Surface Analytical Studies of Gramicidin A in Biomimetic Model Membrane Systems

by

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ABSTRACT

SURFACE ANALYTICAL STUDIES OF GRAMICIDIN A IN BIOMIMETIC MODEL MEMBRANE SYSTEMS

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This thesis is an investigation of the properties of gramicidin A in model membrane systems. This study investigates a solid supported bilayer lipid membrane comprised of 1,2-dimyrystoyl-sn-glycero-3-phosphocholine (DMPC) and gramicidin A deposited on a thioglucose modified Au(111) electrode. A “floating” bilayer lipid membrane was also investigated that was composed of DMPC, cholesterol, glycolipid monosialoganglioside (GM1), and gramicidin A deposited on a thioglucose modified Au(111) electrode. The GM1 was used as a spacer molecule to create a water reservoir between the modified electrode and lipid bilayer. Polarization modulation infrared reflection adsorption spectroscopy (PM-IRRAS) was used to investigate the solid supported and the floating bilayer lipid membrane. The PM-IRRAS studies were completed to probe the electric field driven changes in the orientation and conformation of gramicidin A in the lipid bilayer. Atomic force microscopy was used to investigate the topography and thickness of the floating bilayer lipid membrane system.
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List of Symbols and Abbreviations

Chapter 1

AFM Atomic force microscopy
PM-IRRAS Polarization modulated infrared reflection adsorption spectroscopy

Chapter 2

PC Phosphatidylcholine
DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine
PE Phosphatidylethanolamine
PS Phosphatidylserine
PG Phosphatidylglycerol
GM1 Monosialotetrahexosylganglioside
HD Helical dimer
DH Double helix
NMR Nuclear magnetic resonance
STM Scanning tunneling microscopy
SAM Self-assembled monolayer
IR Infrared
ATR Attenuated total reflection
IRRAS Infrared reflection adsorption spectroscopy
FSD Fourier self deconvolution
2D-COS Two dimensional correlation spectroscopy

Chapter 3

MAC Magnetic AC mode
PM-IRRAS Polarization modulation infrared reflection absorption spectroscopy
G Gibbs free energy per surface area
γ Surface tension
S Surface area
T Temperature
P Pressure
n Composition
Π Surface pressure
w Weight of the meniscus
P Perimeter
θ Static contact angle
S Solid condensed state
LC Liquid condensed state
LE Liquid expanded state
G Gaseous state
LB Langmuir-Blodgett
LS Langmuir-Schaefer
AFM  Atomic force microscopy
k    Spring constant
F    force
x    Distance
E    Young’s modulus
t    Thickness
w    Width
l    Length
vdW  van der Waals
A_H  Hamker constant
R    Radius of tip
D    Distance between the tip and sample
q    Charges of ions
r    Distance between two ions
\varepsilon  Permittivity
\psi_x  Surface potential
E_{pair}  Pair-potential energy
Z_c  Deflection of the cantilever
Z_p  Vertical position of the tip recorded with the piezo
\delta  Elastic deformation
E*  Effective compression modulus
R_p  p- reflectivity
R_s  s- reflectivity
MSEFS  Mean-square electric field strength
SSD  Synchronous sampling demodulator
I_A(\omega)  Intensity average
I_D(\omega)  the intensity difference
I_s  s- polarized light intensity
I_p  p- polarized light intensity
\phi_0  Maximum retardation of the incident radiation
J_0(\omega)  zero-order Bessel function
J_2(\omega)  second order Bessel function
\mu  Transition dipole
A  Intensity of IR band
A_{(random)}  Intensity from simulated spectra
A_{(E)}  Intensity from experimental spectra
S_{C=0}  Order parameter of the amide I band
S_{helix}  Order parameter of the helix
\gamma  Average tilt angle of the helical axis

Chapter 4

DMPC  1,2-dimyristoyl-sn-glycerol-3-phosphocholine
GM1  Monosialotetrahexosylganglioside
AFM  Atomic force microscopy
MAC  Magnetic AC mode
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PM-IRRAS</td>
<td>Polarization modulation infrared reflection absorption spectroscopy</td>
</tr>
<tr>
<td>RE</td>
<td>Reference electrode</td>
</tr>
<tr>
<td>CE</td>
<td>Counter electrode</td>
</tr>
<tr>
<td>WE</td>
<td>Working electrode</td>
</tr>
<tr>
<td>SP</td>
<td>Static Polarizer</td>
</tr>
<tr>
<td>PEM</td>
<td>Photoelastic modulator</td>
</tr>
<tr>
<td>MSEFS</td>
<td>Mean-square electric field strength</td>
</tr>
<tr>
<td>SSD</td>
<td>Synchronous sampling demodulator</td>
</tr>
<tr>
<td>FSD</td>
<td>Fourier self-deconvolution</td>
</tr>
<tr>
<td>A</td>
<td>Band maximum</td>
</tr>
<tr>
<td>γ</td>
<td>Half-width at half-maximum</td>
</tr>
<tr>
<td>HWHM</td>
<td>Half-width at half-maximum</td>
</tr>
<tr>
<td>ν</td>
<td>Wavenumber</td>
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**Chapter 5**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>MAC</td>
<td>Magnetic AC mode</td>
</tr>
<tr>
<td>PM-IRRAS</td>
<td>Polarization modulation infrared reflection absorption spectroscopy</td>
</tr>
<tr>
<td>$A_i$</td>
<td>Areas of pure components</td>
</tr>
<tr>
<td>$X_i$</td>
<td>Mole fraction</td>
</tr>
<tr>
<td>$A_{mix}$</td>
<td>Calculated area of a mixed monolayer</td>
</tr>
<tr>
<td>$A_{exp}$</td>
<td>Experimental area of a mixed monolayer</td>
</tr>
<tr>
<td>$A_{excess}$</td>
<td>Excess area</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>GM1</td>
<td>Monosialotetrahexosylganglioside</td>
</tr>
<tr>
<td>S</td>
<td>Solid condensed state</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid condensed state</td>
</tr>
<tr>
<td>LE</td>
<td>Liquid expanded state</td>
</tr>
<tr>
<td>G</td>
<td>Gaseous state</td>
</tr>
<tr>
<td>$A_{lim}$</td>
<td>Limiting area</td>
</tr>
<tr>
<td>$\nu_{as}$</td>
<td>Asymmetric vibration</td>
</tr>
<tr>
<td>$\nu_{sym}$</td>
<td>Symmetric vibration</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
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**Chapter 6**

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>PM-IRRAS</td>
<td>Polarization modulation infrared reflection absorption spectroscopy</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>GM1</td>
<td>Monosialotetrahexosylganglioside</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
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Chapter 1: Introduction

1.1 Preamble

Biological membranes are inherently complex in nature. Consequently, methods to design biomimetic membranes are in high demand. With a successful model membrane the interaction of proteins and lipids can be investigated. As well, information about specific ion channels can be determined such as orientation and conformational features. This is of great importance due to the fundamental role ion channels serve in everyday life. Ion channels serve in chemical signaling, regulation of cell volume, regulation of cytoplasmic or vesicular ion concentration and pH, and electrical signaling [1]. Ion channel dysfunction can cause many different types of diseases [1]. There is also interest in the potential of ion channels for the development of drugs, in particular for the treatment of antibiotic resistant bacterial infections [2]. Studying antimicrobial peptides in biomimetic membranes will give rise to possible mechanisms in their antibiotic activities, which are largely unknown [3].

Gramicidin A is the most widely studied antimicrobial peptide. Gramicidin is extremely useful in the development of novel model membranes to test suitability for successful incorporation of simple transmembrane proteins. Currently, there is a lack of model membranes that can successfully incorporate transmembrane proteins such that the protein behaves as it would in its natural environment. Successful models can then be selected to study larger transmembrane proteins with the hope of gaining beneficial knowledge of their mechanisms within biological membranes.
1.2 Objectives of this thesis

The overall aim of this thesis was to investigate the incorporation of a transmembrane protein in two different model membranes: solid supported bilayer lipid membrane and ‘floating’ bilayer lipid membrane.

The specific goals of this thesis include:

- Investigate the lateral organization of membrane components
- Investigate the conformation and orientation of the lipid matrix of the ‘floating’ bilayer lipid membrane with the incorporation of gramicidin A
- Investigate the conformation and orientation of gramicidin A in a lipid environment
- Investigate the effect of an applied potential that is comparable to the voltage drop across a natural membrane on the model membrane and protein that is incorporated
- Investigate the effect of a hydrophilic spacer molecule that is not anchored to the metal for the creation of a water reservoir between a metal surface and a bilayer

1.3 Scope of the thesis

Chapter 1 of this thesis outlines the major goals of this work and gives a brief introduction into the motivation of the project. Chapter 2 is a literature review covering past research in areas of biomimetic model membranes, biochemical infrared spectroscopy, and atomic force microscopy of biological samples. Chapter 3 provides a detailed explanation of the theory for all of the techniques used in this work, including Langmuir films, atomic force microscopy, and PM-IRRAS. Chapter 4 gives a detailed
explanation of the experimental procedures used for the deposition of a bilayer on a substrate, as well as the operation of the AFM instrument and the PM-IRRAS equipment. Chapter 5 presents major results obtained for all experiments. Chapter 5 is organized into two groups of results: solid supported bilayer lipid membrane and ‘floating’ bilayer lipid membrane. Chapter 6 provides a summary of all major conclusions as well as some future directions for the project.
1.4 References


Chapter 2: Literature Review

2.1 Properties of Biological Membranes

Cell membranes are a fundamental component of all living organisms. They form the central structure of a cell by creating a barrier between the extra- and intra- cellular environments. The cell membrane and all of its integrated components are important in cellular communication and molecular transport. The cell membrane has been described as a fluid mosaic where the membrane is described as a two-dimensional liquid with proteins that are viewed as icebergs floating in a sea of lipids (Figure 2.1) [1]. However, cell membranes have been found to be highly organized with a variety of lipids and proteins that are intricately held together by mostly non-covalent interactions [2]. Cell membranes are mostly comprised of lipids from three main groups: the phospholipids, sterols, and glycolipids.

Figure 2.1: Schematic of a biological membrane. Adapted from Ref. 3.
2.1.1 Role of Phospholipids

Phospholipids are the most abundant lipids in the biological membrane. Phospholipids are amphiphilic molecules that contain a polar head group with hydrocarbon tails that vary in length (Figure 2.2). The packing of the lipids in the membrane is strongly influenced by the hydrocarbon chains and any additional functional groups attached to the lipid (ex. choline, serine, and glycerol). The packing of the lipids impacts the fluidity of the membrane and the temperature at which the gel to liquid crystalline phase transition occurs.

The diacylglycerol backbone in eubacteria and eukaryotes is composed of a sn-3-glycerol esterified at the first and second positions with long-chain fatty acids (Figure 2.2) [3]. The most common head groups that attach to the negatively charged phosphate group of the phospholipid are choline, ethanolamine, serine and glycerol (Figure 2.2). The phospholipid utilized in this thesis is a phosphatidylcholine (PC) called 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). The PC head group makes up 19% of the lipid composition in human cells [4]. The phase behaviour of PCs has been studied extensively and these studies show the phase preference below the transition temperature of 24 °C as a lamellar, gel phase and above the transition temperature a lamellar, liquid crystalline phase [5, 6, 7]. The gel phase is made of lipids with acyl chains in an all trans configuration whereas the liquid crystalline phase is comprised of disordered acyl chains.
2.1.2 Role of Cholesterol

Cholesterol is a type of steroid with the common structural motif of three six-membered rings and one five-membered ring fused together. Cholesterol has a hydroxyl group at the C-3 position, making it a type of sterol (Figure 2.3). The rigid ring system and the polar alcohol group make the molecule very influential in interactions with neighboring components. Cholesterol is mostly hydrophobic with the exception of the small hydroxyl group and therefore, cholesterol exists mostly in the hydrophobic middle of the bilayer [8]. Due to its ridged, structure cholesterol creates a more ordered bilayer

**Figure 2.2:** Phospholipids with different head groups. Adapted from Ref. 3.
by restricting the conformational degrees of freedom of the hydrocarbon tails of neighboring phospholipids into mostly trans- configurations [9]. Therefore, cholesterol has a ‘condensing effect’ where it effectively decreases the permeability of the bilayer by decreasing the amount of packing defects in the membrane [8]. The increase in lipid chain ordering has been directly measured through the reduction of surface area per phospholipid with cholesterol present [10]. If the phospholipids contain unsaturated hydrocarbon tails, then packing of adjacent hydrocarbon tails is restricted due to the ridged cholesterol and this inhibits the formation of the gel-state [11].

Cholesterol is the most common essential sterol in humans and animals and the role of sterols in the functioning of cells has been widely studied though there still remains many unanswered questions [8]. It is interesting to note that the biochemical synthesis of cholesterol comes at a greater energy cost than the other lipids and therefore, one could deduce that cholesterol must be vital to cell survival [8]. However, accumulation of cholesterol ester and free cholesterol have been found to cause cell death [8]. The role of cholesterol in the membrane is extremely complex.

![Structure of cholesterol](image_url)

**Figure 2.3:** Structure of cholesterol

### 2.1.3 Role of Glycolipids

Glycolipids are a component in lipid membranes that consist of a carbohydrate
group attached to hydrocarbon chains. Glycosphingolipids are a type of glycolipid which contain a ceramide with one or more sugar residues [12]. Gangliosides are a complex subtype of glycosphingolipids that consist of a ceramide backbone with three or more sugars in which one is a sialic acid residue [13]. The large carbohydrate groups branch into the extracellular matrix and the lipid tails integrate into the bilayer [14]. Gangliosides have many different functions and interactions in cells. Gangliosides tend to exist primarily in cholesterol-rich domains called lipid rafts [15]. They function as receptors to several proteins including insulin [14] and bind bacterial toxins such as tetanus and cholera toxin [14]. Protein interactions with gangliosides help regulate cell signaling, control cell growth, and have a major impact in pathological consequences [14]. The ganglioside employed in this research is monosialotetrahexosylganglioside (GM1). The “G” in the name denotes that it is a ganglioside, the “M” (mono) indicates that there is one sialic acid residue in the carbohydrate branch, and the “1” relates to the elution order during separation [16]. GM1 contains four neutral sugar groups and one sialic acid residue (Figure 2.4). GM1 plays an important role in the physiology of the brain and acts as a binding site for cholera toxin and *E. coli* heat-labile enterotoxin.

**Figure 2.4:** Structure of monosialotetrahexosylganglioside (GM1) Adapted from Ref. 17.
2.1.4 Role of transmembrane proteins

There are many different types of proteins associated with biological membranes. For example, peripheral proteins are associated to a side of the bilayer and bind molecules (hormones neurotransmitters, toxins, etc) that are located in the extracellular environment. Some enzymes are considered peripheral proteins by the way they are associated with the membrane. Transmembrane proteins, also known as ion channels, are proteins that are embedded inside the membrane and exist in virtually all cell membranes. Depending on the size, and amino acid sequence of the protein, it may protrude from the membrane on either one or both sides.

Transmembrane proteins permit the flux of ions across a membrane and therefore play a key role in inter- and intracellular communication [18]. There is interest in studying transmembrane proteins as drug targets due to their importance in human physiology [19]. Ion channels have a gating ability where they can exist in an open state or a non-active closed state. When an ion channel is in the open state the ion flux is $10^7$-$10^8$ charges per second, which gives rise to a current of approximately 2-20 pA [20]. Ion channels are highly selective of the ions that pass through the channel. This thesis focuses on the antibiotic ion channel, gramicidin A, and its interactions in a lipid membrane.

2.1.4.1 Gramicidin

Gramicidin is a transmembrane protein that was first isolated from *Bacillus brevis* [20]. It is a small polypeptide that is composed of 15 alternating L- and D- amino acids (Figure 2.5) [21]. All of the amino acid residues are hydrophobic and non-polar. There are three different types of gramicidin and they differ only in the 11th amino acid residue
from the N-terminus. This thesis studies the interactions of gramicidin A, which has tryptophan at the 11th position in the amino acid sequence. Gramicidin is an ion channel that permits the diffusion of monovalent cations through a phospholipid membrane [21]. It is one of the most common model systems for studying ion transport through antimicrobial peptide-based channels [20].

**Figure 2.5: Structure of Gramicidin A**

Gramicidin exists in two different secondary structures that are dependent on the environment. These two structures are known as a helical dimer (HD) and a double helix (DH) (Figure 2.6). Gramicidin exhibits a DH structure when it is in an organic solvent. This structure has been studied through the use of solution NMR [22-26] and x-ray crystallography [27-29]. The DH structure consists of hydrogen bonded β-strands that are intertwined with the tryptophan residues distributed along the bilayer axis. Gramicidin exhibits a HD structure when in a lipid environment and therefore is the structure of the ion channel in its native active form. The HD structure has been studied by solid state NMR [26] and solution NMR [25]. The HD structure consists of two monomeric β-helices joined end-to-end by the N-termini to form a dimer. Each monomeric unit is in both the inner and outer leaflets of the bilayer and the tryptophan groups are clustered toward the membrane interface. The closed and open states of the ion channel occur when the two monomers are separated and attached, respectively. Furthermore, in the open state a monomer from each lipid leaflet join together at the N-termini and are
stabilized by six intermolecular hydrogen bonds (Figure 2.7) [21]. The ion-conducting pathway in the center of the channel is formed from the carbonyl oxygens of the polypeptide backbones [21]. The β-helix in lipid membranes has 6.3 residues per turn and a central cavity of approximately 0.4 nm in diameter [14].

Figure 2.6: Gramicidin structure as a helical dimer (in lipid membrane) and as a double helix (in organic solvents). The hydrogen bonding pattern indicative of β-sheets is depicted on the left. Adapted from Ref. 13.

Figure 2.7: Open and closed state of the gramicidin ion channel. Adapted from Ref. 30.
The ability to modify gramicidin has made it a popular model ion channel to investigate specific structure-function relationships. Specific modifications, such as isotopic labeling, have advanced NMR studies to gain knowledge about the peptide backbone secondary structures and side chain conformations [26, 31]. Hybrid formation channels have been used with side-chain substituted sequences to investigate differences in structures of various gramicidin analogues [32]. Substitution of specific residues in the gramicidin sequence has allowed for the determination of the dipole moment and orientation of the tryptophan residues reported by Koeppe and coworkers. [33]. That study investigated the interactions between an aliphatic or phenyl side chain and an indole ring in lipid membranes. They synthesized gramicidin molecules that replaced the D-Leu$^{10}$ residue with D-Val, D-Ala, and D-Phe and the Trp$^9$ residue was ring labeled. It was shown that the D-Leu$^{10}$ residue is very important for stabilizing the optimal orientation of the Trp$^9$ residue which allows for optimal channel conductance and lifetime. Substitution and/or removal of the N-termini residues were also performed in order to investigate the importance of these residues and their interactions in the dimer channel formation [34]. The result of these investigations provided information about the function of specific structural components for gramicidin and can also be used to hypothesize about other transmembrane proteins.

The observation that proteins are embedded in the lipid membrane has raised questions about the effect of membrane properties on transmembrane proteins. The lipid-protein interactions of gramicidin have been discussed in terms of aggregation in the literature. There have been varying results surrounding the aggregation studies of gramicidin mostly due to differences in sensitivity of the experimental techniques. Some
studies differ in the mole ratio determined for the onset of aggregation and other studies differ in the agreement that aggregation occurs. The technique of fluorescence self-quenching of the tryptophan residues upon lateral aggregation was monitored [35]. The notion behind this technique is that the fluorescence intensity should increase linearly with increasing gramicidin concentration unless aggregation actually occurs. This study resulted in the conclusion that gramicidin aggregation was dependent on lipid chain length and hydrophobic mismatch [35]. The effect of lipid mole fraction on the aggregation of gramicidin was studied with various techniques including x-ray scattering [36] and deuterium NMR studies [37]. Various aggregation models were proposed using freeze-fracture electron microscopy [38] and atomic force microscopy [39]. However, a recent aggregation study of gramicidin was completed with the use of electrochemical scanning tunneling microscopy (STM) [40]. The STM images are far superior to the previous methods used and much better molecular resolution was obtained. These images show that the gramicidin molecules are surrounded by at least one layer of bound lipids that prevent direct peptide-peptide contact. The peptide-lipid interaction is stronger than the peptide-peptide interaction and therefore the gramicidin molecules do not aggregate.

There have been various electrochemical studies on gramicidin. Patch clamp techniques have been the most common methods used to study the electrochemical behaviour of the channel. Patch clamp experiments allow for the measurement of the ion flux occurring through individual ion channels. The specific electrical capacitance of a lipid membrane studied using the patch clamp technique is 0.9 µF cm⁻²; therefore this is a highly sensitive technique to detect changes in conductivity caused by ion channels [41]. Using the patch clamp technique the conductivity was found to be the highest for protons
and cesium ions followed by potassium, sodium, and lithium ions [42]. It is interesting to
determine the specific ions that are selected by transmembrane proteins, as well as the
efficiency of the transport across the membrane.

Another technique to study the electrochemical behavior of gramicidin in lipid
environments is the hanging mercury electrodes. These experiments have been conducted
to study the electrochemical properties of gramicidin with a phospholipid-coated mercury
electrode. The membrane formed on the hanging mercury electrode has similar properties
as biological membranes such as the intrinsic pKa and surface potential of the film. The
kinetics of ion transport through gramicidin has been studied using this configuration [46,
44]. A study by Mauzeroll and coworkers investigated the transport of Tl\(^+\) ions across
phospholipid monolayers with and without gramicidin incorporated [44]. A rate constant
of \(2.8 \times 10^{-4}\) cm/s was measured for the transport of the Tl\(^+\) ions through the gramicidin
embedded in a phospholipid monolayer that was supported on a Tl amalgam hanging
mercury drop electrode.

Gramicidin has been a good candidate for studying transmembrane protein
properties. Although the structure and conformation is unique, the information gained
regarding lipid-protein interactions and the function of structural components can be
applied to other transmembrane proteins. This is very important due to the difficulty of
obtaining information on the native structures of membrane proteins.

2.2 Model Bilayer Lipid Membranes

Biological cell membranes are of great interest; however the inherent complexity
of the natural membrane convolves an accurate understanding of the effect of the
individual components in the membrane. Models of cell membranes need to be used to
simplify the system in order to study a single component in a membrane, such as a protein. Although the models need to be simplified, they also need to resemble the properties of a biological membrane.

The first successful model membrane was a bilayer lipid membrane designed by Mueller and coworkers [45]. This bilayer was deposited over a small hole (~1 mm in diameter) separating two aqueous media [45]. Another common model membrane is the lipid vesicles. Lipid vesicles are spherical lipid bilayers that enclose an aqueous medium [46]. These model membranes are small, fragile, and very sensitive to vibrations and shocks and therefore are not easily integrated with surface sensitive techniques [46].

Non-covalently bonded, model membranes supported on a substrate have been developed. The solid supported bilayer lipid membrane is the most common platform for a model biomimetic membrane and one of the first to be studied. Generally, this model membrane consists of lipids directly deposited onto a metal surface. One of the earliest model membranes was supported on a hanging mercury drop electrode. A non-covalently bound lipid monolayer supported on a hanging mercury drop electrode was introduced by Miller [47] and developed further by Nelson and Benton [48] where the behaviour of the lipids and peripheral proteins can be easily investigated [46]. The advantage of solid supported bilayer lipid membranes is their long term stability which allows for surface analytical techniques to be easily employed. Electrochemical techniques are also easily integrated with this model membrane if the membrane is deposited directly onto a metal or semiconductor. The disadvantage of using a solid supported bilayer lipid membrane is that there is a loss of fluidity because there is an absence of a well-defined water reservoir between the solid substrate and the membrane. The direct contact of the membrane and
solid substrate also creates issues for implementing transmembrane proteins.

There has been interest in developing solid supported model membranes that are covalently bound to metal surfaces. This model is created by a covalent linkage between a self-assembled molecule and the solid support [46]. The most common and best characterized self-assembled monolayers (SAM) are comprised of alkanethiols on a gold surface [49-51]. Alkanethiols form a covalent bond with the gold surface through the thiol group, leaving the hydrocarbon tails directed towards the solution. A lipid monolayer can be added to the SAM by a few different techniques including vesicle fusion. These bilayers are packed very tightly and are not very fluid making it difficult for the transport of biological molecules across the membrane [52]. There is also a lack of a water reservoir between the membrane and the metal surface making it difficult to introduce a transmembrane protein into the bilayer without risking protein denaturation.

To overcome some of the drawbacks mentioned with solid supported bilayers, tethered bilayer lipid membranes have been developed. These bilayers use ‘spacer’ molecules to increase the hydrophilic section between the metal and the bilayer. These ‘spacer’ molecules are termed ‘thiolipids’ and are phospholipid molecules with a covalently linked polyethyleneoxy chain terminated with a thiol group [46]. There is chemisorption of the thiol group to the gold surface where the lipid tails are pointed towards the aqueous solution. The second layer of the bilayer is formed upon lipid vesicle rupture. Various ‘spacer’ molecules have been used at varying lengths and there have been slight differences in packing as well as capacitance based on the selected ‘spacer’ molecule selected [53, 54]. There have been mixtures of ‘spacer’ molecules used such as short hydrophilic spacers attached to the gold through thiolate or dithiolate linkages and a
longer hydrophilic spacer that is covalently attached to a hydrophobic molecule such as cholesterol [55] or phytanol groups [56]. The mixture of spacer molecules creates a water layer between the short and long spacer molecules. There are also polymer tethers that are used to create a polymer cushion between the solid substrate and the bilayer. There are various ‘spacer’ molecules that have been used for this application such as oligo(ethyleneoxide), poly(ethyleneoxide), and oligopeptides with thiol groups [57-59]. However, all of spacer molecules described above tether the model membrane to a solid support and therefore restrict some mobility of the bilayer.

In order to further increase bilayer fluidity, floating bilayer lipid membranes have been utilized in the formation of biomimetic membranes. This model allows for a ‘free’ floating bilayer that is not tethered to the surface of the solid substrate. This permits a fluid bilayer to be present on a water rich cushion and allows for transmembrane proteins to be easily integrated. One approach to obtain a ‘floating’ lipid membrane is to apply an additional bilayer on top of a bilayer that is directly deposited on a solid substrate [60]. Another form of ‘floating’ bilayers is to use large spacer molecules that are not covalently attached to the solid substrate. These spacer molecules can be hydrophilic polymers or contain large oligosaccharide groups [61]. This thesis utilized the ganglioside, GM1, as a spacer molecule that is integrated into the bilayer with the large carbohydrate groups creating a water reservoir between the solid substrate and the bilayer.

2.3 Atomic Force Microscopy Studies of Biomembranes

Atomic Force Microscopy (AFM) can be performed on biological samples that
are on a solid support in an aqueous medium under physiological conditions. AFM has been utilized since the early 1980s and the method has continued to be improved to achieve better images [62]. There have been developments in sample preparation such as different immobilization strategies of biological molecules onto various solid substrates [63, 64]. These developments have allowed for subnanometre resolution [65]. There have been many examples of membrane proteins that were packed into two – dimensional crystalline arrays that have been imaged successfully with subnanometre-scale lateral resolution. These images can be compared to cryo-transmission electron microscopy and x-ray diffraction data with good correlation [65]. Successfully imaged proteins include the porin OmpF [66], bacteriorhodopsin [67], hexagonally packed intermediate [68], ATP synthase [69], Aquaporin 1 [70], Aquaporin Z [71], and Aquaporin 0 [72]. The AFM images of these proteins correlated well with x-ray crystallography data and have the added advantage of being obtained in a more natural environment.

AFM has the ability to monitor the dynamic changes of proteins that are induced by environmental conditions. An investigation of OmpF porins visualized conformational changes that were induced by voltage, pH, and potassium ion concentration gradients [66]. These conformation changes observed with AFM were conformational changes that accompany the closure of the ion channel [66]. Protein diffusion has been tracked with AFM imaging by scanning the same spot on the sample and tracking the diffusion of the protein [73]. Proteins that have been incorporated in bilayer lipid membranes have been investigated with AFM. Biomolecules such as DNA [74], cytochrome c [75], gramicidin [76], and other small peptides [77] have been anchored or incorporated into the lipid membranes and the morphology investigated using AFM. There have also been studies
that use lipid films to crystallize proteins to allow for high-resolution imaging [78]. It is advantageous to study the combination of lipids with proteins and investigate the morphology of the two components in a biologically relevant environment rather than imaging each component individually.

Lipid membranes with various components have been investigated with AFM. Many of these samples are prepared with Langmuir-Blodgett deposition and/or vesicle fusion. These membranes can be made of numerous types of components and therefore a great deal of morphological information can be obtained for various systems. AFM can be used to investigate possible defects in the membrane and the phase separation of lipids [65]. Furthermore, molecular resolution has been possible when imaging bilayers. A study investigating dimyristoylphosphatidylethanolamine bilayers was able to visualize individual head groups that were spaced 0.5 nm apart from one another [79]. Bilayer properties have also been investigated as a function of different Langmuir-Blodgett transfer surface pressures [80] and temperature [81, 82]. There are many parameters that can be investigated for various types of lipid membranes with AFM.

2.4 Infrared Spectroscopy of Biomembranes

Vibrational spectroscopy has played a significant role in the investigation of many interactions that regulate biomembranes. It can be used to investigate lipid-lipid interactions as well as the interactions that arise with the incorporation of proteins. The measurements obtained through vibrational spectroscopy increase our knowledge on the structural architecture of biological membranes as well as the specific function of components in the membrane. A major advantage of vibrational spectroscopy is that the
molecules do not have to be labeled or tagged and can be investigated in many different environments. The vibrational spectroscopy implemented in this thesis is infrared (IR) spectroscopy.

Information about the interactions of lipids and proteins is obtained by monitoring the position and shape of the peaks in the vibrational spectrum. The common vibrational modes that are monitored when investigating biological systems are listed in Table 2.1. Information regarding the structure, conformation, orientation, and phases of the components in the bilayer is attainable from the analysis of the vibrational spectra.

The advance of IR spectroscopy has led to the development of many techniques that can be used to analyze biological systems. Attenuated total reflection (ATR) has been used to study integral membrane proteins in polyethylene glycol-supported bilayers [83]. IR reflection adsorption spectroscopy (IRRAS) has been used to characterize planar-supported model membranes [84]. Polarization Modulation IR reflection adsorption spectroscopy (PM-IRRAS) was used to analyze fusion proteins in various model membranes [85]. Those are just a few examples of the many techniques that can be applied to the spectroscopic investigation of biological samples. IR techniques were applied to two main biological components in this thesis, lipids and proteins, and are discussed in more detail below.
Table 2.1: Characteristic IR bands of lipids and proteins in membranes [86-88]

<table>
<thead>
<tr>
<th>Functional group mode</th>
<th>Approximate Peak Center (cm(^{-1}))</th>
<th>Location/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CH(_3) asymmetric stretch</td>
<td>2950-2975</td>
<td></td>
</tr>
<tr>
<td>-CH(_3) symmetric stretch</td>
<td>2865-2885</td>
<td></td>
</tr>
<tr>
<td>-CH(_2) asymmetric stretch</td>
<td>2915-2940 (strong)</td>
<td>Acyl Chains</td>
</tr>
<tr>
<td>-CH(_2) symmetric stretch</td>
<td>2840-2870 (strong)</td>
<td></td>
</tr>
<tr>
<td>C-H asymmetric deformation</td>
<td>1440-1460</td>
<td></td>
</tr>
<tr>
<td>C-H symmetric deformation</td>
<td>1370-1390</td>
<td></td>
</tr>
<tr>
<td>-CH(_2) scissor vibration</td>
<td>1460-1480</td>
<td>Interfacial glycerol group</td>
</tr>
<tr>
<td>-CH(_2) rocking vibration</td>
<td>720-740 (weak)</td>
<td></td>
</tr>
<tr>
<td>-CH(_2) wagging vibration</td>
<td>1340-1370 (weak)</td>
<td></td>
</tr>
<tr>
<td>C=O stretch</td>
<td>1738-1742, 1724-1729</td>
<td>Interfacial glycerol group</td>
</tr>
<tr>
<td>C-O-C asymmetric group vibration</td>
<td>1160-1180</td>
<td></td>
</tr>
<tr>
<td>-PO(_2) asymmetric stretching</td>
<td>1220-1260 (strong)</td>
<td></td>
</tr>
<tr>
<td>-PO(_2) symmetric stretching</td>
<td>1085-1110 (medium)</td>
<td></td>
</tr>
<tr>
<td>C-OP</td>
<td>1040-1090</td>
<td>Head Group</td>
</tr>
<tr>
<td>CN(^+)(CH(_3))(_3) asymmetric stretching</td>
<td>950-970</td>
<td></td>
</tr>
<tr>
<td>CN(^+)(CH(_3))(_3) symmetric stretching</td>
<td>860-930</td>
<td></td>
</tr>
<tr>
<td>CN(^+)(CH(_3))(_3) bending mode</td>
<td>1470-1490</td>
<td></td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amide A</td>
<td>3300</td>
<td>N-H stretch</td>
</tr>
<tr>
<td>Amide B</td>
<td>3100</td>
<td>N-H stretch</td>
</tr>
<tr>
<td>Amide I</td>
<td>1600-1690</td>
<td>C=O stretch (minor: C-N stretch, N-H bending)</td>
</tr>
<tr>
<td>Amide II</td>
<td>1480-1580</td>
<td>N-H bending (minor: C-N stretching)</td>
</tr>
<tr>
<td>Amide III</td>
<td>1230-1300</td>
<td>C-N stretching, N-H bending, C=O stretch, O-C-N bending</td>
</tr>
<tr>
<td>Amide IV</td>
<td>625-800</td>
<td>O-C-N bending</td>
</tr>
<tr>
<td>Amide V</td>
<td>640-800</td>
<td>Out-of-plane N-H bending</td>
</tr>
<tr>
<td>Amide VI</td>
<td>530-610</td>
<td>Out-of-plane C=O bending</td>
</tr>
<tr>
<td>Amide VII</td>
<td>200</td>
<td>Skeletal torsion</td>
</tr>
</tbody>
</table>
2.4.1 Lipids

The vibrational spectra of a biomembrane can provide structural and dynamic information about the lipids in the system. The acyl chain and polar head group vibrational modes provide a wealth of useful information.

The acyl chain vibrational modes consist of C-H stretching as well as methylene rocking, wagging and scissoring. The most intense bands in the vibrational spectra containing lipids are the methylene stretching vibrations. These stretching bands can be useful when determining the lipid-phase transition of the sample under investigation [88].

The gel to liquid crystalline phase transition of phospholipids has been investigated where the stretching band position and bandwidth have been monitored [89-91]. When the lipids transition from a gel to a liquid crystalline phase, the lipid tails become more ordered. The increase in order corresponds to a decrease in tail mobility. The decrease in mobility is observed in the vibrational spectra as narrowed IR bands and a decrease in peak frequency by 1.5-2.5 cm⁻¹[89-91].

The vibrational bands that arise from the polar head groups of the lipids are phosphate (PO₄⁻) stretching vibrations. The strongest bands are the asymmetric stretching mode followed by medium intensity bands from the symmetric stretching mode. The peaks obtained from the phosphate region give information about the hydration of the polar head groups. The peak position can increase by about 20 cm⁻¹ from a hydrated to dehydrated bilayer [92].

There have been many IR spectroscopic investigations of model membranes that have provided more information about lipids. There was a study that investigated phospholipid membranes that were formed from vesicle fusion [93, 94]. The vibrational
modes from the phospholipids were analyzed and it was noted that head group hydration, the lipid chain organization and the lipid chain conformation were different between fused and unfused vesicles composed of DMPC [93]. A two component lipid vesicle containing cholesterol and DMPC was analyzed and compared to the pure DMPC vesicles [94]. It was found that the hydrocarbon tail tilt angle was less than the vesicles with no cholesterol, leading to the conclusion that the cholesterol increased the thickness and fluidity of the membrane [94]. There was also an investigation of phospholipid membranes prepared with Langmuir-Blodgett/Langmuir-Schaefer techniques [95]. This study used the advantage of building asymmetric bilayers by using deuterated lipids in one leaflet and hydrogenated lipids in the other leaflet [95]. This analysis found differences in tilt angles between the two leaflets of the lipids [95]. There is an abundance of literature on the spectroscopic investigations of lipids in various model membranes.

2.4.2 Proteins

Vibrational spectra can also be used to identify the secondary structure of a protein. In proteins, primary structure is the sequence of amino acids that form the peptide backbone, and the secondary structure is the conformational arrangement of the amino acid sequence. Secondary structure conformations include the α-helix, β-sheet, random coil, etc. A protein can be composed of many different types of secondary structures which form the overall shape of the protein known as the tertiary structure. The most useful vibrational mode in the secondary structure analysis is the amide I band. The amide I band is mostly due to the carbonyl stretching of the peptide backbone as well as the in-plane N-H bending and C-N stretching modes [88]. The band position depends on
the extent of hydrogen bonding with the carbonyl group as well as the coupling between neighboring transition dipoles [88]. If there is more than one secondary structure in the protein, the amide I band is the convolution of all the different secondary structures and therefore can be very intense and broad. Fourier self deconvolution (FSD) and two dimensional correlation spectroscopy (2D-COS) can be used to deconvolute the overlapped bands of the amide I. The deconvolution can be difficult if the approximate structure of the protein is unknown. Another disadvantage that occurs when using the amide I peak for structured analysis is the overlapping water bending vibration that occurs around 1640 cm\(^{-1}\); however, this can be avoided with the use of deuterated solution. When deuterated water is used, there is a minor shift to lower frequencies of the amide I band by less than 3 cm\(^{-1}\).

An investigation of the tertiary structure of a protein can be completed by analyzing the amide II band. The amide II band is comprised of mostly N-H bending and some C-N stretching modes [88]. The amide II band is very sensitive to deuteration, therefore if deuterated solution is used the peak will decrease by about 90 cm\(^{-1}\) [96]. It can be interesting to monitor the hydrogen-deuterium exchange to monitor protein folding. If there is a part of the protein that is inaccessible to solution, such as helices or sheets, then there will be very little hydrogen-deuterium exchange and the peak position will remain consistent with N-H bending [88].

There have been many spectroscopic investigations of gramicidin, the protein of interest in this thesis, in the literature. The first IR measurements of gramicidin were completed using dry films and dry solid samples [97]. Normal mode calculations of the different gramicidin structures (double helix/ helical dimer) [98] have been used for
comparison in more advanced IR spectroscopic investigations. There have been IR investigations of gramicidin in model membranes on metal electrodes [99]. This investigation monitored the amide I peak position as a function of applied potential in a PM-IRRAS setup [99]. The gramicidin was incorporated in a solid supported model membrane and was therefore in direct contact with the metal surface. When a more negative potential (-400 mV to -800 mV) was applied there was increased hydration around the phospholipid head groups and the tilt angle of the gramicidin molecule decreased to ~12 ° from an average tilt angle of ~23 ° at more positive potentials (-300 mV to +400 mV) [99]. Hydration of the membrane and the calculated tilt angles from the analysis of the vibrational spectra provide insight into the interactions of gramicidin with other components in the bilayer.
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Chapter 3: Theory and Techniques

3.1 Introduction

A number of techniques have been developed to construct a variety of biomimetic membrane systems. The approach used in this thesis to construct the desired biomimetic membranes relied on a combination of the Langmuir-Blodgett and Langmuir-Schaefer depositions. These techniques have been proven to be very successful in designing symmetric and asymmetric membranes for studying protein incorporation and as a result this chapter will discuss the theories behind these techniques in further detail.

Investigation of the physical properties and behaviors of biomimetic membranes can be achieved through the use of numerous biophysical methods. This thesis focuses on the application of atomic force microscopy (AFM) and polarization modulation infrared reflection spectroscopy (PM-IRRAS) as surface analytical tools to study the structural properties of the model membrane and interactions between the different phospholipids and peptides located within the membrane. This chapter discusses the theories behind both AFM and PM-IRRAS to provide a better understanding of the information obtained from these two complementary techniques.

3.2 Langmuir Films

3.2.1 Principles and Components of the Langmuir-Blodgett Technique

At the air-water interface, insoluble molecules will spread to form a thin film on the surface known as a Langmuir film. The molecules will spread and occupy all available sites and minimize their free energy on the surface by reorienting into a
monolayer in both gaseous and liquid states. The minimization of the Gibbs free energy per surface area \( (G) \) of the molecules on the subphase is correlated with the surface tension measured in units \( \text{mN m}^{-1} \) \( (\gamma) \) and surface area \( (S) \) by the differential \([1]:\)

\[
\gamma = \left( \frac{\partial G}{\partial S} \right)_{T,P,n}
\]

where temperature \( (T) \), pressure \( (P) \) and composition \( (n) \) remain constant.

In Langmuir-Blodgett techniques, the surface pressure \( (\Pi) \) is measured in terms of a difference in the surface tensions \([1]:\)

\[
\Pi = \gamma_0 - \gamma = -\Delta \gamma
\]

where \( \gamma_0 \) is the surface tension of the pure liquid and \( \gamma \) is the surface tension of the surface covered by a monolayer. The surface pressure is a necessary component to monitor when creating compression profiles of molecules and building biomimetic membranes.

The typical apparatus used for Langmuir-Blodgett techniques is based on the design that was originally introduced by Irving Langmuir and Katharine Blodgett in 1935 \([2-4]\). It consists of moveable barriers placed on top of a Teflon trough and a Wilhelmy plate attached to a balance (Figure 3.1). The trough is filled with the desired subphase (water, acidic solution, basic solution, etc.) and the surface pressure of the subphase is recorded. The molecules of interest are then carefully deposited on top of the subphase using a syringe and the moveable barriers are used to adjust the density of the molecules at the interface to obtain the desired surface pressure.
The Wilhelmy plate, which is attached to a highly sensitive microbalance, is submersed into the subphase and accurately measures changes in the surface pressure. The Wilhelmy plate can be made from different substrates such as platinum, glass, and filter paper. It has been noted in the literature that hysteresis is observed when using platinum and glass Wilhelmy plates [6]. The hysteresis is caused by the change in contact angle at the surface due to the adsorption of molecules to the Pt and glass substrates [6]. When filter paper was used as the Wilhelmy plate the contact angle remained constant [6], therefore filter paper was used for the Wilhelmy plate in this thesis. The forces acting on the plate are gravity and surface tension in the downward direction as well as
buoyancy in the upwards direction [7]. The weight of the meniscus ($w$) is described by [8]:

$$w = P \gamma \cos \theta$$

(3.3)

where $P$ is the perimeter of the Wilhelmy plate and $\theta$ is the static contact angle (Figure 3.2). After the molecules are deposited at the interface, movements in the barriers lead to a change in overall surface area, which causes the external forces acting on the plate to change. The magnitude of this change in force is directly measured by the Wilhelmy balance and converted to surface pressure. This is the most common method for measuring the surface pressure in Langmuir-Blodgett techniques.

![Figure 3.2: A meniscus around a Wilhelmy plate where $\theta$ is the static contact angle.](image)

3.2.2. Compression Isotherms

Langmuir-Blodgett techniques can be used to obtain compression isotherms for pure and mixed molecule solutions. Compression isotherms provide useful information about phase transitions of molecular films. The phase transitions of the monolayer depend on various conditions such as temperature, molecular structure, packing density, and surface pressure. The molecules within a Langmuir film can transition from a
gaseous, to liquid to solid phase when the film at the interface is compressed. At low molecular packing densities, the molecules are very distant from one another and can be considered as a gaseous phase. When the packing density of the molecules become greater, the molecules are closer to each other and the monolayer can exist in either a liquid expanded, liquid condensed state or solid condensed state depending on the surface pressure. The solid condensed state has the highest degree of molecular packing and achieves the highest recorded surface pressure. Further compression leads to film collapse where the molecules begin to spill out of the monolayer film forming a variety of aggregate film structures. The compression isotherm reveals these various phase transitions present in the system and provides valuable information for the development of biomimetic membranes.

A compression profile of the film is obtained by slowly compressing the moveable barriers at a constant rate while monitoring the changes in surface pressure with the Wilhelmy balance. The phase transitions that occur throughout the compression can be observed by plotting the surface pressure as a function of molecular area. A compression isotherm depicting the phase transitions of a typical amphipathic lipid spread at the air-water interface is shown in Fig. 3.3. Initially, the lipids are present in a gaseous state and begin to pack with their hydrophobic tails in a more ordered and upright position as the film is compressed. A negative slope in the compression isotherm is indicative of a phase transition and a plateau in the plot is indicative of two phase transitions that co-exist (ex. liquid-expanded and gaseous). A compression isotherm can display a wide arrangement of plateaus and slopes, which are unique to the specific molecules under investigation and their external environment.
3.2.3 Langmuir Film Depositions onto Solid Substrates

Langmuir film depositions were developed in the interest of transferring monolayers at the air-water interface to solid substrates. These depositions occur at a constant pressure that is determined empirically with the aid of compression isotherms. As previously discussed, phase transitions will occur at specific surface pressures therefore the deposition surface pressure is selected based on the desired phase and structure of the transferred film. The ideal transfer pressure for building biomimetic membranes must be sufficient to ensure that the molecules within the monolayer are in a ‘solid condensed’ state. To successfully deposit a monolayer to a solid substrate, the substrate must first be submerged below the subphase surface and oriented perpendicularly to the interface as shown in Figure 3.4. The molecules of interest are then dispersed onto the subphase surface where they spread out to form a gaseous monolayer.
The barriers on the Langmuir trough are adjusted so that the molecules will stabilize at the selected pressure. Once stabilization of the film is achieved, the solid substrate is slowly withdrawn vertically through the air-water interface transferring the monolayer onto the substrate surface (Figure 3.4). When a hydrophilic solid substrate is chosen, the deposited amphiphatic lipids will adopt an orientation where the polar head groups are in contact with the surface and the hydrophobic tails are directed towards the air. This technique is referred to as a Langmuir-Blodgett (LB) deposition and is responsible for creating the inner leaflet of a biomimetic membrane.

![Figure 3.4: Amphiphatic lipid monolayer transfer from the air-water interface to a hydrophilic solid substrate as the solid substrate moves upwards through the subphase and the barriers move towards one another [Adapted from Ref. 9]](image)

The Langmuir-Schaefer (LS) technique is used to deposit the second bilayer leaflet on top of the previously deposited monolayer. First, the molecules are deposited on to the surface of the subphase and compressed to the specific surface pressure to obtain the desired packing density. Once a stable film is formed at the selected surface pressure, the horizontally oriented solid substrate is lowered towards the molecules at the air-water interface (Figure 3.5). The solid substrate is ‘touched’ softly and quickly to allow the hydrophobic tails of the previously deposited monolayer to interact with those
spread along the subphase surface. The substrate is then withdrawn from the air-subphase interface depositing the outer leaflet of the biomimetic bilayer.

Figure 3.5: Langmuir-Schaefer technique for the formation of a bilayer on a solid substrate [Adapted from Ref. 9]

The combination of Langmuir-Blodgett and Langmuir-Schaefer techniques give additional control over the final membrane structure by providing a means of creating multilayer structures with varying molecular compositions and packing densities at each individual layer. Additionally, the Langmuir-Blodgett/Langmuir Schaefer technique is one of the best techniques for accurately controlling the monolayer thickness and ensuring homogenous distribution of the monolayer on a variety of substrates. It is the leading technique for the popular development of mimetic membranes.

3.3 Atomic Force Microscopy

Atomic Force Microscopy (AFM) was invented by G. Binning, C.F. Quate, and C. Gerber in 1986 [10] to investigate nanoscale structures, dynamics, and forces at interfaces. This technique is used to gain information about a variety of different samples including polymers, biological samples, and both insulating and conducting samples. AFM scans a sharp tip across the surface of a sample and measure the forces interacting between the tip and sample to construct a topographic image of the sample surface.
3.3.1 Components of the Instrument

The principles behind AFM imaging shares many similarities to that of a stylus profiler, however, numerous refinements have been made in order to achieve nanoscale resolution. The key elements used to attain this remarkable resolution are precise tip-sample positioning, a sensitive deflection sensor, and experiment specific cantilevers with ultra-sharp tips [11]. The basic instrumentation for AFM (Figure 3.6) is arranged in a manner such that a laser is reflected off the top of the cantilever towards the center of a position sensitive, four-quadrant photodiode. The following section will explore the major components of a typical AFM setup, specifically the cantilever, piezoelectric scanner, and the photodiode, in further detail.

Figure 3.6: Basic schematic of the instrumentation used for AFM

3.3.1.1 Cantilever

There are many commercially available cantilevers that can suit the needs of most experiments. These cantilevers differ in size, materials, and stiffness. The stiffness of a
cantilever is defined by its spring constant. Hooke’s Law defines the spring constant of a cantilever \((k)\) as the force required to bend the cantilever \((F)\) per unit of distance \((x)\):

\[
F = -kx
\]  

(3.4)

The spring constant of the cantilever depends on both the material and dimensions of the cantilever. Therefore, the spring constant, \(k\) (N/m), of a cantilever can be defined by the following expression:

\[
k = \frac{Et^3w}{4l^3}
\]  

(3.5)

where \(E\) is the Young’s modulus (N m\(^{-2}\)) of the cantilever material, and \(t\), \(w\), and \(l\) are the thickness, width, and length of the cantilever in meters, respectively [12].

Cantilevers with low spring constants are ideal for contact mode imaging, which will be discussed later in this chapter. In order to obtain an AFM image with atomic resolution, the spring constant of the cantilever must be lower than the bonding force constant that keeps the atoms confined together on the surface [12]. For the stiffness of the cantilever to be less than the bonding force constant (about 1 N m\(^{-1}\)), the cantilever must have dimensions on the micron scale [12]. For example, a typical silicon nitride cantilever \((E=1.5x10^{11}\text{ N m}^{-2})\) with dimensions of \(0.6 \times 40 \times 205 \mu\text{m}\) has a spring constant of 0.03 N m\(^{-1}\).

Low spring constant cantilevers may be optimal for imaging samples in contact mode; however, these soft cantilevers are not ideal for dynamic imaging modes because they often have low resonant frequencies. In order to obtain a high resonant frequency, the cantilever length must be reduced to increase the stiffness of the cantilever. In addition, imaging in solution also requires a high resonant frequency due to the change in
the mechanical response of the cantilever. The viscosity of a liquid is much higher than air, which reduces the resonant frequency due to the additional mass that needs to be moved [12]. In Eq. 3.5, it is clear that the spring constant is described in terms of the geometrical dimensions and material properties, therefore these parameters can be adjusted to attain the desired resonant frequency.

The spring constant of a cantilever can be determined by empirical methods. The thermal tune method is most commonly used to calculate the spring constant of an individual cantilever. This method describes the cantilever as a harmonic oscillator through the use of the equipartition theorem from fundamental thermodynamic theory [13]. This theorem relates the kinetic energy stored in the system, at the position that the cantilever has displaced, to half the thermal energy of the system [14]. As previously mentioned, the displacement of the cantilever \( x \) is linearly related to the force required to deflect the cantilever \( F \) according to Hooke’s Law where the cantilever is treated as an ideal spring. In theory, the displacement of the cantilever and the temperature can be measured and used to calculate the spring constant. However, in reality, the cantilever does not actually behave as an ideal spring and therefore the temperature, resonant frequency and deflection sensitivity are used to obtain a more accurate cantilever spring constant. A power density spectrum of the vibrational noise is measured to obtain the resonant frequency of the cantilever. The deflection sensitivity of the cantilever can be determined by taking the reciprocal of the slope on a Deflection vs Distance plot. This plot is obtained by pushing the cantilever tip on a hard flat surface and measuring the deflection response on the photodiode. Software provided by Agilent is used to calculate the spring constant with the experimental temperature, resonant frequency, and deflection
sensitivity.

Optimization of the buffer concentration is extremely important for imaging in solution. Electrostatic interactions play a central role in tip-surface interaction when imaging in liquid, in contrast to imaging in air where capillary forces are the most relevant. The charge of the cantilever in solution is screened by mobile ions that are in the surrounding electrolyte [12]. Ions with the same sign of charge are repelled from the surroundings of the tip, and cantilever and ions with opposite charge are electrostatically attracted to the cantilever [12]. The spatial distribution of the ions is a compromise between the two opposite tendencies of attraction and repulsion, and the screening charges establish an electric double layer at the surface of the cantilever and tip [12]. High resolution imaging is closely related to the distance between the tip and the sample [12]. In solution, high resolution depends on the arrangement and screening of charges and therefore there must be contact between the tip and sample to obtain high resolution in buffer solutions [12]. Direct contact between the tip and sample is problematic when imaging soft materials because of the presence of shear and lateral forces that could damage the sample. The development of cantilevers with low spring constants and high resonant frequencies has allowed the use of dynamic imaging modes [12]. To achieve high resolution images with dynamic mode the electrolyte concentration must be fine-tuned to minimize the electrostatic interactions between the tip and sample.

### 3.3.1.2 Piezoelectric Scanner

AFM scanners are capable of imaging a sample in one of two ways. One method uses a sample scanner where the cantilever and tip are kept stationary while moving the
sample in the x, y and z directions. The second method uses a tip scanner where the entire cantilever is raster scanned across the sample in the x and y direction and moves the tip in the z-direction depending on the topography of the surface. Since the work in this thesis was performed on a tip-scanning AFM, the properties of these scanners will be explored in more detail.

A piezoelectric tip scanner can be used in AFM to precisely position the tip of the cantilever with the sample. Piezoelectric materials are highly sensitive to small changes in the applied stress and will generate a potential difference at the opposing ends of the nonconductive crystal due to the redistribution of charges caused by the external stress [12]. The opposite is also possible and is referred to as the reverse piezoelectric effect where a change in the voltage across the two faces of the crystal causes the piezoelectric material to change its spatial dimensions [12]. Therefore, piezoelectric materials are ideal for creating AFM scanners since very small changes in the applied voltage will allow for precise movements on subnanometer length scales. The piezoelectric materials used in AFM scanners are generally ceramics made of lead zirconate titanates [15].

The scanner tube consists of inner and outer tubes. The outer tube is divided into four longitudinal segments of equal size and electrodes are welded to the internal and external faces [12]. A bias voltage is applied to the inner and outer electrodes to make the piezoelectric tube contract or extend [12]. To create the scan movement, a bias voltage is applied to one longitudinal segment and the opposite segment has a voltage applied with opposite sign [12]. Piezoelectric scanners can be purchased in different sizes that can scan a variety of ranges. There is a drawback to using the scanner at the maximum range
because the scanner moves on an arc plane rather than a straight line. This is not very noticeable when imaging a much smaller area than the maximum range although this effect can be corrected by subtracting a polynomial function to the entire scan [12]. Piezoelectric scanners also suffer from hysteresis in the forward and backward traces. Although these scanners are inherently non-linear and suffer a few drawbacks they are incredibly sensitive. The same piezoelectric scanner is used for imaging in air and in liquid. Some precautions must be made so that liquid does not affect the electronics of the scanner.

3.3.1.3 Photodiode and the Laser Deflection Method

The most common method to monitor the cantilever deflection is the laser deflection method. This method uses a laser, which is reflected off the backside of the cantilever and onto a quadrant photodiode where the light intensity is then converted to a voltage signal [12]. The photodiode is split into four quadrants, which allows for the differentiation of lateral and vertical movement of the cantilever [12]. The torsional movement of the cantilever is determined by the difference between the left and right quadrants of the photodiode [12]. The torsional movement of the cantilever is generated by frictional forces between the tip and sample [12]. The vertical movement of the cantilever is determined by the difference in the upper and lower quadrants of the photodiode [12]. The vertical movement of the cantilever is generated by the normal forces between the sample and tip [12]. For example, in Figure 3.6 the torsional movement would be calculated from $(A+C)-(B+D)$ and the vertical movement would be calculated from $(A+B)-(C+D)$. The small movement of the cantilever is amplified and
3.3.2 Forces

The tip detects changes in the force as it scans across the sample surface. These forces are either repulsive or attractive in nature; therefore, different imaging modes can be applied to the system in response to the specific forces that are present. The forces acting on the cantilever in air differ greatly from those present in a liquid environment. Understanding the nature of these forces and their influence on the cantilever is important for optimizing the resolution and quality of the AFM image as well as providing a physical meaning behind the observed structures. The following sections will examine the forces acting on the cantilever in both an air and liquid environment in more detail.

3.3.2.1 van der Waals forces

There are three different types of forces that give rise to the van der Waals force, which are referred to as the orientation force (dipole-dipole interactions), induction force (dipole-induced dipole interaction) and dispersion force (induced dipole-induced dipole). A dipole occurs in a molecule when the distribution of charge is not symmetrical and an induced dipole is when the charge of a given molecule is induced into a similar charge imbalance by a neighboring molecule [15]. The van der Waals interaction between the tip and sample can be approximated as a sphere approaching a flat infinite surface:

$$F_{vdW} = -\frac{A_H R}{6D^2} \quad (3.6)$$

where $A_H$ is the Hamker constant, $R$ is the radius of the tip, and $D$ is the distance between the tip and sample [16].
3.3.2.2 Electrostatic force

The electrostatic (or Coulombic) force is the force that exists between two oppositely charged ions [15]. The force between these two ions follows the Coulomb force law:

\[ F = \frac{1}{4\pi\varepsilon_0} \cdot \frac{q_1 q_2}{r^2} \] (3.7)

where \( q \) is the charges of the ions, \( r \) is the distance between the two ions, and \( \varepsilon_0 \) is the permittivity of free space [15]. The two oppositely charged ions are attracted towards each other until the separation between the two atoms becomes so close that the outer shell of electrons between each ion will interact. Once the electrons have interacted between the two ions the force becomes repulsive due to the Pauli Exclusion Principle [15]. The ions will begin to experience ‘core repulsion’ where the two ions cannot be pushed together any closer without a large input of energy [15].

3.3.2.3 Double Layer Forces

Electrostatic double layer forces are present when imaging in solution. The double layer is always present when the sample being imaged has an excess surface charge (ex. mica, glass, and charged biomolecules) in an electrolyte solution. A high concentration of oppositely charged ions are attracted towards the surface [15] and balanced by the ions remaining in the bulk of the solution. This creates a double layer where the electrostatic potential is the strongest when it is closest to the surface and decays exponentially away from the surface [15]. The electrostatic double layer forces arise when the two independent electrical double layers of the tip and sample overlap with one another [15]. The distance that the double layer force extends is known as the Debye length. The
surface potential at a given distance ($\psi_x$) is approximated through the Debye-Hückel approximation:

$$\psi_x \approx \psi_0 e^{-\kappa x}$$

(3.8)

where the potential at the surface is defined as $\psi_0$ and the Debye length is defined as $(1/\kappa)$. The Debye length depends on the ionic strength of the electrolyte solution used in the experiment [15]. If the ionic strength is high the electrostatic repulsion that the AFM tip experiences as it approaches the substrate is decreased. This makes it possible to electrically shield the AFM tip from large forces that are present while imaging in solution [15]. For optimized imaging various force-distance curves should be collected at varying ionic concentrations to select the optimized conditions.

### 3.3.2.4 Capillary and adhesive forces

There are capillary and adhesive forces that arise between the tip and the sample when imaging in air. These forces arise due to the inevitable condensation that can occur on the tip of the cantilever as well as a layer of condensation on the surface. The capillary force is the force that pulls the cantilever down towards the surface due to the strong meniscus force from the condensation [15]. This strong force has the ability to damage the surface of the sample and is therefore extremely problematic for imaging soft biological samples [15]. To overcome this issue, many experiments are performed in solution where the force of the meniscus is not present since the cantilever is completely submerged in the solution [15]. Adhesive forces mainly arise from damaged or contaminated tips [15]. The presence of adhesive forces can be monitored with the use of a force-distance curve where any asymmetry in the approach and retract implies the
presence of an adhesive force [15].

3.3.3.5 ‘Leonard Jones’ Function and Force-Distance curves

The Leonard Jones function can be used to model the behavior of the attractive and repulsive regimes of the force-distance relationships between the tip and sample. To model this behavior, the potential energy is monitored between the apex of the tip and a particle on the sample surface [15]. The potential energy is dependent on the distance between the tip and the sample and therefore the potential energy changes as the tip approaches the sample surface [15]. This is described in the Leonard-Jones function:

\[
E_{\text{pair}}(r) = 4\varepsilon \left[ \left( \frac{\sigma}{r} \right)^{12} - \left( \frac{\sigma}{r} \right)^{6} \right]
\]  

(3.9)

where \( E \) is the pair-potential energy between two atoms, \( r \) is the separation between the tip and the sample, and \( \varepsilon \) and \( \sigma \) are constants determined by the material used in the experiment [15]. In Figure 3.7, the pair-potential energy varies with separation distance. When the separation distance is small, there is a strong repulsion between the two atoms due to the Pauli Exclusion Principle [15]. This steep increase in potential energy is described in the Leonard-Jones function (Eq. 3.9) as the \( \left( \frac{1}{r} \right)^{12} \) term [15]. At increasingly larger separation distances, the pair-potential energy is slowly increasing due to van der Waals forces that dominate in the attractive environment [15]. The attractive behavior is described by the \( \left( \frac{1}{r} \right)^{6} \) term in the Leonard-Jones function (Eq. 3.9) [15].
Figure 3.7: Schematic depiction of the Leonard-Jones function

The deflection of a cantilever and its vertical position can be converted into a force-distance curve. The force is obtained using Hooke’s law, multiplying the spring constant of the cantilever \( k \) with the deflection of the cantilever \( Z_c \):

\[
F = kZ_c
\]  
(3.10)

The vertical position of the tip that is recorded with the piezo \( Z_p \) needs to be added to the displacement of the cantilever \( Z_c \) to calculate an overall separation distance \( D \) between the tip and sample [17].

\[
D = Z_p + Z_c
\]  
(3.11)

A typical force distance curve is shown in Figure 3.8 of a hard surface. There are no forces interacting between the tip and the sample when the cantilever is far from the surface. As the cantilever approaches the surface, van der Waals attractive forces and electrostatic interactions increase rapidly. At a specific tip-sample distance, there is a ‘jump to contact’ where the cantilever is in direct contact of the surface. In this position,
the cantilever is bent towards the surface and begins to bend in the opposite direction as the scanner continues to approach the surface. As the cantilever continues to push on the surface there is a steep, linear increase in repulsive forces due to the Pauli repulsion forces. When retracting the cantilever from the surface, the cantilever remains in contact with the sample at distances larger than the original jump to contact point due to the force of adhesion. The tip remains in contact with the sample until the force of the cantilever spring is sufficiently large to overcome the total magnitude of the adhesion forces. Once the tip breaks free of the surface, it returns to a position where there are no further interactions between the tip and the sample.

**Figure 3.8:** Force-distance curve describing the tip-sample interactions on a hard sample

Force-distance curves that are completed on soft samples, such as a membrane, have a unique characteristic. This characteristic in the force-distance curve is indicative of penetration through the sample to the hard surface beneath. This penetration is displayed as a discontinuity in the force distance curve (Figure 3.9). The discontinuity in
the slope of the force distance curve at the point of bilayer penetration can be used to measure the compressed thickness of bilayers. Numerous force distance curves must be completed at various positions on the sample to provide accurate statistics for determining the thickness of the bilayer. The initial indentation of the sample will cause an error in this penetration distance and therefore a correction must be made to take into account this elastic deformation. The elastic deformation (δ) caused by the AFM tip under a known load can be calculated based on the Hertzian model [18]:

\[
\delta = \left( \frac{9F^2}{16RE^*2} \right)^{1/3}
\]

where \( R \) is the radius of the tip curvature, \( E^* \) is the effective compression modulus, and \( F \) is the average load force. The average load force is determined from the statistical histogram of the penetration force measured on the bilayer. An assumption is made when applying the Hertzian model that the deformation is purely elastic in nature and the film structure does not change during the compression. Therefore, the film thickness is equal to the sum of the thickness of the compressed film and the elastic deformation.

Figure 3.9: Force-distance curve describing tip-sample interactions on a soft sample
3.3.3 Imaging Modes

An imaging mode is carefully selected based on the type of sample under investigation and the information that needs to be obtained. The imaging modes differ between the different force interactions used to monitor the tip-sample interaction [15]. Below is a description of two imaging modes used in this thesis.

3.3.3.1 Contact Mode

Contact mode is a common AFM imaging mode that involves direct contact between the tip and sample. The forces monitored using contact mode are typically repulsive in nature [15]. Contact mode requires a pre-set applied force that effectively maintains a constant deflection of the cantilever. The applied force can be adjusted to increase the image contrast, but there is a risk of damaging the sample with increasing force [15]. The major advantages of contact mode imaging are that the tips are relatively inexpensive and the instrumentation is fairly basic [15]. A disadvantage when using contact mode for biological samples and other soft samples is the potential of shear and lateral forces that may alter the original surface topography. Overall, contact mode is the most widely used method for imaging hard surfaces in air.

3.3.3.2 MAC Mode

Magnetic AC Mode (MAC) mode is a dynamic imaging mode that involves the oscillation of a cantilever near its fundamental resonance frequency. The cantilever is oscillated magnetically with the aid of a thin magnetic coating on the top of the cantilever. A solenoid is placed below the sample and an oscillating magnetic field is
used to drive the coated cantilever. The drive frequency is set slightly lower than the natural resonant frequency of the cantilever. When the oscillating cantilever is brought close to the sample surface, the tip oscillation will become dampened due to attractive interactions with the sample surface. The attractive forces make the cantilever ‘feel’ heavier and therefore the resonant frequency is slightly lower than when the cantilever is oscillating in free space, far from the sample [15]. By choosing a drive frequency slightly lower than the resonant frequency the cantilever will have slightly greater amplitude than it did in free space (Figure 3.10). The drive frequency and resonant frequency will be close together, therefore there will an increase in efficiency of the energy transfer. The amplitude continues to increase as the cantilever approaches the surface until the tip ‘taps’ the surface, at this point, the amplitude reduces back to a lower value. The amplitude reduces to a pre-set percentage of the free amplitude, the harder the tap the larger the reduction in amplitude [15]. This allows for control on how soft or hard the imaging is on the soft sample. The isolated oscillation of the cantilever is advantageous over other dynamic modes which create additional vibrations in the cantilever nose head that can create large disturbances when imaging in liquid.
3.4 Polarization Modulation Infrared Reflection Absorption Spectroscopy (PM-IRRAS)

3.4.1 Principles of PM-IRRAS

Infrared Reflection Absorption Spectroscopy (IRRAS) has proven to be a useful technique for analyzing ultrathin films deposited on a metallic substrate since the theoretical and experimental techniques of Greenler [19, 20]. This technique provides the advantage of an increased signal to noise due to the surface electric field enhancement and can be used to determine the orientation of different molecular groups based on the direction of their dipole moment [21]. One disadvantage of this technique arises when the substrate is a dielectric material since there is no surface field enhancement and the electric field vectors of the incident and reflected beams are not equal making it very difficult to determine the orientation of the molecules [21]. This is further complicated when completing an experiment at the air-water interface because of the strong
absorption from water molecules and surrounding water vapour [21]. It is possible to obtain a spectrum using IRRAS at the air-water interface, however, long acquisition times and accurate background measurements are necessary [21].

PM-IRRAS was developed in the 1990s and proves to be a beneficial method for collecting spectra at the air-water interface [21]. PM-IRRAS was used for the study of a Langmuir monolayer in 1993 and has since been a very popular tool for analysis of biosystems [22]. PM-IRRAS is a highly sensitive technique that can detect surface species on metals. This is possible with the combination of Fourier transform mid-IR reflection spectroscopy with fast modulation of the incident beam between s- and p-polarized light [21].

The photoelastic modulator (PEM) generates the alternating s- and p-polarized incident radiation from the incoming linearly polarized light. The circularly polarized IR beam from the optical bench travels through a static polarizer, where it becomes linearly polarized and is directed to the center of the PEM, which is mounted at a 45 ° angle. [23]. The PEM is made of a piezoelectric transducer that is attached to a ZnSe crystal that can compress or expand based on the applied voltage [23]. The crystal experiences birefringence that is proportional to the mechanical stress and can impede one of the linearly polarized light components (s- or p-) more than the other component [23]. The applied voltage is set such that each oscillation of the PEM changes the polarization of the incident beam by 90 °, which is the maximum relative phase shift [23]. The maximum relative phase shift is one half-wave retardation therefore, for one full cycle the half-wave retardation occurs twice [23]. When the crystal is completely expanded the incoming light is linearly polarized at an angle of +45 ° with respect to the incident beam and when
the crystal is completely compressed the incoming light is linearly polarized at an angle of -45° with respect to the incident beam.

Surface selection rules dictate that when p-polarized light is reflected off a metal surface at a grazing angle of incidence, the electric field component perpendicular to the substrate is enhanced due to constructive interference (Figure 3.11) [23]. When s-polarized light is reflected off of a metal surface at a grazing angle of incidence, the electric field component parallel to the substrate nearly vanishes due to destructive interference (Figure 3.11) [23]. Therefore, the reflectivity of the s-polarized light is insensitive to the adsorbed species on the metal and can be used for the background spectrum. The reflectivity of the p-polarized light is sensitive to the adsorbed species on the metal surface and therefore can be used to obtain the adsorbed film spectrum. It is advantageous to collect the background and adsorbed species spectra simultaneously because it is nearly impossible to reproduce the experimental conditions for individual s- and p-polarized spectra. The difference in the polarized reflectivities also eliminates the complications of absorptions due to atmospheric molecules, such as water and carbon dioxide, because these absorptions are effectively subtracted [23].

![Figure 3.11: Surface selection rules of p- and s-polarized light](image)

**Figure 3.11:** Surface selection rules of p- and s-polarized light
The difference and the average between the s- and p-reflectivities \( (R) \) are used to calculate a normalized measured reflection absorption spectrum. This reflection absorption spectrum is defined as:

\[
\frac{\Delta R}{\langle R \rangle} = \frac{|R_p - R_s|}{\left(\frac{R_p + R_s}{2}\right)}
\]  

(3.13)

PM-IRRAS can be applied to either a metal-solution interface or a metal-air interface with some additional considerations. Aqueous electrolytes are strong absorbers of IR radiation and therefore needs to be minimized [23]. This can be minimized through the use of a thin cell configuration where the metal surface is pressed against an IR transparent prism and separated by a layer of electrolyte that is only a few micrometers thick [23]. It is impossible to have complete cancellation of the signal from solvent molecules, [24] however with an optimization of the optical alignment, good cancellation of the p- and s- polarized photons for solvent molecules can be achieved [23]. This technique is useful for studying insoluble films and irreversibly adsorbed biological samples [23] and is not ideal for systems that contain analyte molecules in the bulk solution due to its surface selectivity [25].

3.4.2 Optimizing angle of incidence and thin cavity thickness

The optimal angle of incidence and thin cavity thickness are used to achieve the best possible signal-to-noise ratio for the IR spectra. These parameters are determined through the calculation of the mean-square electric field strength (MSEFS) of the incident IR photons where the MSEFS is proportional to the intensity of the IR band of adsorbed molecules [23]. In-house software written by Vlad Zamlynny [24, 26] is used to calculate the theoretical MSEFS for the system. The incident IR radiation passes through a three
phase system, which includes the transparent prism, electrolyte solution, and the adsorbed insoluble film before reflecting off the metal surface towards the IR detector (Figure 3.12). Each of these layered phases has pre-determined optical constants that are used to describe the optical properties of each individual layer [24]. The Fresnel equations are solved for this layered system using additional input parameters, such as the frequencies of IR photons, angle of incidence of the incoming radiation, and thickness of the thin layer of electrolyte in addition to the previously mentioned optical constants [23]. The solved Fresnel equations are then used to solve for the MSEFS [23]. This information about the MSEFS can be plotted as a function of gap thickness and angle of incidence (Figure 3.13). The optimal gap thickness and angle of incidence is different for each wavenumber region. Figure 3.13 shows an example of the calculated MSEFS for the 1600 cm$^{-1}$ region. The global maximum on the MSEFS plot is used to select the experimental gap thickness and angle of incidence. According to Figure 3.13, the optimal set-up would require a gap thickness of 3.6 µm and an angle of incidence of 66°. To achieve the desired MSEFS on the surface of the metal, the optimal angle of incidence is easily fixed in the experimental set-up and the gap thickness needs to be measured and carefully adjusted.

Figure 3.12: Layered medium used to model of the thin layer cavity of the PM-IRRAS spectroelectrochemical cell
Figure 3.13: Mean-square electric field strength (MSEFS) calculated for 1600 cm\(^{-1}\) at a gold surface with a solution of D\(_2\)O and a CaF\(_2\) prism for a p-polarized beam as a function of gap thickness and angle of incidence

3.4.3 PEM Response Functions

The PEM may cause artifacts in the spectra that are introduced at wavelengths other than the selected wavelength. At the selected wavelength the PEM generates alternating p- and s- polarized light and at all other wavelengths the polarization of the incident beam is circular, with both s- and p- components [23]. For a given frequency region of interest, the artifacts introduced by the PEM must be removed before quantitative analysis can be performed [23]. This procedure to obtain the PEM response functions is similar to the procedure employed by Buffeteau et al. [27].

The synchronous sampling demodulator (SSD) outputs two signals, the intensity average and the intensity difference. These two signals are described by the following
equations [28]:

\[ I_A(\omega) = \frac{I_s(\omega) + I_p(\omega)}{2} + \frac{I_p(\omega) - I_s(\omega)}{2}J_0(\varphi_0) = \langle I \rangle + \frac{\Delta I J_0(\varphi_0)}{2} \]  \hspace{1cm} (3.14)

and

\[ I_D(\omega) = |I_p(\omega) - I_s(\omega)|J_2(\varphi_0) = \Delta I J_2(\varphi_0) \]  \hspace{1cm} (3.15)

where \( I_A(\omega) \) is the intensity average, \( I_D(\omega) \) is the intensity difference, \( I_s \) and \( I_p \) are the s- and p- polarized light intensities, \( \varphi_0 \) is the maximum retardation of the incident radiation, and \( J_0(\omega) \) and \( J_2(\omega) \) zero-order and second order Bessel functions known as the PEM response functions. These PEM response functions are calculated by collecting two spectra of the IR transparent prism with a static polarizer in front of the PEM where the PEM is turned off in one spectrum and turned on for the second spectrum. The static polarizer is arranged so that the IR radiation is p-polarized. When the PEM is turned on, there are two outputs, average and difference intensities, \( (I_A(\omega))^{cal}, (I_D(\omega))^{cal} \) and when the PEM is turned off, there is only one output of an intensity of p-polarized light \( (I_p(\omega))^{cal} \) because the other output \( I_s(\omega))^{cal} \), is equal to zero. Therefore, Eq. 3.14 and 3.15 can be simplified:

\[ I_A(\omega)^{cal} = \frac{I_p(\omega)^{cal}}{2} + \frac{I_p(\omega)^{cal}}{2}J_0(\varphi_0) \]  \hspace{1cm} (3.16)

\[ I_D(\omega)^{cal} = I_p(\omega)^{cal}J_2(\varphi_0) \]  \hspace{1cm} (3.17)
Eq. 3.16 and Eq. 3.17 can be rearranged for the determination of the PEM response functions:

\[ J_0(\varphi_0) = 2 \frac{I_A(\omega)^{cal}}{I_p(\omega)^{cal}} - 1 \]  
\[ J_2(\varphi_0) = \frac{I_D(\omega)^{cal}}{I_p(\omega)^{cal}} \]  

The PEM is set for a selected wavelength and therefore the PEM response functions must be calculated for each selected wavelength.

### 3.4.4 Determination of Tilt Angles

To quantify the orientation of molecules in a membrane, the integrated intensities of the IR bands are determined and related to the angle between the transition dipole moment of a given vibration and the electric field of the incident radiation. This relationship is described [29, 30]:

\[ \int A \, dv \propto |\vec{\mu} \cdot \vec{E}|^2 = |\mu|^2 \langle E^2 \rangle \cos^2 \theta \]  

where \( A \) is the intensity of the absorption band, \( \mu \) is the transition dipole vector, \( E \) is the electric field, and \( \theta \) is the angle between the dipole moment and the electric field. A p-polarized photon has an electric field vector that is normal to the metal surface. Therefore, the angle between the direction of the transition dipole and the surface normal can be determined according to the following expression:

\[ \cos^2 \theta = \frac{1}{3} \frac{A(E)}{A_{(random)}} \]  

where \( A_{(random)} \) is the integrated intensity of an IR band in a simulated monolayer of randomly oriented molecules and \( A(E) \) is the experimental integrated intensity of an IR
band. The integrated intensity of an IR band in a monolayer of randomly oriented molecules is calculated from the optical constants under the same experimental conditions (i.e. gap thickness, angle of incidence, optical window material, and electrolyte). The optical constants are obtained from the literature for the IR transparent prism, electrolyte, and metal [31-34]. The optical constants of the membrane film are obtained by collecting a transmission spectrum of the analyte molecules in an aqueous electrolyte [23]. Once the angle is determined, it can be related to the direction of the transition dipole of the vibrational mode with respect to the coordinates of the adsorbed molecule on the metal surface [23]. The direction of the transition dipole of the vibrational mode is found in the literature or it is calculated using computational methods [23].

The average tilt angle of the acyl chains in lipid molecules can be calculated using the previously described method. The peaks found at the vibrational modes for symmetric and asymmetric methylene stretching can be used to calculate the tilt angle of the hydrocarbon chains in lipid molecules. The direction of the asymmetric and symmetric stretching vibration lies along the plane of the methylene group (Figure 3.14). The direction of the dipole moment for the symmetric stretching frequency is along the bisector of the methylene plane and the direction of the dipole moment for the asymmetric stretching lies along the line joining the two hydrogen atoms of the methylene group. The plane of the methylene group is perpendicular to the trans fragment of the hydrocarbon chain. To calculate the average tilt angle of the trans fragment the following equation can be used:

\[
\cos^2 \theta_{vas} + \cos^2 \theta_{vs} + \cos^2 \theta_{chain} = 1
\]  

(3.22)
Figure 3.14: Diagram depicting the transition dipole moments (red arrow) for symmetric and asymmetric methylene stretching vibrations as well as a schematic for the tilt angle of acyl chains.

The average tilt angle of a peptide/protein helix can be calculated based on the order parameters of the amide I band. The predominate vibration in the amide I band is due to C=O stretching in the amide group. The order parameter of the amide I band ($S_{C=O}$) can be calculated using this equation [35]:

$$S_{C=O} = \frac{1}{2} (3\cos^2\theta_{C=O} - 1) \quad (3.23)$$

The order parameter of the amide I band that was calculated can be used to calculate the order parameter of the helix ($S_{helix}$) with the below equation and a reference angle ($\alpha$) between the dipole moment and the helical axis obtained from literature [35-37]:

$$S_{helix} = \frac{2S_{C=O}}{(3\cos^2\alpha - 1)} \quad (3.24)$$

From the calculation of the order parameter of the helix, the average tilt angle of the helical axis ($\gamma$) can be determined [38]:

$$S_{helix} = \frac{1}{2} (3\cos^2\gamma - 1) \quad (3.25)$$

This quantitative analysis is useful in analyzing the behavior of proteins and lipids in different environments.
3.5 References


[38] Leitch, J.J. Brosseau, C.L.; Roscoe, S.G.; Bessonov, K.; Dutcher, J.R. Lipkowski, J. *Langmuir*, 2013, 29, 965
Chapter 4 Materials and Methods

4.1 Reagents, solutions, and glassware

All glassware was cleaned using a concentrated, hot acid bath containing a solution of \(1\ \text{HNO}_3 : 3\ \text{H}_2\text{SO}_4\) for about 60 minutes, rinsed with Milli-Q water and then dried in an oven at \(~40\ ^\circ\text{C}\). Teflon pieces and the liquid cell for the AFM were washed in a piranha solution made of \(1\ \text{H}_2\text{O}_2:3\ \text{H}_2\text{SO}_4\) and then rinsed with copious amounts of Milli-Q water. The Langmuir trough was cleaned by alternating multiple Milli-Q water and methanol rinses.

The chemicals were purchased in powder form and used without further purification. Chemicals to form the bilayer including: 1,2-dimyristoyl-sn-glycerol-3-phosphocholine (DMPC), cholesterol, gramicidin A, and 1-thio-\(\beta\)-D-glucose were purchased from Sigma-Aldrich. The ganglioside (GM1) (99%) was purchased from Avanti Polar Lipids. The powder form of all chemicals was made into stock solutions of 1-2 mg/mL using HPLC grade solvents purchased from Fisher. All chemicals were dissolved in chloroform with the exception of GM1 that was prepared in a 75:25 chloroform:methanol solution. All mixed solutions have concentrations of \(~1\ \text{mg/mL}\) and were prepared from the stock solutions. AFM and PM-IRASS measurements were completed with 1mM and 0.1M NaF solutions, respectively. The NaF (99%) was purchased from Sigma Aldrich.

4.2. Au(111) Substrate Preparation

The Au(111) substrate for AFM studies was made with a standard clean
microscope slide. These slides were cleaned using a hot chloroform:methanol mixture and a series of water and methanol rinses. The clean microscope slides were blown dry using argon gas and placed in an instrument used for titanium and gold deposition. This instrument was pumped down under vacuum before deposition occurred onto the glass slides. The first layer that was deposited was 3 nm of titanium and the second layer was 200 nm of gold. The gold coated slides were cut into 1x1 cm squares and annealed using a muffle furnace at 675 °C for 65 seconds to form Au(111) terraces. After annealing, the gold coated slides were placed in a 1M HCl solution for 15 minutes at 80 °C. The gold coated glass slides were soaked in acid to remove titanium that was exposed at the terrace edges after annealing. The gold slides were then placed in a 2 mM 1-thio-β-D-glucose solution made with water for about 15 hours to form a hydrophilic self-assembled monolayer on the surface of the gold. The slides were rinsed with water and allowed to dry before further surface preparation was completed.

The Au(111) substrate for PM-IRRAS studies was a single crystal electrode. The electrode was cleaned by flame annealing and was placed in a hanging meniscus configuration in a 2 mM 1-thio-β-D-glucose solution for 15 hours to form a hydrophilic self-assembled monolayer.

4.3 Langmuir-Blodgett / Langmuir-Schaefer Techniques

4.3.1 Instrumentation

All compression isotherms and monolayer transfers were completed with a KSV LB Minitrough (Figure 4.1). The movement of the barriers and dipper arms were controlled with the KSV LB5000 software. The trough was filled with a Milli-Q water
subphase and was heated to 30 °C for all measurements and transfers. A Whilhelmy plate made of filter paper was saturated with the water subphase before any measurements were taken.

4.3.2 Compression Isotherms

A compression isotherm was collected for all pure components as well as every mixed solution used in the formation of the bilayers. The barriers were compressed in a symmetric manner at a speed of 30 mm/min for all solutions containing GM1 and 10 mm/min for all other solutions. The compression isotherm measurements were completed from gaseous phase to the collapse of the monolayer. All isotherms were repeated a minimum of three times.

4.3.3 Bilayer Deposition

Two different model membrane systems were studied in this thesis: solid supported and floating bilayer lipid membranes. Both of these model membranes were constructed using Langmuir-Blodgett techniques.

Monolayer Transfer: Inner Leaflet

The inner leaflet of the floating bilayer is comprised of GM1, cholesterol, DMPC, and gramicidin for the floating bilayer system. Previous studies of floating bilayer lipid membranes have concluded that the most uniform composition of the mixture DMPC:cholesterol:GM1 was a 40:30:30 mole percentage [1]. This project integrates the peptide gramicidin into the bilayer therefore the compositions were adjusted to accommodate for a small hydrophobic transmembrane protein. Due to the similar
hydrophobicity of gramicidin and cholesterol, the mole percentage of cholesterol was lowered when gramicidin was added. The composition for the floating bilayer containing gramicidin was 40:20:30:10 mole percentage of DMPC:cholesterol:GM1:gramicidin. The floating bilayer was compared to a solid supported bilayer that contained 1:9 mole ratio of Gramcidin:DMPC. This mole ratio was the same as used in a previous thesis [2].

Langmuir-Blodgett techniques were applied to deposit the inner leaflet to the substrate. The gold substrate was lowered into the Langmuir trough before the mixed solution was added to the surface. The mixed solution was added very carefully to the surface of the subphase using a microsyringe above the submerged substrate. After fifteen minutes, to ensure complete solvent evaporation, the barriers moved to adjust the surface pressure to a target pressure of 35 mN/m. Once the target pressure was reached the surface was allowed to stabilize for fifteen minutes before drawing the gold substrate upward through the subphase. The upward speed was varied depending on the system, and was adjusted based on the calculated transfer ratio. If the transfer ratio was below one the speed was lowered and if the transfer ratio was above one the speed was increased to ensure complete coverage of the substrate by only one monolayer. The gold coated glass slides and the gold single crystal electrode were raised at a speed of ~25 mm/s and 15 mm/s, respectively. The monolayer deposits onto the substrate such that the polar head group is directed toward the substrate and the hydrocarbon tails are pointing towards the air. The monolayer was allowed to dry for two hours before an additional layer was applied.
Monolayer Transfer: Outer Leaflet

The outer leaflet of the bilayer was completed with the Langmuir-Schaefer touch. Previous studies constructing bilayer lipid membranes found DMPC and cholesterol have the best composition be a 70:30 mole percentage for DMPC:cholesterol, that ensured a homogenous distribution of components [1]. This project incorporated gramicidin into the outer leaflet therefore the molar fraction of cholesterol was adjusted to account for the added hydrophobic gramicidin. The composition used for the outer leaflet of the floating bilayer was 70:20:10 for DMPC:cholesterol:gramicidin. The floating bilayer was compared to a symmetric solid supported bilayer that had an outer leaflet composition of 1:9 for gramicidin:DMPC.

The gold coated electrode with the dried monolayer was placed on the dipper arm so that the monolayer was facing parallel to the surface of the subphase. The mixed solution for the outer leaflet was placed drop wise on the subphase and was left for fifteen minutes before the barriers moved to allow for complete evaporation of the chloroform and methanol. The barriers were used to obtain the target pressure of 35 mN/m and the surface was allowed to stabilize before the “touch” was performed. The electrode was lowered and withdrawn from the subphase on the slowest dipper option. The bilayer was allowed to dry for a minimum of three hours before being handled for experiments.

4.4 Atomic Force Microscopy Experimental Design

4.4.1 Sample Holder

The sample holder was designed specifically for solution imaging. The holder was ample size for the imaging of our biological samples that were on 1x1cm gold coated
glass slides (Figure 4.1). The design allowed for the sample to be placed snug inside a rubber o-ring and a Teflon block to be screwed on top of the rubber o-ring. The cantilever nose holder fit inside the hole in the Teflon block that was filled with 1 mM NaF solution. The design allowed for a simple setup and the pieces were easy to clean which allowed for a clean working environment.

![Sample holder diagram](image)

**Figure 4.1:** Sample holder used for imaging in solution. Adapted from Ref. 3.

### 4.4.2 Imaging conditions

All images were taken in MAC mode with an *Agilent Technologies* 5500 Scanning Probe Microscope using *PicoScan 5.2* software. The *Agilent* scanner that was used for all imaging was model N9520A-US07480132. The cantilevers were rectangular and made of silicon with a magnetic coating on top. The dimensions of the cantilever were 3 µm, 30 µm, and 225 µm for the length, width, and thickness respectively. They were purchased from *ATS Scientific* and were type I MAC levers. The cantilever resonant frequency was 75 kHz in air and 25 kHz in solution and the nominal spring constant was ~2 N/m. The spring constant of each cantilever was determined individually using the thermal tune method.

The sample prepared on the gold coated glass slide was secured on the sample
plate and then filled with 1 mM NaF solution. The sample plate was attached directly underneath the scanner and was not touched for half an hour to ensure the system was completely stabilized. A solenoid was used to apply an AC magnetic field which oscillated the cantilever. The amplitude set point was set between 75-90% of the free amplitude and therefore the cantilever approach would stop when the amplitude was dampened to the predetermined percentage. Various dimensions of scan size were used and the integral and proportional gains were adjusted for optimized imaging. The proportional was generally ten times greater than the integral value. The scan speed was 2.0-3.5 lines per second. Data analysis was completed using Gwyddion v2.19 software to filter and/or level images only when necessary.

4.4.3 Conditions for Force-distance Curves

Triangular shaped cantilevers made of silicon nitride were used to perform force-distance curves on all bilayer samples. These cantilevers were the SNL-10 model and were purchased from Bruker. The spring constant of these cantilevers were determined using the thermal tune method and was found to be ~0.08 N/m. The cantilever width, length, and thickness were 25 µm, 205 µm, and 0.6 µm respectively. The sample was imaged in contact mode to locate a large flat Au(111) terrace where the force-distance curves could be completed. To approach the surface in contact mode the force set-point was set to -1V until contact was made and then the set-point was made more negative until the tip detached from the surface. At the point where the tip detaches from the surface the force set-point is made slightly more positive to re-engage the tip to the sample at a very soft contact position. To complete the force distance curve the tip was
approached to the surface and then retracted from the surface in contact mode. The approach and retract was about 400 nm in distance and took about 1.59 seconds to complete. Many force curve measurements were completed in various locations on the sample and a statistical analysis was completed from all measurements [4]. The cantilever deflection was measured as a function of piezo position and then converted into force vs. tip-sample distance using software written in house [4].

4.4.4 Thermal tune method

To calculate the spring constant of the cantilevers using the thermal tune method the mechanical response of the cantilever was measured. A force-distance curve was obtained by measuring the deflection of the cantilever versus the position of the sample. The sample used for the force distance curves was a freshly cleaved piece of mica. The deflection sensitivity was calculated from the force-distance curve where the reciprocal of the contact portion of the force-distance curve is equal to the deflection sensitivity. The resonant frequency was determined by measuring the power density spectrum of the vibrational noise. An example of the power density spectrum is shown in Figure 4.2, where the curve has a Lorentzian form and the peak is the resonant frequency of the cantilever. The resonant frequency, deflection sensitivity, and the temperature were variables that were input into software provided by Agilent to calculate the spring constant.
4.5 PM-IRRAS Experimental Design

4.5.1 Spectroelectrochemical Cell

The spectroelectrochemical cell was custom designed to allow for the spectroscopic investigation of species adsorbed on a gold electrode, in solution, with an applied potential. The glass cell has an inlet and outlet port to attach gas tubes, an opening to place a reference electrode, as well as an opening on both ends of the cell. One end of the cell was sealed with the glass tube that contained the gold working electrode and the other end of the cell was sealed with a CaF$_2$ IR prism (Harrick, Scientific Corp.). A platinum wire is attached to the cell as the counter electrode. A schematic of the spectroelectrochemical cell is shown in Figure 4.3.
4.5.2 Optical Set-up for PM-IRRAS Measurements

The optical set-up for PM-IRRAS was modeled after Barner et. al [6] and Green et. al [7] (Figure 4.3). The spectroelectrochemical cell was placed on a stage that can be adjusted in height and angle with respect to the spectrometer and detector. The angle of incidence was set based on the Mean-square electric field strength (MSEFS) calculation and the stage height was tweaked to obtain the most optimized signal. The beam from the Nicolet Nexus 870 spectrometer was focused into a static polarizer by a flat and a parabolic mirror. The linearly polarized light was then directed into the photoelastic modulator (PEM) (PM-90 with II/ZS50 ZnSe 50 kHz optical head) from the static polarizer. The PEM generated alternating s- and p-polarized light that was directed
through the CaF₂ prism and reflected off of the gold electrode. The reflected beam was then directed through a ZnSe lens into the MCT-A detector that was cooled with liquid nitrogen. A synchronous sampling demodulator (SSD) obtained the signal from the detector as an input with a signal from the PEM to use as a reference. The PEM and SSD were phased together with the use of an oscilloscope. The SSD demodulated the signal into two outputs, the average and difference signals of the p- and s- polarized light. These outputs were obtained by a computer and OMNIC was used to analyze the spectra. The potential was controlled by a potentiostat where the working electrode was the modified gold electrode, the reference electrode was Ag/AgCl and the counter electrode was platinum. The potential was stepped by 100 mV from +400 mV to -1000 mV in both the cathodic and anodic direction. The PEMs retardation was optimized by taking the ratio of a spectrum collected while the PEM is on to a p-light spectrum where the PEM is turned off. The peak of that ratio was monitored and the retardation was adjusted such that the peak center was on the wavenumber of interest. The optimized retardation was found to be 0.530. All background spectra were collected at a resolution of 16 cm⁻¹ for 100 scans and all other spectra were collected at a resolution of 4 cm⁻¹ for 4000 scans.

4.5.3 Optimization of Angle of Incidence and Thin-Layer Cavity

In-house software written by Vlad Zamlynny [5,8] was used to calculate the theoretical MSEFS for the system. The input parameters for the three phase system were the CaF₂ prism, deuterated water, and gold. The MSEFS was calculated for two wavenumber regions, 1600 cm⁻¹ and 2900 cm⁻¹. The optimized MSEFS angle of incidence and gap thickness was calculated to be 66 ° and 3.6 µm (Figure 4.4) for the
1600 cm\(^{-1}\) region and 57° and 2.4 µm for 2900 cm\(^{-1}\) region. The experimental setup was adjusted as closely as possible to these ideal parameters.

**Figure 4.4**: Mean-square electric field strength (MSEFS) calculated for 1600 cm\(^{-1}\) at a gold surface with a solution of D\(_2\)O and a CaF\(_2\) prism for a p-polarized beam as a function of gap thickness and angle of incidence.

### 4.5.4 Determination of Thin-Layer Cavity Thickness

The thin layer cavity thickness was measured by comparing the experimental reflectivity spectra to a theoretical spectrum. The theoretical spectrum was calculated using Fresnel equations for the same three phase system as the experiment using in-house software. The ratio between the measured spectrum with no electrolyte present to the measured spectrum with electrolyte present was taken as the experimental reflectivity spectrum. A comparison between the theoretical and experimental reflectivity spectra were repeated until a good agreement was achieved between the two curves. The
electrode was adjusted manually for a new approach when good agreement was not achieved, after which a new experimental spectrum was calculated. The thin layer cavity thickness was generally 2-8µm thick. In-house software calculated a numerical gap thickness from the comparison of the theoretical and experimental spectra [5,8].

4.5.5 Spectral Processing

The spectra collected need to be background corrected due to the broad-band absorption of the solvent molecules. It is nearly impossible to collect a background spectrum with identical conditions as the conditions used to collect the spectrum of the analyte film therefore, a method similar to Barner and coworkers was employed [6]. This method involved designing a template such that a spline interpolation was fit to the baseline of the raw spectra (Figure 4.5a). The baseline template was carefully selected and required knowledge on the peak positions of the system. After the background was selected based on a successful spline interpolation the raw spectra was finished being processed (Figure 4.5b).
Figure 4.5: a) Baseline correction of raw spectra: the spline interpolation to create baseline (blue), selected template (orange dots), raw spectra (black); b) background corrected raw spectra

The background corrected raw spectra were deconvoluted using Fourier self-deconvolution (FSD). FSD is a great technique to mathematically process raw spectra to sharpen the band profile as well as a tool for first approximation of number and position of overlapping peaks [9]. A good understanding of the system under investigation is necessary for the initial prediction of band position and number of bands. FSD describes an IR band as a Lorentzian shape described by [9]:

$$A(v) = \frac{\gamma^2 + (\nu - \nu_0)^2}{A_0 \gamma^2}$$  \hspace{1cm} (4.1)

where $A_0$ is the band maximum, $\nu$ is the wavenumber, $\nu_0$ is the wavenumber at band maximum, and $\gamma$ is the half-width at half-maximum (HWHM). The software PeakFit was used for all FSD analysis. The bandwidth was set between 10-12 cm$^{-1}$ and refers to an estimation of the HWHM. A resolution enhancement was set at 1.5 or less and refers to the degree to which the spectral features are resolved. The FSD analysis was completed
in triplicate and the standard deviation was calculated. The FSD analysis calculated an area under the curve that allowed for quantitative analysis of percent helical dimer and tilt angles where the error bars represent the standard deviation of the data analysis.

To calculate the orientation of molecules a spectrum of a randomly oriented molecules needs to be calculated. This spectrum is calculated from the optical constants of the molecules obtained from a transmission experiment. The optical constants used in this thesis were obtained from a previous study of gramicidin and DMPC [2]. The transmission spectrum was of a solution of (1:9) gramicidin and DMPC vesicles.
4.6 References


Chapter 5: Results and Discussion

The results and discussion chapter is divided into two sections: solid supported bilayer lipid membrane and floating bilayer lipid membrane. These are two different model membranes containing the same antibiotic peptide, gramicidin A that are studied in this thesis. Initial experiments were performed on a solid supported bilayer lipid membrane composed of a simple binary mixture of gramicidin and DMPC. There were further developments of the model membrane towards a ‘floating’ bilayer lipid membrane that consisted of a water reservoir between the gold surface and the membrane with a more complex matrix of lipids (cholesterol, phospholipids, and gangliosides) that is more realistic to a natural biological membrane.

5.1 Construction of a Solid Supported Bilayer Lipid Membrane Containing DMPC and Gramicidin A

A solid supported bilayer lipid membrane was constructed with a mixture of 90% DMPC and 10% gramicidin A and was supported on a thioglucose modified Au(111) surface. This bilayer lipid membrane was constructed and characterized with Langmuir-Blodgett/ Langmuir-Schaefer techniques and PM-IRRAS.

5.1.1 Compression Isotherms

The monolayers of pure and mixed solutions were characterized by the temperature, surface pressure, surface area, and number of molecules. The surface area and number of molecules is described as the mean area per molecule where the total area of the surface is divided by the number of molecules deposited on the surface. The mean
area per molecule is plotted against surface pressure to obtain a compression isotherm. The compression isotherms were obtained at 30 °C at the air/water interface for all pure and mixed solutions that were used in the construction of the solid supported bilayer lipid membrane in a water subphase with a barrier compression speed of 10 mm/min. Analysis of the shape of the isotherm provides insight towards phase transitions and molecular reorganization that the monolayers undergo as it is condensed. The collapse pressure provides insight in stability of the monolayer. The molecular area provides insight on how tightly packed the molecules are as well as the space the molecules occupy at the air/water interface (ex. parallel or perpendicular to surface).

The miscibility of mixed monolayers is investigated with the use of compression isotherms. The molecular area at a given surface pressure is calculated as the sum of the areas of all the pure components \( (A_i) \) multiplied by its mole fraction \( (X_i) \):

\[
A_{mix} = \sum_i X_i A_i
\] (5.1)

\[
A_{excess} = A_{exp} - A_{mix}
\] (5.2)

The sign and magnitude of the difference in the area of the mixture calculated \( (A_{mix}) \) and the area measured experimentally using an isotherm \( (A_{exp}) \) provides information about the interactions between molecules in the mixture. If the components are immiscible or the mixture is ideal there will be no difference between the calculated and experimentally determined areas. If the difference is above zero there are repulsive interactions between the components and if the difference is below zero there are attractive intermolecular interactions between the components. The excess area is calculated for each mixed isotherm at the surface pressure of 35 mN/m, which is the transfer pressure used in the deposition of the monolayers to construct the model membranes.
The compression isotherm of pure DMPC is shown in Figure 5.1. The isotherm of DMPC behaves as a condensed monolayer with a ‘lift-off’ area at ~100 Å² molecule⁻¹ and a limiting area of 43 Å² molecule⁻¹ at a collapse pressure of 43 mN/m. The isotherm was measured at 30 °C which is above the transition temperature of DMPC (24 °C) therefore the monolayer is comprised of melted hydrocarbon chains and is in the liquid crystalline state. There are no plateaus visible of the phase transitions from L-E to L-C to S states that were noted in previous literature at lower temperatures [1]. The collapse pressures noted in previous literature with similar experimental conditions were ~42-45 mN/m with a mean molecular area per molecule of ~50 Å² molecule⁻¹ [1,2]. The shape of the isotherm is in good agreement with previous literature [1,2].

![Image](image.png)

**Figure 5.1:** Compression isotherm at the air/water interface of pure DMPC at 30 °C

The compression isotherm of pure gramicidin A is shown in Figure 5.2. It has two regions of compressibility separated by a plateau where the plateau occurs between pressures of 21-23 mN/m. The shape of the isotherm is consistent with the literature and is attributed to the reorganization and reorientation of gramicidin molecules [3]. At
surface pressures below 21 mN/m there is a reorganization of the secondary structure of the gramicidin such that as the monolayer is compressed the gramicidin is gradually transforming from an unfolded protein into a β-helix [4]. The plateau is caused from an increase in packing density of gramicidin molecules oriented parallel to the surface [1]. At surface pressures above 23 mN/m there is an increase in surface pressure that is attributed to the tilting of the β-helix to a more vertical orientation.

Figure 5.2: Compression isotherm at the air/water interface of pure gramicidin A at 30 °C

The compression isotherm of the mixed (1:9) gramicidin A and DMPC solution is shown in Figure 5.3. The compression isotherm shows a ‘lift-off’ area per molecule ~130 Å² molecule⁻¹. After ‘lift-off’, the compression moves from a gaseous state to a more condensed monolayer with a limiting area (A_{lim}) of 37 Å² molecule⁻¹ at a surface pressure of 53 mN m⁻¹. The isotherms were compared to previous studies completed under similar experimental conditions. A previous study by Vitovic and coworkers measured an isotherm of mixed DMPC and gramicidin A (100:17 mol ratio) at a temperature of 28 °C
where a phase transition from LC state to solid state was also observed at a surface pressure ~ 25 mN/m in a previous study [1]. There is a slight change in the slope of the isotherm around ~30 mN/m in the results obtained for this thesis which indicate a phase transition from LC to a solid condensed state. The collapse pressure measured in the studies by Vitovic and coworkers was ~45 mN/m [1], which is slightly lower than the pressure observed in this study.

![Compression isotherm at the air/water interface of a mixture of DMPC and Gramicidin A (9:1) at 30 °C (black line) and the calculated isotherm from pure components (red dots)](image)

**Figure 5.3:** Compression isotherm at the air/water interface of a mixture of DMPC and Gramicidin A (9:1) at 30 °C (black line) and the calculated isotherm from pure components (red dots)

The miscibility of DMPC and gramicidin at a 9:1 mole ratio was investigated with the use of compression isotherms. The mean molecular area at 35 mN/m was 97.3 Å² molecule⁻¹, 49.5 Å² molecule⁻¹, 51.4 Å² molecule⁻¹ for pure gramicidin, pure DMPC, and a (9:1) DMPC and gramicidin mixture, respectively. The difference between the calculated and experimental area was -2.9 Å² molecule⁻¹ at 35 mN/m, therefore there is partial miscibility with attractive intermolecular interactions between the components to
allow for higher packing density in the mixture than in the pure components. The calculated and experimental areas are shown in Figure 5.3, the calculated area overestimates the molecular area at most surface pressures. The data show that the deviations of the experimental compression isotherm from the calculated curve are not too large indicating that deviations from the ideal miscibility of the mixture are not too large.

5.1.2 PM-IRRAS

An electrochemical PM-IRRAS study was completed on the solid supported bilayer lipid membrane. The bilayer was deposited onto a Au(111) electrode and was placed inside a glass cell filled with a solution of 0.1 M NaF made with deuterated water. The effect of the electric field, with a magnitude comparable to the electric field acting on natural biological membranes, was investigated by collecting spectra at many applied potentials. A qualitative investigation of the conformation of gramicidin A in the bilayer was investigated by analyzing the amide I band. The hydration of the bilayer was studied through the analysis of the glycerol ester group on the DMPC.

5.1.2.1 Amide I Band

It is common to analyze the amide I band to deduce the secondary structure of the protein under investigation. The vibrational motions of the peptide backbone of gramicidin A are primarily in the amide I band. The amide I band is mainly composed of the C=O stretching with out-of-phase CN stretching, CNN deformation, and NH in-plane bending being minor contributors [5]. Figure 5.4 shows the amide I band at selected potentials that were collected in the cathodic direction first, followed by the anodic
direction. The amide I band in the spectrum collected at +100 mV, in the cathodic direction, is a broad band centered at 1658 cm\(^{-1}\) (Figure 5.4). As the potential decreases the shape and peak center of the amide I band shift to lower frequencies. Stacking the spectra shows a clear trend, as the peak position at 1658 cm\(^{-1}\) decreases the peak position at 1631 cm\(^{-1}\) increases. Based on normal mode calculations completed by Naik and Krimm, the amide I position at ~1631 cm\(^{-1}\) is indicative of the helical dimer conformation (\(\beta^{6.3}\)) and at higher frequencies it is indicative of the intertwined double helix (\(\beta^{5.6}\)) [6]. Based on this analysis, at the beginning of the experiment, the structure of the gramicidin is predominantly double helix and then switches to a predominantly helical dimer conformation as more negative potentials are applied. When the potential is applied in the anodic direction, majority of the amide I peak is at the 1631 cm\(^{-1}\) position with a slow transition back to the 1658 cm\(^{-1}\) peak.

Applying a more negative potential causes the bilayer to detach from the gold surface, creating a thin cushion of electrolyte between the membrane and the metal. As the bilayer is detached from the metal the majority of the protein is in the helical dimer conformation. It is well known that the preferred conformation of gramicidin in a lipid environment is the helical dimer conformation [7]. Therefore, when the protein is in direct contact with the metal surface it is in a stressed state, but as a thin electrolyte cushion is formed between the bilayer and the metal surface the protein converts to its expected conformation in a lipid bilayer.
Figure 5.4: PM-IRRAS spectra of the solid supported bilayer lipid membrane in the $\nu$(C=O) stretching region. Spectra collected at an applied potential in the cathodic direction (top) and anodic direction (bottom)
5.1.2.1 Lipid Head Group Region

The hydration of the lipid head groups can be investigated by analyzing the stretching vibration of the glycerol ester group. Figure 5.4 shows the band associated with the glycerol ester group of DMPC at a peak center ~ 1735 cm\(^{-1}\). This peak is formed by two overlapping peaks corresponding to both hydrogen bonding (1730 cm\(^{-1}\)) and non-hydrogen bonding 1740 cm\(^{-1}\) ester groups [8]. There is a slight broadening and a slight red shift in the peak center at more negative potentials in both the cathodic and anodic direction. This indicates the lipid head groups are more hydrated at more negative potentials, which is expected as the bilayer is desorbed from the metal surface at more negative potentials. This also indicates that the C=O groups of the lipid are more involved in hydrogen bonding at more negative potentials.

The increase in hydrogen bonding of the DMPC head group can be caused from water molecules as well as amino acid side chains from the gramicidin. A recent report by Chaudhuri and coworkers, investigated the importance of the tryptophan residues in hydrogen bonding patterns with lipids from fluorescence and circular dichroism spectroscopy studies [9]. In this study, the indole hydrogen bonding was monitored by investigating the effect of a 1-methylindole on the conformation and activity of gramicidin compared to gramicidin in its natural form [9]. Their finding was that the 1-methylindole substituted gramicidin molecules formed an intertwined double helical structure with the tryptophans distributed along the axis of the membrane (Figure 5.5). The unsubstituted gramicidin formed a helical dimer in the membrane with the tryptophan residues in the interfacial region of the membrane allowing for hydrogen bonding between the lipid carbonyl groups and interfacial water [9]. Raman techniques
have also confirmed the importance of strong hydrogen bonding between the tryptophan residues and lipid carbonyl groups for the formation of the $\beta^{6.3}$ helical dimer conformation [10]. It is interesting to note that the increase in the amide I band at 1631 cm$^{-1}$, in Figure 5.4, is indicative of the $\beta^{6.3}$ helical dimer conformation which correlates well with the red shift in the C=O stretching of the lipid molecules due to an increase in hydrogen bonding between both the gramicidin and interfacial water molecules and the carbonyl lipids groups. It is likely the bilayer deposited directly on the metal surface was ridged and there was not sufficient hydrogen bonding to stabilize the helical dimer conformation until at more negative potentials where desorption of the bilayer created a more relaxed state.

Figure 5.5: Gramicidin conformation based on available hydrogen bonding between tryptophan residue and lipid carbonyl groups A) indole B) 1-Methyldiemole [Adapted from Reference 9]
5.2 Construction of a Floating Bilayer Lipid Membrane Containing DMPC, Cholesterol, GM1, and Gramicidin A

A floating bilayer lipid membrane was constructed with a mixture of DMPC, cholesterol and gramicidin A with GM1 in the inner leaflet to form a water reservoir between the metal surface and the membrane. The ‘floating’ bilayer lipid membrane was supported on a thioglucose modified Au(111) surface. This bilayer lipid membrane was constructed and characterized with Langmuir-Blodgett/ Langmuir-Schaefer techniques, AFM, and PM-IRRAS.

5.2.1 Compression Isotherms

The monolayers of pure and mixed solutions were characterized by the temperature, surface pressure, surface area, and number of molecules. The compression isotherms were obtained at 30 °C at the air/water interface for all pure and mixed solutions that were used in the construction of the floating bilayer lipid membrane in a water subphase. The barrier compression speed was 30 mm/min for solutions containing GM1 and was 10 mm/min for all other solutions. The isotherm of pure DMPC has been discussed previously (Figure 5.1). All other isotherms are discussed below.

The compression isotherm for pure GM1 is shown in Figure 5.6. The compression isotherm is indicative of a liquid expanded state from ‘lift-off’ to a pressure of ~25 mN/m where there is a phase transition from LE to LC state. The phase transition has been investigated using PM-IRRAS where there is a reorientation of the polar head group aiming in the reduction of the space occupied by the molecule [11]. There is a high ‘lift-off’ mean molecular area at ~136 Å² molecule⁻¹ due to the large head group of the GM1 molecule which is consistent with literature values of ~140 Å² molecule⁻¹ for a similar
experimental set-up [12]. As the compression progresses there is steric repulsion between the large bulky head group and electrostatic repulsion from the negatively charged sialic residue preventing the hydrocarbon tails to align to form a crystalline domain [12]. A collapse pressure was obtained at 60 mN/m with a limiting area of 47 Å² molecule⁻¹ which is consistent with literature values of a limiting area of 50 Å² molecule⁻¹ and a collapse pressure of 64 mN/m [12].

![Graph showing compression isotherm at the air/water interface of pure GM1 at 30 °C](image)

**Figure 5.6:** Compression isotherm at the air/water interface of pure GM1 at 30 °C

The compression isotherm of pure cholesterol is displayed in Figure 5.7. There is a steep ‘lift-off’ with a molecular area of 44 Å² molecule⁻¹ from the gaseous state to the liquid-condensed phase. The monolayer is in a condensed phase from lift-off to the collapse pressure that occurs at 45 mN/m with a limiting area of 41 Å² molecule⁻¹. The shape of the isotherm correlates very well with the literature where “lift-off” areas have been found of 40 Å² molecule⁻¹ with a limiting area of 38 Å² molecule⁻¹ at a collapse
pressure of 42 mN/m [13].

**Figure 5.7:** Compression isotherm at the air/water interface of pure cholesterol at 30 °C

The condensing effect of cholesterol on DMPC monolayers was investigated by analyzing a compression isotherm of a (7:3) DMPC:Cholesterol mixed monolayer shown in Figure 5.8. The isotherm has a steady incline from the ‘lift-off’ area of 67 Å² molecule⁻¹ to collapse to a limiting area of 36 Å² molecule⁻¹ at a pressure of 52 mN/m. The condensing effect of cholesterol on DMPC monolayers has been investigated previously and the isotherm obtained in this thesis correlates well with the literature [14]. Previously reported limiting area for a 7:3 DMPC:Cholesterol monolayer under similar experimental conditions was 38.5 Å² molecule⁻¹ at a surface pressure 52 mN/m [14]. The limiting area that was determined for the pure DMPC monolayer was 43 Å² molecule⁻¹, therefore the addition of the cholesterol had a condensing effect that forms a tightly packed liquid ordered phase. The collapse pressure is also greater as a mixture and
therefore the mixed monolayer is more stable than a monolayer of either of its pure components.

The miscibility of the pure components in the mixed monolayer was calculated by comparing the mean molecular area per molecule at a pressure of 35 mN/m of the mixed isotherm to the mean molecular area per molecule calculated based on the isotherms of the pure components. The difference between these two values was -7.1 Å² molecule⁻¹, which describes a miscible monolayer with attractive interactions between the components at a surface pressure of 35 mN/m. As shown in Figure 5.8, the calculated isotherm overestimates the mean molecular area at all surface pressures investigated and therefore experiences the condensing effect at all surface pressures.

Figure 5.8: Compression isotherm at the air/water interface of a mixture of DMPC and Cholesterol (7:3) at 30 °C (black line) and calculated isotherm based on pure components (red dots)
The effect of GM1 in a monolayer of DMPC and cholesterol was analyzed with the use of the compression isotherm shown in Figure 5.9. The shape of the isotherm is similar to the isotherm without GM1; however, the mean molecular area per molecule is much larger. The ‘lift-off’ area was ~120 Å² molecule⁻¹ and decreased to a limiting area of 48 Å² molecule⁻¹ at the collapse pressure of 53 mN/m. The larger molecular area is expected due to the bulky head group of the GM1 that prohibits tight packing of the monolayer. The miscibility of the components in the monolayer was investigated by calculating the difference between the measured area at a surface pressure of 35 mN/m and the area calculated from the pure components. The difference in the area was +2.5 Å² molecule⁻¹, which is indicative of a monolayer with repulsive interactions between the components in the mixture. It is known that cholesterol forms cholesterol-rich domains in the presence of glycolipids [15]; therefore it is expected to have some cholesterol-rich domains that are homogeneously distributed throughout the monolayer. As shown in Figure 5.9, the experimental isotherm has a higher mean molecular area than predicted based on the calculated mean molecular area at all analyzed surface pressures. Therefore, there are repulsive forces present for all surface pressures that create a larger than expected molecular area.
Figure 5.9: Compression isotherm at the air/water interface of a mixture of DMPC, GM1, Cholesterol (4:3:3) at 30 °C (black line) and calculated isotherm from its pure components (red dots)

The inner leaflet of the floating bilayer lipid membrane contained gramicidin, DMPC, GM1, and cholesterol in a 1:4:3:2 mole ratio, respectively. The isotherm of this mixture is in Figure 5.10. The ‘lift-off’ area was ~160 Å² molecule⁻¹ and decreased to a limiting area of 54 Å² molecule⁻¹ at a collapse pressure of 72 mN/m. The monolayer is very stable which is reflected in the high collapse pressure. There are two regions of compressibility separated by a slight plateau between 24 mN/m and 32 mN/m. The two regions of compressibility arise due to reorientation of the polar head group of GM1 as well as a reorganization of gramicidin molecules from a horizontal to vertical orientation that was seen at similar surface pressures in the pure isotherms of GM1 and gramicidin.

The miscibility was determined through the difference in areas, at 35 mN/m, for
the isotherm of the mixed solution as well as the calculated area determined with the isotherms of the pure components. The difference in area was $+14.1 \text{Å}^2 \text{molecule}^{-1}$, which indicates repulsive interactions between the components in the monolayer reflected in the high molecular area in the experimental isotherm. The calculated areas based on the pure components are shown in Figure 5.15, all calculated areas are lower than the experimental isotherm.

![Figure 5.10: Compression isotherm at the air/water interface of a mixture of DMPC, GM1, Cholesterol, gramicidin A (4:3:2:1) at 30 °C and the calculated isotherm from its pure components (red dots)](image)

The outer leaflet of the floating bilayer lipid membrane contained gramicidin, DMPC, and cholesterol in a 1:7:2 molar ratio, respectively. The isotherm of this mixture is in Figure 5.11. The ‘lift-off’ area was $\sim120 \text{Å}^2 \text{molecule}^{-1}$ and the monolayer was compressed to a limiting area of $37 \text{Å}^2 \text{molecule}^{-1}$ at a surface pressure of 55 mN/m. The
two regions of compressibility are still present and are indicative of the reorientation of the gramicidin molecules from a horizontal to vertical orientation. The overall molecular area is smaller than when GM1 is present.

The miscibility of the monolayer was investigated through the difference in the area of the measured mixed monolayer at 35 mN/m and the calculated area from the pure isotherms. The difference was found to be -2.8 Å² molecule⁻¹, which indicates attractive interactions between the components of the mixed monolayer. The calculated isotherm is shown in Figure 5.11 and is close to the experimental isotherm with slight variation of repulsive and attractive forces at different surface pressures. It is noted that there are less repulsive interactions occurring when GM1 is not present. The small deviations of the calculated from the experimental curves indicate small deviations of the mixture from ideal behavior.
Figure 5.11: Compression isotherm at the air/water interface of a mixture of DMPC, Cholesterol, gramicidin A (7:2:1) at 30 °C (black line) and the calculated isotherm from the pure components (red dots)

5.2.2 PM-IRRAS

An electrochemical PM-IRRAS study was completed on the floating bilayer lipid membrane. The bilayer was deposited onto a Au(111) electrode and was placed inside a glass cell filled with a solution of 0.1 M NaF made with deuterated water. The effect of the electric field, with a magnitude comparable to the electric field acting on natural biological membranes, was investigated by collecting spectra at many applied potentials. A qualitative and quantitative analysis of the conformation of gramicidin A in the bilayer was investigated by analyzing the amide I band. The hydration of the bilayer was studied through the analysis of the glycerol ester group on the lipids. The orientation of the lipid chains was investigated with quantitative and qualitative analysis of the acyl chain vibrational region.
5.2.2.1 Amide I Region

The amide I band was analyzed to determine the orientation and conformation of gramicidin A in the floating bilayer lipid membrane. The amide I band is shown in Figure 5.12 and was collected at various applied potentials. There are two peak centers found for the amide I band; 1631 cm\(^{-1}\) and 1658 cm\(^{-1}\). As stated previously (section 5.1.2.1), gramicidin A has multiple conformations and the conformation that is present can be determined based on the peak center of the amide I band. The peak center at 1658 cm\(^{-1}\) is indicative of the intertwined double helix and is present at the beginning of the experiment at more positive potentials [6]. As the potential decreases in the cathodic direction, the peak position shifts to 1631 cm\(^{-1}\), which is indicative of the active conformation for the ion transport channel called helical dimer [6]. As the potential is decreased to more negative values the bilayer begins to desorb off of the metal surface. This is the same occurrence as discussed previously in the solid supported bilayer lipid membrane experiments. Although the aim was to create a floating bilayer lipid membrane that contains a water reservoir since the amide I peak position at the beginning of the experiment remains consistent with a non-active ion channel conformation. This implies that perhaps the floating bilayer lipid membrane still lacks mobility in the lipids and therefore the gramicidin molecule is unable to form the helical dimer until the bilayer is slightly desorbed from the metal surface. When the potential is then applied in the anodic direction towards more positive potentials, the amide I peak stays at 1631 cm\(^{-1}\) with minimal shifting back towards the 1658 cm\(^{-1}\) peak center. The gramicidin can maintain the active helical dimer conformation as the bilayer settles back onto the metal surface.
Figure 5.12: PM-IRRAS spectra of the floating bilayer lipid membrane in the $\nu$(C=O) stretching region. Spectra collected at an applied potential in the cathodic direction (top) and anodic direction (bottom)
The amide I band is a convolution of overlapping peaks of both the helical dimer and the double helix conformations. Fourier self-deconvolution was used to determine the peak position of these overlapping peaks and the area under the curve for the peak at 1631 cm⁻¹ was used to estimate a percentage of the helical dimer present in the floating bilayer lipid membrane. The number of peaks and relative peak positions were determined from a previous study that indicated six overlapping peaks at positions 1631, 1649, 1656, 1662, 1668, and 1675 cm⁻¹ and are shown in Figure 5.13 [16]. Hydrogen bonding within the intertwined double helix give rise to the bands under the higher frequency shoulder of the amide I band; 1649, 1656, 1662, 1668, and 1675 cm⁻¹ [6, 17]. The percentage of helical dimer was calculated by dividing the area under the peak at 1631 cm⁻¹ by the total area. The relative percentage of helical dimer present at each potential is shown in Figure 5.14. When the bilayer is directly deposited onto the metal surface there is about 5% helical dimer conformation and as a more negative potential is applied the percentage of helical dimer increases to about 60%. When the potential is then applied in an anodic direction to more positive potentials the percentage of helical dimer remains at about 45-55% of the total conformations present.
Figure 5.13: The PM-IRRAS spectra in the $\nu$(C=O) stretching region (blue) with the deconvoluted spectrum of $\nu$(C=O) region (black and red)
Figure 5.14: An estimate of the percentage of gramicidin in the helical dimer conformation of a PM-IRRAS spectrum collected an applied voltage in the cathodic direction (top) and anodic direction (bottom)
5.2.2.2 Lipid Head Group Region

The vibrational analysis of the glycerol ester group of the DMPC lipid used in the construction of the floating bilayer lipid membrane provides valuable information about the hydration of the bilayer. This region was discussed in more detail in section 5.1.2.1 for the solid supported bilayer lipid membrane. The overall shape and position of the carbonyl stretch depends on the extent of hydration of the ester group on the lipid. The peak is comprised of two overlapping bands with peak centers at 1730 cm\(^{-1}\) indicative of hydrogen bonded ester groups and 1740 cm\(^{-1}\) which is indicative of ester groups that are not hydrogen bonded [8]. This peak is shown in Figure 5.12 and the two overlapping peaks are deconvoluted and shown in Figure 5.13. Fourier self-deconvolution was completed to obtain the peak position and shape. As shown in Figure 5.14, the peak indicative of a hydrogen bonded glycerol group is much larger than the peak indicative of the non-hydrogen bonded glycerol group. The ester carbonyl stretching band becomes slightly broader and shifts slightly to lower frequencies as the applied potential becomes more negative in the cathodic direction. This indicates that the lipid head group has more hydrogen bonding activity as the applied potential becomes more negative. This is to be expected as the bilayer is in a desorbed state from the metal surface at high negative potentials and therefore there is a thin layer of electrolyte between the metal and the bilayer creating more opportunity to hydrogen bond with interfacial water molecules. At more negative potentials, the predominate conformation of the gramicidin is helical dimer which also creates more opportunity for hydrogen bonding due to its interfacial tryptophan residues at the interfacial region of the membrane [9]. The tryptophan residues help stabilize the gramicidin in a dimer conformation through hydrogen bonding.
As the spectra are collected at more positive potentials in the anodic direction the shape and position of the ester carbonyl stretching band remains quite constant (Figure 5.12). It is interesting to note that the percentage of helical dimer calculated at potentials measured in the anodic direction is also relatively constant and therefore it correlates well with the constant hydrogen-bonded glycerol group.

5.2.2.3 Acyl Chain Region

The acyl chain vibrational region is comprised of C-H stretching between ~2800-3000 cm\(^{-1}\). This region provides information about the conformation and orientation of the lipids in the membrane. This region contains many overlapping peaks with two prominent peak maxima at the asymmetric and symmetric stretching vibration. The acyl chain region was measured at various potentials and is shown in Figure 5.15 where the \(v_{\text{as}}(\text{CH}_2)\) was 2923 cm\(^{-1}\) and \(v_{\text{sym}}(\text{CH}_2)\) was 2852 cm\(^{-1}\). The position of these peaks is dependent on the state of the bilayer and therefore it can be determined if the lipids are in gel state with all-trans conformation or if there are some gauche conformation present. If the \(v_{\text{as}}(\text{CH}_2)\) is lower than 2920 cm\(^{-1}\) and \(v_{\text{sym}}(\text{CH}_2)\) is lower than 2850 cm\(^{-1}\) the bilayer is in a gel state with acyl chains in an all trans conformation [18]; therefore the acyl chains in the floating bilayer lipid membrane have some gauche conformation. The order parameters of the lipids were calculated based on a deconvoluted spectrum. A Fourier self deconvolution was completed with seven overlapping peaks and is shown in Figure 5.16. The number and position of the peaks used in this analysis was taken from a previous study [16].
Figure 5.15: PM-IRRAS spectra for the C-H stretching region of the floating bilayer lipid membrane for Spectra collected at an applied potential in the cathodic direction (+100 mV to -900 mV) and anodic direction (-900 mV to -800 mV)

Figure 5.16: Deconvoluted spectrum of the C-H stretching region for the floating bilayer lipid membrane
5.2.2.4 Orientation of Acyl Chains and Gramicidin

The orientation of the acyl chains was calculated based on the order parameters of the asymmetric and symmetric methylene stretching bands. The theory describing the calculation of tilt angles from PM-IRRAS data is discussed in detail in Chapter 3. An example of the deconvoluted spectrum used in these calculations is shown in Figure 5.16. The tilt angle of the trans fragment of the lipid was calculated for every potential and is shown in Figure 5.17. The tilt angle ranges from 22-43 ° with an average tilt angle of 34 ° with respect to the surface normal which is consistent with other bilayer lipid membranes containing proteins [16, 19]. There is a slight decrease in the tilt angle of the trans fragment of the lipid as the potential becomes more negative and the bilayer is in a desorbed state.

![Figure 5.17: Tilt angle of the trans fragment of the lipid in the floating bilayer lipid membrane](image)

**Figure 5.17:** Tilt angle of the trans fragment of the lipid in the floating bilayer lipid membrane
The orientation of gramicidin in the helical dimer conformation was calculated from the order parameters of the amide I band. A detailed description of the calculation of the tilt angle of gramicidin based on order parameters can be found in Chapter 3. The angle between the helical axis and the direction of the transition dipole moment of the amide I vibration was taken from the literature. The literature value of 32° was reported from a polarized ATR investigation of hydrated multibilayers containing DMPC and gramicidin [20]. This investigation also reported a tilt angle of the lipid in the bilayer of 30°, which is consistent to the value calculated in this thesis [20]. The tilt angles calculated for spectra collected at an applied potential in the anodic direction are shown in Figure 5.18. The average calculated tilt angle of gramicidin in the floating bilayer lipid membrane with respect to the surface normal was 14° with a range of 5° - 22°. It is interesting to note that as the bilayer settles back onto the metal surface the gramicidin becomes more perpendicular to the surface.
Figure 5.18: Tilt angle of helical dimer gramicidin with respect to the surface normal in a floating bilayer lipid membrane assuming the orientation of the amide I transition dipole is $32^\circ$.

5.2.3 AFM Studies

The surface of the floating bilayer lipid membrane was characterized using MAC mode imaging and the thickness of the film was determined using force-distance curves. The floating bilayer lipid membrane was deposited on a ~1cmx1cm gold coated glass slide. The gold was annealed to form large Au(111) terraces and then modified with a thioglucose self-assembled monolayer before the bilayer was deposited.

5.2.3.1 MAC Mode Imaging

MAC mode images were obtained at room temperature in a solution of 1 mM NaF. The images were obtained on flat Au(111) terraces and allowed for the visualization...
of the lateral organization of components in the bilayer. The topography and amplitude images of the floating bilayer lipid membrane on a flat Au(111) terrace are shown in Figure 5.19. The amplitude and topography images were obtained simultaneously. The images illustrate that the components in the floating bilayer lipid membrane are homogenously distributed throughout the bilayer. There was no phase segregation revealed in the images. The topography image was used for cross sectional analysis to determine the surface roughness. As displayed in Figure 5.19, the surface roughness of the floating bilayer lipid membrane is less than 1 nm. This indicates a smooth bilayer deposited onto the surface with no ripples, or other features indicating phase segregation.

![Topography and Amplitude images](image)

**Figure 5.19:** MAC mode topography (A) and Amplitude (B) images of floating bilayer lipid membrane. A line profile (C) from the topography image. Images were taken at room temperature in 1 mM NaF.
5.2.3.2 Force-Distance Curves

The thickness of the bilayer was determined by force-distance curves. The force distance curves were obtained in 1 mM NaF solution using a cantilever with a spring constant of 0.08 N/m. The spring constant was measured individually using the thermal tune method. This exact measured spring constant was used in the force-distance curve analysis. A typical force-distance curve obtained of the floating bilayer lipid membrane is shown in Figure 5.20. As shown in Figure 5.20, repulsive interactions are detected at tip-sample separation distances of ~15 nm and at distances ~4-5 nm there is a discontinuity in the slope of the curve. The distance of the discontinuity was taken as the thickness of the bilayer. A large set of force-distance curves were collected to complete a statistical analysis. The statistical analysis is shown in Figure 5.21 in a histogram chart made of 62 measurements. The normal distribution curve was overlaid on the histogram chart to obtain the average thickness as well as the standard deviation. The average thickness was found to be 4.1+/− 1.5 nm.

![Figure 5.20: Force-distance curve measured in 1 mM NaF at room temperature of the floating bilayer lipid membrane](image)
Figure 5.21: Histogram plot of the width of the penetration distance measured in 62 force-distance curves. A normal distribution curve is overlaid on the histogram plot with a mean value of 4.1±1.5 nm

This thickness is smaller than anticipated, however this thickness is of a compressed bilayer due to elastic deformation of the bilayer. The elastic deformation by the tip was estimated using the Hertizian model:

\[ \delta = \left( \frac{9F^2}{16RE^*} \right)^{1/3} \]

where the R is the radius of the tip (20 nm), F is the average penetration force, and E is the effective compression modulus. The effective compression modulus is not known for a bilayer containing GM1, gramicidin, cholesterol, and DMPC, therefore the effective compression modulus of a bilayer containing DMPC and cholesterol was used (7x10^8 N/m^2) [21]. The average penetration force was 3.6 nN for the measured bilayer thicknesses shown in Figure 5.21. The elastic deformation was calculated to be 0.9 nm. This means the thickness from the force-distance curves was underestimated by 22%
which is consistent with literature that reports thicknesses of a similar bilayer underestimated by 16-30% [14]. The corrected bilayer thickness was calculated from the sum of the elastic deformation and the penetration distance from the force-distance curves and found to be 5.0 nm.

The corrected thickness is still smaller than expected for the floating bilayer lipid membrane. The aim of the addition of a GM1, which contains a large bulky head group, was to create a water reservoir between the gold surface and the bilayer that was ~1-2 nm thick. Based on the calculations of the bilayer thickness, it is possible that the head group of the GM1 is not oriented perpendicularly to the surface to create a water reservoir as anticipated.
5.3 References


Chapter 6: Conclusions and Future Directions

6.1 Conclusions

The overall objective of this work was to construct a biomimetic model membrane that could incorporate transmembrane proteins. The model membrane system was an asymmetric ‘floating’ bilayer lipid membrane. The inner leaflet was composed of 30% GM1, 20% cholesterol, 40% DMPC, and 10% gramicidin A; and the outer leaflet was composed of 20% cholesterol, 70% DMPC, and 10% gramicidin A. The bilayer was deposited onto a thioglucose modified Au(111) surface using Langmuir-Blodgett and Langmuir-Schaefer techniques. The Au(111) surface was ideal for this investigation because it is an atomically flat surface and it can function as an electrode for the direct application of electrochemical studies. Electrochemical PM-IRRAS was one of the methods used to characterize the ‘floating’ bilayer lipid membrane with the incorporated transmembrane protein. The applied potentials were comparable to that of biological cell membranes. PM-IRRAS provided insight into the structure of the lipid matrix as well conformational and orientation specific information of gramicidin A. Atomic Force Microscopy was an excellent tool to gain topographic information of the surface of the ‘floating’ bilayer lipid membrane as well as bilayer thickness measurements. Solid supported bilayer lipid membranes are the most common model membranes. A symmetric solid supported model membrane composed of 90% DMPC and 10% gramicidin A was used as a qualitative comparison to the ‘floating’ bilayer lipid membrane.
The major conclusions of this thesis are as follows:

(i) The solid supported bilayer lipid membrane was directly deposited on a thioglucose modified Au(111) electrode. The gramicidin A was in direct contact with the metal surface and was in a non-channel conformation. The application of a high negative potential caused the bilayer to slightly desorb off of the metal surface, creating a small water reservoir. This water reservoir increased the hydration of the lipid head groups as well as allowed important hydrogen bonding between the lipid head groups and the gramicidin A to occur to form an active-channel conformation. The active-channel conformation of gramicidin A was energetically favorable and would keep some of its conformation when adsorbed back onto the metal surface.

(ii) The asymmetric ‘floating’ bilayer lipid membrane was deposited on a thioglucose modified Au(111) electrode. The ‘floating’ bilayer lipid membrane did not have a large water reservoir facilitated by the bulky head group in GM1 between the bilayer and the metal surface as was expected. PM-IRRAS measurements indicated the gramicidin A was in a non-channel conformation when it was adsorbed to the metal surface. Upon the application of increased negative potentials, the bilayer was in a desorbed state and the gramicidin A was facilitated into an active-channel conformation. When the bilayer adsorbed back on the metal surface the majority of gramicidin A remained in an active-channel conformation and the lipid head groups remained slightly more hydrated. This could indicate the creation of a larger water reservoir than initially created during the initial deposit of the bilayer. It is possible the bulky head group of the GM1 reorients to a more perpendicular orientation which would create a larger water reservoir. The average tilt angle of the gramicidin was ~14 ° with the largest tilt angle...
being in the desorbed state and the lowest tilt angle being when the bilayer adsorbs back onto metal surface. The average tilt angle of the trans fragment of the lipid was 34 ° and has the smallest tilt angle in the desorbed state. The lipids were found to be in a less ordered state with some gauche conformation.

(iii) Analysis of AFM images established that the components in the ‘floating’ bilayer lipid membrane are homogenously distributed throughout the bilayer. The average corrected thickness of the bilayer was 5.0 nm. This thickness is smaller than expected if the ‘floating’ bilayer lipid membrane had a larger water reservoir. This suggests the bulky head group of the GM1 is not oriented exactly perpendicular to the metal surface and therefore the bilayer would be thinner.

6.2 Future Directions

There is still a great deal of information that can be gained with additional analysis of the ‘floating’ bilayer lipid membrane in this work. The PM-IRRAS spectra were complicated because many components in the bilayer contain the same IR signatures such as methylene stretching frequency. It would be interesting to use deuterated components to separate the vibrational bands for the GM1 and DMPC. This would allow for more determinations of cause and effect towards specific components rather than general groups. The PM-IRRAS studies of the ‘floating’ bilayer raised questions about the mobility of the lipids in the bilayer. It would be beneficial to complete an investigation that could compare the mobility of the lipids in the solid support bilayer to the ‘floating’ bilayer lipid membranes with fluorescence studies such as fluorescence recovery after photobleaching (FRAP).
The main drawback the ‘floating’ bilayer lipid membrane tried to overcome was the close proximity of the bilayer to a metal surface that restricted the incorporation of a transmembrane protein. The model suggested in this work is one example of increasing the water reservoir between the metal surface and the bilayer. There are many other model membranes that would be interesting to investigate. Tethered bilayer lipid membranes have been widely studied using large hydrophilic spacer molecules that anchor the bilayer to the metal surface [1]. It would also be interesting to study a model comprised of multibilayers [2]. The multibilayers would be stacked on a metal surface to create a separation between the protein and the metal and the bilayer deposited on the top of the stack would not be anchored to the metal and therefore it would be interesting to study the lipid mobility of such a system. There is also interesting research involving polymer cushions used as a water reservoir for the incorporation of transmembrane proteins into a biomimetic membrane [3]. Any of these model membranes would be interesting to investigate with transmembrane proteins.
6.3 References

