## AN ABSTRACT OF THE THESIS OF

<u>Thaddeus W. Golbek</u> for the degree of <u>Master of Science</u> in <u>Chemical Engineering</u> presented on June 3, 2015.

Title: <u>Probing the Biophysical Interactions of Anti-Microbial Peptide WLBU2 Using</u> <u>Model Membrane Systems</u>

Abstract approved:

Joe E. Baio

WLBU2 is an engineered cationic amphiphilic peptide that targets Gram-positive and Gram-negative bacteria, and envelopes endotoxin while avoiding other cell types. The exact mechanism of how WLBU2 targets, binds, and disrupts bacterial cell membranes is still not completely known. Thus, the overall goal of this investigation is to determine the structural basis for recognition and specific interactions between the engineered antimicrobial peptide WLBU2 and cell membranes. Currently, it is believed that WLBU2 binds parallel to the surface of the cell membrane in an  $\alpha$ -helical confirmation, and at a critical interfacial concentration, WLBU2 starts to disrupt the lipids that make up the cell. In this investigation – we tested this proposed mechanism by using a set of surface and interface specific spectroscopy tools to probe the biophysical interactions between the peptide and both zwitterionic and negatively charged model cell membranes.

This surface analysis approach demonstrates that binding between WLBU2 and cell membranes is induced by electrostatic interactions between charged amino acids within the peptide and charged lipids. Our experiments also suggest that for zwitterionic membranes WLBU2 binds to the surface in a  $\beta$ -sheet conformation, while for negatively charged membranes folds in an  $\alpha$ -helical conformation at the interface. The observed difference in folding demonstrates WLBU2 selectivity toward negatively charged membranes (i.e. bacteria) and inactivity toward zwitterionic membranes (i.e. mammalian cells and other cell types). ©Copyright by Thaddeus W. Golbek June 3, 2015 All Rights Reserved

# Probing the Biophysical Interactions of Anti-Microbial Peptide WLBU2 Using Model Membrane Systems

by Thaddeus W. Golbek

# A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Master of Science

Presented June 3, 2015 Commencement June 2015 Master of Science thesis of Thaddeus W. Golbek presented on June 3, 2015

APPROVED:

Major Professor, representing Chemical Engineering

Head of the School of Chemical, Biological, and Environmental Engineering

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Thaddeus W. Golbek, Author

# ACKNOWLEDGEMENTS

I would like to thank my family and my fiancé Hannah for support and encouragement during the past couple of years. Thank you for encouraging me to find my passion. Research is what I want to do with my one wild and precious life.

I would also like to thank my advisor, Dr. Joe Baio, for being a great mentor. You have helped me mature and grow as a researcher. You are a great role model to have, both as an advisor and as a person. Thank you, for having me in you lab these past couple years.

# TABLE OF CONTENTS

	Page
1 Introduction	1
1.1.1 Antimicrobial Proteins	1
1.1.2 Biophysics	
1.2 Surface Analytical Techniques	5
1.2.1 X-ray Photoelectron Spectroscopy (XPS)	5
1.2.2 Sum Frequency Generation (SFG)	6
1.2.3 Near Edge X-ray Absorption Fine Structure (NEXAFS)	8
1.3 References	9
2 Creating a Vacuum Stable Model Lipid Bilayer	15
2.1 Introduction	16
2.2 Materials and Methods	19
2.2.1 Substrate Preparation	19
2.2.2 Self-assembled monolayer (SAM) and vesicle fusion	19
2.2.3 X-ray photoelectron spectroscopy (XPS)	20
2.2.4 Sum Frequency Generation (SFG) Vibrational Spectroscopy	20
2.2.5 Scanning Electron Microscopy (SEM)	
2.2.6 Atomic Force Microscopy (AFM)	22
2.3 Results and Discussion	22
2.3.1 XPS Analysis	22
2.3.2 SFG Analysis	
2.3.3 SEM and AFM Analysis	
2.3 Conclusions	
2.4 References	
3 Probing the Interactions of WLBU2 with Lipid Monolayers	
3.1 Introduction	
3.2 Materials and Methods	39
3.2.1 Experimental Setup for SFG	39
3.2.2 Experimental Setup for NEXAFS	40
3.2.2.1 Substrate Preparation	40

3.2.2.2 Self-assembled monolayer (SAM) and vesicle fusion ...... 40

# TABLE OF CONTENTS (Continued)

	Page
3.2.2.3 Protein Adsorption on the FMST Substrates	41
3.2.3 Sum Frequency Generation (SFG) Vibrational Spectroscopy	41
3.2.4 Near-edge X-ray absorption fine structure spectroscopy (NEXAFS)	42
3.3 Results and Discussion	43
3.3.1 SFG Analysis	43
3.3.2 NEXAFS Analysis	46
3.4 Conclusions	47
3.5 References	48
4 Future Directions	58
4.1 Interaction of LPS with WLBU2 and DPPC	58
4.2 Constant Pressure Langmuir Trough Experiments	48
Bibliography	59

# LIST OF FIGURES

<u>Figure</u> <u>Page</u>
1.1. The three well known models for antimicrobial peptide activity11
1.2 X-ray photon core level energy transfer and emitted photoelectron
1.3. The schematic diagram for SFG
1.4. A diagram denoting the theory of how the NEXAFS K-edge signal is generated14
2.1. The basic diagram of the tBLM
2.2. Full membrane spanning tethered lipid bilayers on gold
2.3. The High resolution XPS C <sub>1s</sub> spectrum of FMST SAM on gold and FMST SAM on gold with vesicle fused DPPC
2.4. The <i>ppp</i> SFG spectra of both FMST SAM on gold and FMST SAM on gold with vesicle fused DPPC
2.5. SEM images of FMST SAM on gold FMST SAM on gold with vesicle fused DPPC
2.6. AFM images of FMST SAM on gold FMST SAM on gold with vesicle fused DPPC
3.1. WLBU2 is depicted in an alpha helical conformation to show the ordering of the amino acids in the sequence
3.2. The surface pressure in the trough measured as a function of time in hours for WLBU2 interacting with DMPG monolayer and DPPC monolayer
3.3. SFG in the amide region is depicted for the <i>ssp</i> polarization of a DPPC monolayer and DMPG monolayer
3.4. SFG in the CD region is depicted for the <i>ssp</i> polarization of a DPPC monolayer and DMPG monolayer
3.5 NEXAFS nitrogen K-edge spectrum for WLBU2 adsorbed onto both FMST SAM and FMST SAM with vesicle fused DPPC bilayers

# LIST OF TABLES

<u>Table</u>		Page
2.1	XPS elemental percent composition	32
3.1	Amide I region <i>ssp</i> SFG data for DPPC and DPPC+WLBU2 systems	. 54
3.2	CD region <i>ssp</i> SFG data for DPPC and DPPC+WLBU2	. 56

#### **Chapter 1 – Introduction**

#### **1.1.1 Antimicrobial Peptides**

All organisms produce cationic antimicrobial peptides (AMPs) as a part of their immediate non-specific defense against infections.<sup>1</sup> AMPs have had a fundamental role in the evolution of multicellular organisms, and have remained effective defense weapons throughout their ancient history.<sup>2</sup> They have the ability to stimulate innate immune reaction while simultaneously dampening inflammatory response.<sup>3</sup> AMPs are derived from larger precursors (*i.e.* similar AMPs can be derived from one larger protein) with such diversity that similar peptide sequences are rarely found in different species of animal.<sup>2</sup> The diversity of peptides arises from single mutations, which can greatly alter biological activity. The derived peptides have a conservation of amino-acid sequencing found in the preproregion, suggesting constraints exist in sequences involved in translation, secretion, and intracellular trafficking. There are common features exhibit by all AMPs.<sup>1,3,4</sup> The typical length of the peptide is 12-50 amino acid residues. A net positive charge is characteristic due to the abundance of lysine and arginine residues, and AMPs have around 50% hydrophobic residues.

There are five subgroups that AMPs can be divided into, and each is known. <sup>4</sup> The first subgroup is anionic peptides found in surfactant extracts, bronchoalveolar lavage fluid, and airway epithelial cells. These peptides are small, require zinc as a cofactor, and are active toward both Gram-negative and Gram-positive bacteria. The second subgroup includes linear cationic  $\alpha$ -helical peptides. These peptides are disordered in solution, but assume an  $\alpha$ -helical conformation when bound to a membrane, having a direct relationship with antimicrobial activity. In general, these peptides characteristically have a kink or hinge in the middle and are less than 40 residues in length. The third subgroup contains linear peptides that have plentiful amounts of specific residues. The fourth subgroup of peptides consists of fragments from larger peptides that have a charge. These peptides have antimicrobial activity and are the same size and conformation as other AMPs. Lastly, the fifth subgroup is called defensins and contains peptides with 6-cystine residue motifs that form  $\beta$ -sheets from disulfide bonds. AMPs can also be classified by their secondary structure, which includes linear,  $\beta$ -sheet,  $\alpha$ -helix, loop, and extended structure.

AMPs have many advantages over conventional antibiotics in regards to medical treatment.<sup>3</sup> Activity toward bacterial infections (both Gram negative and Gram positive), fungal and viral infections, sepsis, inflammation, neoplastic cells (includes chemotactic activity), and the ability to cause chemokine production out performs conventional antibiotics, which have activity toward only selective bacterial infections.<sup>3,5</sup> The uptake targets of antimicrobial peptides are non-specific, based on charge, and often multiple in a cell.<sup>3</sup> Comparatively, conventional antibiotics have a specific mechanism of uptake with one dominating target or class of targets. Resistance to AMPs has been found, but only results in a two- to four-fold increase after several passages of sub minimal inhibitory concentration (sub-MIC). In conventional antibiotics, increased resistance could be 190-fold for only a few sub-MIC passages. Conventional antibiotics still hold the medical advantage due to two main reasons. First, AMPs systemic toxicity is still undefined while conventional antibiotics are considered the safest group of pharmaceuticals. Second, conventional antibiotics can be inexpensive while antimicrobial peptides cost approximately \$50 to \$400 per gram. In order to bridge the gap between

AMPs and conventional antibiotics the kinetics and biophysics of AMPs needs to be addressed.

### **1.1.2 Biophysics**

The selectivity of AMPs is attributed to the composition of the bacterial cell wall versus a multicellular animal membrane.<sup>2</sup> Bacterial cells consist of phosphatidylethanolamine (zwitterionic lipid) and phosphatidylglycerol (negatively charged) phopholipids.<sup>4</sup> The outer leaflet of a eukaryotic cell largely consists of phosphatidylcholine lipids (zwitterionic lipid) and cholesterol. AMPs are selective toward the bacterial cell lipid bilayer (negatively charged outer leaflet) due to a combination between the electrostatic forces and hydrophobic interactions, which out weigh the single hydrophobic interaction with the multicellular animal.<sup>3</sup>

The mechanism of an antimicrobial peptides can be broken-down into steps of attraction, attachment, and insertion.<sup>4,5</sup> Attraction occurs from electrostatic interaction between the cationic AMPs and the negatively charged phospholipids on the bacterial membrane. The degree of attraction is controlled by the charge and the amphipathicity of the AMP. Amphipathicity in a peptide means there is an alternating pattern of polar and non-polar amino acid residues. For Gram-positive bacteria, attraction is due to the negative charge on the teichoic acids located in the membrane. Attraction to Gramnegative bacteria is due to the negative charge associated with lipopolysaccharide phosphate (LPS) and other anionic lipids. Bacterial AMP resistance occurs when the membrane has cationic charges on the surface or anionic phospholipids separated to the cytoplasmic surface of the membrane.

Attachment of the AMP to the bacterial membrane is determined by placement of polar and hydrophobic residues. AMPs with arginine stabilize the peptide-membrane interaction through electrostatic interactions with the phosphate headgroup. AMPs with tryptophan locate themselves in the interfacial region of the bilayer. Typical secondary structures of AMPs after binding to membranes become favorable (*i.e.* alpha-helical), with orientations that are either parallel or perpendicular to the membrane. After binding, when the peptide to lipid ratio is low and increasing, pores in the membrane begin to from. When the ratio is high, the peptide orientates perpendicularly and inserts into the membrane.

Models of insertion have been developed to rationalize the process of AMPs killing organisms (**Figure 1.1**).<sup>4,6</sup> The first model is the carpet model, described by peptide aggregation on the bilayer. As the concentration of peptide increases, the bilayer begins to bend, and at critical concentrations the membrane breaks into micelles. The second model is referred to as the barrel-stave model, where peptides form a bundle in the membrane with a pore in the center. The third and final model is the toroidal pore model, which combines the first two models. Peptide aggregation on the membrane surface leads to perpendicular insertion into the membrane. The polar side of the peptide interacts with the polar head group of the phospholipid causing the two leaflets of the membrane to connect. Pores that represent toroids are created. To probe the interactions between AMPs and membranes, surface analytical techniques will be used.

#### **1.2 – Surface Analytical Techniques**

#### **1.2.1 X-ray Photoelectron Spectroscopy (XPS)**

Also known as electron spectroscopy for chemical analysis (ESCA), XPS samples are placed under ultra high vacuum (UHV) and irritated with a photon source.<sup>7</sup> Photoelectrons are emitted from the core level by transfer of energy from the photon to core-level electrons (Figure 1.2). The energy of the photoelectron is directly related to the atomic molecular environment of the electron, and the quantity of photoelectrons directly relates to concentration of the specific element. High resolution spectra gives information about the molecular environments, including oxidation and covalently bonded atoms. The depth of XPS analysis is 10 nm, which is attributed to the inelastic mean free path (IMFP) of an emitted photoelectron; the average distance that an electron travels between successive inelastic collisions. An emitted electron is caused by a photon interacting with an electron, with total energy transfer, generating a photoelectron from the atom. The emitted photoelectron is detected if it escapes the surface without any energy loss, and any photoelectron that escapes with energy loss contributes to the background signal. XPS can identify all elements, except for H and He, present in quantities greater than 0.1 atomic percent. The binding energy, specific for a given atom, needs to be exceeded by the incoming photon otherwise a photoelectron will not be emitted. Binding energy is defined as the energy required for electrons to be bound to an atom by a proton from the nucleus. The closer the electron is to the nucleus the stronger the electron is bound. Each atom has a different size nucleus resulting in different binding energies. Smaller binding energy shifts, for a particular element, are detected depending on types of chemical bonds and bonds to other atoms.

XPS data can be used to calculate the elemental compositions of samples as well as overlayer thickness of a self-assembled monolayer (SAM) on gold.<sup>7,8</sup> Also, when adsorbing a protein onto a SAM, the thickness of the protein layer can be calculated to determine the percent monolayer or multilayer of coverage. An existing equation has been developed to quantify a monolayer or multilayer thickness of peptide with good accuracy.<sup>9</sup> The nitrogen-gold ratio, compared to previously known values of peptides having similar length, is a quick way to determine a monolayer or multilayer of peptide.

Another method for receiving information about the surface of a sample is depthprofiling.<sup>7</sup> Depth-profiling of a sample by non-destructive angular-dependent XPS studies can give information about the composition of a sample as a function of height. In an angular dependence XPS measurement the sample angle to analyzer is increased while the x-ray source and analyzer are kept in fixed positions. As the angle increases the photoelectrons originate closer to the surface of the sample.

#### **1.2.2 Sum Frequency Generation (SFG)**

Sum frequency generation is a second-order nonlinear optical technique, which is capable of detecting biomolecule adsorption and orientation in sub  $\mu M$  concentrations at physiological pH.<sup>10,11</sup> The technique involves a fixed visible laser that is pulsed in temporal and spatial synchronicity with a tunable infrared laser (**Figure 1.3**). The photons that are ejected from the sample surface are a function of the sum of the incident beam frequencies. SFG stems from light interacting with a medium, which can be expressed by a mathematical expression for the polarization (**Eq. 1.1**).<sup>12</sup>

$$P = \varepsilon_0 \left( \chi^{(1)} : (E_1 + E_2) + \chi^{(2)} : (E_1 E_2 + E_2 E_1) + \cdots \right)$$
 (Eq. 1.1)

where **P**, **E**, and  $\chi^{(i)}$  stand for the polarization vector, the electric field, and *i*<sup>th</sup> order susceptibility tensors of the medium. Experimentally, second order and higher terms are observed when the medium has a high electric field due to multiple intensely pulsed lasers. The term  $\chi^{(1)}$  is mathematically responsible for linear optical-processes (i.e. Raman scattering), which can be avoided because nonlinear SFG generates a coherent signal in a predictable direction. Higher order terms are found in **Eq. 1.1** when measuring in a centrosymmetric medium. The assumption that the third ranked tensor  $\chi^{(2)}$  is non-zero for only electric-dipole mechanisms makes contributions from higher order multipoles and magnetic dipoles negligible.<sup>13</sup> SFG is measured at the interface between two bulk phases, where order can be induced, because there is no centrosymmetry.<sup>12</sup> Contributions from the electric-quadruple and the electric-dipole are generally allowed and should not be ignored.<sup>12</sup> The effective second-order nonlinear susceptibility term is composed of a true bulk and a true surface term.<sup>13</sup> Differentiation and separation of the terms is done by forwards and backwards SFG.<sup>13</sup>

The polarization of each beam can be adjusted relative to the plane of incidence.<sup>12</sup> The visible and infrared beams are either S- or P-polarized, meaning the electric field is perpendicular to or in the plane of incidence, respectively. The SFG beam can also be in either S- or P-polarization before reaching the detector. The S and P combinations of the three beams are referenced in a specific sequence; SFG beam polarization, visible beam polarization, and infrared beam polarization. The intensity of the SFG beam at frequency  $\omega_i$  is dependent upon the probing visible- and IR-beam intensities (**Eq. 1.2**).

$$I(\omega_3 = \omega_1 + \omega_2) \propto \left| \chi_{eff}^{(2)} \right|^2 I(\omega_1) I(\omega_2) \qquad Eq. 1.2$$

where *I* is the beam intensity and  $\chi_{eff}$  is an effective second-order nonlinear susceptibility of the interference. The output information of SFG is a vibrational spectrum sensitive to ordering at the interface. By using different polarization combinations the orientation and secondary structure of ordered biomolecules, such as proteins, can be calculated.<sup>14,15</sup> Peak ratios in the CH and CD region can also be used to calculate orientation of specific function groups and to determine the order at the interface.<sup>16</sup>

#### 1.2.3 Near Edge X-ray Absorption Fine Structure (NEXAFS)

The process of NEXAFS begins with a core electron absorbing an x-ray (**Figure 1.3**).<sup>17</sup> The electron propagates out to a discrete unoccupied energy level creating a core hole. An electron from a higher energy level fills the hole and releases energy, in the form of either a photon or an Auger electron, and the energy corresponds to an energy level. NEXAFS has the ability to calculate the tilt angle of a self assembled monolayer (SAM) or a protein adsorbed to a hard surface or membrane.<sup>18</sup> The tilt angle can only be calculated if the SAM or protein shows order in the NEXAFS spectra, which is attributed to a difference in spectral intensity between two angles when normalized (**Eq. 1.3**).

$$I(\rho) \propto \left(1 + \frac{1}{2}(3\cos^2\theta - 1)(3\cos^2\rho - 1)\right) \qquad Eq. 1.3$$

where  $\theta$  denotes the angle of incidence of the x-ray radiation, *I* is the intensity of the peak, and  $\rho$  is the title angle. Specifically, in the N K-edge region the difference spectra can be used to calculate the tilt angle of the peptide backbone using the amplitudes of the  $\pi^*$ amide moiety.<sup>19</sup> If there is a difference in intensities of the  $\pi^*$  amide moiety, and in none of the other peaks, then the peptide is in a  $\beta$ -sheet conformation. If there is no difference in amplitudes between different angles then the peptide is considered to adopt a

conformation allowing radial dispersion of the probed orbitals (i.e.  $\alpha$ -helix). The two

secondary structures mentioned are the most generic, but NEXAFS can show other

peptide secondary structures as well.

## **1.3 References:**

- 1. Hancock, R. E. W. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* **2001**, *1*, 156-164.
- 2. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature*. **2002**, *415*, 389-395.
- 3. Marr, A. K.; Gooderham, W. J.; Hancock, R. E. W. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Current Opinion in Pharmacology*. **2006**, *6*, 468-472.
- 4. Diamond, G.; Beckloff, N.; Weinberg, A.; Kisich, K. O. The Roles of Antimicrobail Peptides in Innate Host Defense. *Curr. Pharm. Des.* **2009**, *15*, 2377-2392.
- Glukhov, E.; Stark, M.; Burrows, L. L.; Deber, C. M. Basis for Selectivity of Cationic Antimicrobial Peptides for Bacterial Versus Mammalian Membranes. *J. Biol. Chem.* 2005, 280, 33960-33967.
- 6. Chan, D. I.; Prenner, E. J.; Vogel, H. J. Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochimica et Biophysica Acta*. 2006, *1758*, 1184-1202.
- 7. Vickerman, J. C.; Gilmore, I. S. *Surface Analysis: The Principle Techniques*; John Wiley and Sons, 2009.
- Techane, S. D.; Gamble, L. J.; Castner, D. G. Multi-technique Characterization of Self-assembled Carboxylic Acid Terminated Alkanethiol Monolayers on Nanoparticle and Flat Gold Surfaces. *J Phys Chem C Nanomater Interfaces*. 2011, 115, 9432-9441.
- Apte, J. S.; Collier, G.; Latour, R. A.; Gamble, L. J.; Castner, D. G. XPS and ToF-SIMS Investigation of α-Helical and β-Strand Peptide Adsorption onto SAMs. *Langmuir*. 2010, *26*, 3423-3432.
- 10. Shen, Y. R. The Principles of Nonlinear Optics; John Wiley & Sons, 1984.

- Nguyen, K. T.; Soong, R.; Im, S. C.; Waskell, L.; Ramamoorthy, A.; Chen, Z. Probing the Spontaneous Membrane Insertion of a Tail-Anchored Membrane Protein by Sum Frequency Generation Spectroscopy. *J. Am. Chem. Soc.* 2010, *132*, 15112-15115.
- 12. Ahn, D.; Dhinojwala, A. Sum Frequency Generation Vibrational Spectroscopy of Silicone Surfaces & Interfaces; Springer, 2012.
- 13. Shen, Y. R. Basic Theory of Sum-Frequency Generation. J. Phys. Chem. C. 2012, 116, 15505-15509.
- Nguyen, K. T.; Le Clair, S. V.; Ye, S.; Chen, Z. Molecular Interactions between Magainin 2 and Model Membranes in Situ. J. Phys. Chem. B. 2009, 113, 12358-12363.
- Nguyen, K. T.; Le Clair, S. V.; Ye, S.; Chen, Z. Orientation Determination of Protein Helical Secondary Structures Using Linear and Nonlinear Vibrational Spectroscopy. *J. Phys. Chem. B.* 2009, *113*, 12169-12180.
- Messmer, M. C.; Conboy, J. C.; Richmond, G. L. Observation of Molecular Ordering at the Liquid-Liquid Interface by Resonant Sum Frequency Generation. J. Am. Chem. Soc. 1995, 117, 8039-8040.
- 17. Stohr, J. NEXAFS Spectroscopy; Springer-Verlag, 1992.
- Rong, H. T.; Frey, S.; Yang, Y. J.; Zharnikov, M.; Buck, M.; Wuhn, M.; Woll, C.; Helmchen, G. On the Importance of the Headgroup Substrate Bond in Thiol Monolayers: A Study of Biphenyl-Based Thiols on Gold and Silver. *Langmuir*. 2001, *17*, 1582-1593.
- Baugh, L.; Weidner, T.; Baio, J. E.; Nguyen, P. T.; Gamble, L. J.; Stayton, P. S.; Castner, D. G. Probing the Orientation of Surface-Immobilized Protein G B1 Using ToF-SIMS, Sum Frequency Generation, and NEXAFS Spectroscopy. *Langmuir*. 2010, 26, 16434-16441.



**Figure 1.1.** The three well known models for antimicrobial peptide activity; the barrel-stave model (A), the carpet model (B), and the toroidal model (C) are depicted.<sup>6</sup>



**Figure 1.2.** X-ray photon core level energy transfer and emitted photoelectron. The incoming x-ray photon transfers its energy to a core electron that is then emitted. Examples of core level electrons are  $O_{1s}$ ,  $N_{1s}$ ,  $S_{2p}$ , and  $Au_{4f}$ .<sup>7</sup>



**Figure 1.3.** The schematic diagram for SFG is depicted with beam frequencies of  $\omega_1$  for the visible light,  $\omega_2$  for the tunable IR, and  $\omega_1 + \omega_2$  for the SFG signal. Each beam can either be S-polarized or P-polarized by polarizer filters.<sup>12</sup>



**Figure 1.4.** A diagram denoting the theory of how the NEXAFS K-edge signal is generated.<sup>17</sup>

#### Chapter 2 – Creating a Vacuum Stable Model Lipid Bilayer

Thaddeus W. Golbek,<sup>1</sup> Hao Lu,<sup>2</sup> Johannes Franz,<sup>2</sup> J. Elliott Fowler, <sup>1</sup> Tobias Weidner, <sup>2</sup> Joe E. Baio<sup>1</sup>

1. School of Chemical, Biological, and Environmental Engineering Oregon State University, OR, USA.

2. Max Planck Institute for Polymer Research, Mainz, Germany

#### Abstract

Biophysical studies of the interactions between peptides and model membranes provide a simple and effective approach to understanding how proteins target and disrupt cell membranes. Currently model membranes for probing protein interactions are generally only stable in aqueous environments. The use of a vacuum stable lipid bilayer is necessary in order to use surface analysis techniques in ultra-high vacuum (UHV) environments. The goal of this study is the creation of a model lipid bilayer that is stable under UHV by self-assembling monolayers made up of full membrane spanning thiols (FMST-SAMs) and vesicle fused phospholipid vesicles. Fusing phospholipid vesicles will mimic the native cellular membrane fluidity, while keeping the amount of tethers at a minimum, allowing inserting proteins to be generally unobstructed by non-native features. X-ray photoelectron spectroscopy (XPS) determined compositions confirm both the thiol terminated monomers self-assembled into a monolayer at the Au substrate, and that we can incorporate phospholipids into the monolayer. SFG shows decreased ratio between the  $CH_3$  and  $CH_2$  symmetric peak amplitudes, upon vesicle fusion of DPPC, which follows the formation of a lipid bilayer. The fusion of phospholipids was further confirmed by the absence of large vesicles bound to the FMST-SAM surface from

images collected by scanning electron microscopy (SEM) and atomic force microscopy (AFM). Combined, this analysis demonstrates that we can build a UHV stable lipid bilayer that can be used to characterize adsorbed proteins.

#### 2.1 Introduction

Models of membrane systems have been previously developed to investigate many lipid bilayer interfaces such as cell-cell signaling and protein-membrane interactions.<sup>1</sup> The goal is to construct a lipid bilayer that mimics the native cellular membrane of a real cell. A biomimetic model provides a simplified version of the lipid bilayer while maintaining the native cellular essential aspects.<sup>1</sup> The cellular membrane is the barrier between the interior and the exterior of the cell, composed of two lipids; each with a hydrophobic tail and a hydrophilic head group.<sup>2</sup> The lipids composing the bilayer are orientated such that the interior is hydrophobic and the exterior is hydrophilic.<sup>2</sup> Essential aspects of a cellular lipid bilayer include an ion and water reservoir underneath the membrane, natural fluidity, and disorder.<sup>1,2</sup> Membrane disorder and fluidity, induced by cholesterol and receptors, occurs naturally in the cell membrane.<sup>2</sup> Therefore, achieving proper membrane fluidity is essential for the flexibility of the membrane to incorporate larger proteins.<sup>1</sup> Thus, we must avoid building a model membrane with a high membrane packing density, preventing proteins from inserting into the bilayer.<sup>1</sup> While also allowing enough pace underneath the lipid bilayer so that the protein will not come in contact with the planar substrate.

Currently, there are two biomimetic approaches for constructing a lipid bilayer. The first is a supported lipid bilayer membrane (sLBM), which is a planar lipid membrane on a solid planar substrate (i.e. silicon or gold coated silicon).<sup>2</sup> The sLBM was the first biomimetic lipid bilayer membrane constructed, but cannot completely mimic native cellular lipid bilayer membranes.<sup>3</sup> This is because the distance between the lipid bilayer and the substrate is very small, and proteins that insert into the lipid bilayer will touch the substrate, altering the secondary structure, due to the insufficient space.<sup>4</sup> Additionally, the sLBM adopts the properties of the rigid planar substrate, preventing true fluidity of the cellular membrane.

An alternative model membrane, that overcomes the limitations of the sLBM, is the tethered bilayer lipid membrane (tBLM). The tBLM (**Figure 2.1**) provides a spacer between the anchor and the bilayer creating an ionic reservoir underneath the membrane, which provides a natural fluidity to the membrane.<sup>4-6</sup> The ionic reservoir created by the spacer provides an intracellular environment that a protein would encounter upon insertion. Also, the spacer provides hydration and ample room for proteins to insert, unhindered by the planar surface of the substrate.<sup>4</sup> These attributes have provided good biomimetic model membranes and allowed protein-lipid interactions to be probed by surface plasmon resonance, ellipsometry, impedance spectroscopy, quartz crystal microbalance, and sum frequency generation spectroscopy (SFG).<sup>4,7,8</sup> One major limititation of tBLMs is that the membrane will rupture under ultra high vacuum conditions. Hydrophobic forces ensure bilayer stability, therefore, under vacuum the outer leaflet will detach from the inner leaflet collapsing the entire bilayer.<sup>9</sup>

Creating a vacuum stable lipid bilayer for probing protein membrane interactions will enable us to expand our current analytical toolbox to include techniques that require vacuum environments. To address this issue of rupturing under vacuum, a model bilayer surface has been developed based on a full membrane spanning thiol (FMST), instead of two separate leaflets.<sup>7</sup> The FMST covalently attaches the two bilayer leaflets and will not separate under vacuum conditions.<sup>10</sup> The vacuum stable FMST provides support for the addition of other biological molecules to insert into the membrane.<sup>7,10</sup> More recently, a tBLM with a highly ordered FMST self-assembled monolayer (SAM) was characterized under ultra-high vacuum environments by x-ray photoelectron spectroscopy (XPS) and near-edge x-ray absorption fine structure spectroscopy (NEXAFS).<sup>10</sup> The highly packed FMST SAM provided an ideal environment for the characterization of a model membrane binding peptide, sweet arrow peptide, interacting with a model cell membrane binding protein.<sup>10</sup>

We believe that the FMST will provide a stable cell membrane to probe with surface analytical techniques that require ultra-high vacuum conditions (**Figure 2.2**). The FMST has a hydroxyl headgroup that, in solution, presents a negative charge.<sup>11</sup> Using the FMST as support, vesicle fusion of a phospholipid in between the spaces of the low densely packed SAM will provide a tBLM which has fewer tethers. Tethers are non-native in a true cellular membrane environment, and thus, may obstruct natural insertion of proteins into lipid bilayers. The goal of the study is to use self-assembled monolayers of FMST and by vesicle fusion create a UHV stable lipid bilayer for the interaction of protein with various phospholipid headgroups. The vesicle fusion of phospholipids will allow alteration of the membrane charge and headgroup. We will then characterize the formed bilayers by SFG, XPS, scanning electron microscopy (SEM), and atomic force microscopy (AFM).

#### 2.2 Materials and Methods

2.2.1 Substrate Preparation: The substrates in the study were silicon wafers, from Plano GmbH (Wetzlar, Germany), cut to 1 cm x 1 cm. Silicon substrates were cleaned by sequential rinsing and sonication in ultrapure water, acetone (EDM Millipore Corp), dichloromethane (EDM Millipore Corp), acetone, and ethanol (Pharmco-Aaper). Following sonication steps, the substrates were coated first with a thin adhesion layer of titanium (3.5 nm) then a film of Au (100 nm) gold by thermal evaporation. All substrates were then stored under nitrogen until use.

**2.2.2 Self-assembled monolayer (SAM) and vesicle fusion:** FMST samples were prepared by immersing the Au substrates in a 1  $\mu$ M ((3-((14-((4'-((5-methyl-1-phenyl-35-(phytanyl)oxy-6,9,12,15,18,21,24,27,30,33,37-undecaoxa-2,3-dithiahenpentacontan-51-yl)oxy)-[1,1'-biphenyl]-4-yl)oxy)tetradecyl)oxy)-2-(phytanyl)oxy)glycerol) (SDx Tethered Membranes Pty Ltd. ) - ethanol solution for 10 minutes. Following assembly, the samples were washed repeatedly with ethanol removing any non-bound FMST molecules. These samples were then dried and stored using nitrogen. FMST samples fused with vesicles of lipids were prepared by first extruding a solution of DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine) (Avanti Polar Lipids Inc.) in ultrapure water (2.5 mg/ml, Millipore Direct-Q3) thereby, producing uniform vesicles no larger than 50 nm in diameter. Bare FMST SAMs were then incubated for 1 hour in the DPPC vesicle solution (1 mg/ml in ultrapure water), allowing the vesicles to fuse with the FMST. Afterwards, the samples are washed thoroughly with ultrapure water, dried with nitrogen, and stored under nitrogen.

**2.2.3 X-ray photoelectron spectroscopy (XPS):** XPS experiments were performed on a Kratos AXIS Ultra DLD instrument equipped with a monochromatic Al K $\alpha$  X-ray source. All spectra were collected in hybrid mode at a take-off angle of 55° (angle between the sample surface plane and the axis of the analyzer lens). Three spots on each sample were analyzed and compositions were calculated averaging the spectra from the three spots. Analyzer pass energy of 80 eV was used for compositional survey scans of C 1s, N 1s, O 1s, and S 2p. High-resolution scans of the same elements were collected at an analyzer pass energy of 20 eV. Compositions and fits of the high-resolution scans were produced in CasaXPS.

**2.2.4 Sum Frequency Generation (SFG) Vibrational Spectroscopy:** A femtosecond Ti:Sapphire laser oscillator coupled with a Nd:YLF laser pumped regenerative amplifier (Spitfire Ace, Spectra Physics) was used to generate a visible beam (35 fs pulse duration and 4.65 mJ) centered at 791.8 nm. The amplified visible beam was split into two parts. The first was used as the visible pulse for sum frequency generation and was passed through a Fabry-Perot etalon to spectrally narrow the pulse to ~15 cm<sup>-1</sup>. The second part was used to pump the optical parametric amplifier (OPA) system (Light Conversion, TOPAS). The generated tunable infrared pulse (3.1-6.1  $\mu m$ ) was polarized by a half-wave plate before use as the sum frequency generation IR pulse. The IR and visible pulses were overlapped temporally and spatially at the solid-air interface generating an SFG signal. SFG spectra of the samples were collected in the polarization combination *ppp* (*p*-polarized SFG, *p*-polarized visible, and *p*-polarized IR) in the C-H region (2800 cm<sup>-1</sup> – 3100 cm<sup>-1</sup>). The resulting SFG signal was focused onto a spectrograph (Action, Princeton Instruments), dispersed by grating, and re-focused on an electron multiplying

charge coupled device (CCD) camera (Newton, Andor). Spectra were normalized by subtracting a spectrum of deuterated dodecanethiol SAM on gold, to eliminate the non-resonant background, followed by division by a reference quartz spectrum.

The fitting of the resulting SFG spectrum was done using the following equation<sup>12</sup>:

$$I_{SFGS} \propto \left|\chi^{(2)}\right|^2 = \left|\chi^{(2)}_{NR}e^{i\phi} + \sum_{\upsilon}\int_{-\infty}^{\infty}\frac{A_{\upsilon}e^{i\phi}e^{-\left[\omega_L - \frac{\omega_{\upsilon}}{\Gamma_{\upsilon}}\right]^2}}{\omega_L - \omega_{\upsilon} + i\Gamma_{\upsilon}}d\omega_l\right|^2 \qquad (Eq. 2.1)$$

where  $\chi_{NR}^{(2)}$  is the second order nonlinear susceptibility tensor of the non-resonant background,  $\phi$  denotes the phase of the respective mode,  $\omega$ IR refers to the incident IR frequency, and  $A_v$  denotes the strength of the *v*th vibrational mode. The integral is over Lorentzian lines, widths set to 2 cm<sup>-1</sup> and allowing  $\Gamma_v$  to vary, with width  $A_L$  having a Gaussian distribution centered at  $\omega_L$ .

**2.2.5 Scanning Electron Microscopy (SEM)**: SEM imaging was performed on a LEO 1530 GEMINI with an Inlense (SE) dectector. The landing voltage (EHT) was 111V and 700V with an imagining probe current of 50 pA at an aperture size of 50  $\mu m$ . SEM is am imaging technique that produces images by scanning the surface with a beam of electrons.<sup>13</sup> Signals are produced from the surface that give in formation on topography and composition of the surface. The image is taken by rastering the surface with a beam of electrons. The beams positions and the detected signal give rise to an image.

**2.2.6 Atomic Force Microscopy (AFM)**: The measurement device used for AFM is a Dimension icon with ScanAsyst. The Experiment was done using tapping mode air with an Olympus (OMCL-AC160TS) cantilever in non-contact mode with a force constant of 42 N/m (range: 33.5 – 94.1 N/m) and a frequency of 300 kHz (range: 278 – 389 kHz). Images were collected of both topographical and phase contrast. The relative mean roughness (RMS) values were determined by the following equation:

$$RMS = \sqrt{\frac{\sum_{i=1}^{N} (Z_i - Z_{ave})^2}{N}} \qquad Eq. 2.2$$

where  $Z_{ave}$  is the average Z value,  $Z_i$  is the current value, and N is the number of points.

## 2.3 Results and Discussion

#### 2.3.1 XPS analysis

Theoretical and average experimental compositions of the FMST and DPPC vesicle fused FMST are compiled in Table 2.1. Theoretically the atomic percent carbon, upon vesicle fusion of DPPC, is expected to decrease. Experimental results show the amount of elemental carbon is similar between the FMST SAM and the FMST SAM with vesicle fused DPPC. This is the result of the inner and outer leaflets positioned above the polyethylene glycol linker, thus closer to the surface. The large reported standard deviation is from the variation in measured carbon atomic composition between each spot on the sample surface. The low packing density achieves proper membrane fluidity in PBS for a protein to bind, while avoiding a high density that would not allow protein insertion into the hydrophobic interior.<sup>14</sup> The FMST SAM bilayer dried under nitrogen

might not keep the structure found in solution without stabilization by bound protein. Holes could form if the FMST does not fully keep a uniform bilayer out of solution, leading to a less uniform carbon elemental composition. The vesicle fusion of DPPC has a lower standard deviation, between the measured spots on the sample, in the carbon signal attributed the increased lipid bilayer packing density from addition of DPPC to the system.

The high-resolution XPS  $C_{1s}$  spectra are used to determine the individual carbon environments for each sample. Normalized  $C_{1s}$  XPS spectra of the FMST SAM and vesicle fused FMST SAM are compiled in **Figure 2.3** with corresponding fits. The  $C_{1s}$ high-resolution spectra has three prominent features in each sample type. The first is at 284.3 eV and 284.5 eV corresponding to C-C bonds of the FMST SAM and DPPC vesicle fused FMST SAM, respectively.<sup>12</sup> The second feature is a shoulder at 285.8 eV and 286.0 eV assigned to C-O species of the FMST SAM and DPPC vesicle fused FMST SAM, respectively.<sup>12</sup> More oxygen is added to the system when DPPC is vesicle fused, and experimentally the percent area composition of C-O species rises from 20.5% to 25.6%. The third and final feature is a peak at 288.3 eV for FMST SAM and 288.5 eV for DPPC vesicle fused FMST SAM assigned to an ester (C=O) and oxidized carbon. DPPC has two ester groups per molecule, and so the experimentally determined rise in percent atomic composition from 6.18% to 8.43%.

Upon vesicle fusion of DPPC, elemental nitrogen  $(N_{1s})$  and phosphorus  $(P_{2p})$  are added to the system, so detection of these elements provides an indication of DPPC on the surface. The unexpected measured  $N_{1s}$  signal of the FMST SAM can be attributed to contamination. The increased  $N_{1s}$  signal produced by the FMST SAM with vesicle fused DPPC is from the quaternary amine located in the phosphocholine headgroup of DPPC. Only DPPC contains phosphorus in the theoretical system, so the XPS detection of  $P_{2p}$  in only the DPPC vesicle fused FMST SAM further supports DPPC on the surface.

The reduction of the sulfur signal in the experimental results can be explained in two ways. The first explanation is that upon vesicle fusion of DPPC the amount of sulfur in the system is constant while amounts of other elements increase. The other explanation is while the FMST SAM and the FMST SAM with vesicle fused DPPC have the same height in solution, outside of solution both membranes lose their full height. The DPPC increases the packing density of the bilayer and thus increases the thickness of the bilayer out of solution. The subsequent inelastic scattering from the longer overlayer causes the attenuation of sulfur signal.<sup>15</sup> The result is a decrease in elemental sulfur signal seen in the experimental results. Overall, XPS indicates that there is DPPC present in the sample, but determination between free vesicles and fused vesicles is not possible at this point.

#### 2.3.2 SFG analysis

While, XPS is used to determine the elemental composition of the samples, it cannot completely distinguish between free vesicles and fused vesicles. Therefore, in order to try to determine if the vesicles have fused or not we collected SFG spectra from both types of samples. SFG is used to probe the order and frequency modes of the two types of samples. CH region SFG spectra of FMST SAM and DPPC vesicle fused FMST SAM are shown in **Figure 2.4**. The nonresonant signal of the gold has a phase opposite that of a monolayer, resulting in dips instead of peaks from the destructive interference and can be reduced by changing the delay of the IR pulse.<sup>16</sup> The general peak

assignments identified in the stretching region near 2805 cm<sup>-1</sup>, 2852 cm<sup>-1</sup>, 2875 cm<sup>-1</sup>, 2926 cm<sup>-1</sup>, and 2963 cm<sup>-1</sup> correspond to CH, CH<sub>2</sub> symmetric, CH<sub>3</sub> symmetric, CH<sub>3</sub> fermi resonance, and CH<sub>3</sub> anti-symmetric stretching modes, respectively.<sup>17,18</sup> The softened CH stretching assignment can be attributed to CH stretching near the surface of the gold, indicative of a low density SAM.<sup>12</sup> Comparing the SFG spectra of the FMST SAM and the vesicle fused FMST SAM, the spectra with vesicle fused DPPC shows a decreased ratio of the CH<sub>3</sub> and CH<sub>2</sub> symmetric peak amplitudes, which suggests the formation of a lipid bilayer.<sup>19</sup> SFG signal is allowed if there is no inversion symmetry, thus the signal from perfect symmetry bilayer lipids (**Figure 2.2**) would give no SFG signal.<sup>19</sup> The fact that there is a signal in the C-H region (2800 cm<sup>-1</sup> to 300 cm<sup>-1</sup>) is due to a combination of signal from an imperfect SFG signal cancellation and the polyethylene glycol linker of the FMST.

#### 2.3.3 SEM and AFM analysis

The purpose of the SEM images is to probe for morphology differences between the FMST SAM and the FMST SAM with vesicle fused DPPC. The images from SEM provide both a wide scale image and a small scale image for viewing the morphology of each sample (**Figure 2.5**). Theoretically, the vesicle fusion of DPPC will produce a bilayer free of large morphological changes from free vesicles. Experimentally, the SEM image of the FMST SAM with vesicle fused DPPC (**Figure 2.5 A, C**) does not indicate large morphological features that differ from the FMST SAM sample (**Figure 2.5 B, D**). The FMST SAM has a "grainy" image from gold contribution due to a low density SAM, while the FMST SAM with vesicle fused DPPC has a "clumpy" image. The difference in images is attributed to fused vesicles at the surface, forming a higher density lipid bilayer. The absence of large morphological features combined with a "clumpy" lipid like image of the FMST SAM with vesicle fused DPPC suggest that vesicle fusion into the FMST SAM took place.

Similarly, AFM is used to show the comparative morphology of the surfaces between the FMST SAM (Figure 2.6 A, B) and FMST SAM with vesicle fused DPPC (Figure 2.6 C, D). Theoretically, the sample with the low density FMST will have a rougher surface compared to the higher density bilayer of the DPPC vesicle fused FMST SAM. The sample with DPPC should also be free of large topographical changes from free vesicles. Experimentally the vesicles are extruded to have diameters no larger than 50 nm, which in comparison is much larger than the height of the lipid bilayer. The average root mean squared (RMS) value of the two sample types, 500 nm by 500 nm images, is 1.4 nm for the FMST SAM and 1.3 for the DPPC vesicle fused FMST SAM. While the RMS values are very nearly the same, the FMST SAM presents a rougher topography than the FMST SAM with vesicle fused DPPC. In the sample with vesicle fused DPPC there are no topographical abnormalities that would indicate free vesicles on the surface and the smoother topography suggests the formation of a lipid bilayer. Altogether, AFM and SEM both suggest the fusion of the DPPC vesicles with the FMST, indicated by lack of large morphological and topographical features from free vesicles.
## **2.4 Conclusions**

The goal of this study was create a vacuum stable model cell membrane from fusing lipid vesicles to self-assembled FMST SAMs. The combination of phosphorus, nitrogen, and carbon XPS signals confirm that DPPC vesicles fuse to self-assembled FMST SAMs. SFG spectrum collected in the CH region indicates that we assembled a high density, ordered lipid bilayer. The resulting SFG signal is from an imperfect symmetry in the lipid bilayer or from the polyethylene glycol linker. Finally, SEM and AFM images taken from DPPC fused FMST SAMs do not contain any morphological or topographical abnormalities related to free, un-fused, vesicles sitting on top of the FMST SAMs. Altogether, it can be reasoned that the vesicle fusion of DPPC with FMST SAMs is achieved and we have created a vacuum stable bilayer.

#### 2. 5 References

- 1. Junghans, A.; Koper, I. Structural Analysis of Tethered Bilayer Lipid Membranes. *Langmuir.* **2010**, *26*, 11035-11040.
- 2. Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. Molecular Biology of the Cell, 5<sup>th</sup> ed.; Garland Science: New York, 2008; pp 617-650.
- Mashaghi, A.; Swann, M.; Popplewell, J.; Textor, M.; Reimhult, E. Optical Anisotropy of Supported Lipid Structures Probed by Waveguide Spectroscopy and Its Application to Study of Supported Lipid Bilayer Formation Kinetics. *Anal. Chem.* 2008, 80, 3666-3676.
- Atanasov, V.; Atanasova, P. P.; Vockenroth, I. K.; Knorr, N.; Koper, I. A Molecular Toolkit for Highly Insulating tethered Bilayer Lipid Membranes on Various Substrates. *Bioconjuget Chem.* 2006, *17*, 631-637.
- Atanasov, V.; Knorr, N.; Duran, R. S.; Ingebrandt, S.; Offenhausser, A.; Knoll, W.; Koper, I. Membrane on a Chip: A Functional Tethered Lipid Membrane on a Silicon Oxide Surface. *Biophysical Journal*. 2005, *89*, 1780-1788.

- Naumann, R.; Schiller, S. M.; Giess, F.; Grohe, B.; Hartman, K. B.; Karcher, I.; Koper, I.; Lubben, J.; Vasilev, K.; Knoll, W. Tethered Lipid Bilayers on Ultraflat Gold Surfaces. *Langmuir.* 2003, 19, 5435-5443.
- 7. Cornell, B. A.; Krishna, G.; Osman, P. D.; Pace, R. D.; Wieczorek, L. Tetheredbilayer lipid membranes as a support for membrane-active peptides. *Biochemical Society Transactions*. **2001**, *29*, 613-617.
- 8. Ye, S.; Nguyen, K. T.; Le Clair, S. V.; Chen, Z. In situ molecular level studies on membrane related peptides and proteins in real time using sum frequency generation vibrational spectroscopy. *Journal of Structural Biology*. **2009**, *168*, 61-77.
- Michel, R.; Subramaniam, V.; McArthur, S. L.; Bondurant, B.; D'Ambruoso, G. D.; Hall, H. K.; Brown, M. F.; Ross, E. E.; Saavedra, S. S.; Castner, D. G. Ultra-High Vacuum Surface Analysis Study of Rhodopsin Incorporation into Supported Lipid Bilayers. *Langmuir.* 2008, 24, 4901-4906.
- Franz, J.; Graham, D. J.; Schmuser, L.; Baio, J. E.; Lelle, M.; Peneva, K.; Mullen, K.; Castner, D. G.; Bonn, M.; Weidner, T. Full membrane spanning self-assembled monolayers as model systems for UHV-based studies of cell-penetrating peptides. *Biointerphases*. 2015, 10, 1-6.
- 11. Solomons, T. W. G; Fryhle, C. B. Organic Chemistry, 10<sup>th</sup> ed.; John Wiley & Sons Inc., 2009; pp 502-548.
- 12. Baio, J. E.; Weidner, T.; Brison, J.; Graham, D. J.; Gamble, L. J.; Castner, D. G. Amine terminated SAMs: Investigation why oxygen is present in these films. *Journal of Electron Spectroscopy and Related Phenomena*. **2009**, *172*, 2-8.
- 13. Pease, R. F.; Nixon, W. C. High resolution scanning electron microscopy. J. Sci. Instrum. 1965, 42, 81-85.
- 14. Courte, J. E.; Narasimhan, L. R.; Patel, C. K. N.; Kaback, H. R. The lipid bilayer determines helical tilt angle and function in lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **1997**, *94*, 10167-10171.
- 15. Siemeling, U.; Rittinghaus, S.; Weidner, T.; Brison, J.; Castner, D. COOH-terminated SAMs on gold fabricated from an azobenzene derivative with a 1,2-dithiolane headgroup. *Applied Surface Science*. **2010**, *256*, 1832-1836.
- He, Y.; Chen, G.; Xu, M.; Liu, Y.; Wang, Z. Vibrational dephasing of selfassembling monolayer on gold surface. *Journal of Luminescense*. 2014, 152, 244-246.

- 17. Weidner, T.; Apte, J. S.; Gamble, L.; Castner, D. G. Probing the Orientation and Conformation of  $\alpha$ -Helix and  $\beta$ -Strand Model Petides on Self-Assembled Monolayers Using Sum Frequency Generation and NEXAFS Spectroscopy. *Langmuir*. **2010**, *26*, 3433-3440.
- 18. Yamamoto, M.; Sakurai, Y.; Hosoi, Y.; Ishii, H.; Ito, I. E.; Kajikawa, K.; Ouchi, Y.; Seki, K. Physisorption of a long-chain n-alkane on Ag(111) surface: an infra-red reflection absorption spectroscopic study. *Surface Science*. **1999**, *427-428*, 388-392.
- 19. Chen, X.; Chen, Z. SFG studies on interactions between antimicrobial peptides and supported lipid bilayers. *Biochimica et Biophysica Acta*. **2006**, *1758*, 1257-1273.



**Figure 2.1.** The basic diagram of the tBLM depicts the lipids, a linking region that holds the lipids together, a spacer composed of polyethylene glycol (PEG) that acts as the tether, and anchor to bond with the substrates surface. PEG is used for biocompatibility and inertness. The figure is adapted from reference 4.



**Figure 2.2.** Full membrane spanning tethered lipid bilayers on gold. In each image is a silicon wafer coated in titanium, for an adhesion layer, and gold. The left image is the FMST SAM on gold. The right image is the FMST SAM on gold followed by vesicle fusion of DPPC. The result is two model membrane systems for vacuum studies of proteins interacting with model lipid bilayers.

Sample	$C_{1s}$ (%)	$O_{1s}$ (%)	N <sub>1s</sub> (%)	$P_{2p}$ (%)	$S_{2p}(\%)$
Theo. Au/FMST	85.5	13.7			<1
Exp. Au/FMST	77.1 <u>+</u> 5.9	17.7 <u>+</u> 1.7	2.9 <u>+</u> 2.6	0.1 <u>+</u> 0.1	2.2 <u>+</u> 0.6
Theo. Au/FMST/DPPC	81.7	15.3	1.4	1.4	<1
Exp. Au/FMST/DPPC	78.3 <u>+</u> 2.4	16.3 <u>+</u> 1.5	3.5 <u>+</u> 1.5	0.8 <u>+</u> 0.5	1.1 <u>±</u> 0.6

 Table 2.1. XPS elemental percent composition



**Figure 2.3.** The High resolution XPS  $C_{1s}$  spectrum of (A) FMST SAM on gold and (B) FMST SAM on gold with vesicle fused DPPC are depicted normalized to 84.0 eV of Au<sub>4f</sub>.



**Figure 2.4.** The *ppp* SFG spectra of both FMST SAM on gold (bottom) and FMST SAM on gold with vesicle fused DPPC (top) are depicted.



**Figure 2.5.** SEM images of (**A**) FMST SAM on gold (scale bar 1  $\mu m$ ), (**B**) FMST SAM on gold with vesicle fused DPPC (scale bar 1  $\mu m$ ), (**C**) FMST SAM on gold (scale bar 100 nm), (**D**) FMST SAM on gold with vesicle fused DPPC (scale bar 100 nm).



**Figure 2.6.** AFM images (500 nm by 500 nm) of (**A**) FMST SAM topography (scale bar 9.5 nm), (**B**) FMST SAM phase contrast (scale bar 13°), (**C**) FMST SAM with vesicle fused DPPC topography (scale bar 9.5 nm), and (**D**) FMST SAM with vesicle fused DPPC phase contrast (scale bare 8°).

## **Chapter 3- Probing the Interactions of WLBU2 with Lipid Monolayers**

Thaddeus W. Golbek,<sup>1</sup> Johannes Franz,<sup>2</sup> J. Elliott Fowler, <sup>1</sup> Karl Schilke, <sup>1</sup> Tobias Weidner, <sup>2</sup> Joe E. Baio<sup>1</sup>

1. School of Chemical, Biological, and Environmental Engineering Oregon State University, OR, USA.

2. Max Planck Institute for Polymer Research, Mainz, Germany

# Abstract

WLBU2 is an engineered cationic amphiphilic peptide that targets Gram-positive and Gram-negative bacteria, and envelopes endotoxin while avoiding lysing of other cell types. However, the exact mechanism of how WLBU2 targets, binds, and disrupts bacterial cell membranes is still not completely known. Thus, the overall goal of this investigation is to determine the structural basis for recognition between WLBU2 and cell membranes. Currently, it is believed that WLBU2 binds parallel to the surface of the membrane in an  $\alpha$ -helical confirmation, and at a critical membrane concentrations may disrupt the membrane using a carpet, barrel-stave, or toroidal model. In order to validate one of these models we will use near edge x-ray absorption fine structure (NEXAFS) and sum frequency generation (SFG) spectroscopy to probe the interactions between WLBU2 and two model lipid bilayers made up of both positive and negatively charged lipids. SFG studies demonstrate that binding of WLBU2 induces increased lipid monolayer order. That is until at a certain interfacial concentration of peptide, the peptide induces disorder within the lipid monolayer. Ordering of the monolayer was assessed by taking the ratio of the CD<sub>3</sub> (~2070 cm<sup>-1</sup>) and CD<sub>2</sub> symmetric (~ 2100 cm<sup>-1</sup>) peaks. Differences in the observed changes in pressure at the lipid – air interface suggest that WLBU2

selectively binds to negatively charged membranes via electrostatic interactions between positively charged amino acids and negatively charged lipids. NEXAFS tilt angle calculations for the peptide bound to vacuum stable model bilayers (see chapter 2) FMST SAM and FMST SAM with vesicle fused DPPC are 71° ± 2° and 70° ± 1°, respectively. NEXAFS and SFG together suggest that WLBU2 binds to the surface of the lipid bilayer in a mostly  $\beta$ -sheet (peaks near 1636 cm<sup>-1</sup> and near 1668 cm<sup>-1</sup>) conformation for zwitterionic membranes and in a  $\alpha$ -helical (peak near1638 cm<sup>-1</sup>) conformation for

## **3.1 Introduction**

WLBU2 is a synthetic amphiphilic cationic AMP, derived from lentivirus lytic peptide 1(**Figure 3.1**).<sup>1,2,3</sup> Like most cationic AMPs, WLBU2 shows activity toward both Gram-negative and Gram-positive bacteria under physiological conditions. In its  $\alpha$ helical conformation, WLBU2 folds so that positively charged amino acids (arginine) and hydrophobic side chains (valine and tryptophan) point out in opposing faces. This separation is important for the ability to disrupt or transverse phospholipid membranes.<sup>4</sup> In water WLBU2 adopts a disordered conformation, but in membrane mimicking solvents, in the presence of counter ions, and at bacterial membranes WLBU2 adopts an  $\alpha$ -helical conformation.<sup>1,2</sup> Circular dichroism shows that WLBU2 reaches 78%  $\alpha$ -helix content in the presence of lipopolysaccharide (LPS, endotoxin) allowing the peptide to insert into LPS vesicles.<sup>5</sup> Unknown information about WLBU2 includes the mechanism the AMP uses when disrupting bacterial membranes and the location of WLBU2 when bound to a membrane. Currently, it is hypothesized that WLBU2 binds parallel to the surface of the membrane, and when enough peptides have bound to the membrane surface, WLBU2 can use either the carpet model or torodial model to disrupt the membrane.<sup>6</sup> Determination if WLBU2 is  $\alpha$ -helical or a  $\beta$ -sheet is important to weather WLBU2 is active or inactive, respectively. Therefore, we will probe the interactions between the lipids and WLBU2 with sum frequency generation (SFG) spectroscopy and near edge x-ray absorption fine structure (NEXAFS). NEXAFS and SFG will allow us to assess the conformation of WLBU2 when interacting at the lipid interface.

SFG has the ability to probe kinetic interactions and conformations of proteins interacting with various phospholipids.<sup>7-9</sup> The experimental setup involves a trough with a monolayer of phospholipids at the air-water interface. WLBU2 is injected into the subphase and allowed to interact with the lipid monolayer. The charge interaction that drives the membrane selectivity of AMPs will be viewed in successive experiments (*i.e.* only one type of phospholipid per experiment). After WLBU2 is injected into the subphase the AMP wants to lower its Gibbs free energy, and thus is driven to the lipid-air interface. In this experiment we are using two different lipid monolayers of DMPG and DPPC. DMPG is a phospholipid that, at physiological pH, assumes a negative charge<sup>10</sup> and we believe models the negative charge of lipopolysaccharide, found in gram negative bacteria.<sup>11</sup> DPPC is a zwitterionic phospholipid, which is the characteristic membrane charge of a mammalian cellular membrane.<sup>11</sup> We believe that compared to a negatively charged membrane of DPPG, WLBU2 will bind slowly to a phosphocholine zwitterionic head group of DPPC. Once WLBU2 is injected into the subphase SFG spectrum will be collected in a periodic fashion in order to view the kinetics and conformation change of WLBU2 interacting with each lipid monolayer.

NEXAFS spectra provides details of specific bond types, lengths of the intramolecular bonds, orientation of these molecular bonds and functional groups on solid surfaces.<sup>12,13</sup> NEXAFS requires samples to be placed in a ultra-high vacuum environment; therefore, unlike the SFG experiments a lipid bilayer that is vacuum stable must be used. During the NEXAFS experiments we will probe the interactions between WLBU2 and the vacuum stable FMST models discussed in Chapter 2. Again, the FMST has a hydroxyl headgroup that in solution will present a negative charge, while vesicle fusion of DPPC into the FMST system will create a zwitterionic membrane, characteristic of a mammalian cellular membrane.<sup>10</sup>

# **3.2 Materials and Methods**

### 3.2.1 Experimental Setup for SFG

An aluminum trough coated in Teflon was used for all experiments. A tensiometer (KBM 315 Sensor Head, Kibron Inc.) is linked to FilmWareX software used to measure the surface pressure as a function of time. To keep the water level consistent, a sterile syringe needle (0.80 x 120 mm, Braun Medical Inc.) hooked up to a pump (Brazel, Fisher Bioblock Scientific) by sterile medical tubing (mediWare), was submerged to the bottom of the trough. Approximately 20 mL of PBS (0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4, Sigma Aldrich), made with D<sub>2</sub>O (Carl Roth GmbH), for Amide region spectral collection, or Ultra-pure H<sub>2</sub>O (Millipore Direct-Q3 System), for CD region spectral collection, filled the trough. The phospholipid (DPPC (1,2-Dipalmitoyl-D62-*sn*-Glycero-3-Phosphocholine, Avanti Polar Lipids Inc.) or DMPG (1,2-Dimyristoyl-D54-*sn*-Glycero-3-[Phospho-*rac*-(1-Glycerol)] (sodium salt)) (Avanti Polar Lipids Inc.)) was dissolved in chloroform (HPLC grade, Avantor Performance Materials) and by using a syringe (25  $\mu$ L, Hamilton Company) droplets are touched to the surface of the PBS buffer until a surface pressure of approximately 20 mN/m is reached. Addition of WLBU2 (RRWVRRVRRWVRRWVRRWVRR, purity 97.2%) (GenScript) was done first by dissolving WLBU2 in 400  $\mu$ L PBS buffer, and second injecting the solution into the subphase of the trough. The final concentration of WLBU2 in the trough was 0.1 mg/mL.

# **3.2.2 Experimental Setup for NEXAFS**

**3.2.2.1 Substrate Preparation:** The substrates in the study were silicon wafers, from Plano GmbH (Wetzlar, Germany), cut to 1 cm x 1 cm silicon substrates were cleaned by sequential rinsing and sonication in ultrapure water (Millipore Direct-Q3n system), acetone (EDM Millipore Corp), dichloromethane (EDM Millipore Corp), acetone, and ethanol (Pharmco-Aaper). Following sonication steps, the substrates were coated first with a thin adhesion layer of titanium (3.5 nm) then a film of Au (100 nm) gold by thermal evaporation. All substrates were then stored under nitrogen until use.

**3.2.2.2 Self-assembled monolayer (SAM) and vesicle fusion:** FMST samples were prepared substrates by immersing the Au substrates in a 1  $\mu$ M ((3-((14-((4'-((5-methyl-1-phenyl-35- (phytanyl)oxy-6,9,12,15,18,21,24,27,30,33,37-undecaoxa-2,3- dithiahenpentacontan-51- yl)oxy)-[1,1'-biphenyl]-4-yl)oxy)tetradecyl)oxy)-2- (phytanyl)oxy)glycerol) (SDx Tethered Membranes Pty Ltd. ) - ethanol solution for 10 minutes. Following assembly - the samples were washed repeatedly with ethanol, removing any non-bound FMST molecules. These samples were then dried and stored using nitrogen. FMST samples fused with vesicles of lipids were prepared by first extruding a solution of DPPC (1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine, Avanti

Polar Lipids Inc.) in ultrapure water (2.5 mg/ml) thereby, producing uniform vesicles no larger than 50 nm in diameter. Bare FMST SAMs were then incubated for 1 hour in the DPPC vesicle solution (1 mg/ml in ultrapure water) allowing the vesicles to fuse with the FMST. Afterwards, the samples are washed thoroughly with PBS, dried with nitrogen, and stored under nitrogen.

**3.2.2.3 Protein Adsorption on the FMST Substrates:** Substrates coated with FMST SAMs were immersed in a PBS solution of WLBU2 (0.1 mg/ml). After 3 hours PBS was pipetted in to break up the protein at the air-water interface, avoiding a Langmuir deposition of additional peptide on the sample when it was removed from the protein solution. The samples were then submerged in sequential baths for 1 minute of vortexed PBS, 50/50 PBS and ultrapure water, and 100% ultrapure water. Immediately the samples are dried with nitrogen and stored under nitrogen.

**3.2.3 Sum Frequency Generation (SFG) Vibrational Spectroscopy:** A femtosecond Ti:Sapphire laser oscillator coupled with a Nd:YLF laser pumped regenerative amplifier (Spitfire Ace, Spectra Physics) was used to generate a visible beam (35 fs pulse duration and 4.65 mJ) centered at 791.8 nm. The amplified visible beam was split into two parts. The first was used as the visible pulse for sum frequency generation and was passed through a Fabry-Perot etalon to spectrally narrow the pulse to ~15 cm<sup>-1</sup>. The second part was used to pump the optical parametric amplifier (OPA) system (Light Conversion, TOPAS). The generated tunable infrared pulse (3.1-6.1  $\mu m$ ) was polarized by a half-wave plate before use as the sum frequency generation IR pulse. The IR and visible pulses were overlapped temporally and spatially at the liquid-air interface generating an SFG signal. SFG spectra of the samples were collected in the polarization combinations

*ssp and sps* (*s*-polarized SFG, *p*-polarized visible, and *s*-polarized IR) in the C-D region  $(2000 \text{ cm}^{-1} - 2300 \text{ cm}^{-1})$  and Amide I region  $(1500 \text{ cm}^{-1} - 1800 \text{ cm}^{-1})$ . The resulting SFG signal was focused onto a spectrograph (Action, Princeton Instruments), dispersed by grating, and re-focused on an electron multiplying charge coupled device (CCD) camera (Newton, Andor). Spectra were normalized by division of a clean gold reference.

**3.2.4 Near-edge X-ray absorption fine structure spectroscopy (NEXAFS):** NEXAFS experiments were conducted at the National Synchrotron Light Source (NSLS) U7A beamline at Brookhaven National Laboratory, using an elliptically polarized beam with ~85% *p*-polarization. The beamline is equipped with a 600 lines/mm grating monochromator providing a FWHM resolution of ~0.15 eV at the carbon K-edge. The energy scale of the monochromator was calibrated by using the  $C_{1s} \rightarrow \pi^*$  transition (285.35 eV) of a graphite high transmission grid positioned in the path of the X-rays. Normalization of the C K-edge spectra were done by a clean gold surface prepared by evaporation of gold *in vacuo*. The signal and the reference were both divided by the NEXAFS signal of a gold-coated mesh, placed upstream, to adjust for beam intensity variations.<sup>12</sup> A detector monitored partial electron yield with the bias voltage maintained at -150 V for the C K-edge and -360 V for the N K-edge. Samples were mounted to allow rotation with respect to the vertical axis and to change the angle between the surface of the sample and the synchrotron x-rays. The NEXAFS angle by definition is the angle between sample and the incident light.

#### **3.3 Results and Discussion**

#### 3.3.1 SFG Analysis

During the SFG experiments the surface pressure is recorded for WLBU2 interacting with DPPC and DMPG lipid monolayers at the lipid-air interface (Figure 3.2). Surface pressure is used first to probe the amount of lipids and eventually proteins deposited at the interface. As more protein reaches the surface, the surface pressure increases until system equilibrium is reached. The needle used to measure the pressure is very sensitive and spectra artifacts are generated when the system is disturbed and as the water height is adjusted to keep a constant solution level. The surface pressure of the DPPC monolayer is approximately 20 mN/m, and is stable over time indicating the lasers are not damaging the lipid monolayer (Figure 3.2 A). Upon injection of WLBU2 into the trough (Figure 3.2 B) the pressure increases to an equilibrium value near 37.0 mN/m. The initial surface pressure of the DMPG lipid monolayer is approximately 24.0 mN/m before injection of WLBU2 (Figure 3.2 C). After the injection of WLBU2 the pressure raises to 39.0 mN/m, at equilibrium (Figure 3.2 D). The rate at which the pressure equilibrium is reached can be largely attributed to the electrostatic interactions of WLBU2 with the lipid monolayer. At physiological pH the head groups of DMPG and DPPC assume a negative and a net zero charge – respectively. WLBU2 is a cationic AMP, due to the net positive charge from its arginine's, and has been shown to be strongly attracted to the negative charge of the DMPG lipid monolayer (shorter time to pressure equilibrium) demonstrating the AMP charge based selectivity.

SFG spectra in the Amide I region (1500 cm<sup>-1</sup> -1800 cm<sup>-1</sup>) were collected to monitor the lipid peak and observe the secondary structure of WLBU2 interacting with

the lipid monolayer. The Amide I region SFG spectra of WLBU2 interacting with a DPPC lipid monolayer at physiological pH are shown in Figure 3.3 A-D and amplitudes of the spectral features found in the data can be found in Table 3.1. After 2.5 hours, two weak spectral features appear near 1636 cm<sup>-1</sup> and 1668 cm<sup>-1</sup>, largely attributed to ordering  $\beta$ -sheets (Figure 3.3 B). The SFG peak near 1668 cm<sup>-1</sup> remains at the same frequency, and the peak amplitude increases over the course of the experiment. Previous work involving Lysine-Leucine peptides (similar length to WLBU2) shows the first peak in the  $\beta$ -sheet doublet to have a larger amplitude than the second peak.<sup>14</sup> A similar spectrum is seen for SFG results 3.5 hours after injection of WLBU2 (Figure 3.3 C-D), the peak near 1633  $\text{cm}^{-1}$  has a larger amplitude than the peak at 1668  $\text{cm}^{-1}$ . The separation of the attributed  $\beta$ -sheet doublet and the amplitudes of the peaks are in agreement with the Lysine-Leucine  $\beta$ -sheet peptides, thus the conformation of WLBU2 interacting with a DPPC lipid monolayer can be greatly attributed to a  $\beta$ -sheet. The DPPC monolayer shows a strong lipid peak near 1725 cm<sup>-1</sup> indicative of the C=O stretching (Figure 3.3 A). The lipid peak remains constant 2.5 hours after injection. After 3.5 hours the lipid peak disappears (Figure 3.3 C) which is viewed as WLBU2 replacing the DPPC lipids in the monolayer and the replaced lipids from a bilayer with the remaining lipids at the airwater interface. In a lipid bilayer one lipid signal (C=O stretching) is cancelled by a phase opposite signal of another lipid. SFG experiments involving lipid bilayers do not give a peak near 1730 cm<sup>-1</sup> (lipid peak).<sup>15</sup> The other possible explanation for the disappearance of the lipid peak is that the lipid monolayer is destroyed by the activation of WLBU2, which is not the case as seen in the CD spectrum (Figure 3.4 A-D).

SFG spectra are taken periodically to probe the ordering of the lipid monolayer fatty acid chains as a function of time. CD region SFG spectra of WLBU2 interacting with a DPPC lipid monolayer at physiological pH are shown in Figure 3.4 and peak locations, amplitudes, and assignments are compiled in Table 3.2. Order is compared between spectrum by using the ratio between  $CD_3$  (peak near 2077 cm<sup>-1</sup>) and the  $CD_2$ (peak near 2105 cm<sup>-1</sup>) symmetric intensities.<sup>16</sup> The DPPC lipid monolayer has a ratio value of 2.7 before the injection of WLBU2. The ratio 2 hours after injection of WLBU2 becomes 4.1, indicating increased order. The increase in order before the pressure equilibrium suggests that WLBU2 is interacting at the monolayer interface causing the hydrocarbon chains of DPPC to pack tighter together. After 4 hours the ratio decreases to 2.4, indicating that there is decreased order in the lipid monolayer. The disorder in the lipid monolayer occurs at the same time as the disappearance of the lipid signal in the Amide I region, thus the disorder suggests the replacement of DPPC lipids by WLBU2. At long times the order is increased suggesting that more WLBU2 is interacting with the lipid monolayer without the replacement of DPPC lipids.

The Amide I region SFG spectra of WLBU2 interacting with a DMPG lipid monolayer at physiological pH are shown in **Figure 3.3 E-H**. The DMPG monolayer shows a strong lipid peak near 1728 cm<sup>-1</sup> indicative of the lipid peak (C=O stretching), and is spectrally seen throughout the length of the experiment. The persistence of the lipid peak throughout the experiment suggests that the DMPG monolayer is neither destroyed by WLBU2 or replaced forming a lipid bilayer. A pronounced spectral feature near 1635 cm<sup>-1</sup> after 1 hour is observed and is attributed to a predominately  $\alpha$ -helical conformation of WLBU2 at the monolayer interface.<sup>17</sup> The intensity of the  $\alpha$ -helical peak increases over time and shifts to near 1638 cm<sup>-1</sup> attributed to more WLBU2 assuming an  $\alpha$ -helical conformation. At long incubation times the emergence of a spectral feature near 1670 cm<sup>-1</sup> appears, which is attributed to a random coil because of the broadness of the peak and the fact that WLBU2 is disordered in PBS solutions.<sup>5</sup> The dominant  $\alpha$ -helical conformation of WLBU2 suggests activity toward the DMPG monolayer.

CD region SFG spectra of WLBU2 interacting with a DPPC lipid monolayer at physiological pH are shown in **Figure 3.4 E-G**. Comparing the peak ratios of the CD<sub>3</sub> symmetric peak near 2070 cm<sup>-1</sup> and the CD<sub>2</sub> symmetric peak near 2100 cm<sup>-1</sup>, order is decreased 1 hour after injection of WLBU2 suggesting insertion of WLBU2 into the monolayer. Initial insertion of WLBU2 into the monolayer causes disorder in the system. At long times (2 hours after injection of WLBU2) order is increased from WLBU2 interaction with the DMPG lipid monolayer.

## **3.3.2 NEXAFS Analysis**

The NEXAFS nitrogen K-edge spectra of WLBU2 adsorbed onto two membrane systems (FMST SAM and FMST SAM with vesicle fused DPPC) at 55° show broad peaks at 406.4 eV and 416.0 eV, which are attributed to  $\sigma^*$  N-C bonds and  $\sigma^*$  N-CO bonds, respectively (**Figure 3.5**). The spectra also show a sharp peak at 401.4 eV attributed to amide  $\pi^*$  bonds. The resulting difference spectrum between 90° and 20° produces a negative dichroism at 401.4 eV (amide  $\pi^*$ ), which is attributed to ordered amide bonds within the  $\beta$ -sheet form of WLBU2.<sup>18</sup> An  $\alpha$ -helical signal is generally not observed because of the broad distribution of orientations.<sup>19</sup> These peaks do not show up in the difference spectrum. The average tilt angle of WLBU2 in each system is calculated normal to the substrate using standard theoretical framework with the assumption that the amide  $\pi^*$  orbitals are perpendicular to the backbone (N-C-O) of the peptide.<sup>12</sup> The averaged tilt angles of WLBU2 are 71° ± 2° for the FMST SAM and 70° ± 1° for the FMST SAM with vesicle fused DPPC. A uniform distribution is assumed for the calculations, but in a real system  $\beta$ -sheets have a range of orientations. The large angle of the peptide, relative to the surface normal, indicates low insertion properties.<sup>20</sup>

Together NEXAFS and SFG suggest the assignment of a  $\beta$ -sheet, attributed from the negative dichroism and the  $\beta$ -sheet doublet of the SFG spectrum, for WLBU2 interacting with DPPC. The negative dichroism of the FMST SAM in combination with the broad SFG peak at 1668 cm<sup>-1</sup> (**Figure 3.3 H**) suggests that the peak has contributions from both random coil (similar to WLBU2 in solution) and  $\beta$ -sheet secondary structures. Altogether, WLBU2 binds with a predominately ordered  $\beta$ -sheet secondary structure for zwitterionic lipids and an ordered  $\alpha$ -helical secondary structure for negatively charged lipids. The presence of the lipid signal in the DMPG lipid monolayer indicates that the lipid monolayer is still intact, which is important for WLBU2 binding and inserting with the lipid monolayer and not ordering at the air-water interface.

# **3.4 Conclusions**

It is believed that WLBU2 binds parallel to the surface of the bacterial cell membrane in a  $\alpha$ -helical confirmation, and at a critical interfacial concentration, WLBU2 inserts into the cellular membrane. Surface pressure results indicate that WLBU2 is selective toward negatively charged membranes. Together SFG spectrum in the CD region and the Amide I region of WLBU2 interacting with a DPPC lipid monolayer indicate that WLBU2 binds in a predominately  $\beta$ -sheet secondary structure and replaces the lipids causing a lipid bilayer to form. Comparatively, SFG spectra of the CD region and the Amide I region of WLBU2 interacting with a DMPG monolayer suggest that WLBU2 binds in an  $\alpha$ -helical conformation and inserts into the membrane using a nondestructive mechanism. NEXAFS further supports the SFG data by measuring ordered  $\beta$ -sheet conformation contributions of WLBU2 at the surface of model bilayer systems. Altogether, WLBU2 binds to the surface of zwitterionic membranes in a  $\beta$ -sheet conformation, while for negatively charged membranes folds in an  $\alpha$ -helical conformation and, at critical peptide concentrations, inserts into the lipid monolayer by either individual insertion or by using either the barrel-stave or toroidal model. The observed difference in folding demonstrates WLBU2 selectivity toward negatively charged membranes (*i.e.* bacteria) and inactivity toward zwitterionic membranes (*i.e.* mammalian cells and other cell types).

# 3.5 References:

- Lampi, M. C.; Wu, X.; Schilke, K. F.; McGuire, J. Structural attributes affecting peptide entrapment in PEO brush layers. *Colloids and Surfaces B: Biointerfaces*. 2013, 106, 79-85.
- Wu, X.; Ryder, M. P.; McGuire, J.; Schilke, K. F. Concentration effects on peptide elution from pendant PEO layers. *Colloids and Surfaces B: Biointerfaces*. 2014, 118, 210-217.
- McClanahan, J. R.; Peyyala, R.; Mahajan, R.; Montelaro, R. C.; Novak, K. F.; Puleo, D. A. Bioactivity of WLBU2 peptide antibiotic in combination with bioerodible polymer. *International Journal of Antimicrobial Agents*. 2011, *38*, 530-533.

- 4. Ryder, M. P.; Wu, X.; McKelvey. G. R.; McGuire, J.; Schilke, K. F. Binding interactions of bacterial lipopolysaccharide and the cationic peptides polymyxin B and WLBU2. *Colloids and Surfaces B: Biointerfaces*. **2014**, *120*, 81-87.
- 5. Stohr, J. NEXAFS Spectroscopy; Springer-Verlag, 1992.
- 6. Messmer, M. C.; Conboy, J. C.; Richmond, G. L. Observation of Molecular Ordering at the Liquid-Liquid Interface by Resonant Sum Frequency Generation. *J. Am. Chem. Soc.* **1995**, *117*, 8039-8040.
- Ding, B.; Laaser, J. E.; Liu, Y.; Wang, P.; Zanni, M. T.; Chen, Z. Site-Specific Orientation of an α-Helical Peptide Ovispirin-1 from Isotope-Labeled SFG Spectroscopy. J. Phys. Chem. B. 2013, 117, 14625-14634.
- Baio, J. E.; Zane, A.; Jaeger, V.; Roehrich, A. M.; Lutz, H.; Pfaendtner, J.; Drobny, G. P.; Weidner, T. Diatom Mimics: Directing the Formation of Biosilica Nanoparticles by Controlled Folding of Lysine-Leucine Peptides. *J. Am. Chem. Soc.* 2014, *136*, 15134-15137.
- 9. Chan, D. I.; Prenner, E. J.; Vogel, H. J. Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochimica et Biophysica Acta*. 2006, *1758*, 1184-1202.
- Baugh, L.; Weidner, T.; Baio, J. E.; Nguyen, P. T.; Gamble, L. J.; Stayton, P. S.; Castner, D. G. Probing the Orientation of Surface-Immobilized Protein G B1 Using ToF-SIMS, Sum Frequency Generation, and NEXAFS Spectroscopy. *Langmuir*. 2010, 26, 16434-16441.
- Weidner, T.; Apte, j. S.; Gamble, L. J.; Castner, D. G. Probing the Orientation and Conformation of α-Helix and β-Strand Model Peptides on Self-Assembled Monolayers Using Sum Frequency Generation and NEXAFS Spectroscopy. *Langmuir.* 2010, 26, 3433-3440.
- 12. Coutre, J. E.; Narasimhan, L. R.; Patel, C. K. N.; Kaback, H. R. The lipid bilayer determines helical tilt angle and function in lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **1997**, *94*, 10167-10171.
- Deslouches, B.; Phadke, S. M.; Lazarevic, V.; Cascio, M.;Islam, K.; Montelaro, R. C.; Mietzner, T. A. De Novo Generation of Cationic Antimicrobial Peptides: Influence of Length and Tryptophan Substitution on Antimicrobial Activity. *Antimicrob. Agents Chemother.* 2005, 49, 316-322.

- Ye, S.; Nguyen, K. T.; Le Clair, S. V.; Chen, Z. In situ molecular level studies on membrane related peptides and proteins in real time using sum frequency generation vibrational spectroscopy. *Journal of Structural Biology*. 2009, 168, 61-77.
- Nguyen, K. T.; Soong, R.; Im, S. C.; Waskell, L.; Ramamoorthy, A.; Chen, Z. Probing the Spontaneous Membrane Insertion of a Tail-Anchored Membrane protein by Sum Frequency Generation Spectroscopy. *J. Am. Chem. Soc.* 2010, *132*, 15112-15115.
- Thennarasu, S.; Huang, R.; Lee, D. K.; Yang, P.; Maloy, L.; Chen, Z.; Ramamoorthy, A. Limiting an Antimicrobial Peptide to the Lipid-Water Interface Enhances Its Bacterial Membrane Selectivity: A Case Study of MSI-367. *Biochemistry*. 2010, 49, 10595-10605.
- 17. Solomons, T. W. G; Fryhle, C. B. Organic Chemistry, 10<sup>th</sup> ed.; John Wiley & Sons Inc., 2009; pp 502-548.
- 18. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature*. **2002**, *415*, 389-395.
- Rong, H. T.; Frey, S.; Yang, Y. J.; Zharnikov, M.; Buck, M.; Wuhn, M.; Woll, C.; Helmchen, G. On the Importance of the Headgroup Substrate Bond in Thiol Monolayers: A Study of Biphenyl-Based Thiols on Gold and Silver. *Langmuir*. 2001, *17*, 1582-1593.
- 20. Chen, X.; Chen, Z. SFG studies on interactions between antimicrobial peptides and supported lipid bilayers. *Biochimica et Biophysica Acta*. **2006**, *1758*, 1257-1273.



**Figure 3.1.** WLBU2 is depicted in an alpha helical conformation to show the ordering of the amino acids in the sequence. The AMP has 24 residues of which 13 are positively charged arginine residues, 8 are hydrophobic valine residues, and 3 are hydrophobic tryptophan residues. Adapted from reference 2.



**Figure 3.2**. The surface pressure in the trough measured as a function of time in hours for WLBU2 interacting with (red) DMPG monolayer and (black) DPPC monolayer.



**Figure 3.3**. SFG in the amide region is depicted for the *ssp* polarization of (A) DPPC monolayer, (B) DPPC monolayer 2.5 hours after WLBU2 injection, (C) DPPC monolayer 3.5 hours after WLBU2 injection, (D) DPPC monolayer 8.5 hours after WLBU2 injection, (E) DMPG monolayer, (F) DMPG monolayer 1 hour after WLBU2 injection, (G) DMPG monolayer 3 hors after WLBU2 injection, and (H) DMPG monolayer 4.5 hours after WLBU2 injection.

Mode	DPPC		DPPC+WLBU2 (2.5 hrs)		DPPC+WLBU2 (3.5 hrs)		DPPC+WLBU2 (8.5 hrs)	
	$\lambda (\text{cm}^{-1})^{a}$	A <sup>b</sup>						
Protein			1636	0.38	1633	4.82	1632	7.01
Peak 1								
Protein			1668	0.43	1668	2.77	1668	2.54
Peak 2								
Lipid	1725	1.9	1725	1.94				
Peak		5						
(C=O)								

**Table 3.1.** Amide I region ssp SFG data for DPPC and DPPC+WLBU2 systems

<sup>a</sup> Frequency of the peaks in the SFG spectrum. <sup>b</sup> Amplitude of peak.



**Figure 3.4.** SFG CD region *ssp* spectra for (A) deuterated DPPC monolayer, (B) DPPC monolayer 2 hours after WLBU2 injection, (C) DPPC monolayer 4 hours after WLBU2 injection, (D) DPPC monolayer 9 hours after WLBU2 injection, (E) deuterated DMPG monolayer, (F) DMPG monolayer 1 hour after WLBU2 injection, and (G) DMPG monolayer 2 hours after injection.

Mode	DPPC		DPPC+WLBU2		DPPC+WLBU2		DPPC+WLBU2	
			(2 hrs)		(4 hrs)		(9 hrs)	
	$\lambda (\text{cm}^{-1})^{\text{d}}$	A <sup>e</sup>	$\lambda (\text{cm}^{-1})^{\text{d}}$	A <sup>e</sup>	$\lambda (\mathrm{cm}^{-1})^{\mathrm{d}}$	A <sup>e</sup>	$\lambda (\text{cm}^{-1})^{\text{d}}$	A <sup>e</sup>
<b>CD</b> <sub>3</sub> –	2077	1.33	2076	2.43	2076	2.10	2075	1.12
sym. <sup>a</sup>								
CD <sub>2</sub> –	2105	0.49	2102	0.59	2102	0.89	2106	0.29
sym. <sup>a</sup>								
CD <sub>3</sub> -	2132	4.15	2129	4.52	2130	4.49	2129	0.91
<b>FR</b> <sup>b</sup>								
CD <sub>2</sub> –	2198	0.81					2198	1.08
asym. <sup>c</sup>								
<b>CD</b> <sub>3</sub> –	2221	0.67	2223	1.20	2223	1.02	2220	0.18
asym. <sup>c</sup>								

 Table 3.2. CD region ssp SFG data for DPPC and DPPC+WLBU2

<sup>a</sup> Symmetric Stretching. <sup>b</sup> Fermi Resonance. <sup>c</sup> Asymmetric Stretching. <sup>d</sup> Frequency of the peaks in the SFG spectrum. <sup>e</sup> Amplitude of peak.



**Figure 3.5.** NEXAFS nitrogen K-edge spectrum for WLBU2 adsorbed onto both FMST SAM and FMST SAM with vesicle fused DPPC bilayers shows spectrum for 55° and for the difference spectrum of 90°-20°.

### **Chapter 4 – Future Directions**

# 4.1 Interaction of LPS with WLBU2 and DPPC

The interaction of WLBU2 with zwitterionic and negatively charged phospholipid headgroups provides valuable information about how antimicrobial peptides like WLBU2 bind to both mammalian and model bacterial cellular membranes. The next step would be to observe how WLBU2 interacts with a lipopolysaccharide (LPS, endotoxin) monolayer, thereby, truly mimicking the environment that WLBU2 has be designed to be active toward. Specific information about the activity and mechanism of WLBU2 toward LPS can be gathered. LPS has not been characterized by SFG before, so experiments of LPS monolayers and the interaction of peptides will provide insight on LPS as well. Deuterating various amino acid side chains in WLBU2 will allow probing of the side chain interaction with LPS in the CD region. This will provide further detailed information about the binding mechanism of WLBU2 and the orientation of its side chains. It is also known that LPS disrupts and destroys mammalian cell membranes but the kinetics are unknown, so an experiment to monitor the kinetics of LPS interacting with a deuterated DPPC phospholipid monolayer will provide such information.

## **4.2 Constant Pressure Langmuir Trough Experiments**

Advancing from the trough experiments to a Langmuir trough with barriers will provide the ability to do constant pressure experiments. Increasing or decreasing surface pressure of the lipid monolayer allows mimicking of various lipid bilayer environments (*i.e.* lungs). The biophysics of proteins relevant to the lipid pressure environment can then be properly probed using SFG.

# **Bibliography**

Ahn, D.; Dhinojwala, A. Sum Frequency Generation Vibrational Spectroscopy of Silicone Surfaces & Interfaces; Springer, 2012.

Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. Molecular Biology of the Cell, 5<sup>th</sup> ed.; Garland Science: New York, 2008; pp 617-650.

Apte, J. S.; Collier, G.; Latour, R. A.; Gamble, L. J.; Castner, D. G. XPS and ToF-SIMS Investigation of  $\alpha$ -Helical and  $\beta$ -Strand Peptide Adsorption onto SAMs. *Langmuir*. **2010**, *26*, 3423-3432.

Atanasov, V.; Atanasova, P. P.; Vockenroth, I. K.; Knorr, N.; Koper, I. A Molecular Toolkit for Highly Insulating tethered Bilayer Lipid Membranes on Various Substrates. *Bioconjuget Chem.* **2006**, *17*, 631-637.

Atanasov, V.; Knorr, N.; Duran, R. S.; Ingebrandt, S.; Offenhausser, A.; Knoll, W.; Koper, I. Membrane on a Chip: A Functional Tethered Lipid Membrane on a Silicon Oxide Surface. *Biophysical Journal.* **2005**, *89*, 1780-1788.

Baio, J. E.; Weidner, T.; Brison, J.; Graham, D. J.; Gamble, L. J.; Castner, D. G. Amine terminated SAMs: Investigation why oxygen is present in these films. *Journal of Electron Spectroscopy and Related Phenomena*. **2009**, *172*, 2-8.

Baio, J. E.; Zane, A.; Jaeger, V.; Roehrich, A. M.; Lutz, H.; Pfaendtner, J.; Drobny, G. P.; Weidner, T. Diatom Mimics: Directing the Formation of Biosilica Nanoparticles by Controlled Folding of Lysine-Leucine Peptides. *J. Am. Chem. Soc.* **2014**, *136*, 15134-15137.

Baugh, L.; Weidner, T.; Baio, J. E.; Nguyen, P. T.; Gamble, L. J.; Stayton, P. S.; Castner, D. G. Probing the Orientation of Surface-Immobilized Protein G B1 Using ToF-SIMS, Sum Frequency Generation, and NEXAFS Spectroscopy. *Langmuir*. **2010**, *26*, 16434-16441.

Chan, D. I.; Prenner, E. J.; Vogel, H. J. Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action. *Biochimica et Biophysica Acta*. **2006**, *1758*, 1184-1202.

Chen, X.; Chen, Z. SFG studies on interactions between antimicrobial peptides and supported lipid bilayers. *Biochimica et Biophysica Acta*, **2006**, *1758*, 1257-1273.

Coutre, J. E.; Narasimhan, L. R.; Patel, C. K. N.; Kaback, H. R. The lipid bilayer determines helical tilt angle and function in lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **1997**, *94*, 10167-10171.

Cornell, B. A.; Krishna, G.; Osman, P. D.; Pace, R. D.; Wieczorek, L. Tethered-bilayer lipid membranes as a support for membrane-active peptides. *Biochemical Society Transactions*. **2001**, *29*, 613-617.

Deslouches, B.; Phadke, S. M.; Lazarevic, V.; Cascio, M.;Islam, K.; Montelaro, R. C.; Mietzner, T. A. De Novo Generation of Cationic Antimicrobial Peptides: Influence of Length and Tryptophan Substitution on Antimicrobial Activity. *Antimicrob. Agents Chemother.* **2005**, *49*, 316-322.

Diamond, G.; Beckloff, N.; Weinberg, A.; Kisich, K. O. The Roles of Antimicrobail Peptides in Innate Host Defense. *Curr. Pharm. Des.* **2009**, *15*, 2377-2392.

Ding, B.; Laaser, J. E.; Liu, Y.; Wang, P.; Zanni, M. T.; Chen, Z. Site-Specific Orientation of an α-Helical Peptide Ovispirin-1 from Isotope-Labeled SFG Spectroscopy. *J. Phys. Chem. B.* **2013**, *117*, 14625-14634.

Franz, J.; Graham, D. J.; Schmuser, L.; Baio, J. E.; Lelle, M.; Peneva, K.; Mullen, K.; Castner, D. G.; Bonn, M.; Weidner, T. Full membrane spanning self-assembled monolayers as model systems for UHV-based studies of cell-penetrating peptides. *Biointerphases.* **2015**, *10*, 1-6.

Glukhov, E.; Stark, M.; Burrows, L. L.; Deber, C. M. Basis for Selectivity of Cationic Antimicrobial Peptides for Bacterial Versus Mammalian Membranes. *J. Biol. Chem.* **2005**, *280*, 33960-33967.

Hancock, R. E. W. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* **2001**, *1*, 156-164.

He, Y.; Chen, G.; Xu, M.; Liu, Y.; Wang, Z. Vibrational dephasing of self-assembling monolayer on gold surface. *Journal of Luminescense*. **2014**, *152*, 244-246.

Junghans, A.; Koper, I. Structural Analysis of Tethered Bilayer Lipid Membranes. *Langmuir.* **2010**, *26*, 11035-11040.

Lampi, M. C.; Wu, X.; Schilke, K. F.; McGuire, J. Structural attributes affecting peptide entrapment in PEO brush layers. *Colloids and Surfaces B: Biointerfaces*. **2013**, *106*, 79-85.

McClanahan, J. R.; Peyyala, R.; Mahajan, R.; Montelaro, R. C.; Novak, K. F.; Puleo, D. A. Bioactivity of WLBU2 peptide antibiotic in combination with bioerodible polymer. *International Journal of Antimicrobial Agents*. **2011**, *38*, 530-533.

Marr, A. K.; Gooderham, W. J.; Hancock, R. E. W. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Current Opinion in Pharmacology*. **2006**, *6*, 468-472.

Mashaghi, A.; Swann, M.; Popplewell, J.; Textor, M.; Reimhult, E. Optical Anisotropy of Supported Lipid Structures Probed by Waveguide Spectroscopy and Its Application to Study of Supported Lipid Bilayer Formation Kinetics. *Anal. Chem.* **2008**, *80*, 3666-3676.

Messmer, M. C.; Conboy, J. C.; Richmond, G. L. Observation of Molecular Ordering at the Liquid-Liquid Interface by Resonant Sum Frequency Generation. *J. Am. Chem. Soc.* **1995**, *117*, 8039-8040.

Michel, R.; Subramaniam, V.; McArthur, S. L.; Bondurant, B.; D'Ambruoso, G. D.; Hall, H. K.; Brown, M. F.; Ross, E. E.; Saavedra, S. S.; Castner, D. G. Ultra-High Vacuum Surface Analysis Study of Rhodopsin Incorporation into Supported Lipid Bilayers. *Langmuir.* **2008**, *24*, 4901-4906.

Naumann, R.; Schiller, S. M.; Giess, F.; Grohe, B.; Hartman, K. B.; Karcher, I.; Koper, I.; Lubben, J.; Vasilev, K.; Knoll, W. Tethered Lipid Bilayers on Ultraflat Gold Surfaces. *Langmuir.* **2003**, *19*, 5435-5443.

Nguyen, K. T.; Le Clair, S. V.; Ye, S.; Chen, Z. Molecular Interactions between Magainin 2 and Model Membranes in Situ. *J. Phys. Chem. B.* **2009**, *113*, 12358-12363.

Nguyen, K. T.; Le Clair, S. V.; Ye, S.; Chen, Z. Orientation Determination of Protein Helical Secondary Structures Using Linear and Nonlinear Vibrational Spectroscopy. *J. Phys. Chem. B.* **2009**, *113*, 12169-12180.

Nguyen, K. T.; Soong, R.; Im, S. C.; Waskell, L.; Ramamoorthy, A.; Chen, Z. Probing the Spontaneous Membrane Insertion of a Tail-Anchored Membrane Protein by Sum Frequency Generation Spectroscopy. *J. Am. Chem. Soc.* **2010**, *132*, 15112-15115.

Pease, R. F. W.; Nixon, W. C. High resolution scanning electron microscopy. J. Sci. Instrum. 1965, 42, 81-85.

Rong, H. T.; Frey, S.; Yang, Y. J.; Zharnikov, M.; Buck, M.; Wuhn, M.; Woll, C.; Helmchen, G. On the Importance of the Headgroup Substrate Bond in Thiol Monolayers: A Study of Biphenyl-Based Thiols on Gold and Silver. *Langmuir*. **2001**, *17*, 1582-1593.

Ryder, M. P.; Wu, X.; McKelvey. G. R.; McGuire, J.; Schilke, K. F. Binding interactions of bacterial lipopolysaccharide and the cationic peptides polymyxin B and WLBU2. *Colloids and Surfaces B: Biointerfaces*. **2014**, *120*, 81-87.

Shen, Y. R. Basic Theory of Sum-Frequency Generation. J. Phys. Chem. C. 2012, 116, 15505-15509.

Shen, Y. R. The Principles of Nonlinear Optics; John Wiley & Sons, 1984.

Siemeling, U.; Rittinghaus, S.; Weidner, T.; Brison, J.; Castner, D. COOH-terminated SAMs on gold fabricated from an azobenzene derivative with a 1,2-dithiolane headgroup. *Applied Surface Science*. **2010**, *256*, 1832-1836.

Solomons, T. W. G; Fryhle, C. B. Organic Chemistry, 10<sup>th</sup> ed.; John Wiley & Sons Inc., 2009; pp 502 – 548.

Stohr, J. NEXAFS Spectroscopy; Springer-Verlag, 1992.

Techane, S. D.; Gamble, L. J.; Castner, D. G. Multi-technique Characterization of Selfassembled Carboxylic Acid Terminated Alkanethiol Monolayers on Nanoparticle and Flat Gold Surfaces. *J Phys Chem C Nanomater Interfaces*. **2011**, *115*, 9432-9441.

Thennarasu, S.; Huang, R.; Lee, D. K.; Yang, P.; Maloy, L.; Chen, Z.; Ramamoorthy, A. Limiting an Antimicrobial Peptide to the Lipid-Water Interface Enhances Its Bacterial Membrane Selectivity: A Case Study of MSI-367. *Biochemistry*. **2010**, *49*, 10595-10605.

Vickerman, J. C.; Gilmore, I. S. *Surface Analysis: The Principle Techniques*; John Wiley and Sons, 2009.

Weidner, T.; Apte, J. S.; Gamble, L.; Castner, D. G. Probing the Orientation and Conformation of  $\alpha$ -Helix and  $\beta$ -Strand Model Petides on Self-Assembled Monolayers Using Sum Frequency Generation and NEXAFS Spectroscopy. *Langmuir*. **2010**, *26*, 3433-3440.

Wu, X.; Ryder, M. P.; McGuire, J.; Schilke, K. F. Concentration effects on peptide elution from pendant PEO layers. *Colloids and Surfaces B: Biointerfaces*. **2014**, *118*, 210-217.

Yamamoto, M.; Sakurai, Y.; Hosoi, Y.; Ishii, H.; Ito, I. E.; Kajikawa, K.; Ouchi, Y.; Seki, K. Physisorption of a long-chain n-alkane on Ag(111) surface: an infra-red reflection absorption spectroscopic study. *Surface Science*. **1999**, *427-428*, 388-392.

Ye, S.; Nguyen, K. T.; Le Clair, S. V.; Chen, Z. In situ molecular level studies on membrane related peptides and proteins in real time using sum frequency generation vibrational spectroscopy. *Journal of Structural Biology*. **2009**, *168*, 61-77.

Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature*. **2002**, *415*, 389-395.
