ABSTRACT

A STUDY OF SUPPORTED LIPID MULTILAYERS IN A HUMIDITY-CONTROLLED ENVIRONMENT

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A topic of interest in the field of biophysics is understanding interactions between cell membranes. Thermal fluctuations within each membrane contribute to these interactions. Thus, understanding how thermal fluctuations play a role in the dynamics of a single membrane will lead to a better understanding of membrane interactions. It is difficult, however, to study dynamics of a single membrane without restricting its mobility. A membrane will have its motion dampened by neighboring ones, and will be further restricted if in contact with the substrate. A solution to this problem is to arrange layers of membranes (also known as a lipid multilayer) and maximize the spacing between the layers. Studying a stack of membranes is also easier than studying a single membrane, due to the stronger intensity of reflected light waves. The first step is to determine how multilayer spacing can be controlled, and this research is presented to demonstrate that multilayer spacing can be regulated via relative humidity of the sample’s environment. DMPC multilayer samples are measured via x-ray reflectivity with relative humidities of 99%, 99.9%, 99.99%, and 100% at 30°C. The data presented in chapter 4 implies that the accuracy of spacing control is sensitive to temperature gradients existing within the sample’s environment. These results suggest that further study requires an improved apparatus design which maintains uniform temperature.
A STUDY OF SUPPORTED LIPID MULTILAYERS IN A HUMIDITY-CONTROLLED ENVIRONMENT

BY

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DEDICATION

This work is dedicated to my friends and family for their continued love and support
# TABLE OF CONTENTS

LIST OF TABLES. ................................................................. vi
LIST OF FIGURES. ............................................................ vii

Chapter

1 INTRODUCTION. ............................................................. 1
   1.1 Lipids and Structure .............................................. 1
   1.2 Forces and Multilayer Spacing ................................... 3
   1.3 The Vapor Pressure Paradox ...................................... 4

2 THERMODYNAMIC EQUILIBRIUM AND THE HUMIDITY CELL ............ 5
   2.1 Thermodynamic Equilibrium ..................................... 5
   2.2 Humidity Cell Design ............................................ 9
       2.2.1 Temperature Control ..................................... 13
       2.2.2 Windows ..................................................... 14
   2.3 Temperature Gradient Measurement .............................. 17

3 SAMPLE PREPARATION ..................................................... 19
   3.1 Materials ........................................................... 19
   3.2 Cleaning Methods ................................................ 19
       3.2.1 Piranha Etch Method ................................... 20
       3.2.2 UV Cleaning Method ..................................... 20
   3.3 Sample Preparation Methods ..................................... 20
       3.3.1 DOPC Sample Preparation Method ......................... 21
       3.3.2 DMPC Sample Preparation Method ......................... 21

4 MEASUREMENTS AND RESULTS. ......................................... 23
   4.1 X-rays ............................................................... 23
   4.2 Bragg’s Law .......................................................... 23
   4.3 Reflectivity Measurements ........................................ 25
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4 Measurements and Data</td>
<td>26</td>
</tr>
<tr>
<td>5 IDEAL RESULTS AND CONCLUSIONS.</td>
<td>30</td>
</tr>
<tr>
<td>REFERENCES.</td>
<td>34</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>27</td>
</tr>
<tr>
<td>5.1</td>
<td>32</td>
</tr>
</tbody>
</table>

4.1 Peak position and repeat spacing for various humidities  

5.1 A table comparing relative humidities and corresponding spacings to effective humidities and adjusted spacings
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 A lipid molecule with a hydrophilic headgroup and hydrophobic tails</td>
<td>2</td>
</tr>
<tr>
<td>1.2 An image showing a stack of the bilayer multistack structure</td>
<td>2</td>
</tr>
<tr>
<td>1.3 A diagram describing the water spacing between neighboring stacks of bilayers</td>
<td>3</td>
</tr>
<tr>
<td>2.1 Top view of the humidity cell</td>
<td>9</td>
</tr>
<tr>
<td>2.2 The outermost chamber of the humidity cell</td>
<td>10</td>
</tr>
<tr>
<td>2.3 The middle chamber of the humidity cell</td>
<td>11</td>
</tr>
<tr>
<td>2.4 Top view of the innermost chamber of the humidity cell</td>
<td>12</td>
</tr>
<tr>
<td>2.5 The innermost chamber of the humidity cell</td>
<td>12</td>
</tr>
<tr>
<td>2.6 The lid of the innermost chamber is also a sample holder which suspends the sample in the cell</td>
<td>13</td>
</tr>
<tr>
<td>2.7 A window of the innermost chamber of the cell</td>
<td>13</td>
</tr>
<tr>
<td>2.8 Circular window of radius R with annulus of inner radius r and outer radius r + dr</td>
<td>16</td>
</tr>
<tr>
<td>4.1 A diagram demonstrating Bragg's Law</td>
<td>24</td>
</tr>
<tr>
<td>4.2 Reflectivity measurement</td>
<td>25</td>
</tr>
<tr>
<td>4.3 A plot of repeat spacing versus relative humidity of DMPC at 30°C for the first sample</td>
<td>28</td>
</tr>
<tr>
<td>4.4 A plot of repeat spacing versus relative humidity of DMPC at 30°C for the second sample</td>
<td>28</td>
</tr>
<tr>
<td>4.5 A plot of repeat spacing versus relative humidity of DMPC at 30°C for the first sample</td>
<td>29</td>
</tr>
<tr>
<td>4.6 A plot of repeat spacing versus relative humidity of DMPC at 30°C for the second sample</td>
<td>29</td>
</tr>
<tr>
<td>5.1 A plot of repeat spacing as a function of relative humidity for DMPC at 30°C</td>
<td>30</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

The most basic functional unit of living organisms is the cell. Full understanding of a cell requires an understanding of each of its components. An area of particular interest in biophysics is the interaction between cell membranes, since these interactions are involved in various essential biological processes (Petrache et al., 1998). These interactions can be studied from a physical standpoint, specifically, by using thermodynamics to study thermal fluctuations of the membranes. The research and experiments documented herein are focused on determining how the spacing of neighboring stacks of lipid membranes depend on the relative humidity of the sample environment. Demonstrating such a relationship helps shed light on membrane interactions through use of thermodynamics, and is a stepping stone to further understanding of the cell as a whole.

1.1 Lipids and Structure

Phospholipids consist of a hydrophilic head group and two hydrophobic tails (Figure 1.1). When introduced to water or a humid environment, these phospholipids arrange themselves into structures such that the tails point inward, to the core of the structure, while the heads point outward, to the water or exterior of the structure. Of the most typical structures formed, the focus of this research deals with bilayer sheets. These lipid bilayer sheets can then be stacked on top of one another to form a multilayer stack of lipids which will be referred to as “multilayers” (Figure 1.2). The spacing of lipid multilayers depends on both the forces acting on the bilayers and the water in contact with the bilayers. This will be discussed in section 1.2.
Figure 1.1: A lipid molecule with a hydrophilic headgroup and hydrophobic tails. Image from (Salditt, 2005).

Figure 1.2: An image showing a stack of the bilayer multilayer structure. The arrows indicate incoming and reflected x-rays, which are used to measure the repeat spacing of the multilayers. Image from (Salditt, 2005).
1.2 Forces and Multilayer Spacing

Once the multilayers come into contact with water or a humid environment, they will equilibrate to some repeat spacing, $D$. This includes the water spacing between the layers, $D_W$, plus the thickness of a single bilayer, $D_B$. Refer to Figure 1.3. It is the combination of the forces acting on the bilayers and the amount of water in contact with the multilayers that regulate this spacing. The forces considered include a van der Waals force, a hydration force, and Helfrich repulsion. There is, however, an additional force which arises possibly due to steric interactions of polar groups as the surfaces of neighboring membranes approach one another (Rand and Parsegian, 1989). This force is not considered since it only plays a significant role for high pressures and small water spacings (Petrache et al., 1998).

Figure 1.3: A diagram describing the water spacing between neighboring stacks of bilayers. The repeat spacing, $D$, is given by $D_W + D_B = D = D'_W + D'_B$. The interbilayer water spacing is denoted by $D_W$ or $D'_W$; the bilayer thickness is denoted by $D_B$ or $D'_B$. Image from (Petrache et al., 1998).

The attractive van der Waals force limits the hydration of bilayers (Rand and Parsegian, 1989). It accounts for the fact that bilayers will achieve a limiting repeat spacing, $D_0$, and the spacing will not continue to increase beyond this value as more water is added. The lipids are said to be fully hydrated once the spacing reaches the value $D_0$ (Petrache et al., 1998). Since there is a non-zero water spacing, there must be a repulsive force that counteracts the van der Waals force,
and accounts for the lowered energy of the head groups when surrounded by water molecules. This is known as the hydration force.

The last force considered here is the Helfrich repulsion, which is an effective entropic force due to thermal undulations of the bilayers (Rand and Parsegian, 1989; Katsaras et al., 2008) and is present when they are flexible (Petrache et al., 1998). If the undulation of the membranes is restricted and they become rigid, then they will not swell nearly as much (Katsaras et al., 2008).

In addition to these forces, the amount of water in contact with the bilayers will also play a role in the repeat spacing. Ultimately, controlling the relative humidity of the vapor in contact with the bilayers allows control of the bilayer spacing. A lipid multilayer exposed to an environment with some relative humidity can be thought of as a two-phase equilibrium consisting of water in vapor phase and liquid water in the multilayer. The amount of water in each phase depends on the chemical potential of the water, which is regulated by the relative humidity. Controlling the repeat spacing via relative humidity is the goal of this research and will be discussed in further detail in Chapter 2.

1.3 The Vapor Pressure Paradox

The vapor pressure paradox is a topic of importance for those studying membranes in humidity-controlled environments, and it is addressed specifically by this research. The paradox can be described as follows: Consider a lipid multilayer placed in bulk water, and compare this to placing the same multilayer in an environment with 100% relative humidity (r.h.). One would expect that since the chemical potentials of bulk water and water vapor with 100% r.h. are the same, that both cases would result in the same repeat spacing (Katsaras et al., 2008). However, this is not observed. A sample placed in vapor with 100% r.h. will not fully hydrate, i.e., it will not achieve the same repeat spacing as when placed in bulk water (Rand and Parsegian, 1989), thus giving rise to the vapor pressure paradox. Results have been published which claim that there is in fact no paradox, and that temperature gradients within the sample environment can account for the difference in the repeat spacing that is observed (Nagle and Katsaras, 1999; Katsaras, 1998). The effects of temperature gradients on repeat spacing and, particularly, how these gradients affect the data collected during this research will be discussed in more detail in Chapter 5.
CHAPTER 2
THERMODYNAMIC EQUILIBRIUM AND THE HUMIDITY CELL

Performing the experiments properly requires careful control of the relative humidity of the sample's environment. To achieve proper control of humidity, multilayer samples require a sample chamber that can maintain a uniform and stable temperature. Achieving thermal equilibrium has been one of the greatest challenges and obstacles of the experiments, and it is not a trivial task. According to Katsaras (1998), a temperature gradient of only 0.01°C decreases the humidity from 100% to \( \sim 99.9\% \). According to data presented by Chu et al. (2005), this difference in humidity could decrease the repeat spacing of DMPC samples by 5-6 Å. Thus, it is important to have a well-designed sample cell to ensure minimal temperature gradients.

2.1 Thermodynamic Equilibrium

According to Levine (2009), an isolated system is in equilibrium when its macroscopic properties remain constant with time. Equilibrium can be divided into three different types—mechanical equilibrium, material equilibrium, and thermal equilibrium. A system achieves mechanical equilibrium when the net force acting on the system, or within the system, is zero. Material equilibrium is achieved when there are no net chemical reactions occurring within the system, and there is no net transfer of matter within the system or from the system to its surroundings. The concentrations of species within the system must not change with time. For thermal equilibrium to exist, there must be no change in the properties of the system or surroundings when parts of the system or the system and its surroundings are separated by a thermally conducting wall. When all three types of equilibria are achieved, a system is said to be in thermodynamic equilibrium (Levine, 2009).

Suppose a lipid multilayer is placed in an isolated container at a constant temperature \( T \). When this system reaches thermodynamic equilibrium, the lipid multilayer will reach and maintain some repeat spacing \( D \). To change this repeat spacing, one must disturb the equilibria of the system. For the purposes of this research, temperature was kept constant, and there were no external forces
applied directly on the samples involved. The method used to change the spacing was disturbing the material equilibrium via introducing an aqueous salt solution. In terms of chemical potential, $\mu$, the requirement for a two-phase material equilibrium is

$$
\mu_1^\alpha = \mu_1^\beta \\
\mu_2^\alpha = \mu_2^\beta \\
\vdots \\
\mu_n^\alpha = \mu_n^\beta 
$$

with phases $\alpha$, $\beta$, and species 1, 2, ..., $n$, where $\mu_i$ is given by

$$
\mu_i = \mu_i^0 + RT \ln(x_i) \tag{2.1}
$$

In other words, if a system is in material equilibrium, then all phases of the same species have the same chemical potential (Levine, 2009).

Consider the system described above, with the addition of an aqueous salt solution sitting in a reservoir, which contains two species—$\text{K}_2\text{SO}_4$ and $\text{H}_2\text{O}$. Also, consider the volume of the container to be made up of species “air” and $\text{H}_2\text{O}$ vapor. The “air” species denotes the sum of all species other than water in the vapor phase. This is used for convenience since it is only necessary to know the mole fraction of water vapor to determine the relative humidity of the container. The system as a whole consists of a liquid phase (aqueous solution) and a vapor phase (air and water vapor). The mole fractions in each phase must sum to 1 (Levine, 2009):

$$
x_1^\alpha + x_2^\alpha + \cdots + x_n^\alpha = 1
$$

where $x_i^\alpha$ is the mole fraction of the $i^{th}$ species in phase $\alpha$. This implies that the mole fraction of water in solution must be equal to the mole fraction of water in vapor:

$$
x_{\text{H}_2\text{O}}^{\text{liquid}} = x_{\text{H}_2\text{O}}^{\text{vapor}} = 1 - \phi \tag{2.2}
$$
with $\phi = x_{\text{K}_2\text{SO}_4}^{\text{liquid}}$ for convenience. The relative humidity of the container is determined by the ratio of the partial pressure of water vapor, $P_{\text{H}_2\text{O}}$, to the saturated vapor pressure of water $P_{\text{H}_2\text{O}}^*$ (Chu et al., 2005). Assuming ideal behavior, this ratio can be expressed as (Levine, 2009)

$$\frac{P_{\text{H}_2\text{O}}}{P_{\text{H}_2\text{O}}^*} = x_{\text{H}_2\text{O}}^{\text{vapor}}$$  \hspace{1cm} (2.3)

By equation 2.2, this gives

$$\frac{P_{\text{H}_2\text{O}}}{P_{\text{H}_2\text{O}}^*} = x_{\text{H}_2\text{O}}^{\text{liquid}} = 1 - \phi$$  \hspace{1cm} (2.4)

Thus, the mole fraction of salt in solution determines the relative humidity within the container. Since $P_{\text{H}_2\text{O}} < P_{\text{H}_2\text{O}}^*$, the saturated vapor pressure of water can be written as

$$P_{\text{H}_2\text{O}}^* = P_{\text{H}_2\text{O}} + \Delta P$$  \hspace{1cm} (2.5)

where $\Delta P$ is a small change in pressure associated with adding a mole fraction $\phi$ of salt to the liquid water. Equating this to equation 2.4 gives

$$\phi = \frac{\Delta P}{P_{\text{H}_2\text{O}}}$$  \hspace{1cm} (2.6)

Next consider the variation of chemical potential with pressure (for convenience let $P = P_{\text{H}_2\text{O}}$) given by

$$\left( \frac{\delta \mu}{\delta P} \right)_T = \beta$$  \hspace{1cm} (2.7)

Then, by equations 2.6 and 2.1, a finite change in chemical potential can be expressed as the mole fraction of salt:

$$\Delta \mu = \beta \Delta P = \beta P \phi \approx RT \phi$$  \hspace{1cm} (2.8)

Suppose initially, that $\phi = 0$. Then the initial relative humidity of the container is at 100% r.h. and the lipid multilayer will reach its maximum repeat spacing. At equilibrium,

$$\mu_{\text{H}_2\text{O}}^{\text{vap}} = \mu_{\text{H}_2\text{O}}^{\text{liq}}$$
Adding salt to pure water, with mole fraction $\phi$ of salt, will decrease $\mu_{\text{liq}}^{\text{H}_2\text{O}}$. The water vapor will go to solution until the chemical potentials are equal and equilibrium is restored. The chemical potential of water between the lipid multilayers must also reach the same chemical potential as the water in solution and vapor. The multilayer will then lose water and the repeat spacing will decrease until the chemical potential of water is the same in all phases.

It is also convenient to express temperature gradients in terms of an “effective” mole fraction of salt, $\phi'$. This can then be related to an “effective” relative humidity via equation 2.4. To derive this relationship, first consider the Clausius-Clapeyron equation for a liquid-gas equilibrium:

$$\frac{d \ln P}{dT} \approx \frac{\Delta H_m}{RT^2} \quad (2.9)$$

where $\Delta H_m$ is the heat of vaporization of water and $T$ is not near the critical temperature $T_c$ (Levine, 2009). This can be written as

$$\frac{d \ln P}{d(1/T)} \approx -\frac{\Delta H_m}{R} \quad (2.10)$$

Approximating $\Delta H_m$ to be constant along the liquid-gas equilibrium line and integrating equation 2.10 gives (Levine, 2009)

$$\ln \left( \frac{P}{P + \Delta P} \right) \approx -\frac{\Delta H_m}{R} \left( \frac{1}{T + \Delta T} - \frac{1}{T} \right) \quad (2.11)$$

Next, rewrite equation 2.11 using equations 2.4 and 2.5:

$$\ln(1 - \phi') = -\frac{\Delta H_m}{R} \left( \frac{1}{T + \Delta T} - \frac{1}{T} \right) \quad (2.12)$$

If $r$ is the relative humidity measured as a fraction of unity, then this can be written as

$$\ln(1 - (\phi + \Delta \phi)) = \ln(r + \Delta r) = \ln(r) + \ln(1 + \frac{\Delta r}{r}) = -\frac{\Delta H_m}{R} \left( \frac{1}{T + \Delta T} - \frac{1}{T} \right) \quad (2.13)$$

Since measurements were taken with a relative humidity near 100%, and since the sample chamber is designed to minimize temperature gradients, this can be approximated as
\[ \Delta r \approx -\frac{\Delta H_m \Delta T}{RT^2} \]  

(2.14)

To determine the effective relative humidity as a fraction of unity, take

\[ r' = (r + \Delta r) = r - \frac{\Delta H_m \Delta T}{RT^2} \]  

(2.15)

Thus, temperature gradients within the sample environment will decrease the relative humidity. If temperature gradients can be minimized, then humidity can be controlled more accurately. The apparatus is designed with this in mind and is discussed in the following section.

### 2.2 Humidity Cell Design

The humidity cell consists of a series of three cylindrical chambers (Figure 2.1), and has been designed and modified to support the use of a water circulator and a PID temperature controller. The task of troubleshooting and changing samples can be quite tedious with such a complex design; however, it allows accurate temperature control and precise monitoring of temperature.

![Figure 2.1: Top view of the humidity cell.](image)

The outermost chamber is made from copper with a height of 25.5 cm. It has an inner radius of 4.7 cm, with a 0.5 cm thickness. Quarter-inch copper tubing is attached using lead tin solder and wound around the exterior which is used with the water circulator and acts as a cooling system (Figure 2.2).
Inside the exterior chamber sits an aluminum chamber, which acts as a heater (Figure 2.3). It has a height of 20.3 cm and an inner radius of 7.7 cm with a 0.5 cm thickness. The windows of this chamber are made from aluminum foil and are epoxied to the chamber using thermal epoxy. A 50 Ω heater coil is wrapped around the exterior of this chamber and is controlled with a PID temperature controller. The heater and cooling system work together to maintain a stable and uniform temperature. Details of this will be discussed in Section 2.2.1.
The innermost chamber holds the multilayer sample and an aqueous salt solution used to control the humidity. It is made from stainless steel; it has a height of 12.4 cm and an inner radius of 3.5 cm with a 2.0 cm thickness (Figure 2.4). Holes are drilled into the sides of the chamber from the top and bottom to allow for temperature sensors. Two temperature sensors are epoxied with thermally conductive epoxy inside these holes; one is placed on the top and one is placed on the bottom (Figure 2.5). Only one sensor can be used at a time to control temperature, but two sensors can be monitored simultaneously to measure gradients within the cell. The sample is suspended in the center of the chamber via a vacuum-sealed sample holder which connects to the lid of the chamber (Figure 2.6). The windows are circular with a radius of 4.1 cm, and are made from aluminum foil (Figure 2.7). Although the window design might appear insignificant, they can have a profound effect on measurements. The importance of windows will be discussed in section 2.2.2. The lids and windows of this chamber are sealed with O-rings to keep the environment isolated to maintain the desired relative humidity.
Figure 2.4: Top view of the innermost chamber of the humidity cell.

Figure 2.5: The innermost chamber of the humidity cell.
2.2.1 Temperature Control

The ability to control temperature is necessary to maintain a stable temperature. The temperature control “system” consists of two parts—a heater and a cooling system. The middle chamber of the humidity cell has a 50 Ω heater wound around its exterior. The outermost chamber acts as the cooling system and has copper tubing wound around its exterior. Water, set at a constant user-defined temperature, is pumped through the tubing using a water circulation bath. The temperature sensors used are precision epoxy NTC thermistors (Thermistor Specifications, 2008).
They are placed inside the walls of the innermost chamber of the humidity cell and are connected to a Lake Shore Model 331 temperature controller (Lake Shore Temperature Controller Manual, 2000).

The temperature controller uses an algorithm known as PID control, which stands for proportional, integral, and derivative. This algorithm regulates the heater output via the following equation:

\[
\text{Heater Output} = P \left[ e + I \int (e) dt + D \frac{de}{dt} \right]
\]  

(2.16)

where \( e \) is the error, defined as \( e = \text{Setpoint} - \text{Feedback Reading} \). The temperature controller allows the user to manually set the PID values and also allows for auto-tuning. The details of PID control will not be discussed here, but can be found in the Lake Shore Temperature Controller Manual (2000).

The temperature controller reads in the resistance of the thermistors and converts it to a temperature using a user-defined temperature curve. The resistances and corresponding temperatures can be found in the Thermistor Specifications (2008). The temperature controller then displays the corresponding temperature in either degrees celsius or kelvin, and up to a precision of 0.001°C (0.001K). The user must specify a desired temperature or “setpoint.” The temperature controller will control the heater output using equation 2.16. The temperature of the system will initially oscillate until the desired setpoint is achieved. It should be mentioned that the controller can only control using a single thermistor at any given time, which must be specified by the user.

The water circulation bath is set to a temperature below the desired setpoint. For the purposes of this research, a setpoint of 30.000°C was used for most experiments. With this setpoint, the temperature of the cooling system was typically set to approximately 22.0°C.

### 2.2.2 Windows

The windows are an important part of the cell design. Without proper windows, gradients can exist within the cell which would suppress the desired bilayer spacing. If the windows are made from a material that conducts heat poorly, then thermal radiation falling on the windows will not be carried away from the center of the window to its edges. This leads to temperature gradients
within the sample chamber. The original design used Kapton as windows. This material works well with x-ray experiments since it is transparent to x-rays. However, it conducts heat poorly, which leads to gradients within the cell. Thermal gradients can be estimated through comparison of heat flow onto the window via thermal radiation to heat conducted away from the window via conduction through the window. The following calculation demonstrates how the properties of the windows relate to thermal gradients:

Consider a circular window of radius $R$ that is in contact with a humidity cell on its perimeter. The window is exposed to thermal radiation from the external environment at temperature $T_1$, and the cell is at a temperature $T_2$. The window conducts thermal radiation away to the perimeter of the window. The temperature of the window at any radius can be written as $T(r) = T_1 + t(r)$.

Next, consider an annulus of inner radius $r$ and outer radius $r + dr$ of the window (Figure 2.8). The heat conducted to this annulus from the interior of the window is given by

$$Q_1 = -2\pi r w \kappa \cdot \frac{dt(r)}{dr} \quad (2.17)$$

where $\kappa$ is the thermal conductivity and $w$ is the thickness of the window. The heat conducted out of the annulus at its perimeter is given by

$$Q_2 = 2\pi (r + dr) w \kappa \cdot \frac{dt(r + dr)}{dr} \quad (2.18)$$
Heat radiated into the annulus due to thermal radiation is given by

\[ Q_3 = \sigma \left[ T_1^4 - (T_1 + t)^4 \right] \cdot 2\pi r dr \]  

(2.19)

where \( \sigma \) is the Stephan-Boltzmann constant. In the steady state, heat flow must be zero, so adding equations 2.17, 2.18, and 2.19 gives

\[ Q_1 + Q_2 + Q_3 = 0 \]  

(2.20)

When combined, and by use of the definition of the second derivative, this becomes

\[ 2\pi w \kappa dr \cdot \left[ \frac{d^2 t(r)}{dr^2} + \frac{1}{r} \frac{dt(r)}{dr} \right] + \sigma[T_1^4 - (T_1 + t)^4] \cdot 2\pi r dr = 0 \]  

(2.21)

Assuming that \( t \) is small, simplifying yields

\[ \frac{d^2 t(r)}{dr^2} + \frac{1}{r} \frac{dt(r)}{dr} - Ct = 0, \quad \text{with} \quad C = \frac{\sigma T_1^3}{\kappa w} \]  

(2.22)

Note that this is a second order ordinary differential equation of the form

\[ x^2 y'' + xy' - (x^2 + n^2)y = 0, \quad \text{with} \quad n = 0 \]
The solution is a modified bessel function of either the first or second kind,

\[ t(r) = c_1 I_0(r\sqrt{C}) + c_2 K_0(r\sqrt{C}) \]  \hspace{1cm} (2.23)

Since the modified bessel function of the second kind, \( K_0 \), diverges at \( r = 0 \), the solution must be the modified bessel function of the first kind. Applying boundary conditions gives

\[ c_1 = \frac{T_2 - T_1}{I_0(R\sqrt{C})} \]  \hspace{1cm} (2.24)

For a window made from aluminum foil, take \( T_1 = 298K \) (25°C) and \( T_2 = 303K \) (30°C), with window thickness \( w = 0.016 \) mm. With \( \sigma = 5.67 \times 10^{-8} \frac{W}{m^2K} \) and \( \kappa = 235 \frac{W}{mK} \), this gives \( \sqrt{C} = 20.0 \) m\(^{-1} \). Using \( R = 0.041 \) m yields \( c_1 = 4.3K \). This result means that the center of the window is at a temperature \( T(r = 0) = 29.3°C \).

Contrast this when the calculation is repeated using the properties of Kapton film. Kapton has \( \kappa = 0.12 \frac{W}{mK} \) and a thickness of \( w = 2.5 \times 10^{-10} \) m. This gives \( R\sqrt{C} = 917 \), resulting in \( c_1 \approx 0K \). This result implies that the center of the Kapton window stays at approximately room temperature.

Note that this calculation is not necessarily valid for the humidity cell that is used. The cell consists of three chambers at varying temperatures, whereas this calculation is for a simplified situation. Instead, the result of this calculation is used as an insight to improve the cell design and minimize temperature gradients. As mentioned, the original design of the cell used Kapton windows. However, after performing this calculation, it is made clear that using a material with a large thermal conductivity for windows will reduce thermal gradients within the humidity cell which will improve the accuracy of measurements.

### 2.3 Temperature Gradient Measurement

To determine the likely size of temperature gradients, a set of four temperature sensors were used to determine the temperature at various locations within the sample chamber. The sensors were first calibrated to one another by measuring the temperature at a single location within the chamber. Next, they were separated around the sample chamber and the temperature of each
sensor was measured. Among these measurements, the largest gradient from the center of the chamber was approximately $\Delta T = 0.092^\circ C$ when the chamber is maintained at 30$^\circ C$.

Recall that equation 2.15 relates the effective relative humidity to a temperature gradient:

$$ r' = (r + \Delta r) = r - \frac{\Delta H_m \Delta T}{RT^2} $$

For $T = 30^\circ C = 303K$, the heat of vaporization of water is $\Delta H_m \approx 44 \ \frac{kJ}{mol}$ (Levine, 2009). Using $R = 8.314 \ \frac{J}{mol \cdot K}$, this gives

$$ \Delta r = 0.0053 $$

For a sample in 100% r.h., the effective humidity becomes 99.47%, which is a significant difference. The effects of reduced humidity will be discussed in Chapter 5.
CHAPTER 3
SAMPLE PREPARATION

Two lipids were studied during the course of this research—dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC). Initially, experiments were performed using DOPC, but the primary focus of the research changed to DMPC. The methods described in this chapter were developed via trial and error starting with the “rock and roll method” as a basis (Tristram-Nagle, 2007).

3.1 Materials

The samples used during this research were made by depositing lipids onto glass substrates. To deposit lipids, they first must be dissolved in a solvent. The DOPC and DMPC lipids, as purchased, are dissolved in chloroform with a concentration of $20 \, \text{mg/mL}$. For the purpose of making DMPC samples, DMPC lipids were dissolved in isopropanol. From trial and error, $20 \, \text{mg/mL}$ solutions of DOPC in chloroform and DMPC in isopropanol produced the best results. For the purposes of cleaning glass substrates, the following materials were used: high purity grade methanol, millipore water, sulfuric acid, and hydrogen peroxide.

3.2 Cleaning Methods

Before lipids can be deposited onto a substrate, the substrate must first be cleaned. All beakers and substrates used were cleaned twice with methanol and twice with millipore water, alternating between water and methanol. The following cleaning methods were tested; however, there were no noticeable differences between the measurements of samples prepared with either method. For this reason, the “UV” cleaning method was the primary method used to clean substrates.
3.2.1 **Piranha Etch Method**

The Piranha Etch method for cleaning substrates must be performed with caution. For safety, it is performed in a fume hood while wearing safety glasses, gloves, a lab coat, and closed-toe shoes. The procedure is as follows:

1. Clean and fill three 150 mL beakers with millipore water.
2. Clean and fill a 150 mL beaker with 60 mL sulfuric acid and place on a hotplate.
3. Heat the sulfuric acid until it reaches 60°C.
4. Once the sulfuric acid reaches 60°C, turn off the hotplate and add 20 mL of hydrogen peroxide.
5. Place glass substrate into the sulfuric acid/hydrogen peroxide solution for at least 3 minutes.
6. Rinse substrate in the first two water beakers. Place the substrate into the last water beaker and leave until needed for sample preparation.
7. Properly dispose of sulfuric acid, and water used to clean the substrate.

3.2.2 **UV Cleaning Method**

Gloves are worn while performing the UV cleaning method. The procedure is as follows:

1. Clean glass substrate once with millipore water and once with methanol. Repeat.
2. Sonicate glass substrate in methanol for 15 minutes and water for 15 minutes.
3. Dry the glass substrate with nitrogen gas and place in UV chamber for 3–5 minutes.

3.3 **Sample Preparation Methods**

Initial attempts at the “rock and roll” method did not produce uniform samples. Through trial and error, alternative methods were discovered that produced uniform samples. There are
two separate methods used—one for DOPC samples and one for DMPC samples. Both methods deposit lipids using pipettes. The differences are mostly in the environment the sample is placed in after lipids are deposited on the substrate. The reason for needing separate methods for different lipids is due to the problem of the substrate “de-wetting.” This phenomenon was observed while developing methods for DMPC samples, but was not observed for DOPC samples. After the lipid solution is placed on the substrate, it appears that the solution is pushed to the edges of the substrate as the solvent evaporates. Either no lipid is left on the substrate, or there is a minimal amount visible near the edges of the substrate. The methods described here attempt to avoid this problem so that uniform samples are produced.

3.3.1 DOPC Sample Preparation Method

It was found through trial and error that chloroform evaporates rather quickly. If a substrate is level, then depositing lipid solution on the substrate and allowing it to sit will often result in uniform samples. Placing the substrate under a fume hood or in a vacuum chamber helps quicken the process. The procedure used for making DOPC samples is as follows:

1. Place substrate in a petri dish on a level surface in either a fume hood or vacuum chamber.
2. Deposit enough lipid solution (20 mg/mL DOPC in chloroform) so that the entire substrate is covered.
3. Let sample sit while chloroform evaporates.

3.3.2 DMPC Sample Preparation Method

At first, the sample preparation method used for DOPC samples (Section 3.3.1) was attempted with DMPC samples. As previously discussed, de-wetting of the substrate occurs and there is little to no lipid left on the substrate once the solvent fully evaporates. There were three major concerns that were thought to contribute to this problem—the transition temperature of DMPC, the concentration of the lipid solution, and using chloroform as a solvent. Since DMPC has a transition temperature at $T_M \approx 24^\circ C$, and since samples were made near this temperature, new
attempts to make samples above or below this temperature were tested, along with using varying concentrations and using isopropanol instead of chloroform. A total of 8 different methods were tested. This is the procedure that produced the most uniform samples:

1. Fill a large beaker with ice. Wrap the entire beaker in aluminum foil to insulate it.

2. Level and place a petri dish in the ice. Place the substrate in the petri dish.

3. Deposit enough lipid solution (20 mg/mL DMPC in isopropanol) so that the entire substrate is covered.

4. Cover beaker with aluminum foil and let sample sit while isopropanol evaporates.
4.1 X-rays

X-rays are produced via an x-ray tube which includes a source of electrons and an anode and cathode (Cullity, 1978). A voltage of roughly 36 kV is maintained across the electrodes and draws electrons to the anode at which they strike a molybdenum target. Two types of radiation are produced—continuous radiation and characteristic radiation.

A molybdenum target will produce characteristic radiation of wavelengths 0.709 Å, 0.714 Å, and 0.632 Å (Cullity, 1978). Measurements are taken using a 0.709 Å wavelength (also known as $K_α$ radiation) since it has the largest intensity. Other characteristic radiation and continuous radiation is filtered out by using a crystal monochromator and beam collimators. This allows use of x-ray reflectivity and Bragg’s Law to study the structure of samples.

4.2 Bragg’s Law

The lipid structures studied in this research fall into a category of liquid crystals known as smectic liquid crystals (Jones, 2002). Since these structures are ordered, they can be studied with x-ray reflectivity. As previously mentioned, the repeat spacing of these structures is of particular interest to this research, which can be determined using Bragg’s law (Cullity, 1978):

$$n\lambda = 2d\sin\theta$$  \hspace{1cm} (4.1)

where $n$ is an integer, $\lambda$ is the wavelength of the x-rays, $d$ is the repeat spacing, and $\theta$ is the angle of the incident x-rays with the horizontal (Figure 4.1).
Figure 4.1: A diagram demonstrating Bragg’s Law. For the ideal case, x-rays reflect at the same angle as the incident x-rays. Bragg’s Law can then be used to solve for the repeat spacing $d$. Image from (Krumeich, 2009).

Typical measurements have Bragg peaks occurring in the order of tenths of a degree. For small angles, a shift in the Bragg peak occurs due to refraction (Birkholz et al., 2006). This can have a significant effect for first and second order peaks, and can usually be neglected for peaks of order greater than 2. According to Birkholz et al. (2006), the corrected angle is given by

$$\theta' = \sqrt{\theta^2 - \theta_c^2}$$

where $\theta$ is the observed angle and $\theta_c$ is the critical angle. The critical angle can be determined via the following calculation:

Consider Bragg’s law for the $n = 1$ and $n = 2$ cases:

$$\lambda = 2d \sin \sqrt{\alpha^2 - \theta_c^2} \quad (n = 1)$$
$$\lambda = d \sin \sqrt{\beta^2 - \theta_c^2} \quad (n = 2)$$

Dividing and rearranging these equations gives

$$\frac{\sin \sqrt{\alpha^2 - \theta_c^2}}{\sin \sqrt{\beta^2 - \theta_c^2}} = \frac{1}{2}$$

If the angles are given in radians, then this can be simplified using the small angle approximation. This gives

$$\frac{\alpha^2 - \theta_c^2}{\beta^2 - \theta_c^2} \approx \left(\frac{1}{2}\right)^2$$
Solving for $\theta_c$ yields

$$\theta_c \approx \sqrt{\frac{4\alpha^2 - \beta^2}{3}}$$  \hspace{1cm} (4.3)

For a particular measurement, $\alpha = 0.0069$ rad and $\beta = 0.0137$ rad. This gives $\theta_c \approx 0.001$ rad or 0.057$^\circ$. Although the critical angle changes depending on properties of the sample, it is approximately 0.057$^\circ$ for all measurements presented in this thesis. Determining the angle $\theta$ from a reflectivity measurement is discussed in the next section.

### 4.3 Reflectivity Measurements

Photons from the reflected x-rays are converted to an electrical signal via a photomultiplier tube. The photon events are counted, and the number of counts reflects the intensity of the x-ray beam. For a typical measurement, the intensity as a function of the angle from the beam fits a gaussian distribution. Figure 4.2 is a plot of one such measurement.

![Figure 4.2: Reflectivity measurement of DMPC at 30$^\circ$C. It is also called a two-theta rocking scan.](image)
The position of the peak and its uncertainty can be determined by fitting the plots to a gaussian distribution of the form

\[ y = A \times \exp \left( -\frac{1}{2} \left( \frac{x - B}{C} \right)^2 \right) \]

All reflectivity measurements were fit using a free online curve fitting resource (Phillips, 2011). Using equation 4.1, the angle \( \theta \) and its uncertainty can be converted to repeat spacing \( D \) and its corresponding uncertainty. The following section contains data and results of the reflectivity measurements. This includes the peak position \( \theta \) and the corrected peak position \( \theta' \) for each measurement, along with the corresponding repeat spacing and uncertainty for each.

### 4.4 Measurements and Data

The purpose of this section is to simply present data from the measurements taken during the course of this study. The interpretation and discussion of these measurements and results will be presented in Chapter 5.

Measurements of DMPC were taken for two samples at a temperature of 30°C at humidities near 100% r.h. Specifically, humidities of 100%, 99.99%, 99.90%, and 99.00% were used. The measurements of the first sample began with 100% r.h. Humidity was then cycled to 99.00% and back to 100%. For the second sample, measurements began with 99.00% r.h. and cycled to 100% r.h. and back to 99.00% r.h. The purpose of cycling humidity is to detect a time dependence on spacing measurements. Theoretically, the equilibrium repeat spacing is regulated by the mole fraction of salt in the water bath. However, the samples are exposed to these humidities for extended periods of time, ranging anywhere from days to weeks. If the spacing of a particular sample is significantly different for multiple measurements at the same relative humidity, then it might be reason to suspect a time dependence, or it might suggest that the sample has not yet reached equilibrium. The data presented here do not appear to have such dependencies, so it will be assumed that no time dependence exists within the data.

Table 4.1 contains data for the positions of bragg peaks for the various humidities of two samples in order of when they were taken. The table includes the bragg peak angles with their uncertainties, and the corrected bragg peak angles. The repeat spacing \( D \) and the uncertainty
of $D$, $\sigma_D$, were calculated from these bragg peak angles. The ideal repeat spacings for the given humidities are the $D_{\text{ideal}}$ values listed in the table. The $D_{\text{adj}}$ data represent adjusted ideal repeat spacings, given a temperature gradient of $\Delta T = 0.092^\circ C$. Both $D_{\text{ideal}}$ and $D_{\text{adj}}$ were determined based on ideal results, which will be discussed in Chapter 5. Also, some of the measurements were taken over the second order bragg peak. These can be identified as measurements with $\theta \approx 0.7^\circ$.

Table 4.1: Peak position and repeat spacing for various humidities

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>R.H. (%)</th>
<th>$\theta$ (°)</th>
<th>$\theta'$ (°)</th>
<th>$\sigma_\theta$</th>
<th>$D$ (Å)</th>
<th>$\sigma_D$</th>
<th>$D_{\text{ideal}}$ (Å)</th>
<th>$D_{\text{adj}}$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.00</td>
<td>0.366</td>
<td>0.362</td>
<td>0.150 $\times 10^{-3}$</td>
<td>56.2</td>
<td>1.3</td>
<td>62.7</td>
<td>53.7</td>
<td></td>
</tr>
<tr>
<td>99.99</td>
<td>0.760</td>
<td>0.758</td>
<td>0.274 $\times 10^{-3}$</td>
<td>53.6</td>
<td>1.1</td>
<td>60.2</td>
<td>53.7</td>
<td></td>
</tr>
<tr>
<td>99.90</td>
<td>0.769</td>
<td>0.767</td>
<td>0.276 $\times 10^{-3}$</td>
<td>53.0</td>
<td>1.1</td>
<td>56.8</td>
<td>53.4</td>
<td></td>
</tr>
<tr>
<td>99.00</td>
<td>0.394</td>
<td>0.390</td>
<td>0.231 $\times 10^{-3}$</td>
<td>52.1</td>
<td>1.7</td>
<td>52.5</td>
<td>51.7</td>
<td></td>
</tr>
<tr>
<td>99.90</td>
<td>0.384</td>
<td>0.380</td>
<td>0.333 $\times 10^{-3}$</td>
<td>53.5</td>
<td>2.6</td>
<td>56.8</td>
<td>53.4</td>
<td></td>
</tr>
<tr>
<td>99.99</td>
<td>0.758</td>
<td>0.756</td>
<td>0.364 $\times 10^{-3}$</td>
<td>53.7</td>
<td>1.5</td>
<td>60.2</td>
<td>53.7</td>
<td></td>
</tr>
<tr>
<td>100.00</td>
<td>0.373</td>
<td>0.369</td>
<td>0.231 $\times 10^{-3}$</td>
<td>55.1</td>
<td>1.9</td>
<td>62.7</td>
<td>53.7</td>
<td></td>
</tr>
</tbody>
</table>

Sample 2

<table>
<thead>
<tr>
<th>R.H. (%)</th>
<th>$\theta$ (°)</th>
<th>$\theta'$ (°)</th>
<th>$\sigma_\theta$</th>
<th>$D$ (Å)</th>
<th>$\sigma_D$</th>
<th>$D_{\text{ideal}}$ (Å)</th>
<th>$D_{\text{adj}}$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99.00</td>
<td>0.786</td>
<td>0.784</td>
<td>0.246 $\times 10^{-3}$</td>
<td>51.8</td>
<td>0.9</td>
<td>52.5</td>
<td>51.7</td>
</tr>
<tr>
<td>99.90</td>
<td>0.382</td>
<td>0.378</td>
<td>0.361 $\times 10^{-3}$</td>
<td>53.8</td>
<td>2.9</td>
<td>56.8</td>
<td>53.4</td>
</tr>
<tr>
<td>99.99</td>
<td>0.389</td>
<td>0.385</td>
<td>0.224 $\times 10^{-3}$</td>
<td>52.8</td>
<td>1.7</td>
<td>60.2</td>
<td>53.7</td>
</tr>
<tr>
<td>100.00</td>
<td>0.383</td>
<td>0.379</td>
<td>0.307 $\times 10^{-3}$</td>
<td>53.6</td>
<td>2.4</td>
<td>62.7</td>
<td>53.7</td>
</tr>
<tr>
<td>99.99</td>
<td>0.386</td>
<td>0.382</td>
<td>0.299 $\times 10^{-3}$</td>
<td>53.2</td>
<td>2.3</td>
<td>60.2</td>
<td>53.7</td>
</tr>
<tr>
<td>99.90</td>
<td>0.387</td>
<td>0.383</td>
<td>0.283 $\times 10^{-3}$</td>
<td>53.1</td>
<td>2.2</td>
<td>56.8</td>
<td>53.4</td>
</tr>
<tr>
<td>99.00</td>
<td>0.398</td>
<td>0.394</td>
<td>0.222 $\times 10^{-3}$</td>
<td>51.6</td>
<td>1.6</td>
<td>52.5</td>
<td>51.7</td>
</tr>
</tbody>
</table>

Repeat spacing versus relative humidity of the first sample is plotted in Figure 4.3. Figure 4.4 is a plot of data for the second sample. The solid line in the plots represents the expected repeat spacing as a function of humidity. This expected data is presented by Chu et al. (2005), and will be discussed in Chapter 5. Circles represent the measurements for the first cycle of humidities, while stars represent the second part of the cycle.
Figures 4.5 and 4.6 are again plots of the first and second sample respectively, but they also include the ideal repeat spacing adjusted to a temperature gradient of 0.092°C. Circles represent sample measurements, and X’s represent the adjusted ideal repeat spacings.

Figure 4.3: A plot of repeat spacing versus relative humidity of DMPC at 30°C for the first sample.

Figure 4.4: A plot of repeat spacing versus relative humidity of DMPC at 30°C for the second sample.
Figure 4.5: A plot of repeat spacing versus relative humidity of DMPC at 30°C for the first sample. Circles represent measurements of the sample; X’s represent the ideal spacing adjusted to a temperature gradient of 0.092°C.

Figure 4.6: A plot of repeat spacing versus relative humidity of DMPC at 30°C for the second sample. Circles represent measurements of the sample; X’s represent the ideal spacing adjusted to a temperature gradient of 0.092°C.
CHAPTER 5
IDEAL RESULTS AND CONCLUSIONS

The goal of this research is to demonstrate the ability to control lipid multilayer repeat spacing via humidity at a fixed temperature. One would expect that if a reservoir of pure water is placed inside the humidity cell, then the multilayer sample will achieve full hydration. For DMPC at 30°C, the repeat spacing at full hydration is 62.5~63.0 Å (Chu et al., 2005). Petrache et al. (1998) present data for lipid multilayers placed in bulk water in which an immiscible polymer is added to produce an osmotic pressure. The osmotic pressure can be related to an equivalent relative humidity. A plot of repeat spacing as a function of this equivalent relative humidity is presented by Chu et al. (2005), and has become the primary means of comparison for data presented in Chapter 4. This plot is shown in Figure 5.1 and is the ideal result when measuring DMPC repeat spacing at 30°C in a humidity-controlled environment.

![Figure 5.1: A plot of repeat spacing as a function of relative humidity for DMPC at 30°C. Figure from (Chu et al., 2005).](image)

To determine the relationship between repeat spacing and relative humidity in Figure 5.1, data points were estimated from the plot and then fit to an exponential curve (Phillips, 2011). The
resulting fit expresses the relative humidity in terms of repeat spacing, \( D \). Rearranging this to find \( D \) in terms of r.h. gives the following relationship:

\[
D_{\text{ideal}} = -1.9 \times \ln (100.01 - \text{R.H.}) + 52.5 \quad (5.1)
\]

The first sample measured follows a similar relationship. This observed relationship between repeat spacing and relative humidity can be approximated using a similar fit:

\[
D_{\text{obs}} = -0.4 \times \ln (100.00 - \text{R.H.}) + 52.1 \quad (5.2)
\]

The data presented in Table 4.1 and in Figures 4.3 and 4.4 are less than the expected results for all humidities. As the relative humidities approach 100%, the differences become more significant. The data from the first sample follow a trend similar to that expected, but with decreased values for repeat spacing. This trend is not evident for the second sample; however, it appears that spacing is slightly greater for humidities closer to 100%.

Recall that equation 2.15 relates the effective relative humidity to a temperature gradient:

\[
r' = (r + \Delta r) = r - \frac{\Delta H_m \Delta T}{RT^2}
\]

As discussed in Section 2.3, a temperature gradient of \( \Delta T = 0.092^\circ \text{C} \) results in \( \Delta r = 0.0053 \). An effective relative humidity is then determined by taking \( r' = r + \Delta r \). Then, using equation 5.1 gives the adjusted spacing. These are presented in Table 4.1 and Table 5.1. Table 5.1 shows the effective relative humidity and corresponding spacings expected for DMPC at \( T = 30^\circ \text{C} \). Also, to find a direct relationship between temperature gradients and adjusted repeat spacing \( D_{\text{adj}} \), equations 2.15 and 5.1 can be combined to give

\[
D_{\text{adj}} = -1.9 \times \ln \left(100 \times \left(1 - r + \frac{\Delta H_m \Delta T}{RT^2}\right)\right) + 52.5 \quad (5.3)
\]

Since data has only been taken at \( T = 30^\circ \text{C} \), it can only be said that this relationship is valid at this temperature. Simplifying then gives

\[
D_{\text{adj}} = -1.9 \times \ln (100 \times (1 - r + 0.058\Delta T)) + 52.5 \quad (5.4)
\]
Table 5.1: A table comparing relative humidities and corresponding spacings to effective humidities and adjusted spacings. The adjusted values correspond to a temperature gradient of $\Delta T = 0.092^\circ C$.

<table>
<thead>
<tr>
<th>R.H. (%)</th>
<th>Effective R.H. (%)</th>
<th>$D$ (Å)</th>
<th>$D_{adj}$ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.00</td>
<td>99.47</td>
<td>62.7</td>
<td>53.7</td>
</tr>
<tr>
<td>99.99</td>
<td>99.46</td>
<td>60.2</td>
<td>53.7</td>
</tr>
<tr>
<td>99.90</td>
<td>99.37</td>
<td>56.8</td>
<td>53.4</td>
</tr>
<tr>
<td>99.00</td>
<td>98.47</td>
<td>52.5</td>
<td>51.7</td>
</tr>
</tbody>
</table>

The adjusted spacings, $D_{adj}$, are compared to measured values in Table 4.1, and they are plotted in Figures 4.5 and 4.6. For the first sample, the adjusted spacings are less than the spacings determined via measurements. This suggests that the average temperature gradient present during measurements of the first sample were smaller than $\Delta T = 0.092^\circ C$. On the other hand, a gradient of this size can account for discrepancies of the second sample measurements when compared to the ideal results. Each of the adjusted spacings fall within the uncertainties of the spacings determined for the second sample.

If it is assumed that these temperature gradients are in fact responsible for the spacing discrepancies, then it can be concluded, from these measurements, that repeat spacing for a multilayer sample can be controlled via humidity of the sample’s environment, provided that temperature gradients are minimized. More measurements, including careful temperature gradient measurements for each measurement, would be required to confirm this, since the temperature gradients during each measurement were not measured.

Additionally, making the above assumption is implicitly denying the existence of a vapor pressure paradox. Based on the results of these measurements, it is plausible that such a paradox does not exist and that temperature gradients alone can account for the observed discrepancies in repeat spacing. Experiments aimed to specifically address the existence of a vapor pressure paradox require an accurate measurement of temperature gradients throughout the sample chamber. For this reason, an improved apparatus design is necessary.

An obvious flaw in the current design is the size. Having such a large apparatus makes temperature gradient measurements difficult. It would be ideal to measure the temperature at all points within the sample chamber, but this is not feasible. Monitoring and controlling temperature within
a smaller volume is much easier than with a larger one. However, it is still necessary to isolate the sample environment from the surroundings in order to maintain a stable and uniform temperature. If the apparatus is too small, then variations in temperature of the surroundings might affect the temperature of the sample chamber. Ideally, the best design would be as small as possible, while still isolating the sample environment from its surroundings.

A second improvement of the design would be to use multiple high-precision temperature sensors, which continuously monitor temperature over time. The temperature gradients themselves cannot be held accountable for discrepancies in the measurements unless the gradients are known at the time of each measurement. It is likely that the temperature gradients are in fact responsible for the differences in the data presented in Chapter 4; however, since the gradient measurement was only taken once, and it was not taken simultaneously with each reflectivity measurement, it cannot be said that the temperature gradients are solely responsible for all discrepancies for all measurements. The differences between data for the two samples measured indicate that the temperature gradients vary. Some measurements have larger gradients than others, but this cannot be determined unless temperature throughout the chamber is known at the time of each measurement.

To conclude, the results of this project motivate further study of lipid multilayers in humidity-controlled environments. Specifically, the results have shown that it is plausible that accurate control of repeat spacing is attainable through minimization of temperature gradients. Thus, it may be worthwhile to pursue further experimentation; however, it would require an improved apparatus design, or a newly designed apparatus altogether. In regards to the vapor pressure paradox, the results indicate that it does not likely exist. Discrepancies between observed repeat spacings can be accounted for through temperature gradients alone. However, the current apparatus design did not allow continuous and accurate monitoring of temperature gradients during each measurement, and therefore, this claim can only be verified by repeating the measurements with an improved apparatus.
REFERENCES


