STUDIES OF MOLECULAR MECHANISMS OF SYNTHETIC ANTIMICROBIAL COMPOUNDS USING SUM FREQUENCY GENERATION VIBRATIONAL SPECTROSCOPY

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Chemistry) in The University of Michigan 2011

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Calvin and Hobbes

Everything familiar has disappeared! The world looks brand-new!

Wow, it really snowed last night. Isn't it wonderful?

A new year. A fresh, clean start.

It's like having a big white sheet of paper to draw on.

A day full of possibilities.

It's a magical world, Hobbes. Oh, Buddy...

...let's go exploring!
To my mother, who forced me to always believe;  
my sister, who celebrated what I’ve achieved;  
my father, who helped me to trust in my smarts;  
and my brother, who taught me to follow my heart.
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Not what we give, but what we share,
For the gift without the giver is bare.
~James Russell Lowell

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<th>Description</th>
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<tr>
<td>AIBN</td>
<td>2,2’-azobisisobutyronitrile</td>
</tr>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>attenuated total reflection Fourier transform infrared</td>
</tr>
<tr>
<td>BMA</td>
<td>butylmethacrylate</td>
</tr>
<tr>
<td>Boc-AEMA</td>
<td>N-tert-butoxycarbonyl-aminoethylmethacrylate</td>
</tr>
<tr>
<td>BzMA</td>
<td>benzylmethacrylate</td>
</tr>
<tr>
<td>DFG</td>
<td>difference frequency generation</td>
</tr>
<tr>
<td>DMA</td>
<td>dopamine methacrylamide</td>
</tr>
<tr>
<td>DMAEMA</td>
<td>2-(dimethylamino)ethyl methacrylate</td>
</tr>
<tr>
<td>DMAEMAC(_{12})</td>
<td>dodecyl quaternary ammonium monomer</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylfuran</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
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<td>DOPG</td>
<td>1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>d-DPSG</td>
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<tr>
<td>DSPG</td>
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</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>HBS</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid</td>
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<td>IR</td>
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<td>K*DP</td>
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<td>LB</td>
<td>Langmuir-Blodgett-Schaefer method</td>
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<td>Muller Hinton</td>
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<td>minimum inhibitory concentration</td>
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<td>MMA</td>
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</tr>
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<td>MMP</td>
<td>methyl 3-mercapto-propionate</td>
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<tr>
<td>SFG</td>
<td>sum-frequency generation</td>
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<tr>
<td>TFMBA</td>
<td>4-(trifluoromethyl)benzyl alcohol</td>
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<td>XPS</td>
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ABSTRACT

Studies of Molecular Mechanisms of Synthetic Antimicrobial Compounds Using Sum Frequency Generation Vibrational Spectroscopy

by

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The rise in bacterial resistance to antibiotics has spurred a significant amount of research intended to find an alternative approach, one of the most promising being antimicrobial peptides (AMPs). AMPs exhibit diverse structures, broad-spectrum activity, and a unique interaction mechanism. However, AMPs are also expensive to synthesize or isolate, susceptible to proteolysis, and have poorly understood pharmacokinetics. As a result, synthetic antimicrobial compounds exhibiting similar activity to their natural predecessors have become attractive as potential antibiotics. In this thesis, guidelines for future rational design and development of antimicrobial compounds are developed through studies of synthetic antimicrobial compounds.

First, antimicrobial arylamide oligomers, structured to mimic the inherent global amphiphilic structure of naturally-occurring AMPs are characterized, using both
biological and spectroscopic analyses. The interaction mechanism of each oligomer is
determined using bacterial, erythrocyte and mixed membrane models, and orientation of
each active oligomer as it interacts with the bilayer is determined. Oligomers are shown
to orient approximately parallel to the bilayer surface normal, cutting into the bilayer in a
“molecular knife” style of interaction, showing these oligomers mimic the functionality
of naturally-occurring AMPs. Second, antimicrobial random co-polymers containing
varying lipophilic side chains are characterized using both biological and spectroscopic
methods. Polymers with different lipophilic chains show vastly different interaction
patterns, indicating the importance of the identity of the lipophilic chain, and providing
insight into rational design considerations. Lipophilic groups structurally similar to
membrane constituents are best able to effectively disrupt the lipid bilayer, and tuning
amphiphilic balance can both help and hinder the polymer’s ability to interact with the
bilayer. Finally, a random tri-polymer is coated onto a surface and characterized.
Results indicate a strong tendency for surface dominance by lipophilic alkyl chains, with
increasing surface coverage of those chains resulting in increased disorder both in terms
of defects within the alkyl chain and orientation of the terminal methyl group. This
disorder is attributable to steric hindrance, which may have significant implications for
the surface’s antimicrobial character. Taken together, these studies provide a model for
the rational design of synthetic antimicrobial compounds for a variety of applications.
CHAPTER 1

INTRODUCTION

1.1 Motivation and Background

An interface is defined as “a surface regarded as the common boundary of two bodies, spaces, or phases.”\(^1\) In a sense, most chemical reactions can be understood to exist at surfaces, as a majority of reactions occur between two or more molecules upon contact. Interfacial properties have long been a focus in colloidal science, and as biotechnology has rapidly advanced, biointerfaces (interfaces between biomolecules) have gained importance in many fields. In medical science, much research is underway that focuses on the biocompatibility of artificial materials. Biocompatibility is controlled by the interfacial interactions between the biomaterial and the body, which is crucial in determining the performance of artificial implant devices in the bodies of patients.\(^2-^4\) In biochemistry, interfacial structure, organization, and interaction mechanisms are being studied to understand the basis of interactions of cellular membranes with the surrounding environment. Understanding these interactions, and where and how they can break down, is critical to our understanding of diseases.\(^5-^6\) Marine biofouling, or the gradual accumulation of organisms such as algae, bacteria, barnacles and protozoa on underwater equipment, pipes or other artificial surfaces, can cause corrosion and ultimately failure of these structures.\(^7\) It has been estimated that costs associated with
biofouling, such as maintenance of ship hulls and the degradation of fuel efficiency as a result of the loss of hydrodynamic efficiency, are annually in the billions.\textsuperscript{8}

Surfaces and interfaces, including polymer-based examples, have been studied extensively by a variety of methods. However, most conventional techniques are unable to provide detailed molecular information specifically and solely about that surface or interface. X-ray photoelectron spectroscopy (XPS), an elemental technique, is one of the most surface-sensitive techniques available to researchers. Unfortunately, regular XPS requires a high vacuum environment, rendering it unusable for biological studies of the interaction mechanisms between cellular membranes and their aqueous surroundings \textit{in situ} in real time. Vibrational techniques, such as attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) and Raman spectroscopy have been used to study surfaces and interfaces, however neither of these techniques have a high intrinsic surface or interfacial sensitivity. Further, many interactions between cellular membranes and membrane-active peptides are thought to be dependent upon orientation of the peptide relative to membrane, which is difficult to probe using such techniques. While some techniques do exist to address this, such as polarized IR and Raman, their sensitivity is less than ideal.

Sum frequency generation (SFG) vibrational spectroscopy is a powerful analytical technique to study surfaces and buried interfaces at the molecular level \textit{in situ}. SFG has been used to study antimicrobial peptides, polymer surfaces and buried interfaces, polymer restructuring in water, and lipid bilayers, among other applications. Sections 1.1.1 through 1.1.4 detail the unique properties of each of these systems. The details of SFG are presented in Section 1.2, describing how the inherent surface selectivity of SFG
can be attributed to its selection rules. These rules make SFG an ideal tool for studying antimicrobial peptide-mimetics on surfaces and in cellular membranes. Section 1.3 reviews prior research on peptide mimetics, providing the foundation for the current work presented in this dissertation, which is summarized in Section 1.4.

1.1.1 Antimicrobial Peptides

Interactions between a membrane bilayer and biological molecules such as peptides and proteins are omnipresent throughout a cell. Each one of those proteins and peptides is designed to interact with lipid bilayers in a precise manner. Some proteins interact very weakly or even repel bilayers, while others are integral to the membrane itself and bind very tightly. Antimicrobial peptides (AMPs) are inherently structured to interact very strongly with certain types of membrane bilayers so that they can disrupt the membrane and disable their natural function as a barrier to the external environment. However, the actual structures of AMPs are complex, and to use such molecules in applicable studies can be quite expensive. The high manufacturing cost, susceptibility to proteolysis, and lack of understanding of their pharmacokinetics are roadblocks to implementation of AMPs in a broader capacity. It is therefore desirable to use synthetic compounds that mimic the structure of AMPs without the complexity of structure and experimental use associated with AMPs. It has been demonstrated that such structures can be designed with broad-spectrum activity and potency, and that these molecules can discriminate between eukaryotic and prokaryotic cells.

1.1.2 Peptide-mimetic Synthetic Antimicrobial Compounds

The growth in research of AMPs has happened in parallel to research into antimicrobial polymers for use as surface coatings. These polymers tend to have a high
molecular weight and use cationic quaternary ammonium salt units with long hydrophobic alkyl chains as biocidal moieties. These polymers are often referred to as “polymer disinfectants” and kill bacteria via the same mechanism as AMPs: membrane disruption. A wide variety of these polymers have been documented as antimicrobials. Typically, these polymer disinfectants are immobilized on a surface, yielding a self-sterilizing antibiotic surface, which makes them ideal for use in a myriad of applications, such as sterile hospital surfaces, water purification devices, or disinfecting textiles. The advantage of using these types of compounds is that they are not hindered by the limitations currently preventing use of AMPs on a broader scale. However, use of high molecular weight polymers is limited in that they are often indiscriminately toxic to all cells, not selective to bacterial cells over erythrocytes, as are AMPs.

Peptide-mimetic antimicrobial compounds are a new class of antimicrobial synthetics that combine the design principles gleaned from AMP research with the inexpensive and highly customizable methods of production for high molecular weight polymers. Some initial research on these compounds indicate that they exhibit the same selectivity as do naturally occurring AMPs, and often so do at a higher activity level. These compounds hold a great deal of promise for future development of biocidal materials. However, in order to be rationally designed for implementation, their mechanisms need to be better understood at a molecular level. Such information will allow for a more targeted, intentional approach when applying these compounds in a real-world setting.
1.1.3 Lipids and Lipid Bilayers

Lipids are a broad group of naturally occurring molecules that play a vital role in biological systems. They are the main structural components of cellular membranes, serving as the basis for the bilayer system. Structurally, lipids are amphiphilic molecules, containing a hydrophilic (typically charged or zwitterionic) headgroup and hydrophobic tail groups. These molecules have a natural tendency to coalesce and segregate into structures that allow the hydrophobic sections to associate with each other while exposing only the hydrophilic headgroup to the general aqueous environment. This can take the form of vesicles (micelles and liposomes) or bilayer sheets, as shown in Figure 1.1. Bilayer sheets contain two oppositely oriented lipid leaflets, resulting in a hydrophobic core sandwiched between two hydrophilic layers.

Figure 1.1 Diagram of phospholipids self-organizing into one of several states: a) a liposome, a spherical structure with a double lipid layer and a hydrophilic core; b) a micelle: a spherical structure with hydrophobic core; and c) a bilayer sheet, a planar structure with a hydrophobic core between two hydrophilic layers.

Lipids are not static within a bilayer; they are capable of moving and do diffuse throughout the system. This movement can be in two forms: lateral diffusion within an individual leaflet or transverse diffusion from one leaflet to the other. Lateral diffusion is common, as lipids are highly mobile in the plane of the bilayer, as has been demonstrated
via fluorescence photobleaching recovery studies.\textsuperscript{19} Transverse diffusion, often referred to as “flip-flop,” is the movement of a lipid molecule across the core of the bilayer to the opposing leaflet. In biological systems, this is an extremely rare event. Flip-flop requires the hydrophilic headgroup to pass through the hydrophobic core, which is energetically unfavorable. Reaction rates of flip-flop in biological systems have been measured by half-times that are minimally several days.\textsuperscript{19}

Bilayers can be defined to exist in specific “phases,” differentiated by these diffusion rates. Rates of movement of lipids within a bilayer is a powerful way to compare different bilayer systems, because these rates are reflective of inherent properties of the bilayer itself. Broadly, a bilayer can essentially exist in a solid phase or a liquid phase, both of which are defined by the mobility constraints placed upon the lipids. The solid phase is often referred to as the “gel” phase. These phases are separated by what is called the characteristic transition temperature ($T_m$) for the bilayer. Above the $T_m$, the bilayer exists in the liquid phase; lipids have a high degree of fluidity and are highly mobile in the bilayer plane. Below the $T_m$, the bilayer becomes a gel-like solid, with lipids far more densely packed and impeded in their lateral movement. It is worth noting that this change in state is not representative of the technical definition of a phase change. Rather, this is an order-disorder transition. The phase a bilayer exists in can have massive repercussions on the interaction mechanism of AMPs, and will be taken into account in the research presented here.

\textbf{1.1.4 Solid-Supported Lipid Bilayers}

Lipids are highly insoluble in aqueous solutions and have a strong tendency to form either vesicles or to segregate to a surface and form a lipid monolayer. If lipids
dissolved in an organic solution (chloroform is commonly used) are spread over an aqueous surface, a lipid monolayer will readily form as the organic solvent evaporates. This is the basis of the Langmuir-Blodgett-Schaeffer (LB) method, a well-known method of applying a lipid monolayer to a particular surface or substrate. With a Langmuir trough, surface pressure of the lipid film is monitored with a Wilhelmy plate while the molecular area of the surface is controlled via movable barriers along the aqueous surface. Phospholipids in biological studies are usually in a liquid-crystal or gel phase, as described in Section 1.1.3. Typical surface pressures in biological studies are around 30-34 mN/m.\\(^{20}\)

First introduced by Tamm and coworkers in 1985, solid-supported lipid bilayers have been the subject of a vast array of studies, and have been proven to be excellent models for studying biological membranes.\\(^{20-25}\) The two most commonly used methods for preparing solid-supported lipid bilayers are the LB method (which is used extensively in Chapters 2 and 3) and vesicle fusion (not presented here). Under the normal conditions of an SFG bilayer experiment, the SFG process is forbidden because a symmetric bilayer contains inversion symmetry. Bilayers containing two structurally different lipid leaflets can often get around this issue, however even in this case the alkyl chains will still likely have inversion symmetry. Isotopic labeling can break the inversion symmetry of a bilayer, allowing SFG to be used to study the system. Deuteration of the alkyl chains of one leaflet, leaving the other leaflet's alkyl chains hydrogenated, is therefore a common technique to generate SFG signal from a bilayer. Such a structured bilayer is referred to as “asymmetric.”
The use of such bilayers has become a common technique. Multiple studies have been performed in recent years using both symmetric and asymmetric bilayers as models for cell membranes with AMPs. Several of these studies form the basis of the research presented here, and are discussed in detail in Section 1.3.

1.2 Sum Frequency Generation Vibrational Spectroscopy

SFG vibrational spectroscopy is a highly useful, unique technique that has emerged as a valuable tool to study surfaces and interfaces at the molecular level.\textsuperscript{26-28} It combines two other well-known spectroscopic techniques, infrared (IR) and Raman spectroscopy to yield a new technique that is capable of looking at very specialized aspects of chemical systems. More recently, SFG has been applied to studies of polymer surfaces and interactions between lipid bilayers and their surrounding environment. As it is a laser technique, any surface or interface accessible by light can be probed using SFG, making it an ideal tool for investigating the interactions under study here.

Polymers, and more recently lipid bilayers, have been extensively studied in the past few decades by several different methods, including XPS, ATR-FTIR, and Raman spectroscopy. While all of these methods are useful, each has some drawback, be it physically, such as requiring a vacuum environment in the case of XPS, or lacking intrinsic surface or interface sensitivity, as in the cases of ATR-FTIR and Raman.\textsuperscript{29-31} Since the focus of this research is to investigate interactions between antimicrobial agents and polymer or bilayer surfaces, the ability to probe surface interactions with a high degree of sensitivity \textit{in situ} is crucial to properly understanding those interactions.
1.2.1 Brief Overview

Optical SFG describes the process in which two input laser beams of frequencies $\omega_1$ and $\omega_2$ overlap in a medium, which then generates an output beam with the sum of the two input frequencies ($\omega_{SF} = \omega_1 + \omega_2$).\textsuperscript{32-33} This process is diagrammed below in Figure 1.2. For IR-visible SFG, $\omega_1$ describes the input visible beam, which is maintained at a fixed frequency, and $\omega_2$ describes the tunable input IR beam. When $\omega_2$ is scanned over the vibrational resonances of the molecules of the material under study, the SFG signal becomes resonantly enhanced, thus generating a vibrational spectrum of the sample.

![Figure 1.2 The SFG geometry. The reflected and transmitted IR and visible beams and the transmitted SFG beam have been omitted for clarity.](image)

Sum frequency generation is a second-order nonlinear optical process, and under the electric-dipole approximation, SFG is forbidden in media that possess inversion symmetry. Most bulk material, such as polymers or pure liquid samples are centrosymmetric and therefore do not exhibit an SFG signal. However, at interfaces between sample layers, or at sample surfaces, the symmetry of the bulk is broken and an SFG signal can be detected. SFG is also known to be submonolayer surface-sensitive, a determination that was made based on both theoretical calculations and experimental evidence.\textsuperscript{27-28,32} In addition, the orientation and orientational distribution of functional
groups on the sample surface can be deduced by collecting SFG spectra using different polarization combinations of the three input and output beams.

1.2.2 Basic Theory

When a molecule is placed in a weak electric field, the polarization $P$, also called the oscillating dipoles per unit volume of molecule, is proportional to the strength of the electric field $E$. This relationship is described by

$$P = \chi^{(1)} E$$

(1.1)

where $\chi^{(1)}$ is the linear susceptibility. In the intense laser light, the external electric field becomes comparable to the fields experienced by an electron in a molecule, and the linear polarization of matter is no longer an appropriate approximation. Higher order terms must then be taken into account. Using the electric dipole approximation, the total polarization can be described by

$$P = P^{(1)} + P^{(2)} + P^{(3)} + ... = \chi^{(1)} E + \chi^{(2)} : EE + \chi^{(3)} : EEE + ...$$

(1.2)

where the second-order nonlinear susceptibility, $\chi^{(2)}$, is a third-rank tensor, the third-order susceptibility, $\chi^{(3)}$, is a fourth-rank tensor, as so on.\textsuperscript{34} When two light waves, one of frequency $\omega_1$ and amplitude $E_1$, the other with frequency $\omega_2$ and amplitude $E_2$, mix in a medium, a nonlinear polarization is induced. This is described by

$$P^{(2)} = \chi^{(2)} : E_1 \cos(\omega_1 t) E_2 \cos(\omega_2 t)$$

(1.3)

By applying the correct trigonometric identities, the following expression can be derived from Equation (1.3):

$$P^{(2)} = \frac{1}{2} \chi^{(2)} : E_1 E_2 \left[ \cos(\omega_1 + \omega_2) t + \cos(\omega_1 - \omega_2) t \right]$$

(1.4)
Equation (1.4) shows that, in this situation of two large external electric fields, two oscillating dipoles form at frequencies $\omega_1 + \omega_2$ (the sum) and $\omega_1 - \omega_2$ (the difference). This gives rise to SFG and difference frequency generation (DFG) signals, respectively.\textsuperscript{34}

The intensity, $I$, of the emitted light depends on $\left| P^{(2)} \right|^2$. So, for SFG:

$$I(\omega_{\text{SF}}) \propto \chi^{(2)}_{ij} I(\omega_i) I(\omega_j)$$

(1.5)

where $\omega_{\text{SF}} = \omega_1 + \omega_2$, $I(\omega_i) = |E_i|^2$, and $I(\omega_j) = |E_j|^2$.\textsuperscript{33}

As a third-rank tensor, $\chi^{(2)}$ contains 27 elements. An SFG experiment essentially measures the non-zero components of $\chi^{(2)}$. In a centrosymmetric environment the inversion operation mandates that

$$\chi^{(2)}_{ijk} = -\chi^{(2)}_{-i-j-k}$$

(1.6)

where $ijk$ refers to coordinates in the laboratory’s frame of reference. However, $\chi^{(2)}_{ijk}$ is a third-rank polar tensor, so a change in the sign of the three subscripts is simply equivalent to reversing the axis system, and therefore the physical phenomenon $\chi^{(2)}_{ijk}$ describes must reverse sign. Equation (1.7) shows this relationship.

$$\chi^{(2)}_{ijk} = -\chi^{(2)}_{-i-j-k}$$

(1.7)

Therefore, in a centrosymmetric medium, $\chi^{(2)}_{ijk}$ must be equal to zero to satisfy both Equations (1.6) and (1.7), and by extension, SFG is forbidden. The majority of bulk phases are centrosymmetric, but the boundary between two materials is inherently non-centrosymmetric and consequently is SFG active.

In addition to the symmetry constraints, it is necessary that the two input beams also overlap spatially and temporally to attain an SFG signal. SFG is a coherent process
and accordingly, the generated light has a definite direction. Specifically, the output SFG beam is generated at an angle $\theta_{\text{SF}}$ to the surface normal, as shown in Figure 1.2. By applying conservation of momentum parallel to the interface, also known as the phase-matching condition, we get

$$n_{\text{SF}} \omega_{\text{SF}} \sin \beta_{\text{SF}} = n_1 \omega_1 \sin \beta_1 \pm n_2 \omega_2 \sin \beta_2$$  \hspace{1cm} (1.8)

or

$$n_{\text{SF}} k_{\text{SF}} \sin \beta_{\text{SF}} = n_1 k_1 \sin \beta_1 \pm n_2 k_2 \sin \beta_2$$  \hspace{1cm} (1.9)

where $n$ is the refractive index of the medium through which the relevant beam propagates, $\omega$ is the beam’s frequency, $\beta$ is the angle of each beam with respect to the surface normal (the $z$-axis in Figure 1.2), and $k$ is equal to $\omega/c$, where $c$ is the speed of light. When the two input beams are arriving at the surface from the same $x$-direction (called co-propagating), as they are in Figure 1.2, the positive sign is used in Equations (1.8) and (1.9). The negative sign is used for counter-propagating input (arriving from opposite $x$-directions) beams. SFG light is then both transmitted into and reflected from the surface.

In the reflected direction, the intensity of the sum frequency is given by

$$I(\omega_{\text{SF}}) = \frac{8 \pi^3 \omega_2 \sec^2 \beta_{\text{SF}}}{c^3 n_1(\omega_{\text{SF}}) n_1(\omega_1) n_1(\omega_2)} |\chi^{(2)}_{\text{eff}}|^2 I_1(\omega_1) I_2(\omega_2)$$  \hspace{1cm} (1.10)

where $n_i(\Omega)$ is the refractive index of medium $i$ at frequency $\Omega$, $\beta_{\text{SF}}$ is the reflection angle of the sum frequency field, and $I_1(\omega_1)$ and $I_2(\omega_2)$ are the intensities of the two input beams. The effective second-order nonlinear susceptibility tensor, $\chi^{(2)}_{\text{eff}}$, of the surface is related to the second order nonlinear susceptibility tensor, $\chi^{(2)}$, in the lab coordinate system and takes the form
\[ \chi^{(2)}_{\text{eff}} = \left[ \hat{e}(\omega_{\text{SF}}) \cdot L(\omega_{\text{SF}}) \right] \cdot \chi^{(2)} \cdot \left[ L(\omega_i) \cdot \hat{e}(\omega_i) \right] \left[ L(\omega_j) \cdot \hat{e}(\omega_j) \right] \]  \hspace{1cm} (1.11)

with \( \hat{e}(\Omega) \) being the unit polarization vector and \( L(\Omega) \) being the Fresnel factor at frequency \( \Omega \). Therefore, different tensor components of \( \chi^{(2)} \) can be elucidated from different components of \( \chi^{(2)}_{\text{eff}} \). Although \( \chi^{(2)} \) is a third rank tensor (thus giving it 27 different elements), in the case of azimuthally isotropic interfaces there are only four independent non-vanishing components of \( \chi^{(2)} \). When choosing lab coordinates such that the z-axis is along the interface normal and the x-axis is in the incident plane (as in Figure 1.2), these components are \( \chi_{xx} = \chi_{yy}, \chi_{xx} = \chi_{yy}, \chi_{zz} = \chi_{yy} \) and \( \chi_{zz} \). These four components of \( \chi^{(2)} \) can be deduced by collecting SFG spectra with four different polarization combinations of the input and output beams. These polarization combinations are ssp (s-polarized SFG output, s-polarized \( E_1 \) input, and p-polarized \( E_2 \) input), sps, pss, and ppp. The expressions for the effective second-order nonlinear susceptibilities for each of these four polarization combinations are:

\[ \chi^{(2)}_{\text{eff,ssp}} = L_{yy}(\omega_{\text{SF}}) L_{yy}(\omega_1) L_{zz}(\omega_2) \sin \beta_2 \chi_{yyz} \]  \hspace{1cm} (1.12a)

\[ \chi^{(2)}_{\text{eff,sps}} = L_{yy}(\omega_{\text{SF}}) L_{zz}(\omega_1) L_{yy}(\omega_2) \sin \beta_2 \chi_{yy} \]  \hspace{1cm} (1.12b)

\[ \chi^{(2)}_{\text{eff,pss}} = L_{zz}(\omega_{\text{SF}}) L_{yy}(\omega_1) L_{yy}(\omega_2) \sin \beta_2 \chi_{yy} \]  \hspace{1cm} (1.12c)

\[ \chi_{ppp} = -L_{xx}(\omega_{\text{SF}}) L_{xx}(\omega_1) L_{xx}(\omega_2) \cos \beta_1 \sin \beta_2 \chi_{xx} \\
- L_{xx}(\omega_{\text{SF}}) L_{zz}(\omega_1) L_{xx}(\omega_2) \cos \beta_1 \sin \beta_2 \chi_{xx} \\
+ L_{zz}(\omega_{\text{SF}}) L_{xx}(\omega_1) L_{xx}(\omega_2) \sin \beta_2 \chi_{xx} \\
+ L_{zz}(\omega_{\text{SF}}) L_{zz}(\omega_1) L_{zz}(\omega_2) \cos \beta_1 \cos \beta_2 \chi_{zzz} \]  \hspace{1cm} (1.12d)

In all the above equations, the \( \beta_i \)'s are the incident angles of the relevant optical fields \( E_{i} \), and \( L_{xx}(\Omega), L_{yy}(\Omega) \) and \( L_{zz}(\Omega) \) are the Fresnel coefficients for beam \( \Omega \), which are given by
\[ L_{xx}(\Omega) = \frac{2n_1(\Omega)\cos \gamma}{n_1(\Omega)\cos \gamma + n_2(\Omega)\cos \beta} \]  \hspace{1cm} (1.13a)

\[ L_{yy}(\Omega) = \frac{2n_1(\Omega)\cos \beta}{n_1(\Omega)\cos \beta + n_2(\Omega)\cos \gamma} \]  \hspace{1cm} (1.13b)

\[ L_{zz}(\Omega) = \frac{2n_1(\Omega)\cos \beta}{n_1(\Omega)\cos \gamma + n_2(\Omega)\cos \beta} \left( \frac{n_1(\Omega)}{n'(\Omega)} \right)^2 \]  \hspace{1cm} (1.13c)

where \( n'(\Omega) \) is the refractive index of the interfacial layer, \( \beta \) is the incidence angle of the beam in question, and \( \gamma \) is the refracted angle given by Equation (1.14).\(^{35}\)

\[ n_1(\Omega)\sin \beta = n_2(\Omega)\sin \gamma \]  \hspace{1cm} (1.14)

In the case where SFG is being used to study molecules at an interface or surface, \( \chi^{(2)} \) is related to the molecular nonlinear polarizability, \( \alpha^{(2)} \), through a coordinate transformation averaged over the molecular orientational distribution. This distribution is given by

\[ \chi^{(2)}_{ijk} = N_s \sum_{lmn} \left( \hat{i} \cdot \hat{a} \right) \left( \hat{j} \cdot \hat{b} \right) \left( \hat{k} \cdot \hat{c} \right) \alpha^{(2)}_{lmn} \]  \hspace{1cm} (1.15)

where \( N_s \) is the surface density of molecules and (i,j,k) and (a,b,c) are the unit vectors along the lab and molecular coordinates, respectively. For IR-visible SFG when the IR frequency (\( \omega_2 \)) is near a vibrational resonance \( \alpha^{(2)} \) and \( \chi^{(2)} \) can be written as

\[ \alpha^{(2)} = \alpha^{(2)}_{NR} + \sum_q \frac{\alpha_q}{\omega_2 - \omega_q + i\Gamma_q} \]  \hspace{1cm} (1.16)

and

\[ \chi^{(2)} = \chi^{(2)}_{NR} + \sum_q \frac{\chi_q}{\omega_2 - \omega_q + i\Gamma_q} \]  \hspace{1cm} (1.17)

where the subscript NR designates the nonresonant contribution, \( \alpha_q \) (and \( \chi_q \)), \( \omega_q \), and \( \Gamma_q \) denote the strength, resonant frequency, and damping constant of the \( q^{th} \) vibrational...
mode, respectively. By obtaining different \((\chi_q)_{ijk}\) components from the resonant features associated with any particular molecule or chemical group on the surface in the SFG spectra collected using different polarization combinations of the input and output beams, the average orientation of that moiety can be determined if \((\alpha_q)_{abc}\) is known.

As an example, a common application of this method in this lab is to use the above information to determine the orientation of terminal methyl groups on a surface. It is reasonable to assume that the polymer surface of something such as poly(methyl methacrylate) (PMMA) is azimuthally isotropic; therefore the angle \(\theta\) between the surface normal and the principal axis of the methyl group can be used to describe its orientation. In addition, the polymer surface may not be fully ordered, so the molecular orientation can be characterized by the distribution function \(f(\theta)\) of the orientation angles.

As shown in Equation (1.15), the measured elements of \(\chi^{(2)}_{ijk}\) determined in the laboratory frame can be expressed in terms of the molecular hyperpolarizability tensor elements \(\alpha_{abc}\) described with respect to the molecular frame. The resulting expressions carry the orientation information obtainable from SFG measurements, and are shown below. Equations (1.18) and (1.19) describe the symmetric stretching mode of the methyl group.

\[
\chi_{yyz,s}^{(2)} = \int_0^\pi \frac{1}{2} N_s \alpha_{ccc,s} \left[ \cos \theta (1 + r) - \cos^3 \theta (1 - r) \right] f(\theta) \sin \theta d\theta
\]

\[
\chi_{yyz,as}^{(2)} = \int_0^\pi \frac{1}{2} N_s \alpha_{cann,as} \left[ \cos \theta - \cos^3 \theta \right] f(\theta) \sin \theta d\theta
\]

Equations (1.19) and (1.20) describe the asymmetric stretching mode.

\[
\chi_{yyz,as}^{(2)} = \int_0^\pi \frac{1}{2} N_s \alpha_{cann,as} \left[ \cos \theta - \cos^3 \theta \right] f(\theta) \sin \theta d\theta
\]
\[ \chi_{y\gamma,\alpha}^{(2)} = \int_0^{\pi} \frac{1}{2} N_s \alpha_{\alpha,\alpha,\alpha} (\cos^3 \theta) f(\theta) \sin \theta d\theta \] (1.21)

where \( \alpha_{\alpha,\alpha,\alpha} \) and \( \alpha_{\alpha,\alpha,\alpha} \) are nonvanishing stretching vibration hyperpolarizability tensor elements of the methyl group, \( r = \alpha_{\alpha,\alpha,\alpha} / \alpha_{\alpha,\alpha,\alpha} \), and \( N_s \) is the surface density of methyl groups. From the evaluation of Equations (1.18) and (1.19) it follows that the measured tensor elements \( \chi_{ij}^{(2)} \) are linear combinations of \( \left\langle \cos \theta \right\rangle \) and \( \left\langle \cos^3 \theta \right\rangle \). The brackets reflect the fact that the tensor elements \( \chi_{ij}^{(2)} \) are made of up average values of the cosine functions, rather than single values. Thus, evaluation of the above equations allows for the average value of the cosine of the angle relative to the surface normal of the moiety under study to be obtained, and the value of \( \theta \) can be determined.

The resonant portion of Equation (1.16) is directly proportional to the product of the IR dipole derivative and the Raman polarizability tensor of the vibrational mode \( q \). This relationship is described below.

\[ \alpha_{R}^{(2)} \propto \frac{\partial \mu_{IR}}{\partial Q} \frac{\partial \alpha_{Raman}}{\partial Q} \] (1.22)

As a result of what is seen above in Equation (1.22), only those vibrational modes that are both IR-active and Raman active will be SFG-active. Figure 1.3, shown below, depicts a schematic energy level diagram for the IR-visible SFG process.
1.2.3 Experimental System

A schematic diagram of the SFG laser system used in this lab is displayed below in Figure 1.4. It is a custom-designed EKSPLA SFG spectrometer. The 20 picosecond mode-locked Nd:YAG laser has a fundamental output of 1064 nm and a repetition rate of 20 Hz. The fundamental beam from the laser is directed to the harmonics unit, where two K*DP nonlinear crystals produce the second and third harmonics, which are at 532 nm and 355 nm, respectively. The 532 nm beam serves as the visible beam for the SFG experiment. The 355 nm beam, along with the fundamental 1064 nm beam pump the optical parametric generation/amplification and difference frequency generation system (OPG/OPA/DFG) which is based on LBO and AgGaS$_2$ crystals. The IR beam generated from the OPG/OPA/DFG is tunable from 2.3 to 10 µm (4300 to 1000 cm$^{-1}$).$^{37-39}$
The SFG system can be conceptually divided into two sections. The first section, shown in the right in Figure 1.4, is the fundamental portion of the system which actually generates the laser beams used in the course of SFG experimentation. The three main components of this first section are the Nd:YAG laser, the harmonics unit, and the optical parametric oscillator/amplifier and difference frequency generator, referred to in Figure 1.4 as the OPG/OPA/DFG. The second section, shown on the left in Figure 1.4, is the section where the sample itself is exposed to the beams and SFG signal is generated and collected, and the geometry of this section can be changed depending on what experiments are being run. What follows is a brief description of how the three fundamental components of the first section are and how they are used.

1.2.3.1 Nd:YAG Laser

Neodymium-doped yttrium aluminum garnet (Nd:Y\textsubscript{3}Al\textsubscript{5}O\textsubscript{12}) is a crystal widely used as the active laser medium for solid-state lasers. The laser is optically pumped using
flashlamps and emits light with a wavelength of 1064 nm. As a Nd:YAG laser, it is operated using passive mode-locking. The light generated in the laser cavity is passed through a laser dye, which acts as a saturable absorber. The dye exhibits an intensity-dependent transmission, meaning it behaves differently depending on the intensity of the light passing through it. For passive mode-locking, ideally a saturable absorber will selectively transmit high intensity light while absorbing low intensity light. However, when the laser is initially triggered, it is not initially mode-locked. Any un-modelocked laser will experience random, intense spikes in radiation, and those spikes will be preferentially transmitted by the dye. As the light in the laser cavity oscillates, this process repeats, which leads to the selective amplification of the high intensity spikes and the suppression by absorption of the low intensity light. After many round trips in the cavity, this leads to a train of pulses and the mode-locking of the laser. In addition, Pockel cells are also used in the laser cavity to assist in the generation a fundamental laser beam at a sufficiently high intensity.\textsuperscript{37}

1.2.3.2 Harmonics Unit

The harmonics unit is important to the SFG laser setup because it generates the visible beam for SFG experiments. It also produces a 355 nm beam for the OPG/OPA/DFG system to generate the frequency tunable mid-IR beam. To do this, the fundamental wavelength emitted from the Nd:YAG laser is frequency doubled and tripled by having the beam pass through the nonlinear crystals generating a corresponding harmonic. The wavelengths are summarized below in Table 1.1.
<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Harmonic</th>
<th>Spectral Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1064</td>
<td>Fundamental</td>
<td>Infrared</td>
</tr>
<tr>
<td>532</td>
<td>2\textsuperscript{nd}</td>
<td>Visible (green)</td>
</tr>
<tr>
<td>355</td>
<td>3\textsuperscript{rd}</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>

Table 1.1 Summary of wavelengths generated and used in SFG laser setup.

The harmonic radiation propagates collinearly with the fundamental beam. To maximize the efficiency of the conversion to harmonics, it is necessary for the path of the input fundamental beam to lie along a unique axis of fixed orientation relative to the axis of the crystal. This condition is referred to as phase matching. The harmonics generation is done in K\textsuperscript{*}DP (potassium dideuterium phosphate) nonlinear crystals, and the second and third harmonics are obtained in the harmonics unit. The purity of the third harmonic is enhanced using a system of two reflections from two mirrors, a step which is necessary because the process of converting to the third harmonic is not 100\% efficient.\textsuperscript{38}

1.2.3.3 OPG/OPA/DFG

The OPG/OPA/DFG system is the device used to generate a tunable IR beam. As previously stated, a tunable IR beam is necessary for SFG experimentation to determine the vibrational resonances of the molecule under study. It functions by combining a lithium borate (LBO) crystal-based optical parametric generator/amplifier and a difference frequency generator on an AgGaS\textsubscript{2} (silver gallium sulfate) crystal, and uses the fundamental and third harmonic beams to generate the tunable IR signal. The 355 nm beam passes through the nonlinear LBO crystal and is separated into two beams, a signal beam and an idler beam. The wavelengths of the two generated beams are determined by the phase matching condition, which can be changed simply by rotating the axis of the crystal. This allows for the tuning of the eventual final beam to a desired wavelength. Once the signal beam is separated from the parametric generator outputs, the desired
wavelength is then passed through the AgGaS$_2$ crystal collinearly with the fundamental beam, resulting in a beam at the difference of the two frequencies. This is also controlled by tilting the axis of the crystal to ensure the beams are collinear. The resulting beam is the tunable IR beam used in the SFG experiment, which ranges from 4300-1000 cm$^{-1}$.\(^{39}\)

1.2.4 Experimental Setup

The visible and tunable IR beams are overlapped both temporally and spatially on the sample with incident beams of 60° and 54°, respectively, versus the surface normal. The energy of the visible beam is approximately 200 $\mu$J, and the energy of the IR beam is approximately 100 $\mu$J. Both beams have a diameter of roughly 0.5 mm. The SFG signal from the sample surface or interface is then collected by a photomultiplier tube and processed with a gated integrator. Two photodiodes are used to monitor the input visible beam and IR beam powers by collecting the back reflections of those two beams from focus lenses. This is extremely important for data collection because the SFG spectra can be normalized by the powers of the input lasers. This allows for comparison between spectra taken at different times and days. The SFG intensity is measured as a function of the input IR frequency, generating a vibrational spectrum of the sample surface or interface. The polarization of the input and output beams can be altered, and by changing the polarizations of those beams, SFG spectra with different polarization combinations can be collected.\(^{28}\) The combinations ssp (s-polarized SFG output, s-polarized visible input, and p-polarized IR input), sps, and ppp are most commonly collected in this lab.

Different geometries can be employed to collect SFG spectra, and the geometry of the sample can have a significant effect on the signal detected. For example, a common method of sample preparation in this lab is spin-coating a thin polymer film onto an
appropriate IR transparent window. That sample may then have signal collected in a face-up or face-down geometry. In the face-up geometry, the input beams overlap on the polymer surface on the top of the window, whereas in the face-down geometry, the two input beams travel through the window and then overlap on the polymer film. This set up is diagrammed below in Figure 1.5.

![Figure 1.5 The face-down SFG experimental geometry. The left side diagrams geometry using a window setup, and the right side diagrams a prism setup.](image)

All the spectra collected during the course of this experimentation were taken in the face-down geometry. This geometry not only allows SFG spectra to be obtained from polymer films in air, but it also allows contact between the polymer film and a liquid,
thus enabling the collection of spectra from polymer/liquid interfaces. Lipid bilayers may also be constructed on the window or prism surface and the face-down geometry facilitates spectra collection from a bilayer interface as well. In addition, the SFG signals using the face-down geometry have been shown to be larger than those using the face-up geometry as a consequence of the Fresnel factors. This signal increase is an especially useful aspect of this experimental setup, because it facilitates detection of signals that would, in any other experimental setup, be too weak to detect. This is especially true for SFG signals using proteins or similar large molecules.

1.3 Prior Work: Molecular Knife-style Interactions of Antimicrobial Compounds with a Lipid Bilayer

Much of the previous work done using SFG to monitor AMP-bilayer interactions has focused on naturally occurring AMPs, such as magainin 2, tachyplesin I, and melittin. As many of those experiments have generally indicated that the global amphiphilic structure of the AMP is largely responsible for its interaction capabilities with a membrane, interest in development of synthetic mimics of antimicrobial peptides has grown. Some initial work has shown that a specifically-designed rigid oligomer with an amphiphilic global structure can interact with a lipid bilayer in a manner similar to a naturally occurring AMP.

Figure 1.6 a) Molecular structure of 1, showing facial amphiphilicity. b) green groups below the central line of the molecule represent hydrophobic groups; blue groups above the central line of the molecule represent positively charged (hydrophilic) groups.
Oligomer 1, shown in Figure 1.6, was intentionally and rationally designed to structurally mimic the amphiphilic structure of natural AMPs – cationic segments are spatially separated from the nonpolar segments. This creates a facially amphiphilic structure, mimicking in a smaller scale the induced amphiphilicity of a natural AMP.²⁰

Figure 1.7 SFG spectra collected from a DPPG/d-DPPG bilayer in the C-D stretching region (left panel) and the C-H/O-H stretching region (right panel) before (a, e), and after contacting 1 at three solution concentrations: (b, f) 0.1 µg/mL; (c, g) 0.8 µg/mL; and (d, h) 4.0 µg/mL.

A 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol/1,2-dipalmitoyl-D62-sn-glycero-3-phosphoglycerol (DPPG/d-DPPG) bilayer was used as a bacterial membrane model for these experiments. The spectral separation of the two leaflet layers allowed for observation of independent effects of 1 on the lipid bilayer. The bilayer was then exposed to concentrations of 1 below, at, and above the minimum inhibitory concentration (MIC) of 1, as shown in Figure 1.7. Spectral signals between 2800 cm⁻¹ and 3000 cm⁻¹ are primarily due to symmetric stretching of the terminal methyl groups of the acyl chains in DPPG (the inner leaflet), while signals between 2000 cm⁻¹ and 2300 cm⁻¹ are contributed from the CD₃ groups on d-DPPG (the outer leaflet). SFG signals of CD₃ decrease for all
concentrations, even at 0.1 µg/mL, indicating that 1 can disrupt the outer leaflet in all the cases. The decrease in SFG signals from CH₃ groups is substantial for high concentrations, but the decrease is minimum at 0.1 µg/mL, indicating that the inner leaflet was not affected at 0.1 µg/mL.

Figure 1.8 SFG spectra collected from 1 adsorbed on to a bilayer. (Left panel) C=O stretching signals collected at three solution concentrations of 1: (a) 0.1 µg/mL; (b) 0.8 µg/mL; and (c) 4.0 µg/mL. (Right panel) C-H stretching signals from 1 at a solution concentration of 0.8 µg/mL using (d) ppp, (e) ssp, and (f) sps polarization combinations. A d-DPPG/d-DPPG symmetric bilayer was used to avoid spectral confusion. The SFG signals are multiplied by 14, 2.5, and 10 respectively for (a), (b), and (f) for comparison purposes.

Because of the regular, predictable structure of 1, all the carbonyl groups and t-butyl groups along the backbone must orient in a single direction. Therefore, if 1 has adopted a specific orientation relative to the bilayer surface normal, SFG signal from those specific moieties should be theoretically observable. Shown in Figure 1.8, signal from each of these moieties was observed, both at multiple concentrations of compound 1 and in spectra collected using different polarization combinations. To determine the orientation of 1 in the bilayer, signal from the methyl symmetric stretch (2907 cm⁻¹) of the t-butyl groups was used. More specifically, the ratio of $\chi_{yyz,as}/\chi_{yyz,s}$ was used to
determine orientation and orientation distribution. The relative values of $\chi_{yzy,as}$ and $\chi_{yzy,s}$ were used to cross-check. The resulting value, along with the possible distributions, are shown in Figure 1.9. Experimentally calculated to be -0.72, the measured ratio corresponds to a restricted range of both the orientation angle and angle distribution around the orientation angle. Oligomer 1 orients at a slight tile away from the surface normal, between two extremes of 35° of the tilt angle with a delta distribution and 0° tilt angle with an orientation distribution of 30°. A similar analysis of the C=O stretching signals confirmed these results. This more or less perpendicular orientation of 1 indicates a very specific interaction mechanism, one where the oligomer inserts into the bilayer in a similar way to a knife cutting into food. We have therefore termed this a “molecular knife” interaction mechanism.  

Figure 1.9 Relation between the $\chi_{yzy,as}/\chi_{yzy,s}$ and the orientation distribution of the tert-butyl group. $\chi_{yzy,as}/\chi_{yzy,s}$ was experimentally found to be -0.72, which is marked in the figure by the shaded area.

1.4 Presented Research in This Dissertation

The presented research in this dissertation continues these initial investigations of synthetic antimicrobial compounds. The inherent surface and interface sensitivity of SFG
will be used to understand how synthetic antimicrobial mimics of AMPs interact with a solid-supported lipid bilayer and what effect different function groups can have on the mimic’s activity and structural organization in situ.

In Chapter 2, several antimicrobial mimics similar in structure to oligomer 1 will be exposed to isotopically-labeled bacterial and erythrocyte membrane models to investigate activity and toxicity. Bilayers of mixed phosphoglycerol (PG), phosphoethanolamine (PE) and phosphocholine (PC) lipids will also be used to understand this effect. Orientation of the oligomers in bacterial membrane models will be determined by analysis of multiple polarization combinations of SFG signal observed from the carbonyl groups aligned along the oligomer backbone. Trifluoromethyl signal will also be observed from oligomer 3. It will be determined that oligomers 2 and 3 are active, whereas 4 is non-active, and all three oligomers are non-toxic to erythrocyte cells. Further, orientation analysis will show that 2 and 3, when interacting with a bilayer, orient in such a way as to align the carbonyl groups along the oligomer backbone with the surface normal of the bilayer. This mode of interaction was also previously seen in studies of 1, which is referred to as a “molecular knife” interaction.

The work in Chapter 2 shows that by intentionally altering the bilayer-active moieties along the outer edge of the oligomer, activity and toxicity of the membrane-active compound can be controlled. Chapter 3 considers this and expands upon it, investigating whether the central core of the oligomer, which acts as a basic structure upon which the moieties around it built upon, is necessary for a compound to have specific interactions with lipid bilayers.
Chapter 3 thus investigates the interaction between bacterial membrane models and antimicrobial polymers. Random co-polymers were synthesized to have both cationic and lipophilic subunits. The identity of the lipophilic subunit will be varied to determine the effect different moieties may have on polymer activity. Dye-leakage and biological studies will show clear differences in polymer activity, and SFG studies will confirm these results. Isotopically-labeled bilayers will be used to show clear differences in the interaction mechanism of two of the polymers. The polymer containing a benzyl ring as a lipophilic constituent will be shown to interact via a partitioning mechanism, where the outer leaflet is disrupted with no corresponding disturbance of the inner leaflet. In contrast, the polymer containing a butyl chain as a lipophilic constituent will be shown to disrupt both leaflets simultaneously, indicating faster partitioning into the whole of the bilayer. In addition, it will be shown via orientation analysis of the butyl-containing polymer that the lipophilic butyl chain orients parallel to the surface normal of the bilayer. This interaction may indicate that those polymers with lipophilic side chains most similar to the lipophilic core of the bilayer may be most effective at penetrating the membrane. Such an understanding of how the lipophilic constituents organize within the bilayer matrix stands to be useful for future design of these polymers.

Finally, Chapter 4 will investigate and characterize the surface structure of an antimicrobial tripolymer to understand how the polymer organizes itself on that surface at a molecular level. Polymeric coatings are currently the focus of much research as they have the potential to function very effectively as biocidal coatings in marine systems. Five polymers will be discussed, each with differing ratios of the three subunits: one containing a catechol group, one containing a methoxy group, and one containing a
quaternary ammonium group with a long acyl chain. Bacterial characterization tests will confirm the antimicrobial nature of the surface, and suggest a degree of self-organization of the bulk polymer structure, as the antimicrobial moieties would need to be present at the surface. Contact angle and atomic force microscopy (AFM) studies will indicate three of the polymers organize roughly equivalently, with the fourth showing higher hydrophobicity and surface roughness. It is possible that this is due to the complete lack of a methoxy-containing subunit in the fourth polymer, and suggests that all three subunits are needed to generate a smooth surface. SFG studies were also performed in both water and air of the polymers spin-coated on fused silica surfaces. It will be shown that clear SFG signal can be obtained from these polymers at the polymer/air interface, indicating organization of specific moieties at the surface. It will also be shown that the polymer/water interface induces a randomization of the polymeric surface, resulting in a complete loss of SFG signal. However, this will be shown to be a reversible process, whereupon removal from contact with water and subsequent drying of the surface will allow for recovery of SFG signal. Finally, orientation analysis of the terminal CH$_3$ group of the acyl chain will show that this group lies parallel to the surface. Taken together, these results will be used to develop a detailed understanding of how and why the polymeric subunits self-organize into layers of the subunits, which has consequences for the efficacy of the polymer as a biocide.

The presented work investigates the interaction mechanisms between antimicrobial compounds and a variety of environments. By developing a detailed understanding of the molecular interaction mechanisms of such antimicrobial compounds, these studies provide evidence that the details of these interfacial interactions
can have a substantial impact on how a system may interact with a broader environment, aiding in the design and development of antimicrobial compounds with improved properties.
1.5 References


(4) Wang, Y. X.; Roberston, J. L.; Spillman, W. B.; Claus, R. O. Pharmaceutical Research 2004, 21, 1362-1373.


(40) Woodcock, S. E., University of Michigan, 2005.
CHAPTER 2

EFFECT OF ANTIMICROBIAL OLIGOMER STRUCTURE ON SELECTIVITY, POTENCY AND MOLECULAR ORIENTATION WITHIN A LIPID BILAYER

2.1 Introduction

Since the 1930s, with the introduction of sulfonamide drugs for therapeutic applications, antibiotics have become widely used as highly effective tools to combat infectious diseases. However, since their introduction into the standard medical arsenal, bacteria have been developing resistance to many antibiotics. Resistant strains are now commonly seen in hospitals and very few solutions for solving this problem have yielded significant results. As a result, antibiotic resistance is regarded as a massive threat to global public health, and investigation into new antibiotics is drawing new levels of interest.\(^1\)

Antimicrobial peptides (AMPs) represent a particularly interesting new perspective in the development of new drugs.\(^1\)\(^-\)\(^6\) AMPs have large structural diversity and broad-spectrum applicability, but what makes them most interesting is that they have a unique mode of action: they target the lipid membrane itself, rather than proteins or specific receptors contained within the membrane or inside the cell.\(^1\)\(^-\)\(^6\) This effectively minimizes any drug resistance ability of the microbe against AMPs. However, AMPs are inherently complex and before they can be used in any effective manner, the mechanisms
by which they work should be better understood. Extensive research has been performed to understand such mechanisms.$^{1-6}$

It was demonstrated in previous work that simple structures can be made as effective mimics for AMPs, intentionally designing them to have the same potent broad-spectrum activity as their natural counterparts.$^{7-9}$ Additionally, it was shown that these molecules can differentiate between microbial and erythrocyte cellular membrane models. Molecular dynamics simulations showed that molecules with a facially amphiphilic structure similar to those shown in Figure 1.6 would have a molecular plane perpendicular to the bilayer plane, thus inserting into the bilayer similar to a knife cutting into cheese.$^{10}$ These simulations were confirmed by direct experimental evidence using SFG vibrational spectroscopy.$^{11}$

Solid-supported lipid bilayers have long been used as models for cellular membranes.$^{12}$ However, by many methods, studying a single lipid bilayer and any specific interactions between that bilayer and the molecules under study is extremely difficult as a direct result of the small amount of material involved. Bulky labels are often introduced to either the molecules interacting with the bilayer or the bilayer itself, which can have a dramatic effect on the system. SFG has been used as an alternative to these problematic approaches of investigating lipid monolayers and bilayers with great success.$^{13-30}$ SFG has been used to monitor monolayer and bilayer structure,$^{31-32}$ transmembrane movement of lipids,$^{33}$ and transition temperatures of such solid-supported bilayers.$^{34}$ Research has also shown SFG to be an extremely powerful technique in the investigation of bilayer – molecule interaction mechanisms, specifically because of its inherent surface specificity. For example, SFG studies on melittin interacting with
bilayers indicated that melittin can adopt multiple orientations in a lipid bilayer. Its interacting mechanisms or disrupting models depend on the melittin concentration. Time-dependent SFG studies have shown that the kinetics of melittin – lipid bilayer interactions are also determined by the melittin concentration. The outer leaflet of the bilayer was disrupted first, followed by the second leaflet. Conversely, for tachyplesin I, both leaflets were disrupted simultaneously.

2.2 Experimental Methods

2.2.1 Materials

The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DPPE) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), as well as all deuterated versions (named d-DPPG, d-DPPC, d-DPPE and d-DSPC, respectively), were purchased from Avanti Polar Lipids (Alabaster, AL). Oligomers 1, 2, 3 and 4 were all synthesized in the research lab of Dr. Gregory Tew, University of Massachusetts (UMass) at Amherst. Right-angle calcium fluoride substrates were purchased from Altos Photonics, Inc (Bozeman, MT). A neutral CaF$_2$ prism was used as the substrate rather than negatively charged mica or silica prisms, despite the more frequent use of the charged prisms. This was done to avoid the possible interactions between the positively charged oligomers and the mica or silica substrates. It was previously determined that these CaF$_2$ prisms are valid substrates for such supported bilayers. Bilayers were prepared using the Langmuir-Blodgett method, as described in Section 2.2.2.
2.2.2 Bilayer Preparation

Solid-supported lipid bilayers were built as model systems for this study using a Langmuir-Blodgett trough. In each case, a hydrogenated proximal leaflet and a deuterated distal leaflet of the lipid bilayer were used to allow for independent monitoring of the C-H and C-D stretching ranges concurrently using SFG. The lipids DPPG and d-DPPG were used to construct lipid bilayers to model bacterial membranes, as phosphatidylglycerol is the main component of the negatively charged lipids that make up bacterial cell membranes. The lipids DSPC and d-DSPC were used to construct bilayers to model mammalian membranes, as phosphocholine is a significant component of the zwitterionic mammalian cell membranes. In addition, phosphoethanolamine (PE), phosphocholine (PC) and phosphoglycerol (PG) mixtures were used in dye-leakage as well as SFG experiments.

Several CaF$_2$ prisms were suspended just below the surface of water purified by reverse osmosis (RO). A solution of lipids dissolved in organic solvent (typically chloroform) was then spread onto the surface of the water-filled trough. A Langmuir-Blodgett trough allows for control of the surface pressure, measured by a Wilhelmy plate. The monolayers were built at a surface pressure of 34 mN/m, which approximately matches the surface pressure of phospholipids as they exist in biological systems.$^{35-36}$ Lipid monolayers were built 12-24 hours before experimentation to allow any excess chloroform or water molecules intercolated into the acyl chains of the lipid to evaporate.

Prisms with a lipid monolayer were then mounted in the SFG sample stage area and the distal leaflet of the bilayer was built. To do this, a small dish (~3 mL volume) was placed in the lid of a clean Petri dish and the Petri dish was then placed on a lowered
LabJack such that the small dish was vertically aligned with the mounted CaF$_2$ prism. The lid/small dish setup was then filled with RO water and a lipid/chloroform solution was spread across the surface to a surface pressure of 34 mN/m, monitored by a Wilhelmy plate. The lipid layer was then raised until contact with the monolayer on the CaF$_2$ prism was made. The water level in the Petri dish lid was then lower via siphoning until it had dropped below the rim of the small dish. All exposures to the amphiphilic oligomers studied here were performed via sample injection into the ~3 mL subphase in the small dish.

2.2.3 SFG Experimental Setup

SFG theory and the experimental details of the EKSPLA SFG setup were previously described in Section 1.2. The spectra from the monolayer/air interface and the bilayer/water interface were collected with two input beams traveling through the substrate and overlapping on the respective interface. The SFG spectra were collected using the ssp (s-polarized SFG output, s-polarized visible input, and p-polarized IR input), sps and ppp polarization combinations and normalized by the intensities of the input IR and visible beams.
2.3 Results and Discussion

2.3.1 Results from Previous Study on Oligomer 1

Our previous SFG studies on 1 (Figure 1.6), showed that the ability of 1 to interact with bacterial membrane models was strongly dependant on its concentration. In these experiments, a DPPG (proximal leaflet)/d-DPPG (distal leaflet) bilayer was exposed to solutions of 1 with concentrations below, at and above the minimum concentration required for bacterial inhibition (or minimum inhibition concentration, MIC). Isotopic labeling of the bilayer leaflets allowed the signals arising from the C-H and C-D stretches from the terminus of lipid chains in each leaflet to be independently monitored. At concentrations below the MIC, signal from the proximal leaflet remained constant, whereas signal from the distal leaflet decreased. As the concentration was increased, signal from both leaflets decreased to minimal levels, indicating the disruption of the lipid bilayer. Oligomer 1 was thus found to be capable of acting on the cellular membrane to kill bacteria, but that action was highly dependent on the compound’s concentration. In addition, by comparing SFG signals arising from the CH₃ groups and
C=O moieties located along the hydrophobic face of the oligomer using several polarization combinations of the input and output beams, we were able to determine the orientation of 1 while interacting with lipid bilayers. It inserts into the bilayer with a slight tilt from the surface normal, effectively making the oligomer more or less orient perpendicular to the lipid surface. It was this approximately perpendicular orientation that caused 1 to be referred to as a “molecular knife.”

2.3.2 Oligomer Structures for the Current Study

Here, three new oligomers will be studied to understand the effect of the side chain identity on the membrane activity of the oligomer. As shown in Figure 2.2, these oligomers have a central delocalized core with amino side chains identical to the previously studied 1, but with different hydrophilic and lipophilic side chains. Fluoride and trifluoromethyl groups were used as lipophilic groups (different from compound 1, which used t-butyl groups), and amino and carboxylic groups were used as hydrophilic groups. It is also worth noting that the side chains on some oligomers are more flexible than on others, with 4 having the largest amount of flexibility. This could have an effect on the activity and selectivity of the oligomer and should be considered in the design of these molecules.
Colorimetric assays were performed to determine activity, toxicity and selectivity toward bacterial and mammalian cells in the Greg Tew group at UMass. Oligomers 2 and 3 exhibited excellent antibacterial activity against \textit{E. coli} and \textit{S. aureus}; oligomer 4 remained inactive (as shown in Table 2.1). Although the antimicrobial activities of 2 and 3 are similar, 3 is significantly less cytotoxic towards mammalian cells resulting in superior selectivity indices (ratios of $EC_{50}$ values for cytotoxicity to MIC).

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>MIC ($\mu$g/mL)</th>
<th>Cytotoxicity ($EC_{50}$, $\mu$g/mL)</th>
<th>Selectivity ($EC_{50}$/MIC)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>\textit{E. coli}</td>
<td>\textit{S. aureus}</td>
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<tr>
<td>2</td>
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<tr>
<td>4</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>&gt; 1000</td>
</tr>
</tbody>
</table>

Table 2.1 Antibacterial activity and hemolytic activity of oligomers 2, 3 and 4. Cytotoxicity was evaluated in a colorimetric assay using a transformed human liver cell line (HepG2 cells).

Dye-leakage studies were also performed on 2, 3 and 4 to determine their activity toward bacterial and erythrocyte membrane models in the Greg Tew group at UMass. Such experiments were done using four different liposomes having identical PG
concentration but different PE and PC concentrations (see more details about the experiments in supporting information). PE lipids are found at much higher concentrations in the bacterial cell membrane, while PC is widely distributed in erythrocyte membrane. Dye-leakage data from 2, 3 and 4 are shown in Figures 2.3A, 2.3B and 2.3C, respectively. Oligomer 2 remained active even at 40% PE lipid concentration, and its activity diminished as all the PE lipids were replaced by PC lipids. Oligomer 3 required more than 40% PE lipids in the outer leaflet to retain its activity. Oligomer 4 remained inactive against all lipid vesicles. Therefore, 2 was classified as active, 3 was classified as relatively selective, and 4 was classified as not active and not selective. The oligomers that were active enough to interact with PE and PG membranes should show a similar difference in SFG spectra, and those interactions were studied in the following experiments. With comparison to the dye-leakage experiment, SFG should be able to elucidate more molecular level information regarding the cell membrane (lipid bilayer) - oligomer interactions.

Figure 2.3 Percentage of calcein dye leakage from different DOPG/DOPE/DOPC liposomes having same concentration of PG lipids, and varied concentrations of PE and PC lipids. Oligomer 2, plot A; 3, plot B; 4, plot C (DOPG/DOPE/DOPC: 20/80/00, blue; 20/60/20, black; 20/40/40, green; 20/00/80 red). The more selectively active oligomer 3 needed higher percentage of DOPE lipids for their membrane activity. The dye leakage experiments were done in phosphate buffer pH 7.0, at 2.0 µg/mL oligomer concentration (oligomer stock solutions were prepared in DMSO); final total lipid concentration is 5 µM.
2.3.3 SFG Studies on Oligomers Interacting with DPPG Bilayer

Figure 2.4 shows the SFG spectra of a DPPG (proximal leaflet)/d-DPPG (distal leaflet) in both the C-H and C-D stretching regions. Spectrum 2.4a shows the distal leaflet of the bilayer before contacting a solution of \( \text{2} \). The dominant stretch comes at 2070 cm\(^{-1} \) and is due to the symmetric stretching of the terminal deuterated methyl groups in the d-DPPG leaflet. Spectra in 2.4b, 2.4c, and 2.4d were collected at increasing concentrations of \( \text{2} \), until minimal signal from the distal leaflet could be detected. At the lowest concentration, 0.10 µg/mL, no decrease in SFG signal is observed. However, at higher concentrations, the observed signal drops dramatically. The greater the concentration, the greater the signal decrease. This is indicative of the oligomer partitioning into the bilayer and interacting with it. The decrease in signal from the distal leaflet should be due to disordering of the terminal deuterated methyl groups. While it is possible that this decrease could be due to displacement of the distal lipid molecules, it is unlikely. Light-scattering experiments were performed under similar conditions previously, and the results of those similar experiments (not reported in detail here) indicate that substantial destruction of the bilayer does not occur.
Figure 2.4 SFG spectra from a DPPG/d-DPPG bilayer in the CD (left panel) and CH (right panel) stretching regions before (a, e) and after being exposed to oligomer 2 at three solution concentrations: (b, f) 0.10 µg/mL; (c, g) 0.25 µg/mL; and (d, h) 0.44 µg/mL.

Spectra 2.4e, 2.4f, 2.4g, and 2.4h show the proximal leaflet of the same bilayer exposed to the same concentrations of 2. The signals between 2800 cm\(^{-1}\) and 3000 cm\(^{-1}\) are primarily contributed by the methyl end groups of DPPG. The 2875 cm\(^{-1}\) and 2940 cm\(^{-1}\) peaks are contributed by the symmetric CH\(_3\) stretch and Fermi resonance of the methyl group. At the lowest concentration, 0.10 µg/mL, no change is observed in the proximal leaflet signal relative to signal from the initial bilayer. As described previously, there was also no corresponding decrease in distal leaflet signal at this same concentration. Therefore, it seems that once the oligomer has reached a concentration at which it will interact with the bilayer, it affects both lipid leaflets at the same time. At higher concentrations there is significant decrease in both proximal and distal leaflet
signals, indicating penetration of 2 into the inner leaflet. The concentration at which the oligomer begins disrupting bilayer SFG signals is around 0.25 µg/mL.

Figure 2.5 SFG spectra from a DPPG/d-DPPG bilayer in the CD (left panel) and CH (right panel) stretching regions before (a, f) and after being exposed to oligomer 3 at four solution concentrations: (b, g) 0.21 µg/mL; (c, h) 0.41 µg/mL; (d, i) 0.82 µg/mL; and (e, j) 1.2 µg/mL.

A similar study was done on DPPG/d-DPPG bilayers using increasing concentrations of 3, the results of which are shown in Figure 2.5. In the case of 3, the concentrations used ranged from 0.21 µg/mL to 1.2 µg/mL. As for 2, a concurrent decrease in signal from the proximal and distal leaflets of the bilayer was observed. At the lowest concentration, signals from both leaflets remained unchanged, and at higher concentrations, a decrease in SFG signal from the proximal leaflet had a corresponding decrease in distal leaflet signal. A significant difference however was the range of concentrations needed to see the substantial and full disruption of the bilayer. There was only slight disruption for the DPPG leaflet when 3 reaches a concentration at 0.41 µg/mL.
(Figure 2.5h), while for 2, a similar concentration (0.44 µg/mL, Figure 2.4d and 2.4h) completely disrupted both leaflets. Oligomer 3 substantially disrupts the DPPG leaflet when its concentration is at 0.81 µg/mL (Figure 2.4i). While 2 only needed approximately a third of the maximum concentration to substantially disrupt the DPPG leaflet (0.25 µg/mL, Figure 2.4g) comparing to that required for 3. This is spectral evidence of a higher activity for 2 than 3, which was also independently observed in dye-leakage tests previously described.

Figure 2.6 SFG spectra from a DPPG/d-DPPG bilayer in the CD (left panel) and CH (right panel) stretching regions before (a, d) and after being exposed to oligomer 4 at two solution concentrations: (b, e) 2.0 µg/mL; and (c, f) 5.8 µg/mL.

Oligomer 4 was studied in a similar way to 2 and 3, however with significantly different results, shown in Figure 2.6. DPPG/d-DPPG bilayers were exposed to much higher concentrations of 4 (maximum concentration was 5.8 µg/mL) relative to 2 or 3, however no SFG signal decrease was observed in either the C-D or C-H regions. This would indicate that 4 has no activity to this type of bilayer up to 5.8 µg/mL. Support for
such a conclusion was also found in dye-leakage tests. As shown in Figure 2.2c, no dye leakage was detected for 4.

2.3.4 Discussion on Possible Transmembrane Lipid Movement

One other possible explanation for the decrease in bilayer SFG signal in the cases of 2 and 3 could be the transmembrane movement of the lipids induced by these oligomers. Such flip-flop would mix the hydrogenated and deuterated lipids and result in reduced membrane asymmetry and thus a decrease in SFG signal. We believe that under the experimental conditions described here, these oligomers do not induce any significant transmembrane movement of lipids. For previously studied 1, there was an independent decrease in signal from the proximal and distal leaflets. It was our contention that the different SFG signal decrease of the two leaflets of the bilayer after interacting with oligomers probably indicated that the SFG signal decrease was not primarily caused by the flip-flop. This type of signal change was not observed in the case of the oligomers under study here, which saw a simultaneous decrease in SFG signals from both leaflets. The simultaneous decrease in signal could theoretically be from flip-flop among the leaflets. Under the current experimental conditions, the DPPG/d-DPPG bilayer should be in the gel phase, and the intrinsic flip-flop rate should therefore be negligibly slow when not interacting with the oligomers, as was observed (not shown). It has been found that when cell membranes interact with some antimicrobial peptides, transmembrane toroidal pores can be formed. Formation of transmembrane toroidal pores is known to greatly increase the flip-flop process by the creation of an aqueous channel by which polar headgroups can move from one leaflet to another without being forced to pass through the hydrophobic interior of the membrane. It is our belief that the oligomers, as small,
relatively rigid molecules are unlikely to form toroidal pore-like structures in a DPPG/d-DPPG bilayer. It is therefore most likely that these oligomers are directly disturbing the bilayer, rather than forming pore structures to cause flip-flop.

2.3.5 Oligomer Selectivity

As we discussed above, PG lipids are important components for bacterial cell membranes, while PC lipids are significant in mammalian cell membranes. Our previous studies on DPPC/d-DPPC bilayers indicate that the SFG C-H and C-D signals decrease very quickly even without interacting with any oligomers, due to the fast flip-flop. Therefore it is difficult to study oligomer–DPPC/d-DPPC lipid bilayer interactions by observing SFG signal intensity decrease as what we did when DPPG bilayers were used. Here, instead of using a DPPC/d-DPPC bilayer, we prepared a d-DSPC/DSPC bilayer, where no substantial flip-flop occurred. By using such a bilayer, for all three oligomers, even when the concentration is very high (e.g., ~20 µg/mL), no SFG signal decrease for the lipid bilayer was observed. This is correlated to the results shown in Figure 2.3 very well. As demonstrated in Figure 2.3, little dye leakage was observed when the oligomer concentration is at 5 µM for the liposomes containing 20% PG and 80% PC, relative to the other lipid mixtures tested.
Figure 2.7 DSPC/d-DSPC bilayers exposed to high concentrations of oligomers 2 (left panel - (a) 19.9 µg/mL; (b) 9.9 µg/mL; (c) 2.38 µg/mL) and 3 (right panel – (d) 3.85 µg/mL (e) 9.9 µg/mL). All concentrations are far above the concentration at which a DPPG membrane was disrupted.

To further investigate the selectivity of these oligomers between charged and neutral lipids, 2 and 3 were exposed to PC bilayers at higher concentrations and SFG signals in the C=O stretching frequency region were monitored. The C=O signals can be contributed by both lipid bilayer head groups and the carbonyl moieties on the oligomers’ planar core. However, the C=O signal from the oligomers is around 1705 cm$^{-1}$. According to our previous studies, the C=O signal from the lipid is centered at about 1720 cm$^{-1}$. They can be easily differentiated. Without the addition of oligomer, no C=O SFG signal was observed, showing that the lipid bilayer does not contribute to SFG C=O signals. The oligomer solutions with high concentrations were used to see if the oligomer could be forced to interact with the PC membrane at these higher concentrations. The collected spectra are shown in Figure 2.7. In the case of 2, which is the less selectively active compound, a small amount of C=O signal can be seen in spectra 2.7a (with an
oligomer concentration of 19.9 µg/mL) and 2.7b (with an oligomer concentration of 9.9 µg/mL), however at a far lower level than seen for PG membranes, which will be shown below. Also, the concentration where these small signals were observed was at nearly two orders of magnitude higher than that required for PG membranes. Similarly for 3, small C=O signals can be seen at very high concentrations; however they are again very small. These signals are around 1720 cm⁻¹. We believe that they are contributed by C=O signals from the lipid bilayer, due to the symmetry broken by small perturbations. Therefore, we conclude that at very high concentrations, these oligomers could be forced to interact with mammalian cell membranes to only a small degree. As shown later in Figures 2.8 and 2.9, the C=O signals observed in PG lipid membranes are centered at 1705 cm⁻¹, which should be dominated by the contributions from oligomers.

2.3.6 SFG Studies on DPPG/DPPE/DPPC Mixed Bilayers

To make a direct comparison between the dye-leakage test results and the SFG studies performed here, a study was done of DPPG/DPPE/DPPC mixed bilayers exposed to solutions of 2 and 3. Spectra are shown in Figures 2.8 and 2.9, respectively. In all cases, as the percentage of PC lipids in the outer leaflet increased, the C=O stretching signal (peaked at 1705 cm⁻¹) observed from the oligomer decreased. There is a significant decrease in signal from 2 when the %PE drops below 40%, and a similar decrease occurs for 3 when the %PE drops below 60%. Interestingly, the intensity of the C=O signal exactly follows the pattern observed in dye-leakage tests for these two compounds (Figures 2.3A and 2.3B). This would seem to imply that there is a significant correlation between the dye-leakage tests and our SFG results.
Figure 2.8 SFG spectra of d-DPPG/mixed (PG/PE/PC) bilayers exposed to 0.25 µg/mL of 2 (DPPG/DPPE/DPPC: (a) 20/80/0; (b) 20/60/20; (c) 20/40/40; and (d) 20/80/0). Ratios are referenced to the percentage each lipid type has in the outer leaflet.

Figure 2.9 SFG spectra of d-DPPG/mixed (PG/PE/PC) bilayers exposed to 0.41 µg/mL of 3 (DPPG/DPPE/DPPC: (a) 20/80/0; (b) 20/60/20; (c) 20/40/40; and (d) 20/80/0). Ratios are referenced to the percentage each lipid type has in the outer leaflet.

It is theoretically possible that the decrease in signal described above could be due to a reorientation of the oligomer upon interaction with the bilayer and not due to a lessened degree of interaction with PC lipids. To determine if this was a cause of the
signal decrease, the orientation of the oligomers was determined using the method described further in this chapter. In all cases, the orientation of the oligomers in the mixed bilayers was found to be identical to their orientation in DPPG/d-DPPG bilayers which will be reported later in Section 2.3.7. It is therefore unlikely that the SFG signal decrease observed as the %PC of the bilayer increased was due to orientation changes of the oligomers. Details about the analysis of the orientation of these oligomers are discussed below.

It should be noted that all three sets of tests (exposure to PG bilayers, exposure to PC bilayers, and dye-leakage tests) all show similar results. In all cases, 2 is less selectively active than 3, and 4 showed virtually no activity against any membrane type.

2.3.7 Oligomer Orientation

![SFG spectra](image)

**Figure 2.10** SFG spectra collected from a DPPG/d-DPPG bilayer in the CO stretching region. The left panel shows spectra of oligomer 2 at 0.25 µg/mL in (a) ppp (b) ssp and (c) sps polarization orientations. The right panel shows spectra of oligomer 3 at 0.41 µg/mL in (d) ppp (e) ssp and (f) sps polarization combinations.

In the previously described research (Section 2.3.1), we determined the orientation of 1 in the DPPG bilayer by collecting SFG C-H stretching signals from t-butyl groups. It was found that 1 more or less stands up in the lipid bilayer, cutting the
lipid bilayer as a molecular knife. Such an orientation was confirmed by C=O stretching signals collected using different polarization combinations of the input and output laser beams in the SFG experiment.\textsuperscript{11} Since 2 and 3 do not have t-butyl groups, to determine their orientations inserted into the bilayer, orientational analysis of the C=O stretch (1705 cm\textsuperscript{-1}) of the carbonyl moieties on the oligomer was performed (Figure 2.10). Since a near total reflection geometry was adopted in the experiment, ppp spectra are mainly contributed by $\chi_{zzz}$, while the contributions of $\chi_{xxz}$, $\chi_{xzx}$, and $\chi_{zxx}$ can be ignored, due to their negligible Fresnel coefficients.\textsuperscript{47} From the ppp and ssp SFG spectra the $\chi_{zzz}/\chi_{yzz}$ ratio can be deduced directly. According to the symmetry and hyperpolarizability of the C=O stretching mode, the $\chi_{zzz}/\chi_{yzz}$ ratio of the C=O stretching mode as a function of different orientations and orientation distributions can be deduced,\textsuperscript{48} as shown in Figure 2.11. The measured ratios are described by the red and blue bars for 2 (at 0.25 µg/mL) and 3 (0.41 µg/mL), respectively. As shown above, at these concentrations, 2 and 3 have similar disruption activities against PG bilayers. The measured $\chi_{zzz}/\chi_{yzz}$ ratios for 2 and 3 are quite similar, corresponding to a more or less stand-up orientation (less than 35° from the bilayer surface normal) and a limited distribution width (less than 30°) for both oligomers. These results indicate that the plane of the oligomer is roughly perpendicular to the bilayer surface. This is the same method of interaction as was observed for 1, which was found to insert into the bilayer approximately perpendicular to the bilayer plane. As mentioned, we have previously referred to this type of vertical insertion into the bilayer as a “molecular knife.” If we compare 2 and 3, 2 is only slightly less perpendicular to the lipid bilayer, however the difference between 2 and 3 is so minimal it would seem to imply that the two oligomers orient approximately the same. Assuming
that the distribution width is 30°, both 2 and 3 are approximately perpendicular to the lipid bilayer. As mentioned above, the orientation of the oligomers in the mixed bilayers was found to be identical to their orientation in DPPG/d-DPPG bilayers (details were not shown).

Figure 2.11 Relation between $\chi_{zzz}/\chi_{yyz}$ and the orientation distribution of the parallel carbonyl groups. “Delta” means a delta-distribution, with orientation angle distribution width of 0°. For 2, $\chi_{zzz}/\chi_{yyz}$ was experimentally found to be 1.91, and for 3 was found to be 2.05, and are indicated on the above plot by the red and blue bars, respectively.

2.3.8 SFG C-F Stretching Signals

As mentioned, we deduced the orientation of 1 according to its t-butyl groups. Here 2, 3, and 4 do not have t-butyl groups, but have some C-F groups instead. Research was previously conducted in this lab to investigate the spectrally observable effect a phenyl ring can have when attached directly to a trifluoromethyl group. In those experiments, a strong coupling effect was observed between the CF$_3$ group and the phenyl ring, and the observation of SFG signal due to CF$_3$ stretches is highly dependent on that coupling. Those specific effects were observed from 4-(trifluoromethyl)benzyl
alcohol (TFMBA), however the comparison is still relevant here. As shown in Figure 2.2, 2 has a fluorine directly attached to two of the three phenyl rings, whereas 3 has a CF$_3$ group, just as in TFMBA. SFG signal was detected in the CF stretching region for 3 and not for 2, shown below in Figure 2.12. It is also worth noting that 4 also contains a CF$_3$ group attached to two of the three core phenyl rings. No CF SFG signal was detected from the lipid bilayer while in contact with 4 solution. As previously discussed, 4 does not show any interaction to a DPPG bilayer and it does not goes to the interface, so we would therefore not expect to see any CF signal from 4 at the bilayer surface.

![SFG spectra of CF stretching region for oligomers 2 (left panel) and 3 (right panel). Spectra (a) and (c) were taken in the ssp polarization combination whereas (b) and (d) were taken in ppp.](image)

2.4 Conclusions

In these studies, the mechanism of interactions between several AMP mimics and cell membrane models was studied using SFG. Signals from C-H, C-D, C=O and C-F stretches within the bilayer and the oligomer were monitored to characterize and correlate
results seen through SFG to dye-leakage and colorimetric assay experiments. As with results from previous work, disruption of the bilayer leaflets was seen to depend strongly on oligomer concentrations. Three different oligomers have distinctly different activities against PG lipid bilayers. Oligomers 2 and 3 are quite active, while 4 is not. Oligomer 4 has a long and flexible polar/charged chain on one side of the molecule. This lack of rigidity of one side of the molecules may lead to its less active property. For 2 and 3, while they interact with PG bilayers, they more or less stand up in the bilayer, as did 1. SFG studies indicated that under the oligomer concentrations investigated here, all three compounds are not active against PC lipid bilayers. Their activities and selectivities detected from SFG studies were well-correlated to those observed from the dye-leakage and colorimetric assay experiments.

Alteration of the identity of the lipophilic groups along the oligomeric backbone clearly has relevance to the oligomer’s activity and toxicity. Oligomer 2, with a fluoro group functioning as the lipophilic group, showed far less of an ability to differentiate between bacterial and erythrocyte cells than oligomer 3, which had a trifluoromethyl group. Oligomer 1, which had methyl groups as the lipophilic side chains, was less active than 2 as well. The main difference between 1, 2, 3 and 4 were the identity of these lipophilic groups, and differences in their activities must therefore be attributable, at least in part, to these structural differences. It is possible that amphiphilic balance between hydrophilic cationic groups and lipophilic groups must also take into account the physical volume of these membrane-active side chains. Future studies investigating these kinds of oligomer/lipid bilayer interaction mechanisms should take this into account. Additionally,
future experiments should also consider the physical volume each membrane-active side
chain takes up to see if this property must be intentionally accounted for as well.

This research demonstrated that SFG can be used to study the details of
interaction mechanisms between synthetic antimicrobial oligomers designed as mimics
for naturally occurring AMPs. Different lipids can be used to model specific cellular
membrane compositions, and isotopic labeling allows for independent monitoring of each
leaflet within the bilayer, yielding additional mechanistic information. Further, by
intentionally arranging specific SFG-active moieties along the rigid oligomer backbone,
orientation of the oligomer during its interaction with the bilayer can be observed.
2.5 References


CHAPTER 3

DETERMINATION OF THE INTERACTION MECHANISMS OF ANTIMICROBIAL POLYMERS IN LIPID BILAYERS

3.1 Introduction

The synthetic oligomers discussed in Chapter 2 are a promising alternative to native AMPs, in that they have simpler structures, smaller molecular weights and rationally designed global amphiphilicity. However, they still suffer from some of the same shortcomings that handicap AMPs themselves: high manufacturing costs, susceptibility to proteolysis, and poorly understood pharmacokinetics.\textsuperscript{1} Both AMPs and their synthetic mimics will remain largely impractical in an industry setting until further research fleshes out these concerns. Despite the expanded research on AMPs in the last decade, very few real-world applications of that research have been seen to address the underlying problem motivating that research: bacterial resistance.

High molecular weight (MW) polymers have been a separate field of study for antibacterial compounds for some time.\textsuperscript{1-7} Typically these polymers have side chains containing cationic quaternary ammonium salt (QAS) units. They are often referred to as “polymer disinfectants” and kill via a membrane disruption mechanism, much like AMPs. Unlike AMPs, high-MW polymers are typically immobilized on a surface, rather than suspended in solution, making them ideal for disinfecting surfaces, such as textiles, water purification devices, hospital surfaces, or biofouling materials.\textsuperscript{5} They often have
low manufacturing costs, can be synthesized on a large-scale platform and can showcase a diversity of chemical structures. However, these polymers suffer from a crucial shortcoming: they are often indiscriminately toxic, especially so to humans, and many have never been tested for toxicity.

The field of disinfecting polymers developed separately but concurrently with the discovery and investigation of host defense peptides, and renewed interest in AMPs for industrial applications has allowed the fields to begin to merge. Peptide design principles, such as amphiphilic structure, cation charge identity and density, and molecular weight considerations, have begun to be combined with the simplistic and inexpensive methods of production of polymers, giving way to a whole new class of synthetic biocompatible compounds. The merging of these two fields is represented in low-MW antimicrobial polymers. Some research has indicated that the activity of both natural and synthetic polymers is in fact unrelated to the specific defined sequence of subunits or the secondary structure of the peptide. Instead, the physiochemical or global properties dictate a given polymer’s activity. In light of this shift in thinking, development of low cost, biocompatible and highly customizable polymers for biocidal applications has raised questions regarding the role of the functional groups within the polymers. A molecular level understanding of the interaction mechanism between these antimicrobial polymers and cellular membranes has become necessary to fully develop a coherent model to guide future iterations of these polymers.

The study presented here aimed to investigate membrane-interaction mechanisms of several low-MW random co-polymers. These polymers can be classified as peptide-mimetic antimicrobial co-polymers, as they will be shown to display antimicrobial
characteristics. SFG vibrational spectroscopy was used to analyze interactions between solid-supported lipid bilayers acting as models for cellular membranes and several membrane-active random co-polymers with different lipophilic side chains, named 0R (no group), 33Me (methyl group), 11Bz (benzyl group) and 33Bu (butyl group), according to both the identity and percentage of the side chains within the polymer. Biological tests of the minimum inhibitory concentration (MIC) and hemolytic concentration were performed. The inherent surface sensitivity of SFG allowed for independent monitoring of isotopically labeled lipid bilayer leaflets as a function of concentration to study polymer-bilayer interaction mechanisms. Concentrations at which each bilayer leaflet was disrupted were quantitatively determined for each co-polymer. Spectroscopic evidence of interaction with the bilayer below the critical concentrations was observed for the 11Bz polymer. The lipophilic butyl side chain of the 33Bu polymer was found to orient parallel to the surface normal. This research further develops understanding of how rational design of antimicrobial co-polymers can maximize the applicability of membrane-active polymers to therapeutic applications.

3.2 Experimental Methods

3.2.1 Polymer Synthesis

The molecular formulas for the random copolymers studied here are shown in Figure 3.1. They were prepared in the Kuroda Group at the University of Michigan by a previously described technique with minor alterations. Methylmethacrylate (MMA), butylmethacrylate (BMA) or benzylmethacrylaye (BzMA) was dissolved with N-tert-butoxycarbonyl-aminoethylmethacrylate (Boc-AEMA) in acetonitrile (1 mmol total monomers, various ratios, 0.5 mL). 2,2’-Azobisisobutyronitrile (AIBN) at 0.01 mmol and
methyl 3-mercapto-propionate (MMP) at 0.1 mmol were added from concentrated stock solutions. The mixtures were deoxygenated by nitrogen flushing for 2 minutes each and then submerged in a 70 °C oil bath overnight. After concentration under reduced pressure, the Boc-protecting groups were cleaved by stirring in neat trifluoroacetic acid for 1 hour at room temperature and the excess acid was removed by nitrogen flushing. The amphiphilic copolymers were then dissolved in methanol (0.5 mL) and precipitated from diethyl ether (25 mL) three times. The precipitated pellets were then isolated by centrifugation (2000 rpm, 5 min) and the supernatant ether was decanted. The pellets were dried under vacuum overnight and then lyophilized to afford the random copolymers as fine white powders.

![Figure 3.1 Structure of the amphiphilic copolymers. In each polymer, both the identity of the R group and the value of the fraction of side chains containing the R group were altered to tune the biological activity and membrane interactions. Characterization of the copolymers is given in Table 3.1.](image)

3.2.2 Antimicrobial Activity

The lowest polymer concentration required to completely inhibit growth of bacteria, defined as the MIC, was determined by a turbidity-based microdilution assay in Muller Hinton (MH) broth in the Kuroda Group at the University of Michigan. *Escherichia coli* ATCC 25922 in the midlogarithmic phase were diluted to $OD_{600} = 0.001$ in MH broth. This stock suspension of bacteria (90 µL) was mixed with serial dilutions of a polymer
stock solution (10 µL each) in each well of a 96-well microplate (Corning #3359). After incubating for 18 h at 37 °C, the OD$_{600}$ in each well was recorded using a microplate reader (Perkin-Elmer Lambda Reader). The MIC was defined as the lowest polymer concentration at which no turbidity increase was observed relative to the negative growth control, which was sterile MH broth.

### 3.2.3 Hemolytic Activity

Toxicity to human red blood cells (RBCs) was assessed by a hemoglobin release assay by the Kuroda Group. RBCs (1 mL) were diluted into HEPES buffered saline (9 mL; HBS = 10 mM HEPES, 150 mM NaCl, pH 7) and then centrifuged at 1000 rpm for 5 min. The supernatant was carefully removed using a pipette. The RBCs were then washed with HBS for two additional times. The resulting stock (10% v/v RBC) was diluted 3-fold in HBS to give the assay stock (3.3% v/v RBC). The assay stock (90 µL) was then mixed with each of the polymer serial dilutions (10 µL) on a sterile 96-well round-bottom polypropylene microplate to give a final solution of 3% v/v RBC, or $10^8$ red blood cells per mL. HBS (10 µL) or Triton X-100 (10 µL, 1% v/v) were added instead of polymer solution as negative and positive hemolysis controls, respectively. The microplate was secured in an orbital shaker at 37 °C and 100 rpm for 60 min. The plate was then centrifuged at 1000 rpm for 10 min. The supernatant (10 µL) was diluted into PBS (90 µL) and the absorbance at 405 nm was recorded using a microplate reader (Perkin-Elmer Lambda Reader). The fraction of hemolysis was defined as $H = (A - A_0)/(A_{TX} - A_0)$, where $A$ is the absorbance reading of the sample well, $A_0$ is the negative hemolysis control (buffer), and $A_{TX}$ is the positive hemolysis control (Triton X-100). Hemolysis was plotted as a function of polymer concentration and the HC$_{50}$ was defined.
as the polymer concentration which causes 50% hemolysis relative to the positive control. This value was estimated by curve-fitting using Kaleidagraph software with the empirical Hill equation, \( H = \frac{1}{(\text{HC}_{50}/[P])^n + 1} \), where \([P]\) is the total concentration of polymer and the fitting parameters were \(n\) and \(\text{HC}_{50}\).

3.2.4 Sample Preparation for SFG Experiments

3.2.4.1 Bilayer Preparation

Solid-supported lipid bilayer samples were prepared using CaF\(_2\) right-angle prisms via the method outlined in Section 2.2.2. As previously discussed, this method has been well-documented in published research from our lab as an effective means of constructing such a bilayer.\(^{14}\) Both hydrogenated and deuterated DPPG and d-DPPG were alternately used for the proximal (inner) or distal (outer) leaflet. Phosphatidylglycerol, a major component of bacterial membranes, has a negative charge, giving bacterial membranes a fundamentally different character than erythrocyte membranes, which are zwitterionic.\(^{15}\) All lipids used in the research described here were purchased from Avanti Polar Lipids (Alabaster, AL). Neutral CaF\(_2\) prisms were used instead of negatively charged mica or fused silica prisms to avoid charge interaction between the lipid molecules and the substrate itself. Again, previous research has demonstrated that these prisms are valid as substrates for solid-supported lipid bilayers.\(^{14,16-20}\)

3.2.4.2 Polymer Preparation

Dried samples of the polymers were rehydrated using water purified by reverse osmosis (RO) via a Millipore filter. The polymers were dissolved in 1 mL of RO water to create a concentrated stock solution at approximately 1 mg/mL. A diluted solution was
then generated at 50 µg/mL for each polymer. This diluted stock solution was used to inject into the subphase for all SFG experiments described here.

3.2.5 SFG Experimental Setup

An extensive discussion of SFG theory, application, setup and data analysis can be found in Section 1.2 and will not be repeated here. The experimental setup used in these experiments was diagrammed in Figure 2.1. Use of this geometry yielded total internal reflection within the prism substrate. In all cases, a polarization combination of s-polarized SFG output, s-polarized visible and p-polarized IR (ssp) was used.

3.3 Results and Discussion

3.3.1 Antimicrobial and Hemolytic Activities

The antimicrobial activity of the amphiphilic random copolymers was assessed as the minimum polymer concentration to inhibit the growth of *E. coli* in MH broth (Table 3.1). The cationic homopolymer (0R) showed only weak activity, with an MIC value of 500 ug/mL. Increasing the hydrophobicity of the polymers by including 33% methylmethacrylate units (33Me) lead to a four-fold enhancement of the activity with an MIC value of 125 ug/mL. Further increasing the hydrophobicity by elongation of the alkyl groups from methyl to butyl side chains (33Bu) caused a dramatic decrease in the MIC to 15.6 ug/mL. Potent activity against *E. coli* cells was also observed when a small fraction of benzyl methacrylate was included in the copolymer (11Bz), with an MIC value of 62.5 ug/mL. These results are in agreement with previously published data on related polymers.11
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<th>HC_{50} (µg/mL)</th>
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<td>11.5</td>
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Table 3.1 Characterization and biological activity of the amphiphilic random copolymers. Degree of polymerization (DP) was calculated using a number average based on end-group analysis of ^1^H NMR spectra.

Leakage of hemoglobin from human red blood cells induced by each of the polymers was measured to determine the polymer concentrations required to damage mammalian cell membranes. The relatively hydrophilic copolymers 0R and 33Me showed no appreciable hemolytic activity up to the highest polymer concentration tested, which was 2000 µg/mL. On the other hand, the hydrophobic copolymer 33Bu and the copolymer containing aromatic groups 11Bz showed significant hemolytic activity. These trends also agree closely with previously published data.\(^\text{11}\)

3.3.2 SFG Studies on Polymers Interacting with a DPPG Bilayer

Figures 3.2a and 3.2d show the SFG spectra from the deuterated leaflet of both a DPPG/d-DPPG and a d-DPPG/DPPG bilayer in the C-D stretching region. Use of such a bilayer (referred to as “asymmetric” in light of the separation of hydrogenated and deuterated lipids into discrete leaflets) allows for easy spectral separation of the independent leaflets. Further, isotopic labeling allows for polymer/bilayer interactions to be monitored for each leaflet, potentially yielding a far more detailed description of the interaction mechanism.
Figure 3.2 SFG spectra from the deuterated inner leaflet of a d-DPPG/DPPG bilayer (left panel) and the deuterated outer leaflet of a DPPG/d-DPPG bilayer (right panel). Spectra show the C-D stretching region before (a, d) and after exposure to polymer 0R at two concentrations: (b, e) 1.6 µg/mL and (c, f) 3.1 µg/mL.

Spectrum a in Figure 3.2 displays the deuterated proximal leaflet of a d-DPPG/DPPG bilayer before exposure to polymer 0R. Spectra 3.2b and 3.2c show the proximal leaflet of a similarly structured bilayer exposed to concentrations of 0R at 1.6 µg/mL and 3.1 µg/mL, respectively. As can been seen in Figure 3.2, no significant decrease of signal is observed. Similarly, the right panel in Figure 3.2 shows the distal deuterated leaflet of a DPPG/d-DPPG bilayer before (spectrum 3.2d) and after exposure of 0R at 1.6 µg/mL (spectrum 3.2e) and 3.1 µg/mL (spectrum 3.2f). Again, no significant decrease in signal was observed. This lack of decrease in signal indicates that polymer 0R is not partitioning into the bilayer and disrupting the lipid ordering. Here we did not study the SFG C-H stretching signals of the d-DPPG/DPPG or DPPG/d-DPPG bilayers because C-H signal may also be contributed by the copolymer. An identical study was done using the 33Me polymer, the SFG spectra for which is shown in Figure 3.3. As for the 0R
concentration study, the left panel shows the deuterated proximal leaflet and the right panel shows the deuterated outer leaflet. Using the same concentrations, no change was observed in signal strength from the polymers interacting with the bilayer, strongly indicating that the 33Me polymer does not partition into the bilayer. The previously described antimicrobial and hemolytic activity testing showed that these polymers are inactive against bacteria and human cells at low concentrations, which corroborates the SFG result.

Figure 3.3 SFG spectra from the deuterated inner leaflet of a d-DPPG/DPPG bilayer (left panel) and the deuterated outer leaflet of a DPPG/d-DPPG bilayer (right panel). Spectra show the C-D stretching region before (a, d) and after exposure to polymer 33Me at two concentrations: (b, e) 1.6 µg/mL and (c, f) 3.1 µg/mL.

A similar study of the effect of concentration was done using both d-DPPG/DPPG and DPPG/d-DPPG bilayers and increasing concentrations of 11Bz. The results of this study are shown in Figure 3.4. In this case, the concentrations used ranged from 0.08
µg/mL to 0.82 µg/mL, a far lower and narrower concentration range than that shown for 0R and 33Me. However, unlike the 0R and 33Me polymers, a clear loss of signal from each leaflet was observed in the case of 11Bz. Signal loss from the 2070 cm$^{-1}$ symmetric stretch of the CD$_3$ group should be due to the polymer partitioning into the bilayer and disrupting the rigid ordering of the system. While it is possible that signal loss could be due to displacement of the lipids themselves from the substrate surface, such an action is unlikely. Signal from each leaflet did not drop simultaneously. This is most noticeable when comparing spectra 3.4b and 3.4g to 3.4a and 3.4f, respectively. Both bilayers were exposed to the same concentration of 11Bz (0.08 µg/mL for 3.4b and 3.4g), however only the distal leaflet showed disruption and loss of signal (3.4g). Not until a concentration of 0.12 µg/mL was reached did proximal leaflet signal begin to decrease (spectra 3.4c). Even at the highest exposure concentration (0.82 µg/mL, spectra 3.4e and 3.4j), when virtually all distal leaflet signal was lost, some proximal leaflet signal remained. This type of interaction mechanism has been observed in our lab before, in the case of the antimicrobial arylamide oligomer 1, discussed in Section 1.3. This kind of interaction pattern is indicative of a “molecular knife” style of interaction, where antimicrobials partition into external surface of the bilayer before penetrating into the interior.
Figure 3.4 SFG spectra from the deuterated inner leaflet of a d-DPPG/DPPG bilayer (left panel) and the deuterated outer leaflet of a DPPG/d-DPPG bilayer (right panel). Spectra show the C-D stretching region before (a, f) and after exposure to polymer 11Bz at four concentrations: (b, g) 0.08 µg/mL; (c, h) 0.12 µg/mL; (d, i) 0.50 µg/mL; and (e, j) 0.82 µg/mL.

A similar series of exposures to increasing concentration was done for the 33Bu polymer, to both d-DPPG/DPPG and DPPG/d-DPPG bilayers. The SFG spectra for this series of exposures are shown in Figure 3.5. As with 11Bz, clear evidence of interaction with and disruption of the bilayer was observed. However, 33Bu yielded a clearly different interaction pattern. The span of concentrations where full signal loss was not yet achieved is both lower and far narrower for 33Bu, ranging from 0.03 µg/mL to 0.25 µg/mL. Further, in this case, both the proximal and distal leaflets decreased in SFG signal simultaneously, indicating concurrent disruption of both bilayer leaflets. This is spectral evidence that 33Bu has a higher activity than all other polymers studied here, whereas 11Bz is more active than either 0R or 33Me, but less active than 33Bu. This general trend
is also reflected in their antimicrobial activities. Thus, both SFG and antimicrobial and hemolytic testing indicate the same trend in activity; 0R and 33Me are inactive, 11Bz is more active, and 33Bu is the most active. This is especially interesting given the dramatic differences between the systems used for testing. In the case of antimicrobial activity and hemolytic testing (and unlike in the SFG studies), MIC values were determined in broth, but not in buffer, and the number of cells and lipid bilayers are entirely different. In spite of these dissimilarities, the same trend is observed, indicating that the supported lipid bilayers we used in SFG studies are good models as cell membranes.

Figure 3.5 SFG spectra from the deuterated inner leaflet of a d-DPPG/DPPG bilayer (left panel) and the deuterated outer leaflet of a DPPG/d-DPPG bilayer (right panel). Spectra show the C-D stretching region before (a, e) and after exposure to polymer 33Bu at three concentrations: (b, f) 0.03 µg/mL; (c, g) 0.17 µg/mL; and 0.25 µg/mL.
There are several possible reasons for the loss of SFG signal as a function of concentration, as shown above. One, already described, indicates partitioning of the polymer into the bilayer and disruption of the bilayer ordering. Such disruption would strongly decrease SFG signal, as it is dependent upon ordering at the surface. However, another possibility is transmembrane movement of the lipids across the hydrophobic core of the bilayer itself, referred to as flip-flop. Such movement would mix the bilayer lipids, decreasing the signal for each leaflet by decreasing the asymmetric separation of the isotopically labeled lipids. It is our contention that, under the experimental conditions described here, this does not occur. The independent signal loss from each bilayer leaflet in the case of the 11Bz polymer is strong evidence for this, as such a pattern of signal loss is inconsistent with transmembrane movement of lipids. Further, as was discussed in Chapter 2, under these experimental conditions, the DPPG bilayer should be in the gel phase, making the intrinsic flip-flop rate of the bilayer so slow as to be negligible. The formation of transmembrane pores has been documented to occur when a bilayer interacts with a membrane-active peptide, and the formation of such pores would greatly increase the flip-flop rate, as it would provide a hydrophilic gateway for the polar lipid headgroup to pass through the hydrophobic bilayer core. It is our contention that formation of such transmembrane pores is unlikely. Polymer 11Bz shows independent action by the polymer on each of the bilayer leaflets, which is inconsistent with the expected effect of a transmembrane pore. While 33Bu shows a simultaneous loss of signal, which could be consistent with disruption, flip-flop or a combination of both, results from our time-dependent study (shown later) indicate flip-flop is not occurring.
Therefore, it is most probable that 11Bz and 33Bu are interacting with and directly disrupting the bilayer, as opposed to forming pores.

It can be seen that the copolymer concentrations required to disrupt the lipid bilayers for 11Bz and 33Bu are much smaller compared to the MICs listed in Table 3.1. We believe that this is due to the fact that the copolymers studied here can specifically interact with DPPG lipids. While in the bacterial cell membrane, many other lipids and biological molecules exist together, thus requiring a higher copolymer concentration to disrupt the bacterial cell membrane. A similar phenomenon was observed while studying AMP-lipid bilayer interactions using SFG.\(^{44}\)

### 3.3.3 SFG Signals from the Polymers

To determine the orientation of the lipophilic side chains for each of the copolymers, a symmetric d-DPPG/d-DPPG bilayer was built using \(\text{D}_2\text{O}\) as a subphase. To ensure that any signal in the C-H stretching regions would be entirely due to the polymer and not lipids, SFG scans were taken of d-DPPG monolayers and bilayers in the C-H region before polymer was introduced to the subphase. In all cases, no SFG signal from the lipids was observed in this spectral region. No C-H region SFG signals were observed from the 0R and 33Me polymers, which was expected as both polymers did not show activity to a DPPG bilayer, as previously discussed. Additionally, no signal was observed from the 11Bz polymer in the C-H region. This was surprising, because 11Bz was previously clearly shown to be bilayer active. Further, C-D signal due to the breakdown in symmetry of the d-DPPG/d-DPPG bilayer was observed. As shown in the left panel of Figure 3.6, prior to exposure to 11Bz, no bilayer signal was observed. However, after injection of 11Bz into the subphase, some bilayer signal was recovered. This is due to the
polymer partitioning into the distal leaflet of the bilayer and disrupting it, also disrupting the symmetry of the bilayer overall, and allowing for SFG signal to again be observed. Despite this evidence of interaction, no corresponding polymer signal was observed in the C-H stretching region, as shown in the right panel of Figure 3.6. It is possible that the reason for this is both an orientation effect and a number density issue. If 11Bz is not orienting along the bilayer plane in a regular manner, the polymer will not give SFG signal. Additionally, only 11% of the subunits contain the lipophilic benzyl ring. Because 11Bz had a degree of polymerization of roughly 12 (see Table 3.1), this translates to only one benzyl ring per polymer, on average. Clearly, this would have an effect on the orientational conformation of the polymer as it interacts with the bilayer. The benzyl rings may more or less lie down on the lipid bilayer surface. Further, SFG signals are dependent upon the number density of the SFG-active moieties present at a surface; the fewer SFG-active moieties present, the lower the SFG signal observed. It is possible that there are simply not enough SFG-active moieties present at the surface when 11Bz interacts with a lipid bilayer to produce recognizable SFG signal patterns. Another possible source of SFG signal in 11Bz is from the $\alpha$-methyl groups, located along the backbone of the polymer (see Figure 3.1). SFG $\alpha$-methyl stretching modes can be quite strong, however, as can be seen in Figure 3.6, no such signals are observed in this case. Therefore, it seems likely that the $\alpha$-methyl groups on the 11Bz polymer chain have a random distribution, giving them inversion symmetry, resulting in no observable SFG signal. A third possible source of C-H SFG signal in 11Bz are methylene stretches. Again, as no SFG signals are observed at any corresponding methylene stretches, we
conclude that these moieties, which are located close to the backbone of the polymer, have inversion symmetry and are therefore SFG-inactive.

Figure 3.6 (Left panel) Time dependent spectra monitoring the peak intensity at 2070 cm\(^{-1}\) for a d-DPPG/d-DPPG symmetric bilayer with a D\(_2\)O subphase during exposure to 11Bz at 0.50 µg/mL (red curve) and 0.08 µg/mL (black curve). (Right panel) SFG spectra of the C-H/O-H stretching region of a d-DPPG/d-DPPG bilayer with a D\(_2\)O subphase exposed to 11Bz at 0.50 µg/mL (red spectrum) and 0.08 µg/mL (black spectrum).

Exposures of 33Bu to d-DPPG/d-DPPG bilayers with a D\(_2\)O subphase were also performed at several concentrations. Results are shown in Figure 3.7. As in the case of 11Bz, clear evidence of interaction with the bilayer was observed as the symmetry of the deuterated bilayer was broken at both concentrations, shown in the left panel of Figure 3.7. However, signal from the 33Bu polymer itself was also observed, shown in the right panel of Figure 3.7.
Figure 3.7 (Left panel) Time dependent spectra monitoring the peak intensity at 2070 cm$^{-1}$ for a d-DPPG/d-DPPG symmetric bilayer with a D$_2$O subphase during exposure to 33Bu at 0.13 µg/mL (black curve) and 0.03 µg/mL (red curve). (Right panel) SFG spectra of 33Bu at (a) 0.13 µg/mL and (b) 0.03 µg/mL interacting with a d-DPPG/d-DPPG bilayer with a D$_2$O subphase. The results from spectral fittings are shown in red.

Spectrum 3.7b was taken at a subphase concentration of 0.03 µg/mL, at which perturbation of both leaflets had begun to occur, but was still minimal (see Figure 3.5). This was further supported by the time dependent SFG signal of the 2070 cm$^{-1}$ peak of the terminal CD$_3$ group of the d-DPPG lipid molecules, shown by the red curve in the left panel in Figure 3.7. Initially, before 33Bu was injected into the subphase, no bilayer signal was observed. Upon injection, some bilayer signal was recovered as the polymer disrupted the distal leaflet. This signal was due to a break in the bilayer symmetry increased quickly then decreased again, likely because the polymer penetrated further into the bilayer, disrupting the proximal leaflet, which was the source of the CD$_3$ signal spike. Apparently, the increase and then the decrease of the C-D signal should not be due to the flip-flop process, because otherwise the two leaflets should behave more or less similarly. At about thirty minutes shown in the red time-dependent curve, spectra were taken of the C-H stretching region. Spectrum 3.7b showed both symmetric and
asymmetric stretching signals due to the terminal \( \text{CH}_3 \) group on the butyl lipophilic side chain. Similarly, black time-dependent signal curve and 3.7a were taken at a subphase concentration of 0.13 \( \mu \text{g/mL} \), a concentration at which significant penetration into the bilayer’s proximal leaflet had already occurred. Again, upon injection of 33Bu into the subphase, bilayer signal recovered. Perturbation of the distal leaflet broke the symmetry of the \( \text{d-DPPG/d-DPPG} \) bilayer, and again, this signal dropped as the polymer penetrated further into the bilayer. This also indicates that the signal change is not due to the flip-flop. One noteworthy observation here was that the equilibrium signal level for the bilayer was lower for the 0.13 \( \mu \text{g/mL} \) subphase concentration than it was for the 0.03 \( \mu \text{g/mL} \) subphase concentration. For 0.13 \( \mu \text{g/mL} \), the signal returns to zero because finally the two leaflets were disrupted more or less similarly, matching the observations showing in Figure 3.5c and Figure 3.5g. While at 0.03 \( \mu \text{g/mL} \), at 30 minutes, SFG signal still decreases, perhaps does not reach the equilibrium yet. Figure 3.5b also shows a slightly stronger signal than that in Figure 3.5f, further indicating that polymer concentration is likely directly correlated to direct disruption of the bilayer matrix. Also, SFG signal was observed from the polymer itself in the C-H stretching region at the higher concentration.

### 3.3.4 Polymer Orientation

In previous research, we have shown that SFG can quite effectively be used to determine orientation of SFG active moieties on surfaces. We have also demonstrated that this approach can determine orientation of membrane-active compounds via SFG-active moieties within a bilayer. This approach can be used again here by looking at the methyl signals we observed, which can only be due to the terminal methyl group on the butyl lipophilic side chain of the polymer.
To deduce the orientation of the terminal methyl group, orientational analysis of the methyl symmetric (2880 cm\(^{-1}\) and 2940 cm\(^{-1}\)) and asymmetric mode (2960 cm\(^{-1}\)) of the terminal methyl group on the butyl lipophilic side chain was performed.\(^{30}\) It is well known that \(\alpha\)-methyl peaks can be observed by SFG if they are present on the surface and do not lie down, typically located around 2930 cm\(^{-1}\) for the symmetric stretch and 2960 cm\(^{-1}\) and 2990 cm\(^{-1}\) for asymmetric stretches,\(^{29}\) as was previously discussed. According to previous results,\(^{29}\) \(\alpha\)-methyl moieties tend to orient randomly or semi-randomly in these types of systems. Also, the lack of any \(\alpha\)-methyl SFG signal from 11Bz (discussed previously, Figure 3.6), is strong evidence that the \(\alpha\)-methyl moieties along the backbone of these polymers are randomly orienting within the bilayer. If we assume random orientation of \(\alpha\)-methyl moieties, we would not expect the \(\alpha\)-methyl peak to interfere with the asymmetric stretching mode of the terminal methyl group.

<table>
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</tr>
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<td>12.62</td>
<td>(\text{CH}_3) (s)</td>
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<td>37.72</td>
<td>15.46</td>
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</tr>
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<td>2938</td>
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<td>25.66</td>
<td>(\text{CH}_3) Fermi</td>
</tr>
<tr>
<td>2950</td>
<td>-7.131</td>
<td>12.01</td>
<td>(\text{CH}_3) (as)</td>
</tr>
</tbody>
</table>

Table 3.2 Parameters for fitting of 33Bu polymer at 0.03 \(\mu\)g/mL. Fitting was also calculated to have a nonresonance of -6.174 a.u.

<table>
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<th>Width (cm(^{-1}))</th>
<th>Possible Assignment</th>
</tr>
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<tr>
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<td>13.04</td>
<td>(\text{CH}_3) (s)</td>
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<td>(\text{CH}_3) (as)</td>
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<tr>
<td>2950</td>
<td>-16.02</td>
<td>11.99</td>
<td>(\text{CH}_3) (as)</td>
</tr>
</tbody>
</table>

Table 3.3 Parameters for fitting of 33Bu polymer at 0.13 \(\mu\)g/mL. Fitting was also calculated to have a nonresonance of -3.117 a.u.

Under this assumption, the red spectra in Figure 3.7 show the fitting results for 33Bu. The details of the fitting parameters for each concentration are shown in Tables 3.2 and 3.3. Figure 3.8 illustrates \(\chi_{xyz,as}/\chi_{xyz,s}\) as a function of orientation angle with different
orientation distribution widths (assuming Gaussian distributions). The ratio $|\chi_{yyz,\text{as}}/\chi_{yyz,\text{s}}|$ for 33Bu at each of these concentrations is nearly identical. At 0.03 µg/mL, the ratio is calculated to be 0.05, indicated by the green bar in Figure 3.8, whereas at 0.13 µg/mL, the ratio is calculated to be 0.06, indicated by the red bar in Figure 3.8. This corresponds to a limited range of possible orientations ($\theta < 15^\circ$) and orientation distribution width ($\sigma < 10^\circ$). These results point toward a slight tilt from the surface normal with a reasonably narrow orientational distribution, indicating that the butyl side chain is orienting roughly parallel to the bilayer surface normal. These results seem to indicate that 33Bu interacts with the bilayer by penetrating into the lipid bilayer core with the acyl chains. This mechanism does not seem to be concentration dependent, given the similar orientation results at multiple concentrations spanning the range of bilayer-active subphase concentrations.

Figure 3.8 Relation between $\chi_{yyz,\text{as}}/\chi_{yyz,\text{s}}$ and the orientation/orientation distribution of the terminal methyl group of the side chain of 33Bu. “Delta” in this case refers to a delta-distribution, with an orientation angle distribution width of 0°, shown by the solid curve above. The dotted curves correspond to other assumed distribution widths (10°, 20°, 30°, 40° and 50°). The shaded bars represents the calculated ratios at each concentration, which were found to be 0.05 for 0.03 µg/mL (green bar) and 0.06 for 0.13 µg/mL (red bar).
3.3.5 Membrane Disruption

These results yield some interesting clues as to the details of the interaction mechanisms between antimicrobial polymers and lipid bilayers. First, it is clear from the comparison of the activities of polymers with differing R groups that the identity of the antimicrobial moiety has a significant impact on interaction. The polymers with 0R and 33Me groups had far higher MICs than did 33Bu or 11Bz, while the latter two showed far more hemolytic activity.

Of the two polymers which showed activity at lower concentrations (33Bu and 11Bz), significantly different interaction mechanisms were observed. While no specific orientation was able to be determined for 11Bz, it was clearly seen that the inner and outer bilayer leaflets were independently disrupted. The lack of SFG signal from the benzyl rings perhaps indicates that they more or less lie down on the membrane surface. They may interact/disrupt the outer leaflet first, then interact/disrupt the inner leaflet. In contrast, a simultaneous decrease in leaflet signals was observed for 33Bu. This could be due in part to the higher level of activity against the bilayer for 33Bu than for 11Bz; the polymer may be interacting to a further degree that prevents us from observing the separation of effect between leaflets. However, orientation analysis of the terminal methyl group was possible for 33Bu, which provides additional interaction information. The terminal methyl group was found to be orienting roughly parallel to the surface normal of the bilayer. It seems most likely that, to adopt this type of conformation, the terminal methyl group, and by extension the majority of the butyl chain, is intercalating within the hydrophobic core of the bilayer, directly disrupting the lipid acyl chains and causing the loss of SFG signal from the bilayer.
It is particularly interesting that the butyl chains exhibit this interaction mechanism whereas the benzyl rings do not. One possible explanation for the probable lack of quick penetration into the hydrophobic core by the benzyl groups is steric hindrance. In the gel phase, a DPPG symmetric bilayer is tightly packed, and a benzyl group would necessarily require a greater degree of disruption than would a butyl chain. Additionally, a butyl group, while far shorter than the 16-carbon acyl chain on a DPPG lipid, is still far more similar to the chain than a benzyl ring. It is possible that the principle of “like dissolves like” may apply in this instance; polymers with antimicrobial groups structurally similar to structures within the lipids themselves could be more likely to penetrate into the core of the bilayer at lower concentrations.

These results provide useful guidance in the design and development of future antimicrobial polymers. It is very clear that the identity of the lipophilic group plays a major role in determining the antimicrobial character of the polymer, both in terms of its bulk effect (referring to antimicrobial activity and hemolytic toxicity) and its molecular level interaction kinetics. Polymers with acyl chains of at least four carbons show the best ability to interrupt the bilayer of those lipophilic chains tested here. Future studies of the kinetics of these polymers should provide greater insight into the detailed polymer-bilayer interaction mechanism, further yielding guidance in the design of therapeutic polymeric compounds.

3.4 Conclusions

These studies describe a characterization of several different membrane-active copolymers and lipid bilayers via SFG. By monitoring the C-H and C-D stretches within the bilayer and the polymer itself, the interaction between the two was characterized and
correlated with the antimicrobial activity data from biological tests. Disruption of the bilayer was observed as a function of concentration for each membrane-active co-polymer. Polymers 0R and 33Me were membrane-inactive up to 3.1 ug/mL, whereas 11Bz and 33Bu showed potent activity in all tests. Further, spectroscopic confirmation of a higher level of activity for 33Bu than 11Bz was found. The 11Bz polymer is largely made up of cationic subunits, with, on average, only one lipophilic benzyl-containing side chain on each polymer molecule. It is possible that this may be related to its somewhat lower activity. Further tests into determining the importance of differing the ratio of polar and non-polar subunits are warranted.

SFG spectra collected from the 11Bz and 33Bu polymers themselves yielded dramatically different results. The 11Bz polymer yielded no recognizable SFG signals, indicating either a lack of preferential orientation of specific moieties along the bilayer surface normal or a lack of enough polymer at the surface to give rise to significant SFG signal. In contrast, 33Bu showed clear orientational organization, with the terminal methyl group orienting approximately parallel to the surface normal of the bilayer.

Taken together, these results paint an interesting picture of the potential interaction mechanisms by which antimicrobial polymers operate. Clearly, the activity and toxicity levels of the polymer are not sufficient to characterize those mechanisms. The identity of the lipophilic segments also has an obvious effect, as shown by the differences in interaction mechanisms between 33Bu and 11Bz. The antimicrobial and hemolytic studies were not, by themselves, enough to capture the full description of how these polymers interact with lipid bilayers over time. SFG studies were needed to identify the different interaction patterns between 33Bu and 11Bz. The higher activity, toxicity
and selectivity of 33Bu in comparison to 11Bz give insight into several important parameters of the polymers for intentional design. First, the identity of the lipophilic group is incredibly important. The benzyl group in 11Bz is far more bulky than the butyl chain in 33Bu, which strongly implies, given steric hindrance considerations, that the benzyl group will have a far harder time penetrating into the bilayer’s lipophilic core. Thus, lipophilic groups that are structurally similar to lipophilic groups in the bilayer core (in this case, alkyl chains) are best able to intercalate into that core, making them more effective at bilayer disruption. Second, amphiphilic balance may also impact the interaction mechanism. Using the polymers studied here, direct comparison between 33Me and 33Bu, which is the only comparison between two polymers of the same amphiphilic balance, is unhelpful, as the 33Me polymer was membrane-inactive. Comparing 33Bu to 11Bz indicates that number density of the lipophilic groups may impact the extent to which antimicrobial polymers can penetrate into the membrane. These are all low molecular weight polymers, and with only ~11% of the subunits of 11Bz containing the benzyl group, this translates to one benzyl ring per polymer, on average. As discussed previously, this low number density of moieties could explain the lack of observed SFG signal, however increasing the number density of these moieties could impact the interaction mechanism of the benzyl-based polymers. Further studies in this area should take this into account and, while controlling for all other variables, investigate the effect changing amphiphilic balance can have on interaction mechanisms such as those shown here.

While these results are an interesting first step towards development of a complete interaction mechanism between antimicrobial polymers and lipid bilayers, further
research should be done to flesh out these relationships. For example, consideration of the effect of molecular weight of the polymer may be important when considering optimal structures for any particular clinical purpose. Another consideration for future studies is effect of alkyl chain length on the activity and toxicity of these polymers. Importantly, future studies considering differences in structural parameters between membrane-active polymers should also seriously focus on how those differences in structure effect the interaction mechanism with bilayers, and not just bulk properties such as activity, toxicity and selectivity.
3.5 References


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CHAPTER 4

CHARACTERIZATION OF SURFACE STRUCTURES OF A SERIES OF RANDOM ANTIMICROBIAL TRIPOLYMERS

4.1 Introduction

Polymeric materials are a fundamental technology in today’s world, and the surface properties of polymers, such as wettability, friction, biocompatibility and basic organization, all play a significant role in how such polymers interact with the general environment.\textsuperscript{1-5} Understanding of how those surface properties are impacted by the molecular makeup of the polymeric surface could be extremely important to the intelligent design of polymers for multiple applications. Polymers with antimicrobial properties have in particular been receiving increasing attention for two major uses: prevention of infection by bacterial accumulation on medical surfaces, and antifouling or fouling release coatings.

The development of new materials that can effectively inhibit growth of microbes and prevent bacterial accumulation on a surface has received increasing attention because 60-70\% of nosocominal microbial infections are associated with medical devices and implants.\textsuperscript{6} Another major research focus has been to develop antifouling or fouling-release coatings, where organisms are unable to effective adhere to the surface, and are easily removed via mechanical means. One approach involves the use of antimicrobial materials that are capable of inflicting damage on the sessile organism, thus preventing it
from effectively adhering to the surface.7-13 One of strategies to prepare such materials is to incorporate biocidal agents into coating matrices,14-17 which are released from the surface and kill bacteria in solution or a proxy of the surface, preventing bacterial growth.18 Alternatively, biocides can be permanently fixed to create non-leaching surfaces through covalent attachment, killing bacteria on contact.19-24 Synthetic polymers containing alkyl quaternary ammonium groups in their side chains have been widely used to incorporate bactericidal function into material surfaces like glass,25 plastics,26 and cellulosic filter papers.27 Although the biocidal mechanism of these surfaces is still in debate, the polycations are believed to reach bacterial cell membranes and disrupt the membranes integrity, resulting in cell death.24,28 Another proposed mechanism is that, although the polymer chains may not bind to cytoplasmic membranes, the cationic groups of polymers could replace the divalent cationic ions such as Mg$^{2+}$ and Ca$^{2+}$ in bacterial cell membranes, causing the disruption of membrane structures.19,21,25

As was previously discussed in Chapter 1, many of the methods used for characterizations of these types of materials are focused on biological and topological characteristics in aqueous environments, such as marine field tests,29 lab biological studies,30 and AFM.31 These methods may be insufficient to effectively guide the development of polymeric coatings for medical or antibiofouling applications because the correlations between properties and structures of the polymer at the surface are also relevant. If possible, whatever characterization technique used to study such structures should be sensitive to molecular features of the material, especially organization structure, orientation and chemical bonding on the surface or at the interface. Changes in
these specific features are subject to subtle differences in energy at the molecular level, so highly sensitive spectroscopic techniques are ideal for these types of characterizations.

Much research has focused on understanding the polymer/air and polymer/water interfaces. Several techniques exist to do this, but each has drawbacks. Secondary ion mass spectrometry (SIMS) can generate molecular level information, however it requires ultrahigh vacuum to do so, which prevents studies of the hydrated polymer state. X-ray photoelectron spectroscopy (XPS), also a technique requiring ultrahigh vacuum, has been developed to study hydrated surfaces using freeze-dried samples, but it cannot give orientational information regarding surface groups.\textsuperscript{32} Also, the sample preparation method is very complicated. Contact angle goniometry can be used to measure surface hydrophobicity and how polymer surfaces may change when exposed to water, but cannot provide molecular-level information about the material surface under study.\textsuperscript{33}

Sum frequency generation (SFG) vibrational spectroscopy is a powerful, surface-specific spectroscopic tool with submonolayer sensitivity.\textsuperscript{34-51} As discussed previously, it is very different from many other surface analytical tools, such as XPS or SIMS because it does not require a high vacuum and can be applied to any system where the surface or interface of interest is accessible by light. Because of its unique capabilities, SFG has begun to revolutionize surface science. It has also been extensively applied to studies of polymer surfaces.\textsuperscript{52-60} SFG collects a vibrational spectrum of the surface of the polymer material with submonolayer sensitivity, resulting in a spectrum of only those functional groups on the surface, and not in the bulk. By altering the polarization of each of the input and output beams, different polarization combinations can be used to access orientational information about the surface functional groups.
Figure 4.1 Scheme of synthesis of antimicrobial copolymers. Molar ratio of side chains was varied to synthesize each of the polymers.

The research presented in this chapter provides a surface characterization of a series of novel antimicrobial tri-polymer materials, shown in Figure 4.1, at both polymer/air and polymer/water interfaces. The molar ratio of the components in this series of polymers will be varied to determine the effect each moiety had on the surface properties of wettability, surface roughness, antimicrobial activity in solution and on a surface, and the molecular structure as studied via SFG. A summary of the relative mole ratios of each side chain for each polymer is shown in Table 4.1. Several polymers will be studied, each with differing ratios of the three subunits: a catechol group, a methoxy group, and a quaternary ammonium group with a long alkyl chain. Bacterial characterization tests will confirm the antimicrobial nature of the surface. Contact angle and atomic force microscopy (AFM) studies will indicate three of the polymers organize roughly equivalently, with the fourth showing higher hydrophobicity and surface roughness. It is possible that this is due to the complete lack of a methoxy-containing subunit in the polymer, and suggests that all three subunits are needed to generate a smooth surface. SFG vibrational spectroscopy studies will be shown performed in at both polymer/air and polymer/water interfaces. SFG signal was obtained from the polymer surface. Domination of the surface by the alkyl chain subunit will be demonstrated. The
polymer/water interface will be shown to induce randomization of the polymeric surface, resulting in a complete loss of SFG signal. This will be found to be a reversible process, whereupon removal from contact with water and subsequent drying of the surface allows for recovery of SFG signal. Finally, orientation analysis of the alkyl chain terminal \( \text{CH}_3 \) group lies perpendicular to the surface. Taken together, these results will show a detailed, molecular level understanding of how and why the polymeric subunits self-organize into layers of the subunits, which has consequences for the efficacy of the polymer as a biocide.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>x%</th>
<th>y%</th>
<th>z%</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>11</td>
<td>89</td>
<td>0</td>
</tr>
<tr>
<td>P1</td>
<td>12</td>
<td>72</td>
<td>16</td>
</tr>
<tr>
<td>P2</td>
<td>12</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>P3</td>
<td>13</td>
<td>31</td>
<td>56</td>
</tr>
<tr>
<td>P4</td>
<td>12</td>
<td>0</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 4.1 Mole ratio of synthesized antimicrobial copolymers.

4.2 Experimental Methods

4.2.1 Polymer synthesis

Dopamine-HCl, 1-bromododecane, and methoxyethyl acrylate (MEA) were purchased from Acros. 2, 2’-Azobisisobutyronitrile (AIBN) and 2-(dimethylamino)ethyl methacrylate (DMAEMA) were ordered from Sigma-Aldrich (St. Louis, MO). Methacrylate anhydride was purchased from Polysciences (Warrington, PA). Reagent grade solvents were obtained from Fisher (Hanover Park, IL). These chemical agents and solvents were used without further purification. Pre-cleaned glass slides were acquired from Fisher. A live/dead Baclight bacterial viability kit was purchased from Invitrogen (L-7007, Carlsbad, CA). Mueller-Hinton (MH) broth and agar were obtained from Difco Laboratories (Franklin Lakes, NJ). The monomer dopamine methacrylamide (DMA) was
prepared by the procedure described by Lee et al. The dodecyl quaternary ammonium monomer (DMAEMAC$_{12}$) was prepared by the procedure described by Ravikumar et al.

A series of random copolymers were prepared in the Kuroda lab at the University of Michigan by altering the mole ratio of DMA, MEA and DMAEMAC$_{12}$ (Table 4.1). The total amount of monomers was kept constant at 2 mmol. The mol % of DMA was fixed at 15% in the feed monomer compositions. All monomers (typically, 66.5 mg for DMA, 0-182.2 mg for MEA, and 122.1-691.0 mg for DMAEMAC$_{12}$) were dissolved in dimethylfuran (DMF) (5 mL) and then AIBN (3.3 mg, 0.02 mmol) was added. The solution was bubbled with N$_2$ for 5 minutes and heated at 60 °C overnight. The resultant polymers were precipitated in diethyl ether (200 mL). The polymer was dissolved in a small amount of methanol and precipitated again in diethyl ether. This procedure was repeated three times. Random copolymers poly(MEA-DMAEMAC$_{12}$) with the different mole ratio of MEA and DMAEMAC$_{12}$ and homopolymer poly(DMAEMAC$_{12}$) were prepared by the same polymerization conditions.

4.2.2 Sample preparation

Thick polymer films were prepared by spin-coating a 2 wt% solution of P1, P2, P3 or P4 dissolved in water onto fused silica substrates using a spin-coater from Specialty Coating Systems (Indianapolis, IN), which yielded thin films approximately 100 nm thick. The samples were subsequently placed in a 110° C oven for at least 1 hr prior to analysis to ensure removal of any residual solvent.
4.3 Results and Discussion

4.3.1 Biological Studies of Polymers

The antimicrobial activity of the polymers was evaluated in solution by the minimal inhibitory concentration of polymers (MIC), which was determined by the turbidity-based assay method in the Kuroda lab. Because of the low water-solubility of polymers, the assay media contained 5 v/v% dimethyl sulfoxide (DMSO) in which any polymer precipitation was not apparent during the assay, the results of which are shown in Table 4.2. Under these conditions, all of the polymers displayed high activity against *Escherichia coli* (MIC < 7.8 µg/mL). The polymers immediately precipitated in the medium with no or less amount of DMSO and did not display any antimicrobial activity. On the other hand, the polymers showed relatively weak activity against *Staphylococcus aureus* (MIC > 62.3 µg/mL) compared to *E. coli*. Interestingly, the activity against *S. aureus* depends on the monomer compositions; with P2 displaying low MIC values (<125 µg/mL), indicating the amphiphilic balance of polymers may play an important role in the anti-*S. aureus* activity of polymers.

The antimicrobial activity of the polymer coatings was examined in a dynamic solution condition, in which the coated glass-slides were soaked in bacterial dispersions and incubated with gentle shaking. These data are summarized in Tables 4.2 and 4.3. The P0 coating, which has no DMAEMAC\textsubscript{12}, showed no reduction of *E. coli* and *S. aureus* as compared to the control. The activity against *E. coli* depends on the ratio of DMAEMAC\textsubscript{12} and MEA; no detectable *E. coli* cells remained in suspension for the P2 and P3 coatings, while the P1 and P4 coatings displayed only a fraction of reduction in the number of viable *E. coli* cells. These results indicate that the dodecyl quaternary
ammonium groups are responsible for the antimicrobial activity of the coatings. It also seems that an optimal ratio of DMAEMAC\textsubscript{12} and MEA is necessary to achieve potent antimicrobial activity. The polymers also showed the same trend in their activity against \textit{S. aureus} as for \textit{E. coli} although they appear to be less effective as compared to \textit{E. coli}. To determine if any antimicrobials leached from the coatings to kill bacteria in solution, the coated glass slides were soaked in PBS buffer without bacteria, and this solution was added into an \textit{E. coli} suspension. The PBS buffer solution incubated with the coatings of polymers P1-4 showed no activity.

| Polymer | MIC (µg/mL)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{E. coli}</td>
</tr>
<tr>
<td>P1</td>
<td>&lt; 7.8</td>
</tr>
<tr>
<td>P2</td>
<td>&lt; 7.8</td>
</tr>
<tr>
<td>P3</td>
<td>&lt; 7.8</td>
</tr>
<tr>
<td>P4</td>
<td>&lt; 7.8</td>
</tr>
</tbody>
</table>

Table 4.2 Antimicrobial activity of polymers in solution.

<table>
<thead>
<tr>
<th>Polymer coatings</th>
<th>Viable cells (10\textsuperscript{5} cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{E. coli}</td>
</tr>
<tr>
<td>Control</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>P0</td>
<td>3.8±3.0</td>
</tr>
<tr>
<td>P1</td>
<td>1.7±0.4</td>
</tr>
<tr>
<td>P2</td>
<td>0\textsuperscript{*}</td>
</tr>
<tr>
<td>P3</td>
<td>0\textsuperscript{*}</td>
</tr>
<tr>
<td>P4</td>
<td>2.3±0.5</td>
</tr>
</tbody>
</table>

Table 4.3 Dynamic contact antimicrobial activity of polymer coatings. “Control” in this case refers to an uncoated glass slide. *The assay solution for these polymers was plated without dilution, and no colony formation was observed.

\subsection*{4.3.2 Water Contact Angle Measurement on Polymer Films}

To determine the hydrophobicity of P0-P4, static contact angle measurements were obtained from coatings of each polymer on glass slides. Measurements are shown in Table 4.4. The reported contact angle measurements are all significantly different than contact angle measurements of bare glass slides, which was so close to zero as to be
unmeasurable by our instrument. The contact angle for P0, P1, P2 and P3 were all effectively identical, averaging approximately 82°. There was a slight decrease in the contact angle as the percentage of the hydrophilic y-subunit decreased, however this decrease was within the standard deviation of the samples and cannot be used to determine any trend. In contrast, P4 had a drastically different contact angle (105.3°), more than 20° higher than the other three polymers. We therefore conclude that P4 is more hydrophobic than the other four polymers. A possible explanation for this is the fundamental difference in polymer structure between P4 and the other three polymers. As previously described in Table 4.1, P1, P2 and P3 all contain some portion of each of the three side chains, albeit at different relative ratios. Polymer P4 does not contain the y subunit, and it is likely that the absence of this side chain is responsible for the change in surface hydrophobicity. The y-subunit contains a methoxy group that should be hydrophilic, making the polymer more hydrophobic overall without its presence. Interestingly, P0, which has no alkyl chain-containing z-subunits, still shows a similar hydrophobicity to P1-P3. This seems to indicate that it is the alkyl chain that is responsible for the hydrophobic character of the surface. It is worth noting that for P1, P2 and P3, as the methoxy side chain percentage increased, the hydrophobic alkyl chain percentage (from subunit z) decreased, which should theoretically make the polymer surface even more hydrophilic. In spite of this, all four y-subunit containing polymers showed approximately the same contact angle value, so the presence of the hydrophilic side chain at or above any of the tested percentages was enough to impact the hydrophobic nature of the polymer surface.
### Table 4.4 Contact angle measurements for P0-P4. Glass slides were used as substrates.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Water contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>82.9 ± 2.3</td>
</tr>
<tr>
<td>P1</td>
<td>83.7 ± 2.1</td>
</tr>
<tr>
<td>P2</td>
<td>81.7 ± 1.4</td>
</tr>
<tr>
<td>P3</td>
<td>80.4 ± 0.9</td>
</tr>
<tr>
<td>P4</td>
<td>105.3 ± 2.6</td>
</tr>
</tbody>
</table>

4.3.3 Atomic Force Microscopy of Polymer Films

It is possible that the polymers are forming different surface domain structures when applied to a substrate, which could theoretically provide another possible explanation for differing hydrophobicities between the polymers. To investigate this, AFM images were obtained of each of the polymers spin-coated onto fused silica window substrates. The image for each polymer is shown in Figure 4.2. Visually, the images of P1, P2 and P3 are all reasonably similar and reasonably smooth, with no structures of significant note. In comparison, P4 showed clear structuring into polymer domains, rather than a smooth surface. While specific structural components cannot be determined from these images alone, this is further, visual evidence of what was already seen in the contact angle experiments: P4 has a very different surface structure than P1, P2 or P3.
To compare the surface roughness of the three samples, it was necessary to calculate the root mean squared (RMS) roughness value for each sample. Table 4.5 shows the RMS roughness for each polymer calculated from the AFM images shown in Figure 4.2. The RMS roughness of P1, P2 and P3 were all within 1-2 nm, with P1 as the roughest of the three and P3 the smoothest. However, P4 again showed dramatically different results, with an RMS roughness significantly higher than the other three polymers. Given the domains P4 was observed forming, this increase in roughness was
expected. Clearly, the presence of the methoxy-containing y subunit introduces some degree of stabilization to the polymer matrix, allowing for a smoother, continuous surface. However, the effect of this is somewhat limited as well; P3, with the lowest percentage (25%) was the smoothest, and increasing the y-subunit percentage for P2 (40%) and P1 (70%) actually made the surface rougher.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>RMS Roughness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1.83</td>
</tr>
<tr>
<td>P2</td>
<td>1.29</td>
</tr>
<tr>
<td>P3</td>
<td>1.03</td>
</tr>
<tr>
<td>P4</td>
<td>4.35</td>
</tr>
</tbody>
</table>

Table 4.5 RMS roughness calculated from AFM images for polymers P1-P4.

4.3.4 SFG Studies of Polymer Films

Figure 4.3 SFG spectrum of P0 in ssp polarization combination.

Fused silica substrates coated with polymer thin films were placed into the SFG spectrometer with the polymer surfaces located on the underside of the substrate, in what is referred to as the face-down geometry. An SFG spectrum was taken of P0 in ssp polarization, and is shown in Figure 4.3. The peak assignments of C-H stretching modes have been well-studied with SFG. The dominant peak at 2815 cm$^{-1}$ is attributed to the symmetric stretch of the methoxy group within the y-subunit. Since P0 contains only the catechol- and methoxy-containing subunits, this indicates that the methoxy groups
dominate the surface over the catechol groups. The smaller peak at ~2955 cm\(^{-1}\) can most likely be attributed to the asymmetric stretch of the methoxy moiety.

SFG spectra were taken of each polymer in ssp and sps polarizations, shown in Figure 4.4. Peak assignments for the ssp spectra are summarized in Table 4.6. In the ssp spectra (black spectra), three dominant peaks, one with two shoulders, and two weaker peaks can be observed in the spectra. The peak at ~2880 cm\(^{-1}\) is attributed to the symmetric stretch of the terminal CH\(_3\) group of the long alkyl chain. The dominant peak at ~2945 cm\(^{-1}\) is attributed to the CH\(_3\) Fermi resonance. The third peak, located at ~2850 cm\(^{-1}\) in each spectrum, is attributed to the symmetric stretch of CH\(_2\) groups in the long alkyl chain of the z-subunit. The symmetry requirements of SFG dictate that likely the two CH\(_2\) groups present between the backbone and the terminal functional group of the x- and y-subunits will cancel each other out, resulting in no SFG signal. Similarly, the two CH\(_2\) groups between the backbone and quaternary nitrogen of the z-subunit will also likely spectrally cancel. However, of the 11 CH\(_2\) groups in the terminal alkyl chain, only 10 will cancel out, allowing one CH\(_2\) group to generate observable SFG signal. In addition, for this long alkyl chain, gauche defects are more likely to occur, contributing to SFG signals. Given this type of a structure, the relative intensities of the methyl and methylene peaks tell us something about the surface organization of the alkyl chains. In polymer P1, with only a 15% mole ratio of the z-subunit, the alkyl chain is not subject to the same degree of steric hindrance from other alkyl chains at the surface, and is therefore likely to be stretched out, minimizing any gauche defects along the chain that would disrupt the chain’s symmetry and result in greater SFG signal. P2, which has a 45% z-subunit mole ratio, is more likely to have such defects. As can be seen in Figure 4.4B, the
CH₂ peak at 2850 cm⁻¹ is greatly increased relative to its neighboring methyl peak, which has not appreciably dropped in intensity. P3, with a 60% z-subunit mole ratio, again sees an increase in methylene signal due to this disruption of symmetry. Finally, as we would expect, P4, at with an 85% z-subunit mole ratio, sees the greatest amount of gauche defects due to steric hindrance preventing full extension of the alkyl chain. This trend also makes clear that the hydrophobic groups contained within the z-subunit are dominant on the surface in all of the tested polymers.

![Graphs of SFG spectra](image)

**Figure 4.4 SFG spectra of (A) P1, (B) P2, (C) P3, and (D) P4 in ssp (black) and sps (red).**

Several other peaks are observable in the ssp spectra as well. A small negative peak is observable at 2960 cm⁻¹. This is likely due to the asymmetric stretch of the methyl group. A large shoulder on the methyl Fermi resonance is observable at ~2925 cm⁻¹. This
could theoretically be due to a number of sources within our system. One possibility is an α-methyl symmetric stretch, while another possibility is a methylene asymmetric stretch. It is also possible that both of these stretches are contributing to this peak, which is problematic as these stretches should be out of phase. Determination of the exact contribution from each stretch was outside the scope of this research. Rather, a phase was simply chosen to best fit the spectra as observed. Another small peak is observable at ~3040 cm$^{-1}$. This peak is both weak and quite broad, and can be attributed to the catechol group in the x-subunit,$^{66}$ indicating that some catechol groups are present on at the polymer surface.

Finally, a small peak is observable in the ssp spectrum in 4.4A at 2815 cm$^{-1}$. As was demonstrated using P0 in Figure 4.3, this peak can be attributed to the symmetric stretch of the methoxy group in the y-subunit.$^{65}$ The presence of this stretch in the spectrum of P1 indicates that, with a 70% mole ratio, some methoxy groups are organized at the surface. As the mole ratio of the y-subunit decreases, so does the intensity of this peak relative to others in the ssp spectra (Figures 4.4B and 4.4C), indicating a decrease in the presence of methoxy groups at the polymer surface. Interestingly, this peak returns in the ssp spectrum of P4, despite having no y-subunits within its structure (and therefore no methoxy groups). The complete absence of methoxy groups means this peak must be attributed to a different moiety, and it can be attributed to the N-(CH$_3$)$_2$ group within the z-subunit.$^{67-68}$ It is possible that the z-subunit does not pack tightly enough to force the N-(CH$_3$)$_2$ groups into an organized layer at the surface until the subunit reaches a certain mole ratio. Investigation into the necessary density of surface groups to force
organization of the near-backbone moieties would be necessary to satisfactorily answer this question.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Wavenumber (cm$^{-1}$)</th>
<th>Amplitude (a.u.)</th>
<th>Width (cm$^{-1}$)</th>
<th>Possible Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>2818</td>
<td>45.41</td>
<td>11.47</td>
<td>O-CH$_3$ (s)</td>
</tr>
<tr>
<td></td>
<td>2848</td>
<td>26.40</td>
<td>15.50</td>
<td>CH$_2$ (s)</td>
</tr>
<tr>
<td></td>
<td>2918</td>
<td>55.38</td>
<td>33.95</td>
<td>$\alpha$-CH$_3$, CH$_2$ (as)</td>
</tr>
<tr>
<td></td>
<td>2953</td>
<td>21.20</td>
<td>9.928</td>
<td>$\alpha$-CH$_3$ or O-CH$_3$</td>
</tr>
<tr>
<td>P1</td>
<td>2815</td>
<td>3.010</td>
<td>4.005</td>
<td>O-CH$_3$ (s)</td>
</tr>
<tr>
<td></td>
<td>2853</td>
<td>2.374</td>
<td>2.011</td>
<td>CH$_2$ (s)</td>
</tr>
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<td>2882</td>
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<td>2922</td>
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<td>22.77</td>
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<td>2945</td>
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<td>CH$_3$ Fermi</td>
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<td></td>
<td>2960</td>
<td>-6.980</td>
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<td></td>
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<td>78.91</td>
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<td>5.012</td>
<td>6.756</td>
<td>O-CH$_3$ (s)</td>
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<td>4.998</td>
<td>O-CH$_3$(s)</td>
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<td>3020</td>
<td>106.7</td>
<td>50.10</td>
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<td>2813</td>
<td>2.333</td>
<td>5.101</td>
<td>N-(CH$_3$)$_2$</td>
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<td>2940</td>
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<td>7.500</td>
<td>CH$_3$ Fermi</td>
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<td>2960</td>
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<td>CH$_3$ (as)</td>
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<td></td>
<td>3040</td>
<td>158.2</td>
<td>50.02</td>
<td>Benzyl ring</td>
</tr>
</tbody>
</table>

Table 4.6 Fitting results for SFG spectra from polymer/air interface in ssp polarization combination.

Spectra were also collected from each polymer in the sps polarization combination, shown by the red spectra in Figure 4.4. These spectra looked very different than those collected in ssp, and were far weaker. The spectra shown in Figure 4.4 have
been scaled by a factor of 20 to allow for visual comparison. The sps spectra show two dominant peaks. The peak at ~2960 cm$^{-1}$ can be attributed to the asymmetric CH$_3$ stretch, and the peak at ~2920 cm$^{-1}$ can likely be attributed to the asymmetric CH$_2$ stretch. The peak due to the terminal methyl group is especially useful here, as it allows for a more rigorous orientation analysis, which is detailed in the following section.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Wavenumber (cm$^{-1}$)</th>
<th>Amplitude (a.u.)</th>
<th>Width (cm$^{-1}$)</th>
<th>Possible Assignment</th>
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</tr>
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<td>14.89</td>
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</tr>
<tr>
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<td>50.01</td>
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<td>12.45</td>
<td>CH$_3$ (as)</td>
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Table 4.7 Fitting results for SFG spectra from polymer/air interface in sps polarization combination.

4.3.5 Terminal Methyl Orientation

Previous research has shown that SFG can be used to determine orientation of SFG-active moieties organized on a surface.$^{60,69}$ This technique was previously applied in both Chapters 2 and 3, and has again applied here to determine the orientation of the terminal methyl groups of the alkyl chain within the z-subunit in each polymer, which is the only possible source of CH$_3$ signal in these compounds. To deduce the orientation of the terminal methyl group, three different orientational analyses were performed using ratios of the symmetric and asymmetric stretches of the methyl group. It is very clear that the peak at 2880 cm$^{-1}$ comes from the symmetric C-H stretch of the CH$_3$ groups. However, it is possible that the 2960 cm$^{-1}$ contains contributions from methyl, methoxy...
and/or α-methyl asymmetric stretches. Unfortunately we are not able to separate the contributions from methyl, methoxy, and α-methyl asymmetric stretching. Here we do believe that the 2960 cm\(^{-1}\) peak is mainly due to the methyl asymmetric stretching, and approximate the signal strength of the 2960 cm\(^{-1}\) to be contributed by the methyl asymmetric stretching.

The first method to deduce the methyl orientation is to compare the symmetric and asymmetric stretches observed in the ssp spectrum, and Figure 4.5 illustrates the dependence of \(\chi_{yyz,as}/\chi_{yyz,s}\) ratio on methyl orientation angle for different orientation distributions (assuming Gaussian orientation distributions). The ratios \(\chi_{yyz,as}/\chi_{yyz,s}\) for all four polymers are not very different, with P1 having the lowest, at 0.254, and P4 having the highest, at 0.461. Based on these findings, for a delta distribution around the orientation angle, P1 stands the most vertically (\(\theta < 30^\circ\)), followed by P2 (\(\theta < 35^\circ\)), P3 (\(\theta < 40^\circ\)), and finally P4 (\(\theta < 45^\circ\)). It is noteworthy that this is the pattern we see, as this also follows the increasing mole ratio of the alkyl chain-containing z-subunit. This would seem to imply that the alkyl chain tends to orient in a way that allows the terminal methyl group to stand more or less vertically off the polymer-coated surface, however increasing surface coverage of the alkyl chain inhibits or partially disturbs this behavior. This logically follows the previously observed increase in methylene signal due to packing-induced gauche defects seen as the mole ratio of the z-subunit increases as well.
Figure 4.5 Relation between $\chi_{yzy,as}/\chi_{xyz,as}$ and the orientation of the CH$_3$ groups with different orientation distributions. “Delta” in this case refers to a delta-distribution, with an orientation angle distribution width of 0°, shown by the thickly shaded curve above. The subsequent dotted curves correspond to other assumed distribution widths: 10° (−), 20° (···), 30° (−·), 40° (---) and 50°(− −). Ratios were calculated to be 0.254 for P1 (blue bar), 0.267 for P2 (green bar), 0.352 for P3 (yellow bar), and 0.461 for P4 (red bar).

To further investigate the orientation of the terminal methyl groups, two other orientation analyses were performed. The second orientational analysis compares the ratio $\chi_{yzy,as}/\chi_{xyz,as}$ and is shown in Figure 4.6. Again, we see a similar pattern, with the terminal methyl group in P1 standing the most vertically ($\theta < 35^\circ$), and subsequently increasing as the mole ratio of the z-subunit increases (P2, $\theta < 42^\circ$; P3, $\theta < 45^\circ$; P4, $\theta < 50^\circ$). Finally, the third orientation analysis compares the ratio $\chi_{yzy,as}/\chi_{xyz,as}$ and is shown in Figure 4.7. Here again, the terminal methyl group of P1 has oriented closest to the surface normal ($\theta < 30^\circ$), with a subsequent increase in $\theta$ as the mole ratio of the z-subunit increases.
Figure 4.6 Relation between $\chi_{\text{YZZ,as}} / \chi_{\text{YZZ,as}}$ and orientation of the CH$_3$ groups with different orientation distributions. “Delta” again refers to a delta-distribution, with an orientation angle distribution width of 0°, shown by the thickly shaded curve above. The subsequent dotted curves correspond to other assumed distribution widths: 10° (--), 20° (⋯), 30° (---), 40° (---) and 50° (-- --). Ratios were calculated to be 0.399 for P1 (blue bar), 0.335 for P2 (green bar), 0.328 for P3 (yellow bar), and 0.265 for P4 (red bar).

Figure 4.7 Relation between $\chi_{\text{YZZ,as}} / \chi_{\text{YZZ,as}}$ and orientation of the CH$_3$ groups with different orientation distributions. “Delta” again refers to a delta-distribution, with an orientation angle distribution width of 0°, shown by the thickly shaded curve above. The subsequent dotted curves correspond to other assumed distribution
widths: 10° (−), 20° (−−), 30° (---), 40° (----) and 50°(-- −). Ratios were calculated to be 0.175 for P1 (blue bar), 0.207 for P2 (green bar), 0.223 for P3 (yellow bar), and 0.303 for P4 (red bar).

The quantitative methyl orientation angles deduced on different surfaces using different susceptibility component ratios are not identical, perhaps due to minor contributions from α-methyl and methoxy to the asymmetric stretching signal. However, all three of the orientation analyses indicate a similar pattern of alkyl chain structuring on the surface: the terminal methyl groups tend to stand up on the surface. Additionally, the trend of the orientation angles is the same: the angle of the methyl groups relative to the polymer surface normal increases from the P1 surface to the P2 surface and then to the P3 surface. The methyl groups tilt most toward the surface on the P4 surface (with the largest orientation angle). The methyl orientation is highly dependent on surface coverage of the alkyl chain. A higher mole ratio of the z-subunit necessarily results in a more densely-packed surface of alkyl chains, which clearly impacts the orientation of the terminal methyl group. Further, these results agree well with the previously discussed analysis of the change in the relative intensities of the methyl and methylene groups. Clearly, the extent of surface coverage is an important parameter in the overall structure of these polymers on a surface.

4.3.6 SFG Studies of the Polymer/Water Interface

To determine the effect that contact with water can have on the surface organization of the polymer, SFG spectra of the polymer surface were taken before, during and after contact with D₂O. These spectra are shown in Figure 4.8. In all cases, all SFG signal was lost immediately upon contact with water. Once the sample was removed from water contact and allowed to dry, a near complete recovery of signal was observed. This recovery of the polymer signal is indicative of a hydrophobicity-motivated
reorganization of the polymer, which reverts to the structure we observe from a polymer/air interface, as previously described. Therefore, we conclude that the polymer reorganizes upon contact with water to a random orientation, likely because of the prevalence of hydrophobic components in the polymer.

Figure 4.8 SFG spectra of polymers in air (black), in contact with water (red), and in air, after removal from water contact (blue). A) P1; B) P2; C) P3; and D) P4. Samples were air-dried before blue spectra were taken.

4.3.7 Polymer Segregation and Orientation

Given the above results, we can conclude several things about the structure of these polymers on a fused silica surface. First, signal from the terminal methoxy group of the y-subunit would seem to indicate that this group is able to organize at the surface only when the extent of surface coverage of the z-subunit does not preclude it from doing so.
Increasing surface coverage of the hydrophobic z-subunit seems to crowd out the hydrophilic methoxy groups, decreasing their presence at the surface. Second, when no methoxy groups are present to disrupt its organization, the N-(CH\(_3\)_2) group near the backbone on the z-subunit is likely organizing at the surface as well. Third, the terminal methyl group of the z-subunit are highly organized, and are all standing approximately perpendicular from the substrate surface, however the degree to which these groups stand vertically is highly dependent upon surface coverage. Some of the methoxy moieties within the y-subunit do go to the surface, and this has consequences for the hydrophobicity of the surface. However, the extent to which that occurs is dependent upon surface coverage of the more-dominant alkyl chains. Also, it is possible that the z-subunit is orienting in such a way that the short chain connecting the polymer backbone to the N-(CH\(_3\)_2) group and subsequent longer alkyl chain are standing vertically into the air, much like the idealized description of a self-assembled monolayer, however further, targeted studies are needed to confirm this conjecture.

This type of structure could also possibly account for the results seen in the contact angle goniometry and AFM studies. Polymers P0-P3 have similar contact angle and RMS surface roughness values from P4, indicating some kind of different surface environment for P4 in comparison to the other three. P0-P3 all contain some percentage of the y-subunit middle layer, while P4 does not. This lack of the methoxy-containing subunit could account for both the higher level of hydrophobicity and formation of surface domain structures for P4.
4.4 Conclusions

For polymers such as those studied here to function as effective antibiofouling coatings, their surface properties must be well understood. The structure of that surface has a significant impact on those properties. We showed that the ratio of hydrophobic and hydrophilic groups has a significant impact on the surface structuring of the polymers under study here. Static water contact angle measurements indicated that the hydrophobicity of P0-P4 was affected by the presence or absence of the hydrophilic methoxy-containing y-subunit, but not necessarily the percentage of the y-subunit within the polymer. It is possible that reducing the y-subunit percentage lower than 25% may show different results, however, this was not tested in this work. The AFM studies showed surfaces that were reasonably smooth for polymer surfaces, yet still indicated differences between the polymers under study here. Polymers P1-3 showed reasonably similar RMS roughness values, with P2 and P3 being the most similar. Polymer P4 was dramatically different, with an RMS roughness two times rougher than the next roughest polymer, P1. Additionally, a visual inspection of the AFM spectrum indicates a clearly different surface, one which forms into domains, rather than a flat, smooth surface. Clearly, a balance between hydrophilic groups, such as the terminal methoxy group, and hydrophobic groups, such as the long alkyl chain, is needed to generate these kinds of smooth surfaces. A total lack of hydrophilic groups prevents the polymer from forming a smooth surface, which could be potentially very problematic depending on the extent to which domains will form. The rougher the polymer surface, the more likely it becomes that a polymer film will organize in such a way as to expose portions of the support structure. Especially in the case of antimicrobial or antibiofouling films, such polymeric
structuring could undermine the ability of the polymer to act in its intended capacity as a buffer layer between the support structure and the external environment. These kinds of surface roughness properties must be taken into account when considering applications of the polymer to solve real-world problems.

SFG studies of these polymers yield further clues as to the self-organization of the polymers on a surface. Spectra fitting indicates a degree of organization of the N-(CH$_3$)$_2$ groups and terminal methyl groups within subunit $z$. While orientation analysis of the catechol and N-(CH$_3$)$_2$ groups was not possible at this time, orientation analysis of the terminal methyl groups was performed. This analysis indicated that the terminal methyl groups were standing perpendicular to the surface, but this orientation was highly dependent upon the surface coverage of the alkyl chains. As the surface coverage increased, disorder in the chains also increased, as evidenced by the increase in methylene signal. This could have significant impact on the ability of the polymer film to effectively damage the bacterial cells with which it comes into contact. As the polymer itself is immobilized on a structural surface, the alkyl chains responsible for the polymer’s antimicrobial character must have the steric freedom to extend into the bacterial cells to disrupt the membrane. Highly sterically hindered chains may be less able to accomplish this, which could negatively impact the polymers’ intended efficiency.

SFG studies also showed that these polymers become disordered upon contact with water. Despite this, they will recover when exposed to a hydrophobic environment (e.g., air) again. When bacteria contact such polymer surfaces, the surface chain may extend into the hydrophobic core of the cell membrane. In this case, the polymer surfaces may recover to the case when exposed to a hydrophobic environment. Therefore P1, P2
and P3 may extend into the bacteria more than P4. The bacterial inhibition behavior depends on both the surface coverage of quaternary ammonium chain and how far such chains can extend to the bacteria. P1 can extend the most, but the surface coverage must be too low, whereas on P4, the surface chains mostly tilt because they are simply too crowded. Therefore, on P2 and P3, the surface coverage and extension of ammonium chains are optimized, resulting in best antimicrobial activities.

Determination of the organization of antimicrobial polymers on a surface is a useful step in developing compounds to be used as biocides or as biocidal coatings. In this case, it is possible that the biocidal nature of the polymer is partially due to the organization of it; the antimicrobial activity of the polymer would likely be significantly lower if the antimicrobial alkyl chains were not organizing along the polymer/air interface. Self-structuring properties of antimicrobial polymers must be understood if future iterations of polymers are to optimize their applicability in the future.
4.5 References


CHAPTER 5

CONCLUSIONS

Synthetic antimicrobial compounds stand to be useful in a multitude of applications, and could potentially be applied to solve some significant problems in today’s society. The increasing problem of bacterial resistance to traditional antibiotics has created a great need for development of alternatives, whether through drugs, drug delivery mechanisms or different forms of sterilization techniques. Biofouling has long been an issue for marine industries, and loss of billions of dollars in revenue to maintenance and fuel costs has spurred much research into development of coatings. Antibiofouling coatings must not only be useful, but they must also be environmentally friendly and not perpetuate the bacterial resistance issue currently isolated to the medical field. By taking structural clues from naturally-occurring antimicrobial peptides, these studies seek to develop synthetic mimics into alternatives to today’s brute-force methods that have caused significant medical and environmental problems.

The current research using surface analytical techniques, including XPS, ATR-FTIR and Raman spectroscopies, has provided information about the surface structures of polymers and peptides, and these findings have influenced the field. However, none of these techniques are able to provide detailed, molecular-level information about interfaces involving these compounds in situ, making their applicability to these systems limited at best. In the studies presented in the previous chapters, the nonlinear optical
technique SFG has been used to study three different series of peptide-mimetic antimicrobial compounds, each with a very different rationale for their intended structure and function. These SFG studies have provided detailed, molecular-level information of the surface and interfacial properties of these compounds, as well as kinetic information, such as the interaction mechanisms between these compounds and the model cell membranes. Prior SFG studies indicated that SFG could be used to determine these kinds of interaction mechanisms, but did not attempt to elucidate the role that specific moieties within the antimicrobial compounds can have on its activity, toxicity, selectivity and interaction mechanism. The work presented here attempts to fill some of that gap by investigating all of these properties simultaneously. Taken together, this work demonstrated that SFG is an effective tool for detection of changes in interfacial systems such as lipid bilayers and polymer surfaces, and that it can be used to understand how molecular-level details can impact broader system functionality.

The presented studies use SFG to explore three very different synthetic antimicrobials. Chapter 2 built directly on previous work with antimicrobial arylamide oligomers. This work showed that small, rigid structures that are rationally designed to mimic the size and amphiphilicity of naturally occurring AMPs can be used to study interactions between such compounds and lipid bilayers. Chapter 2 took the next step of studying multiple compounds with similar rigid structure, but differing hydrophilic and lipophilic groups. Since these groups are what directly interact with the bilayer, different side chains should result in different interaction mechanisms. In Chapter 2, three different antimicrobial arylamide oligomers were studied in a variety of lipid bilayer systems to model bacterial cell membranes, human cell membranes, and models mixing the
properties of the two. Biological tests indicated that of the tested oligomers, 2 was the most active and most toxic, 3 was active and selective for bacterial membranes, and 4 was simply inactive. SFG studies confirmed the findings of the biological tests, and further determined that 2 and 3 interact with cellular membranes by penetrating fully into the bilayer, rather than partitioning in and disrupting one leaflet at a time. Interaction studies with bilayers mixing bacterial and erythrocyte lipids showed that increasing the relative ratio of bacterial lipids yielded an increasing extent of membrane disruption. These findings were again confirmed by both biological tests and SFG studies. Signal was obtained from carbonyl moieties along the oligomer backbone of both 2 and 3 during interaction with the bilayer, and orientation analysis of those signals showed both oligomers to be standing approximately parallel to the bilayer surface normal. Conceptually, the oligomers orient in a similar manner to a knife cutting into cheese, which has resulted in these compounds being referred to as “molecular knives.” Additionally, SFG signal was observed from the trifluoromethyl groups in oligomer 2, which has not previously been reported.

The work in Chapter 3 explored similar interactions with bacterial membrane models, but with radically different compounds. Four antimicrobial random co-polymers were synthesized, each with a different lipophilic group. These compounds had very different structures than those compounds studied in Chapter 2. The previously discussed oligomers were rigidly structured, whereas these polymers were far more flexible, and able to change confirmation to optimize their polymer-bilayer interaction. Determination of the impact of the lipophilic group on this interaction mechanism was thus the point of this work. This study represented an attempt to develop the understanding of the
interactions of peptide-mimetic antimicrobial compounds with model cellular membranes.

To do this, four antimicrobial polymers were studied using the same techniques described in Chapter 2. The polymers were very similar in size, molecular weight and hydrophilic groups, with a different lipophilic group in each polymer. The four random co-polymers were named 0R (no group), 33Me (methyl group), 11Bz (benzyl group) and 33Bu (butyl group), according to both the identity and percentage of the side chains within the polymer. It was shown that 0R and 33Me were membrane-inactive in both biological tests and SFG studies, whereas both 11Bz and 33Bu were membrane active. Further, it was determined that 11Bz and 33Bu interacted with a membrane in fundamentally different ways. The 11Bz polymer displayed independent loss of signal from each bilayer leaflet, indicating step-wise partitioning into the bilayer. By contrast, 33Bu displayed a simultaneous loss of signal from both bilayer leaflets, indicating deep penetration of the lipophilic groups into the bilayer matrix. Additionally, 33Bu was more active than 11Bz, fully disrupting the bilayer at far lower concentrations. Orientation analysis of the 33Bu polymer determined the terminal methyl group of the butyl side chain oriented parallel to the surface normal of the bilayer.

Taken together, these results indicate certain properties of peptide-mimetic antimicrobial polymers must be specifically considered to optimize these compounds for intentional use. Identity of the lipophilic group is clearly crucially important for activity. It is possible that lipophilic structures that most closely mimic bilayer structures are best able to penetrate the bilayer. Acyl chains, structurally identical to the chains making up the lipophilic core of the bilayer, are best able to intercolate and disrupt. The benzyl ring
in 11Bz is bulky in comparison, and likely has a far greater degree of steric hinderance. However, the length of the chain is clearly relevant to the activity of the polymer, and could possibly affect the interaction mechanism as well. Future studies of this phenomenon could yield a better model for understanding in this case. For example, a series of polymers with increasingly long acyl chains could pinpoint the ideal length for the lipophilic acyl chain to optimize activity and minimize toxicity. Another useful study would be to determine the importance of the size of the lipophilic group in terms of both activity and interaction mechanism.

The study presented in Chapter 4 attempted to understand a series of fundamentally different antimicrobial polymers. Instead of the low molecular weight polymers described in Chapter 3, this considered how higher molecular weight polymers that still maintain antimicrobial character could self-organize on a surface. Such organization would be expected to have consequences for the polymers’ antimicrobial activity. It was shown that the structural makeup of the polymers had dramatic consequences for surface organization. In all five polymers in the series, the methoxy group and the alkyl chain group dominated the surface over the catechol group. Increasing mole ratios of long alkyl chains results in surface domination of these groups. However, increasing surface domination of the alkyl chains resulted in increasing disorder of those chains, as was shown by the increasing orientation angle of the terminal methyl group. Such disorder could have consequences for the antimicrobial activity of these polymers, and potentially their selectivity as well, and future studies should be considered to study these impacts. Additionally, it is likely that the catechol group was binding to the fused silica surface, providing an anchor for the polymer and leaving the
other functional groups to interact with the external environment. However, little SFG signal was observed from the catechol groups themselves and such conclusions are difficult to draw given the lack of direct evidence. Perhaps future studies on these polymers should focus on observing signal from the buried polymer/substrate interfaces, as SFG has been shown to be a powerful analytical technique for observing both organization and orientation of functional groups at buried interfaces.

In total, these studies provide an initial model for understanding how and why these polymers interact with biological systems in the way they do. Future fleshing out of this model should take into account the differences between idealized lipid bilayers and natural cellular membranes. One important difference is membrane constituents. Natural cellular membranes contain many compounds other than lipids, including transmembrane proteins and cholesterol. Future studies could focus on the effect incorporating some of these membrane constituents into the model bilayer on the interaction mechanisms of these polymers. Another important difference is the planar nature of the SFG substrates. Prism substrates have a planar surface upon which the lipid bilayer is built. This is necessary, given the experimental setup currently used for SFG experiments, however it is not entirely accurate to biological reality. It is likely that this difference is largely irrelevant; living cells are large enough relative to a native AMP that the cellular membrane appears flat. However, some initial studies have suggested that membrane curvature could play an important role in determining the interaction mechanism. Future studies should consider this and develop methods of studying lipid bilayers with curvature via SFG.
This work demonstrates that SFG is a powerful and unique tool for studying interaction mechanisms between synthetic antimicrobial compounds and lipid bilayers. Synthetic mimics of AMPs were shown to interact similarly to native AMPs, and in ways that were predictable and understandable given the identity of the side-chains responsible for the amphiphilic character of the compounds. Also, these studies revealed the importance of the identity and structure of the lipophilic groups in determining the activity of both rigid oligomeric and flexible polymeric antimicrobial compounds. Especially useful was the observation of signals from native structures within the compounds themselves, freeing future studies from dependence upon bulky tags which may dramatically affect how such compounds interact with their environment. Further, SFG was used to determine how antimicrobial polymers on a surface can self-segregate into semi-discrete layers, which has implications for the antimicrobial properties and mechanisms of biological interactions. Continued in situ studies of synthetic antimicrobial compounds using SFG may lead to the development of better drug delivery techniques and antibiofouling materials for broad use across multiple industrial, medical, and marine sectors.