct change in chemical shift behavior with increasing DOPC/DOPE molar ratio. In line with experimental observations, let us assume that an upfield shift reflects decreased interaction between xenon and the lipid environment due to reduced van der Waals interactions, and a downfield shift corresponds to increased xenon-membrane interactions, according to the spectroscopic trends shown in Figure 7-5. Using previous chapters as a guide, it would seem that decreased partitioning is observed with increasing PE content when $P_{Xe} \leq 5$ atm; the downfield shift at higher overpressures are indicative of increased partitioning and/or binding.

As the xenon overpressure is increased above 5 atm, the ability of DOPE to inhibit partitioning seems reduced. This is further substantiated by Figure 7-8B, where the spectral intensity is plotted with respect to xenon loading pressure and percent DOPE in a 50 mM lipid mixture with DOPC at 298 K. As the peak intensity is proportional to the area under the peak, one may assume that the amount of xenon associated with the lipid membranes increases with pressure, consistent with single component lipid vesicles, studied in previous chapters. The increased interaction at higher PE may be a result of partitioning into the lipid core due to a disruption in the membrane’s elasticity or changes in xenon diffusion properties—DOPE decreases the effective membrane surface area which could reduce the rate of diffusion.
Recent investigations on the dibucaine-membrane interaction have shown that the anesthetic modifies the molecular packing of monolayer or bilayer surfaces due to interactions at the lipid headgroup region. Increased anesthetic concentrations led to longer incubation times as well as local disruptions at lipid-water interface. Cotta et al. (2009) described this anesthetic induced disruption as a two step process. First, the lipid monolayer becomes packed with anesthetic resulting in a maximum elasticity, at which point dibucaine inserts deeper into the bilayer leading to larger local stresses and changes in curvature. Small angle X-ray scattering and $^2$H NMR experiments have also shown that higher concentrations of inhalation anesthetics i) displace the water molecules at the water-lipid interface (dehydration), ii) solubilize into the hydrophobic core and iii) encourage the loss of curvature. Chloroform, for example, has even been shown to form structures with periodic curvature (e.g. cubic phase) when present at high concentrations in several types of lipid vesicles. As discussed by the authors, the driving force between these transitions is the change in the average molecular shape of the lipid (shape concept of lipid polymorphism, see Chapter 3).

In order to see whether the changing chemical shift behavior is due to structural changes in the lamellar phase, we looked at the $^{31}$P NMR data of xenon dissolved in (1:1) DOPC/DOPE lipid mixture. As seen in Figure 7-8A, the difference in the chemical shift behavior at low and high xenon overpressures is the greatest for this sample. While the $^{31}$P NMR chemical shift at low xenon concentrations resulted in a single, broad isotropic resonance centered around 0 ppm, three distinct resonance peaks were observed in the (1:1) DOPC/DOPE lipid mixture (Figure 7-9). The upfield $^{31}$P resonance at 0 ppm (Figure 7-9A) will now be referred to as the ‘lamellar’ phase as it is similar to isotropic peak observed in the single component DOPC LUV system. The two additional resonances includes one of low intensity at 3.94 ppm (Figure 7-9A),
Figure 7-9. $^{31}$P NMR spectrum of the (1:1) DOPC/DOPE lipid mixture at 298 K and 10 atm. A) characteristic of $H_{II}$ phase while C) is due to extruded LUVs (0 ppm). The intermediate peak B) may represent lateral diffusion of lipids between curved and non-curved states: B) appears at the average of resonance A) and C).

Figure 7-10. Temperature dependence of the observed chemical shift ($\delta_{\text{obs}}$) for various lipid compositions and pressures; (▲) and (◼) are (7:1), (3:1) DOPC/DOPE molar ratios at 10 atm, respectively. Here, (Δ) denotes (7:1) DOPC/DOPE at 2.5 atm of xenon and (*) is lipid-free buffer solution at 10 atm for comparison.
characteristic of the inverted hexagonal phase, and a narrow resonance of greater amplitude at 1.91 ppm (Figure 7-9B) located directly between the ‘lamellar’ (Figure 7-9C) and the ‘curved’ phase (Figure 7-9A). This middle peak may suggest some lateral diffusion between the two states or indicate the formation of an intermediate structure. So, while it is clear that xenon does not induce a full phase transition on its own, it does appear to promote nonbilayer structures when present at higher concentrations. The increased xenon-membrane interaction at higher xenon overpressures may facilitate larger local stress at the membrane interface, leading to our observed changes in curvature.

Since increasing temperature has the same effect as dehydration, we attempted to monitor changes in the interfacial curvature thermotropically. Investigations into the thermal properties of the xenon-membrane interaction are consistent with previously observed trends in single component lipid systems: all samples display a downfield shift with increased xenon loading and exhibit a maximum at 290 K before shifting monotonically upfield. Similar to Figure 7-8B, there is no noticeable difference between the observed chemical shifts at higher loading pressures ($P_{Xe} \approx 10$ atm), which suggests similar thermodynamic behavior at elevated temperatures. Furthermore, the chemical shifts of all samples seem to converge at higher temperatures, signifying faster exchange conditions of xenon between phases.

### 7.3.2 Lamellar-to-Inverted Hexagonal Transition

As mentioned previously, the DOPC/DOPE mixture can be described as a highly cooperative, two-component system; the higher temperature structure possessing higher entropy (DOPE) and the lower temperature structure having lower entropy (DOPC). A macroscopic rearrangement of the lipid morphology occurs when these entropies are energetically equal. The $L_a$-to-$H_{ll}$ phase transition can be induced thermotropically between 323-328 K in fully hydrated
(1:1) DOPC/DOPE mixtures and the process is characterized by slow lipid exchange between the two structures.\textsuperscript{[332, 333]} We attempted to detect this structural rearrangement via $^{129}$Xe NMR by heating the sample to the temperature region specified. In doing so, we observed the emergence of a second peak downfield from the pre-existing chemical shift trend at 318 K (Figure 7-11), likely indicating the coexistence of both lamellar and inverted hexagonal phase. As the temperature was increased, the $^{129}$Xe chemical shift appears to merge with the previously identified upfield trend (Figure 7-10). This experiment was repeated with 56.5% and 60% PE content under identical experimental conditions. The slight increase in the DOPE mole fraction resulted in significant depressions in the $L_\alpha$-to-$H_{II}$ phase transition temperature ($T_h$). Results are summarized in Figure 7-12A. It should be noted that the second resonance was not observable at loading pressures under 10 atm.

In order to verify the reproducibility of the chemical shift behavior at low concentrations of Xe, a 2% mixture of hyperpolarized xenon was bubbled into aqueous dispersions of (1:1) DOPC/DOPE LUVs. The specifics of this process are described within the Experimental section of this chapter. As seen in Figure 7-11B, the $^{129}$Xe NMR resonance shifts downfield at approximately the same temperature for both thermally polarized and hyperpolarized methods. Similar studies have shown depressions in the phase transition temperature to be dependent on the concentration of anesthetic in solution. This appears not to be the case here. In studying the effect of anesthetics on the thermal transitions of DPPC vesicles via $^1$H NMR, Yokono et al. (1981) demonstrated that anesthetic-induced transitions do not occur simultaneously; the headgroup protons responded at lower anesthetic concentrations than the hydrocarbon region.\textsuperscript{[334]} While it is likely that the presence of xenon facilitates structural rearrangement by introducing additional stress to the lipid-water interface, no notable dependence on the xenon concentration...
Figure 7-11. Variation in the observed NMR parameters of xenon dissolved in a (1:1) DOPC/DOPE 50mM lipid mixture with increasing temperature. A) $^{129}$Xe NMR spectra of thermally polarized xenon at an overpressure of $P_{Xe} \approx 10$ atm. B) Summary plot of the temperature dependence of the $L_a$-to-$H_{II}$ transition: (○) and (●) signify experimental results using hyperpolarized $^{129}$Xe NMR bubbling sequence and thermally polarized xenon, respectively.

was observed via $^{129}$Xe NMR. However modest changes in the overall DOPE mole fraction was shown to have large effects on $T_h$; the higher the DOPE content, the greater the depression in the transition temperature.

**Changes in the transition temperature.** A simple relation has been made between the packing parameter and shifts in the $L_a$-to-$H_{II}$ transition temperature by Marsh et al. (1996).

According to this proportionality (Eq. (7-6)), the phase transition temperature should decrease with increasing packing parameter value. Increasing the mole fraction of DOPE within the DOPC lipid matrix enhances the packing parameter, which in turn depresses the transition temperature.

$$
\Delta T_h = \frac{\Delta \mu_{curv}}{\Delta S_h} = -\frac{1}{2} \left( \frac{N_k}{\Delta S_h} \right) \delta \left( \frac{1}{R^2} \right) \alpha - \delta \left( \frac{V}{A} \right) 
$$

(7-6)

Since anesthetics tend to stabilize the higher entropy structure ($H_{II}$ phase), it should not be surprising that xenon does the same. When a solute is incorporated into a lipid matrix its
degree of stabilization is reflected in the transition temperature. Assuming the solute does not exhibit preference for the lamellar or inverted hexagonal phases, the enthalpic contribution of the solute ($\Delta H_s$) to the transition is expressed as curvatures stress. As discussed in detail by Janes (1996), solute induced changes in the packing parameter within the lamellar phase are relaxed and stored as potential energy in the inverted hexagonal phase. Changes in the enthalpic curvature stress imparted on the lipid matrix are manifest in increases/decreases in $T_h$ and can be quantified through changes in the transition entropy ($\Delta S_h$). In accordance with Eq. (7-1), modifications in the chemical potential of the bilayer phase ($\Delta \mu_{\text{bil}}$) due to changes in curvature or packing parameters in the presence of small molecules, has the potential to effectively shift the phase transition temperature. [335]

For a pure DOPE bilayer, the thermal entropy of change from the lamellar to inverted hexagonal phase is 1.03 cal/mol-deg. If the addition of a solute decreases $T_h$ by one degree, $\Delta H_s$

![Figure 7-12](image)

Figure 7-12. Potential effects the addition of a solute can have on the lamellar-to-inverted hexagonal phase transition temperature. A) The $L_{\alpha}$-to-$H_{II}$ transition temperature ($T_h$) as a function of DOPE mole fraction: (*) indicates reported values in fully hydrated (1:1) DOPC/DOPE mixtures without xenon. [332] (●) and (Δ) denote experimentally obtained values via thermally polarized and hyperpolarized $^{129}$Xe NMR, respectively. B) Depression in $\Delta T_h$ as a function of $1/R_o^2$. Results were obtained at an overpressure of 10 atm of thermally polarized xenon. The solid line denotes the fit to Eq.(7-6).
would amount to 1.03 cal. For example, tetradecane has been shown to depress $\Delta T_h$ by 4.5 °C/mol% in DOPE-Me. Thus, the enthalpic contribution of the solute to the transition is 13.5 cal. Using this as a guide, we employed Eq. (7-6) to estimate $\Delta S_h$ by plotting the change in the phase transition temperature as a function of $1/R_o^2$ (Figure 7-12 B). This yields an average value of 4.63 ± 0.23 cal/mol·K for $\Delta S_h$. Similar to the expression provided for the intrinsic curvature in Eq. (7-5), we approximate $A_o^{bil}$ according to the following expression:

$$A_o^{mixture} = A_o^{DOPE}X_{DOPE} + A_o^{DOPC}(1 - X_{DOPE})$$  

(7-7)

where $A_o^{DOPE}$ and $A_o^{DOPC}$ are 82 Å$^2$ and 65 Å$^2$, respectively. The radius of the intrinsic curvature $R_o$ was calculated from Eq. (7-5) since $c_o^{mixture} = 1/R_o^{mixture}$, and the mean-curvature elastic modulus, $k_c$, was set to 9.9 $k_BT$. These results suggest that xenon induces 4.68 cal of enthalpic curvature stress per degree, which corresponds to 37.4 cal/mol for the (1:1) DOPC/DOPE lipid mixture with 10 atm of xenon overpressure.

Looking back at the thermodynamic analysis of the DOPC lipid we are reminded that the maximum entropic contribution to the chemical shift occurs at 317 K and the maximum enthalpic contribution at 324 K. As discussed previously, $\Delta H_m^\circ$ (317 K) defines the maximum partitioning limit and $\Delta S_m^\circ$ (324 K) the minimum in the molar chemical potential of transfer of xenon between the aqueous and bilayer phases. Assuming that the thermal partitioning behavior of xenon in the DOPC/DOPE lipid matrix is comparable to the observed trends in the DOPC lipid system we can expect that the $K_p$ increases with temperature. So, it makes sense that the xenon-induced change in the transition temperature would be near the maximal partition limit since it likely leads to higher degree of dehydration. According to recent X-ray diffraction studies on fusion intermediates, inverted hexagonal phase was formed in at (1:1)
Figure 7-13. Phase diagram of various DOPC/DOPE lipid mixtures. A) Phase dependence as a function of relative humidity (RH) and mole fraction of DOPE at 298 K determined by X-ray diffraction methods. B) Phase dependence of (1:1) DOPC/DOPE lipid mixture with increasing temperature and hydration: $L_\alpha$ - , rhombohedral lattice (R)- , distorted hexagonal ($\delta H_{II}$)- , $H_{II}$ - , and mixed- $\bigtriangleup$ phases. Figures modified from Yang, Ding, and Huang (2003).1337

DOPC/DOPE lipid mixture at and 53-95% relative humidity (Figure 7-13A). This further supports the dehydrating effect of xenon and temperature on the mixed DOPC/DOPE dispersions.

Consistent with Eq.(7-6), increasing the relative amount of DOPE within the lipid mixture increases the chemical potential of the lamellar phase and lowers the transition temperature (Figure 7-12A). While it is expected that the phase transition temperature will decrease with increasing DOPE content, the phase diagram provided by Yang, Ding, and Huang (2003) shows that a significant reduction in the hydration is required for full inverted hexagonal phase formation in PC/PE ratios less than (1:2). According to our results, the solute-induced enthalpic curvature stress increases with DOPE content; $\Delta H_s$ was determined to be 74.8 cal/mol and 84.2 cal/mol for 0.57 and 0.60 mole fraction of DOPE, respectively.
In an attempt to elucidate the downfield shift observed in Figure 7-11A (at 318 K) we dissolved thermally polarized xenon into (1:3) DOPC/DOPE lipid mixture. At low lipid concentrations a single resonance is observed near the lipid-free buffer solution, signifying fast exchange conditions. As the lipid concentration is increased, a second peak is resolved downfield. This trend differs from those observed in the single component DOPC system. Consistent with $^{19}$F NMR studies of anesthetic partitioning into lipid membranes, the $^{129}$Xe NMR resonance associated with the water-lipid interface is near that of the lipid-free buffer solution at dilute DOPC concentrations. As the DOPC concentration is increased, so does the chemical shift difference between.$^{[16]}$ Non-immobilizers, which have been shown to partition within the membrane core, do not exhibit the same chemical shift behavior. Since they do not reflect interactions at the membrane-buffer interface, these resonances appear at distinctly different chemical shifts. According to this logic, the upfield resonance shown in Figure 7-14 can be associated with xenon-membrane interactions at the lipid interface, while the downfield peak can be attributed to xenon partitioning into interstitial voids produced by the inverted hexagonal phase.

The high degree of packing frustration in the $H_{II}$ phase is a result of inefficient geometric packing of the inverted cylinders. Interstitial voids form, requiring the acyl chains to stretch or compress away from their preferred morphology. For the packing energy to stabilize the inverted phase there must be a lowering of $\Delta \mu_{ch}$ (Eq. (7-1)) In order to do so, xenon must populate the interstitial region and relax the acyl chain packing stress. General anesthetics have been shown to remain at the lipid-water interface area without losing contact with bulk water or penetrating into the terminal methyl region of the acyl chains.$^{[143]}$ The upfield resonance in Figure 7-14 suggests that xenon remains in contact with the interfacial region. And as the $H_{II}$ packing efficiency
improves with increasing lipid concentrations, so does the xenon exchange rate between the interfacial and acyl chain domains. It should be noted that while two $^{129}$Xe NMR chemical shifts were observed in the (1:3) DOPC/DOPE lipid mixture at a xenon overpressure of 2.5 atm, only a single resonance was exhibited under similar experimental conditions for xenon dissolved in DOPC lipid dispersions.

Figure 7-14. Concentration dependence of the $^{129}$Xe chemical shift dissolved in (1:3) DOPC/DOPE MLV lipid mixture compared to the chemical shifts of a) 100 mM DOPC LUV sample and B) lipid free buffer solution, under similar experimental conditions. The blue spectrum is 12.5 mM, the red spectrum, 25 mM, and the black, 100 mM concentrations of (1:3) DOPC/DOPE, respectively. All spectra were obtained at 298 K and 2.5 atm of xenon.

7.3.3 Evidence of Kinetically Trapped Structures

Cubic phases refer to a family of ordered nonlamellar, liquid crystalline lipid phases having cubic symmetry. These structures can be formed directly from lamellar phases as end products, or as intermediates in the formation of nonbilayer phases (e.g. $H_{II}$). Though these geometries are difficult to detect due to their sensitivity to temperature, scanning rate and the
sheer variety of cubic phases, some of these structures can remain kinetically trapped once formed, and can only be relaxed after cooling the sample below the gel/lamellar phase transition. As mentioned previously, certain molecules can stabilize the formation of these structures. Experimental evidence suggests that the formation of the cubic phase is accelerated between the $L\alpha$-to-$H_{II}$ transition temperatures and can be induced by cooling or heating.

The rhombohedral phase is another example of a transition intermediate of DOPC/DOPE lipid mixtures. Evidence suggests that this is a stable phospholipid stalk phase, similar to structures shown in Figure 7-2. Like the cubic phase, the rhombohedral phase exists between the lamellar and inverted hexagonal lipid morphologies and is sensitive to both temperature and hydration levels. Molecular dynamics simulations suggest that elongated stalk structures formed in pure DOPE lipids can drive the lipid matrix directly into the inverted hexagonal phase. Unlike the cubic phase, these stalks are ordered in a hexagonal pattern. As shown in the DOPE/DOPC phase diagram (Figure 7-13B), the propensity of mixed phase formation is higher at elevated temperatures.

A $^{31}$P NMR spectrum of the (1:1) DOPC/DOPE LUV lipid mixture was taken after induction of the inverted hexagonal phase (after cooling) in order to confirm the relaxation of the lipid matrix out of the inverted hexagonal phase. Doing so resulted in a lineshape with three distinguishable characteristics: a broad peak upfield associated with the lamellar phase, a smaller resonance at 1.10 ppm, and a third at 3.57 ppm, indicative of the inverted hexagonal phase. The xenon containing sample was then subjected to a series of freeze/thaw cycles one week after cooling; it was immersed in a bath of liquid nitrogen five times per cycle. This resulted in an increase in the lamellar phase and a sharpening of the resonances associated with the nonlamellar...
phases (Figure 7-15C). Thus, it appears that the presence of xenon in the lipid dispersions stabilize these nonbilayer structures long after the inverted hexagonal phase has been induced.

![31P NMR spectra of the different lipid phases in the presence of xenon](image)

**Figure 7-15.** $^{31}$P NMR spectra of the different lipid phases in the presence of xenon. A) Single component lipid DOPC as LUVs, B) Single component DOPC as MLVs. C) (1:1) DOPC/DOPE lipid mixture at 328 K. D) (1:1) DOPC/DOPE mixture at 298 K, 1 week after cooling, following 2 separate freeze/thaw cycles.

### 7.4 Conclusions

In summary, our results show that the interactions of xenon with lipid membranes containing DOPE are much more complex that those in the single DOPC lipid matrix. At low xenon loading the presence of DOPE appears to inhibit the xenon partitioning, decreasing the association constant with increasing PE content. As the concentration of xenon in solution increases, the lipid matrix appears to lose this capability. Dispersions with high DOPE mole ratios were shown to be especially sensitive to the presence of xenon. $^{31}$P NMR of (1:1) DOPC/DOPE mixtures under 10 atm of xenon overpressure shows the existence of nonbilayer phases prior to heating. This is likely attributed to alterations in the lateral bilayer organization.
due to displacement of water molecules at the membrane interface. Furthermore, the presence of xenon was also shown to promote changes in the thermal behavior of the (1:1) DOPC/DOPE lipid. The phase transition temperature was depressed approximately 8 K, consistent with changes in the packing parameter. Since changes in the phase transition temperature indicate changes in the membrane curvature, xenon likely induces curvature defects at high concentrations and temperatures in DOPE containing lipid membranes. Interestingly, once the nonbilayer structures were formed, they were unable to be fully relaxed into the lamellar phase. $^{31}$P NMR suggests the coexistence of three lipid phases even after being subjected to multiple freeze/thaw cycles.

Regulation of the membrane pressure is essential for cellular function. There is a whole family of membrane proteins called mechano-sensitive channels that depend on membrane pressure to open and close. Take for example the bacterial protein MscL. When the bacteria experiences significant stress, it opens in order to relieve the pressure. Agents that induce asymmetry in the membrane environment introduce curvature and thus pressure, which can trigger the opening of the channel. We have shown that xenon can induce a significant defect in the bilayer structure in the presence of nonbilayer lipids and that it stabilizes the formation of curved structures long after they should have been relaxed. It is our view that $^{129}$Xe NMR is uniquely suited for the elucidation of the anesthetic-protein interaction in membrane-supported environments.
CHAPTER 8
LIPID NANOTUBE ARRAYS INVESTIGATED BY HYPERPOLARIZED XE-129 NMR

8.1 Introduction

The biofunctionalization of inorganic substrates has become one of the most attractive methods to study the physiochemical properties of biomedia in confined systems. Interest in assembly and applications of nonmaterials for new technologies has prompted integrative research in chemistry, physics and biology.\textsuperscript{[343-347]} As mentioned previously, the low density and long relaxation time of dissolved thermally polarized \textsuperscript{129}Xe prevents it from becoming a viable tool to study biological and inorganic materials in solution within moderate physical conditions and experiment times. The only way to circumvent this problem is through hyper-polarization techniques. Inorganic substrates, such as Anodic Aluminum Oxide (AAO) membranes, can be functionalized by self assembly of lipid bilayers on their surfaces, creating a convenient model of cellular membranes.\textsuperscript{[348-350]} Although these membrane systems are generally used for filtration purposes (e.g., lipid extrusion), its use in the fabrication of lipid nanotube arrays has emerged as a new trend in biotechnology.\textsuperscript{[351-353]} Their confined surface and increased stability reduces the likelihood of surface perturbation and contamination, making these lipid nanotube arrays more robust than mechanically aligned bilayers. Furthermore, these lipid nanotube arrays can retain water for a longer duration through capillary action; the network of tightly bound water molecules at the lipid interface is not easily removed. And while lipid diffusion between channels does not occur, the surfaces of both leaflets are fully accessible to aqueous solutes.

The increased used of membrane filters in the fabrication of nanostructured materials can be attributed to their i) commercial availability, ii) tunability of its pore dimensions, iii) high thermal stability, iv) relative ease of lipid adherence, v) long shelf life and vi) the ability to dehydrate/rehydrate with full sample viability. As such, the cylindrical lipid bilayers formed
inside AAO nanotubes are ideal for structure-function studies of membrane proteins, cell signaling, and ligand-receptor interactions within a model membrane environment. Herein, we study the effects of nano-scale confinement on the spectral properties of dissolved $^{129}$Xe in self-assembled phospholipid membranes. In addition to verifying effective bilayer formation on the substrate via $^{31}$P MAS, we monitor the xenon gas-to-lipid membrane exchange via 2D EXSY.

8.2 Experimental

8.2.1 Materials

As mentioned in previous chapters, DOPC was purchased from Avanti Polar Lipids (Alabaster, AL). The 50 mM (pH 7.4) Hepes Buffer solution used for hydration was purchased from Sigma-Aldrich. Anodic Aluminum Oxide (aka, ANOPORE inorganic membranes, Aluminum Oxide Membranes, AAO) filters, having 200 nm pore size and 47 mm diameter were purchased from Whatman International Ltd (England). Materials were used as supplied.

8.2.2 Physical Description of AAO

ANOPORE inorganic membranes are available commercially from Whatman in a variety of pore sizes: 20 nm, 100 nm, and 200 nm. The hydrophilic nature of the membrane makes it highly compatible with most aqueous bio-material and solvents. As shown in Figure 8-1(A-B), these aligned, through-film porous structures are macroscopically homogenous and assembled in a hexagonal-like structure in which each pore represents a separate channel (60 μm long). However, recent NMR studies have shown the presence of short, repetitive segments, approximately 3-5 μm in length (Figure 8-1C). The high heterogeneity observed in commercial AAO solid supports often complicates the interpretation of the effects of nanopore-confined lipid bilayers, prompting researchers to synthesize customized support materials. Despite this, we find the commercially available membranes to be adequate for our preliminary investigations. All reported sizes are as specified by Whatman International.
Figure 8-1. The side and top views of an AAO membrane. A) AAO possesses hexagonal packing structure. B) AAO with 200 nm channel diameter, from Whatman International. C) Model for lipid adsorption to the surface of pores in AAO; upper C): lipids adsorb as wavy tubules with water remaining trapped between these tubules and the AAO surface; lower C): the relative shape of the lipid bilayer tubules inside the AAO pore.[354]

8.2.3 Preparation of Lipid Nanotube Arrays

Instructions on how to prepare AAO supported bilayers are well documented in the literature.[355-358] We followed the following guidelines for manual deposition of DOPC onto these supports. First, one side of the AAO support was exposed to an aqueous suspension of MLV which led to an immediate wetting of the AAO disc, making it semitransparent to the eye. The main phase transition temperature of DOPC is well below room temperature, so constant heating was not required. Both sides of the AAO disc were cleaned by repetitive wiping (Kimwipes EX-L) in order to remove any excess lipid from the membrane surface. The sample was then crushed with a motor and pestle and placed into the wideline probe, which held approximately 20 mg of sample (AAO, lipid, and water). In the case of shift-reagent studies, DyCl₃ was dissolved into buffer solution (50 mM) and the disc soaked in the mixture for 30 minutes before being wiped of excess lipid, crushed, and placed within the sample holder.

More than one layer of substrate is often desired for NMR studies as the bound quantity of phospholipids is proportional to the total coverage area (one bilayer per pore).[359] Generally a series of approximately 50 or more commercial AAO discs are stacked and oriented at a particular angle in the magnetic field. For NMR experiments, AAO discs were stacked in the
rotor and this design had previously been used to assess the degree of alignment of the lipid bilayer inside the nanoporous support; having precise control of the alignment with the magnetic field is imperative for more intricate studies. A new sample holder was designed to accommodate the increase in volume required for this process, the details of which can be found in the Appendix. The diameter is approximately 2.9 times larger than the previous sample holder, providing a significant increase in the total available sample volume. This particular design was able to hold vacuum at temperatures \( \geq 208 \text{ K} \).

**8.3 Results and Discussion**

**8.3.1 Adsorption of Single Lipid Bilayers onto Substrates**

Substrate supported bilayers were deposited inside the 200 nm pores of AAO filters using methods described in the previous section. \(^{31}\text{P}\) NMR spectroscopy was employed to verify proper adhesion of bilayer to the inner surface of the pores. Consistent with published results, the \(^{31}\text{P}\) NMR of lipid-free AAO results in a significantly broad spectrum in both the static and MAS spectra.\(^{[358]}\) This is due to the low levels of phosphorus contained in the AAO (7.4 wt\%).\(^{[360]}\) The blue line in Figure 8-2 is the static \(^{31}\text{P}\) spectra of unoriented lipid bilayers incorporated into AAO substrate; neither decoupling nor presence of paramagnetic shift reagent has a noticeable effect on the overall lineshape. Recent studies show that only the first bilayer adheres strongly to the inner surface of the pore. Additional bilayers layers can be easily washed out by water. As discussed previously, paramagnetic shift reagent can significantly broaden the \(^{31}\text{P}\)NMR signal, effectively reducing the contribution of ‘loose’ lipid molecules to the chemical shift if present at high enough concentrations. According to our results, the addition of 50 mM DyCl\(_3\) reduced the overall spectral intensity, but did not result in a noticeable shift in the resonance (Figure 8-3D). The isotropic peaks for both phosphoric acid (standard) and hydrated, AAO supported DOPC for
comparison. Results presented in Figure 8-3 show a number of $^{31}$P NMR spectra obtained over a period of days, in sequential order. The $\sigma_\perp$ and $\sigma_{||}$ values of the 50 mM DOPC MLV $^{31}$P NMR spectra were 24.22 ppm and -15.65 ppm, respectively. A graphical representation of xenon-AAO supported bilayer interaction is given in Figure 8-4. Xenon first dissolves into the water pore, and then diffuses through the lipid bilayer. It may be possible for lipids to become clogged in the pores. This can neither be confirmed nor disproven using $^{129}$Xe or $^{31}$P NMR methods.

### 8.3.2 Evidence for $^{129}$Xe inside AAO Pores

A series of simple 1D NMR spectra of $^{129}$Xe dissolved into various lipid phases is shown in Figure 8-5. To begin we looked at the lipid-free, crushed AAO support to verify limited interaction between its surface and gas phase xenon ($Xe_{(g)}$); the temperature dependence of the xenon-AAO surface interaction is provided within the Appendix. Note the lack of signal in the aqueous region of the spectrum. Thus it can be concluded that Xe-Xe interactions on the AAO
Figure 8-3. $^{31}$P NMR spectra of DOPC under various conditions. A) Chemical shift of phosphoric acid reference: $\sigma_{iso} = 0$ ppm. B) DOPC bilayer on AAO substrate (crushed, 200 nm pore size): $\sigma_{iso} = -0.836$ ppm. C) DOPC bilayers rehydrated with buffer solution. D) Sample washed with 50 mM DyCl$_3$ solution. E) Spectra after sample was soaked in excess buffer solution to remove DyCl$_3$.

Figure 8-4. Illustration describing the diffusion of xenon to AAO supported bilayers. A) From the gas phase to the aqueous pore. B) The subsequent diffusion of $Xe_{(g)}$ in the bilayer plane.
A series of $^{129}$Xe NMR spectra of xenon dissolved/adsorbed gas in various AAO environments. A) Lipid-free, crushed AAO substrate using continuous flow HP $^{129}$Xe NMR methods: 1 scan. B) 50 mM DOPC LUV solution utilizing thermally polarized $^{129}$Xe NMR: 720 scans. C) AAO supported DOPC using continuous flow HP $^{129}$Xe NMR: single scan. D) AAO supported DOPC employing interrupted flow HP $^{129}$Xe NMR technique: single scan.

Figure 8-5. A series of $^{129}$Xe NMR spectra of xenon dissolved/adsorbed gas in various AAO environments. A) Lipid-free, crushed AAO substrate using continuous flow HP $^{129}$Xe NMR methods: 1 scan. B) 50 mM DOPC LUV solution utilizing thermally polarized $^{129}$Xe NMR: 720 scans. C) AAO supported DOPC using continuous flow HP $^{129}$Xe NMR: single scan. D) AAO supported DOPC employing interrupted flow HP $^{129}$Xe NMR technique: single scan.

surface will have negligible contribution to the observed chemical shift near the water and lipid associated peaks. Next we used continuous flow hyperpolarized (HP) $^{129}$Xe NMR methods to verify adsorption into the lipid filled AAO channels—a $^{129}$Xe NMR spectrum of Xe(g) dissolved into aqueous dispersions of lipid vesicles (50 mM DOPC LUVs) is given for comparison. This particular sample (see Figure 2-8B) had less volume in the coil region and possessed an isolated gas peak which was lined up with the $^{129}$Xe(g) of all other spectra and used as a reference.

Spectra (C) and (D) of Figure 8-5 were obtained using continuous and interrupted flow methods, respectively. In the continuous flow mode, the HP gas mixture is continuously recirculated through the coil region at a steady flow rate of 90 ml/min, as recorded from the calibrated flow
meter (see Figure 2-8). The flow rate is briefly interrupted before the detection time in the interrupted flow technique, increasing the residence time of xenon associated with the lipid phase, yielding an increase the signal-to-noise ratio of the $^{129}$Xe dissolved within that nanotube phase. This improvement is seen clearly in Figure 2-8C and Figure 2-8D.

8.3.3 Relaxation Rates of Dissolved Xenon

While we have gained significant improvement in the experiment time (seconds opposed to hours), only a single peak is observed due to fast exchange conditions. We then performed a saturation recovery experiment to get a sense of how relaxation times of xenon changes within the lipid nanotube environment as compared to our previously studied lipid suspensions. Continuous flow HP $^{129}$Xe NMR was used to determine the longitudinal relaxation time on confined lipid phase (Figure 2-8C). Results were fit to both a single and bi-exponential fit (Figure 8-6), yielding values of $0.67 \pm 0.23$ s$^{-1}$ and $8.31 \pm 2.47$ s$^{-1}$ for the bi-exponential fit and $2.10 \pm 0.40$ s$^{-1}$ for the mono-exponential fit. It appears that the bi-exponential fit is slightly better. The higher valued $T_1$ corresponds to the water pore and the shorter to the supported lipid bilayer; the longer the correlation time, the shorter the longitudinal relaxation time.

8.3.4 Indication of Chemical Exchange Between Anopore and Gas Phase $^{129}$Xe

Lastly, a 2D EXSY experiment was performed to see whether we could resolve additional information on $^{129}$Xe exchanging between the lipid/water/gas phases. The complete absence of cross peaks at 50 ms indicates no significant exchange between the lipid and gaseous phase during this period (Figure 8-7A). However, as seen in Figure 8-7B, the exchange between $Xe_{(g)}$ and xenon dissolved within the lipid associated phase gives rise to two small cross peaks at mixing times greater than 100 ms. These mixing times are on the same order as those observed by Tallavaara and Jokisaari (2006), who studied $^{129}$Xe dissolved into thermotropic liquid crystals.
Figure 8-6. \(^{129}\)Xe NMR saturation recovery curves for xenon dissolved in AAO supported DOPC bilayers using continuous flow methods, with a mono-exponential fit (dotted line) versus bi-exponential fit (dashed line). Data fit to broad line in the general vicinity of the fast exchanging lipid/water peak (see Figure 8-5C).

confined to a mesoporous Controlled-Pore Glass (CPG) material.\(^{361}\) Two distinct sites were resolved: xenon dissolved in the bulk liquid crystal between the CPG particles and xenon in the liquid crystal confined to the pores. Two cross-peaks, of equal population were observed at mixing times greater than 80 ms; the diffusion rate was determined to be very slow. The disproportionality of our cross-peak intensities between the gas and adsorbed phase is consistent with Eqs.(2-28) and (2-29).

### 8.4 Conclusions

Recent results suggest that 2D EXSY signals can be amplified under interrupted flow conditions, providing greater sensitivity.\(^ {52, 56}\) The interrupted flow pulse sequence is different than the continuous flow sequence in that the HP gas is halted prior to the first \((\pi/2)\) pulse of the EXSY sequence and is ‘interrupted’ during the mixing time, changing the magnetization dynamics of \(^{129}\)Xe in both the gas and lipid coated nanotubes. Briefly, this interruption allows the
gas sufficient time to accumulate in the more energetically favorable phase by increasing the
residence time of xenon in a particular phase, improving the probability of detecting xenon in the
adsorbed phase during the mixing time. Our results suggest that this method could yield more
detailed information on the kinetic behavior of xenon embedded within the bilayer and the water

Figure 8-7. 2D $^{129}$Xe EXSY spectra. A) AAO supported DOPC at $\tau_m = 50$ ms. B) AAO
supported DOPC at $\tau_m = 120$ ms. $P_{tot} = 2983$ Torr (2% Xe /2% N$_2$ /96% He), and 298
K for both.
pore. Extracted $T_1$ values suggest the presence of two distinct environments within the AAO porous tubule. What’s more, the use of supported bilayers in anesthetic research would be very useful. Incorporating proteins into membrane environments can provide substantial information on the site of anesthetic action—more so than studies involving dissolved proteins in bulk solution.
CHAPTER 9
CONCLUSIONS AND OUTLOOK

The nature of xenon-phospholipid interactions and Xe exchange depend on the structure of the lipid headgroups and acyl chains, the phase state of the lipid bilayer, and the heterogeneity in both vesicle size and overall distribution of lipids with external variables. Our study of the dissolution of Xe into aqueous solutions of MLVs and LUVs provide the basis for Xe exchange dynamics within DOPC lipid membranes. While slow exchange was observed in emulsions of MLVs, rapid chemical exchange was observed in the homogeneously dispersed vesicles of LUVs. However, not all lipid systems exhibit the same trends. For instance, both DMPC and DPPC MLVs have been shown to start as a single peak at room temperature then split into two phases with increasing temperature. Separate Xe-DMPC studies showed opposite behavior. The primary difference between these lipid and those that we have studied is that DPPC and DMPC are saturated lipids, while DOPC and DOPE are unsaturated. Can we extrapolate the contributions from the acyl chain regions and those from the lipid headgroup? Which has a larger contribution to the observed chemical shift? Exploring these differences in terms of Xe exchange and vesicle fluidity is of merit.

As the partition coefficients of Xe in DOPC and DOPE are not known, the expression utilized in anesthetic binding studies is most useful. Not only does it allow for an estimation of the limiting shifts of Xe in the bound environment and determination of the association constant, it also allows for a comparison of the solubility of xenon in the lipid membrane with respect to that in water under fast exchange conditions. Our results indicate that the limiting shift of bound xenon increases substantially with xenon concentration as does the binding constant. The majority of studies investigating hydrophobic cavities of proteins in solution use a slightly more advanced model to differentiate between specific and non-specific interactions. Non-specific
interactions take place at the protein surface, while specific interactions consist of those inside the hydrophobic core. This treatment may prove to be useful in the study of lipid domains (a.k.a. lipid rafts) to determine localized effects of xenon-lipid interactions. Despite all this, neither method takes into account the transverse relaxation ($T_2$), which characterizes the motional restriction of xenon. Conventional Carr-Purcell-Meiboom-Gill (CPMG) spin-echo methods can be used for a more accurate determination of the binding constant.

The present results also show that the interactions of Xe with lipid membranes containing DOPE are much more complex than those in pure DOPC. Whether the observed trends are due to the presence of hydrophobic pockets or decrease in vesicle mobility still needs to be determined. The $^{129}$Xe chemical shift dependence on the xenon loading pressure is of significant interest. Recent molecular dynamics simulations studying the role of lipid membranes on anesthesia may be helpful in the interpretation of our data. Not only did the added presence of xenon lead to an increase in membrane thickness, surface area and acyl chain order, but in the occupation within the inter-leaflet space as well. This is contradictory to experimental studies which suggest that Xe is located at the lipid headgroup region. Our studies indicate that Xe changes the thermal behavior of the (1:1) DOPC:DOPE 50 mM LUV mixture, inadvertently altering the lateral organization. The depression in the transition temperature is consistent with changes in acyl chain packing. As its presence facilitates the $H_{II}$ transition, one might assume that Xe assists by filling the interstitial voids. A second peak near phase transition temperature suggests some change in the structural properties between 40-45°C. This anomalous peak may due to a pre-transition state such as an elongated stalk.

A particular limitation to the studies performed was the long acquisition times. Although the increased solubility of Xe in lipid systems is substantially greater than in water, we were still
forced to find creative ways to reduce the experiment time. $T_1$ estimates of Xe dissolved in lipid vesicle suspensions are on the order of $60 \pm 14$ s. We also attempted to dissolve optically-pumped $^{129}$Xe in solution by bubbling the gas mixture through the lipid emulsions. While we were able to obtain spectra corresponding to Xe under fast exchange conditions with a single scan, modifications are still needed to allow for signal averaging. Spectral intensity was shown to decrease with temperature and broaden with increasing lipid concentration. Lastly, Anodic Aluminum Oxide (AAO) substrates are utilized to stabilize and align bilayers in the magnetic field, facilitating the study of Xe diffusivity between pores via two-dimensional (2-D) exchange spectroscopy (EXSY).

$^{129}$Xe NMR studies of non-specific Xe-lipid interactions in the DOPC/DOPE lipid system will help to develop $^{129}$Xe NMR as a biomolecular probe of packing effects, chemical exchange, factors affecting lateral pressure, and phase transitions in membrane model systems. While our investigations provide a good starting point, additional studies are needed to fully characterize observed effects. Here is a list of experiments that would further elucidate our studies to date as well as some additional systems of interest:

- Make use of SPINOE: enhance the polarization of the $^{13}$C within the acyl-chains to study the dynamics within the hydrophobic interior of the bilayer with increasing stress.

- Membrane defects such as lipid rafts are localized domains of lipids with expressed curvature. As changes in membrane permeability are suggested to change at these boundaries, it would be a good candidate for Xe NMR studies.

- Low concentrations of cholesterol are thought to preferentially partition into lipid domains and enhance lipid membrane permeability. High concentrations of cholesterol increase the lateral packing effectively reducing the free volume of the membrane. This would be useful in the study of lipid domains.

- Our preliminary investigations into the use of inorganic substrates to support model membranes show potential in determining the Xe diffusion coefficients. Not only could this be used to validate the presence of intermediate structures and lipid domains, it can help elucidate binding kinetics at the lipid-water interface with changing lipid composition. This can be used in conjunction with magnetization transfer experiments, measurements of
relaxation times ($T_1, T_{1p}, T_2$)—all of which exploit the dynamics of a system at different time scales.

- Lastly, the antimicrobial peptide MSI-78 has been shown to induce significant changes in the lipid bilayer. In addition to inhibiting the lamellar-to-inverted hexagonal phase formation it has been shown to alter the bilayer morphology such as to resemble the formation of a toroidal pore at low concentrations. We would like to further characterize these phenomena with Xe NMR methods.
APPENDIX

NEWLY DESIGNED SAMPLE HOLDER FOR AAO STUDIES

Schematics for a sample holder custom made for use in wideline probe. Particular focus was made on creating a larger sample space for the use to stacked AAO supports, and allowing the use of an insert (comprised of two halves of a cylinder) to reduce the filling factor, and providing more accurate control of the alignment. As seen below, the apparatus consists of three parts labeled 1, 2, and 3. Part 1 connects the 1/8” outer diameter (O.D.) PFA tubing to the sample region. The second component (Part 2) receives the tubing and creates a seal with a 1/8” ferrule where Part 1 and 2 meet. Part two essential acts as a reducer; it reduces the diameter of the sample region to accommodate the size of the PFA tubing. The seal between Parts 2 and 3 is created by the placement of two o-rings on either side of the sample body (Part 3). As mentioned previously, this particular design allowed for experiments at temperatures \( \geq 208 \text{ K} \).

Figure A-1. Schematic diagram of the custom sample holder designed for aligned lipid bilayers on AAO supports.
Figure A-2. $^{129}$Xe NMR stacked plot showing the xenon-AAO interaction with decreasing temperature. The exchange rate is reduced as the temperature is lowered.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Caroline D. Pointer-Keenan was born in Anchorage, Alaska in 1979. She graduated from Robert Service High School (Anchorage, Alaska) in 1997 and received her B.S. degree in chemistry from Lincoln University, PA, in 2000. She acquired her M.S. degree in physical chemistry from the University of Michigan, Ann Arbor, MI, in 2003 and joined Dr. Russ Bowers’ research group at the University of Florida thereafter to pursue her Ph.D. degree in physical chemistry. Since then she has worked as a full-time graduate student and teaching assistant. While a member of the Bowers’ group, Caroline has designed various experimental apparatus utilized in both gaseous and solid state NMR experiments. Her immediate goal is to continue her scientific development on the postdoctoral level; she is eager to put her experience into practice and enhance her knowledge of applied physical chemistry. Her future goal is to perform research and teach in academia.